

Regulation of behavioral maturation by a primer pheromone produced by adult worker honey bees

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Previous research showed that the presence of older workers causes a delayed onset of foraging in younger individuals in honey bee colonies, but a specific worker inhibitory factor had not yet been identified. Here, we report on the identification of a substance produced by adult forager honey bees, ethyl oleate, that acts as a chemical inhibitory factor to delay age at onset of foraging. Ethyl oleate is synthesized *de novo* and is present in highest concentrations in the bee's crop. These results suggest that worker behavioral maturation is modulated via trophallaxis, a form of food exchange that also serves as a prominent communication channel in insect societies. Our findings provide critical validation for a model of self-organization explaining how bees are able to respond to fragmentary information with actions that are appropriate to the state of the whole colony.

division of labor | *Apis mellifera* | chemical communication | social insects

Many animal species communicate via pheromones. Releaser pheromones cause rapid, transient changes in behavior, whereas primer pheromones cause more long-term changes in both behavior and physiology (1). Hundreds of releaser pheromones are known; in contrast, very few primer pheromones have been identified, primarily because they are much more difficult to assay (2). More information on primer pheromones is needed because they play important roles in the regulation of behavior in many animal societies.

A major function of primer pheromones in both invertebrate and vertebrate societies is to help coordinate the timing of physiological and behavioral development (1). For example, pheromone secretions of the queen honey bee (*Apis mellifera*) are partially responsible for the inhibition of worker ovary development and queen rearing behavior that underlies reproductive division of labor (2).

Another key aspect of colony social organization in honey bees is the division of labor among workers for colony growth and development (3), a feature much less understood in its chemical ecology. Adult worker honey bees perform tasks within the nest such as brood care ("nursing") during the first 2–3 weeks of life and then switch to foraging and colony defense for their final 1–3 weeks. But division of labor in honey bee colonies is not rigid, because bees are sensitive to changes in their social environment, especially colony age structure. One response to changes in colony age structure is a change in the typical pattern of behavioral maturation. For example, in colonies lacking older bees (foragers), some bees initiate foraging when they are as young as 5 days of age, >2 weeks earlier than under more typical conditions.

Research published in 1992 led to the hypothesis that the age at onset of foraging in honey bee colonies is regulated by worker-worker interactions (4). Old bees inhibit the maturation of younger bees. For example, when a portion of a colony's foragers is removed to simulate predation, young bees develop

faster compared with those in a control colony in which the same number of individuals is depleted, but evenly across different age classes (5). Conversely, when foragers are confined to their hive by artificial rain young bees delay, rather than accelerate, their maturation (5). The feasibility of the worker social inhibition concept has been supported both by empirical findings (4–8) and theoretical models (7, 9, 10). However, a specific worker inhibitory factor had not been identified.

Social regulation of the rate of behavioral maturation requires physical contact among bees. Older bees separated from younger bees via a screen that permits some forms of physical contact (food transfer, antennal contact, and licking) are able to inhibit behavioral maturation, but not when they are separated via double screen that prevents these interactions (6, 8). These results suggested that the worker inhibitory factor was either a nonvolatile "contact" pheromone, a behavior, or both, with old bees having greater inhibitory potency than younger bees. The possibility that the worker inhibitory factor might be a pheromone gained strength with the discovery that two other honey bee primer pheromones, queen mandibular pheromone (QMP) and brood pheromone (BP), also play a role in regulating worker behavioral maturation (11, 12). In addition, Pankiw (13) recently reported that a hexane extract of foragers delays age at onset of foraging.

Here, we report on the identification of a substance produced by adult forager honey bees, ethyl oleate (EO), which acts as a chemical inhibitory factor, delaying age at onset of foraging. EO is present in highest concentrations in the honey crop, suggesting that it is transmitted via trophallaxis, a form of food exchange that also serves as a prominent communication channel in insect societies (14).

Materials and Methods

Bees. Colonies were maintained according to standard commercial procedures at the Institut National de la Recherche Agronomique Laboratory of Bee Biology and Protection and the University of Illinois Bee Research Facility. Experiments were performed in 2002 and 2003. In France, we used *A. mellifera ligustica* bees because the inhibition of behavioral maturation appears to be stronger in *ligustica* than in other subspecies in France (15). Subspecies determinations were confirmed by allozyme analysis at the *malate dehydrogenase* locus (ref. 16 and data not shown). In Illinois, bees were a mixture of European subspecies typical of this region, primarily *ligustica*. To provide 1-day-old adult bees, honeycombs containing late-stage pupae were removed from source colonies headed by naturally mated

Abbreviations: EO, ethyl oleate; QMP, queen mandibular pheromone; BP, brood pheromone.

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queens in the field and placed in an incubator (33°C, 50–60% relative humidity).

Identification and Quantification of EO. As discussed in the introduction, foragers inhibit behavioral maturation but young bees do not. We exploited this difference by searching for chemicals present in higher quantities in foragers. This was done by comparing GC/MS profiles of 2-methylpentane extracts from nurses and foragers. 2-Methylpentane was chosen because we were particularly interested in fatty acid esters, given that BP affects behavioral maturation and is composed of 10 fatty acid esters (12). Nurses and foragers were of unknown age, collected from a typical colony in the field headed by a naturally mated queen. They were collected with a modified portable vacuum cleaner (5) and frozen at –80°C.

Whole-body extracts were made in 2 ml of 2-methylpentane with the addition of 100 μ l of internal standard solution [500 ng of (10*E*)methyl-pentadec-10-enoate, 500 ng of methyl heptadecanoate, and 500 ng of methyl nonadecanoate]. Bodies were ground with a glass rod for 10 min and centrifuged (2,000 \times g, 20 min at 4°C); the supernatant was collected, another 500 μ l of 2-methylpentane was added, and centrifugation was repeated. The second supernatant was added to the first, and this solution was concentrated to 100 μ l under nitrogen. The concentrated supernatant was applied to a silica column (silica gel 60, particle size 40–63 μ m, 230–400 mesh) by using 4 ml of solvent mixture 1 [98.5% 2-methylpentane/1.5% diethyl ether (vol/vol)]. This eluate contained a mostly hydrocarbon fraction. Then 2 ml of a second solvent mixture [94% 2-methylpentane, 6% diethyl ether (vol/vol)] was added to the column; the second fraction, containing the fatty acid esters, was eluted. An additional 2 ml of the second solvent mixture was passed through the column and a third fraction, containing more polar compounds, was eluted.

Quantification by GC was performed with a Varian 3800 with flame ionization detector and a split/splitless injector, helium carrier. Separation was done with a capillary HP-Inno-Wax column (25 m length \times 0.2 mm i.d., 0.2 μ m thick). Oven temperature was 60–180°C at 30°C per min and 180–230°C at 1.5°C per min, held at 230°C for 10 min. Injector and detector temperatures were 250°C and 300°C. Data were processed with BORWIN software (Varian); quantification used (10*Z*)methyl-10-pentadecenoate as internal standard. Sample sizes for nurse and forager groups (10 bees per group) were: whole-body extracts ($n = 4$), tissues ($n = 5$), and cuticular extracts ($n = 1$).

Products were identified by GC-MS and quantified by using GC. Analyses were carried out with a Trace 2000 gas chromatograph with a PTV injector and an AS2000 autosampler, coupled to a Polaris ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) operating in electron impact mode at 70 eV. Separation was done with a capillary CP-Sil-8CBMS (Varian) column (30 m length \times 0.25 mm i.d., 0.25- μ m film thickness). Oven temperature was 50°C for 1 min, increasing to 150°C at 40°C min^{-1} , 240°C at 3°C min^{-1} , then 280°C at 30°C min^{-1} . Injector temperature was programmed from 50°C to 260°C at 12°C s^{-1} . Helium was used as carrier gas at 1 $\text{ml}\cdot\text{min}^{-1}$. Transfer line was at 280°C, and MS source was at 200°C. EO [(9*Z*)ethyl 9-octadecenoate] was identified by mass spectrum and retention time.

Pheromone Treatment. EO was obtained from Sigma-Aldrich. Bees were exposed to EO by ingesting EO-containing sugar candy (30% honey, 70% powdered sugar). We thought that candy was necessary because it is not known whether EO is intrinsically attractive to bees. This method allowed for chronic treatment with minimal disturbance but raises the question of whether EO is perceived by bees by taste, smell, or both. One gram of EO-containing candy was replaced daily; there was no trace of candy observed in any colony after 24 h, suggesting that the full amount was eaten daily. Two doses of EO were used: 0.21

and 2.1 mg of EO per g of sugar candy. Doses were based on the effects of BP in another study that used the same form of treatment (12). EO constitutes 21% of the 10-component BP (17), and the doses of BP used in ref. 12 were 1 and 10 mg. With colony sizes set precisely at 1,500 (see *Experimental Bee Colonies*), the average doses of EO per bee per day were 0.140 and 1.4 μ g (low and high, respectively), assuming equal consumption. However, we do not know whether there was interindividual variation in food consumption (and thus dosage).

Experimental Bee Colonies. The effect of EO was tested in the field with triple-cohort colonies. Triple-cohort colonies are composed of three cohorts of bees ($n = 500$ per cohort) of different ages to roughly simulate the normal range of worker ages within a colony, while controlling for variation in demography between colonies (5, 18). Results from triple-cohort colonies have been consistent with those obtained from more typical colonies (18). Each trial was performed with a trio of colonies. Colonies in each trio all were made from the same source colony to control for genotypic variation and in addition were made as similar as possible to each other in every respect (size, demography, honeycomb number and contents, and location in the field). One colony was treated with sugar candy containing the low dose of EO, one received the high dose, and one was given sugar candy alone (control).

Triple-cohort colonies were made with 1-day-old adult bees (focal cohort), nurses, and foragers. One-day-old bees were marked with a paint dot on the thorax, with each cohort a separate color. Nurses and foragers were identified according to standard criteria (4). Nurses were collected with their heads in cells of honeycomb containing larvae, and foragers were collected when returning to the entrance of their colony with either pollen loads in their corbiculae or distended abdomens (nectar or water foragers). All colonies had the same number of bees and were placed in small beehives that contained two honeycomb frames (one with honey, one empty).

Exposure to QMP and BP was controlled in these experiments because both can affect age at onset of foraging (11, 12). Instead of a live queen each colony was given a commercially available plastic strip (Bee Boost, PheroTech, Vancouver, Canada) containing the five-component QMP blend (2) that releases one queen equivalent per day. Because no brood was produced, colonies had no exposure to BP. The pheromone strips were replaced every 2 weeks.

Behavioral Observations. The effect of EO treatment on rate of behavioral maturation was quantified by determining the mean age at which the first 50 bees from each treatment group initiated foraging. In most trials, observations began when focal bees were 5 days old (trials 13 and 14: 10 days old), at least several days before any of them were observed foraging (casual observations). The hive entrance was observed daily during the most active time of day (9 a.m. to 4 p.m.) in four 15-min observation periods per colony, two in the morning and two in the afternoon. A metal screen was placed in front of the hive entrance for 5 min before the start of observations to prevent bees from entering; they accumulated on the screen, and marked focal bees were easily observed (19). Those showing signs of pollen or nectar foraging (described above) were given an additional paint marking (so they would not be counted again) and their ages were recorded. The screen was removed after each observation period. Each trial took \approx 2–3 weeks to complete.

To determine whether there was substantial differential mortality among the focal cohorts within a trial, censuses of each colony were performed 5 days after colony establishment and at the end of each trial. Censuses were performed by removing each frame of honeycomb and counting the number of marked

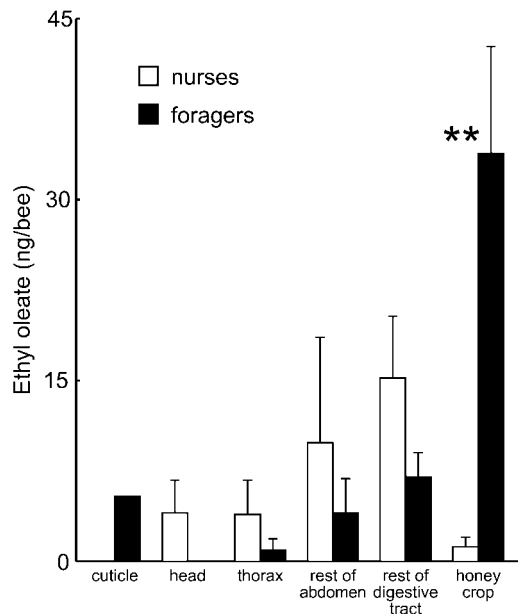


Fig. 1. Behaviorally related differences in quantity and localization of EO in honey bees. Shown are results of GC-MS analysis of tissue-specific analyses ($n = 5$ samples of nurses and foragers, each composed of 10 bees; $n = 1$ nurse and 1 forager sample for cuticle). The crop (specialized for storage of nectar and honey) was the only tissue that showed a significant difference between nurses and foragers (**, $P < 0.05$, Mann-Whitney U test).

age at onset of foraging between bees exposed to low and high doses of EO ($P = 0.08$).

Experiments with Various Biological Extracts and Synthetic Chemicals.

In contrast to the findings for EO, results for these extract/blend/compounds were sometimes suggestive but overall inconsistent (Table 1). In several cases there were trials that showed significant effects and other trials that did not, leading to experimentwide results that showed no significant effect. These

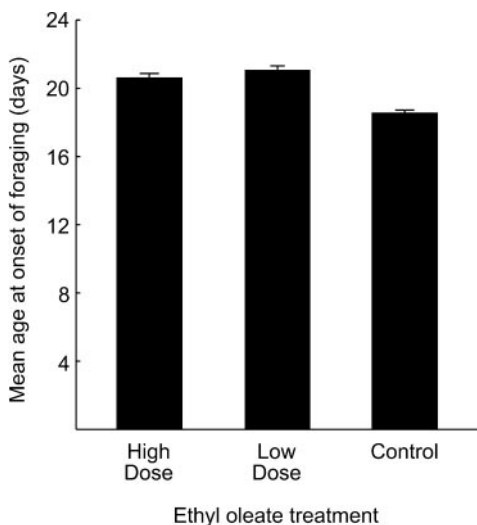


Fig. 2. Effect of EO on age at onset of foraging in honey bee colonies. In each trial ($n = 14$ trials) we calculated the mean age at which the first 50 bees from each focal cohort initiated foraging. Grand means depicted here \pm SE. EO caused a significant delay in the mean age at onset of foraging ($P < 0.0001$, ANOVA). Significant trial and trial by treatment effects were also detected ($P < 0.0001$).

trial-by-trial inconsistencies are not caused by differences in the types of colony used (single cohort or triple cohort) because one colony type was used for each experiment. It is an open question whether one colony type might be better suited for discovering pheromones of this type.

The only exception was the pentane cuticle extract, which overall caused a slight, but significant, delay in the mean age at onset. This finding is consistent with what we reported above (presence of EO on cuticle) and a recent report (13) on a hexane cuticle extract.

Biosynthesis of EO. Only trace amounts of EO were detected in whole-body analyses of 7-day-old bees fed either sugar candy and pollen or candy alone (data not shown). Fourteen-day-old bees had comparable levels of EO fed either sugar candy and pollen (18.9 ± 2.2 ng per bee) or candy alone (51.2 ± 19.6 , $n = 3$). This finding indicates that EO is not derived from dietary pollen.

Labeled EO was found in the honey crop of treated foragers. Fig. 3A shows the MS profile for unlabeled EO from control forager bees, including the characteristic ions 310 and 264. In contrast, bees fed ^{13}C glucose clearly showed ion 311 (Fig. 3B). Labeled EO was not detected in other tissues, in either treated or control bees, except in the labial gland.

EO extracted from the honey crop of ^{13}C -glucose-fed foragers showed incorporation of 1, 2, 3, 4, and 5 ^{13}C atoms and a small amount of 6 ^{13}C atoms from the labeled glucose (Fig. 3A–C). $1\text{-}^{13}\text{C}$ glucose generates one $2\text{-}^{13}\text{C}$ acetate (as acetyl CoA) by glycolysis (23). The acetyl CoA can be incorporated into fatty acids, being synthesized *de novo*. Given that the bees have an endogenous acetate pool, the incorporation within 48 h of mostly 3- and 4- ^{13}C , and up to 6- ^{13}C , suggests that these foragers very actively synthesized the 18-carbon fatty acyl portion of the EO.

Perfusion of isolated forager honey crops with D_6 ethanol gave D_5 EO (Fig. 3D). A control experiment, where synthetic EO was incubated in buffer containing 8% D_6 ethanol, showed no incorporation of deuterium label (data not shown). This finding indicates that no detectable transesterification of existing EO occurred under the experimental conditions; labeled EO must have been formed by an enzyme-catalyzed reaction in the honey crops.

Discussion

This study has identified a primer pheromone, still one of only a few to be chemically characterized from an animal society. Our results provide important validation for a model that explains how social interactions can regulate a key aspect of colony division of labor, the age at onset of foraging (4, 9). Our findings help us to understand how the regulation of the size of the colony foraging force can be controlled by a self-organizing mechanism of social integration. This mechanism enables bees to respond to the needs of the colony even though each individual likely possesses only fragmentary information about these needs.

Our results suggest that older bees inhibit the behavioral maturation of young bees at least in part via EO. Reduced exposure to EO caused by a lack of foragers would lead to accelerated behavioral maturation by some younger bees, providing for an adaptive colony response. It is possible that EO acts with other, still unidentified, compounds produced by the workers; multicomponent pheromones are common in insects, especially social insects (3). The other two known honey bee primer pheromones, QMP and BP, are multicomponent pheromone blends. EO itself is part of BP (17), but the other components of BP were either not found on foragers or found not to differ between nurses and foragers (20).

Given that EO also is found on the brood, what is the evidence to support the conclusion that it is both a brood and adult worker pheromone? The evidence is circumstantial, but strong. First, regulation of age at onset of foraging by worker-worker inter-

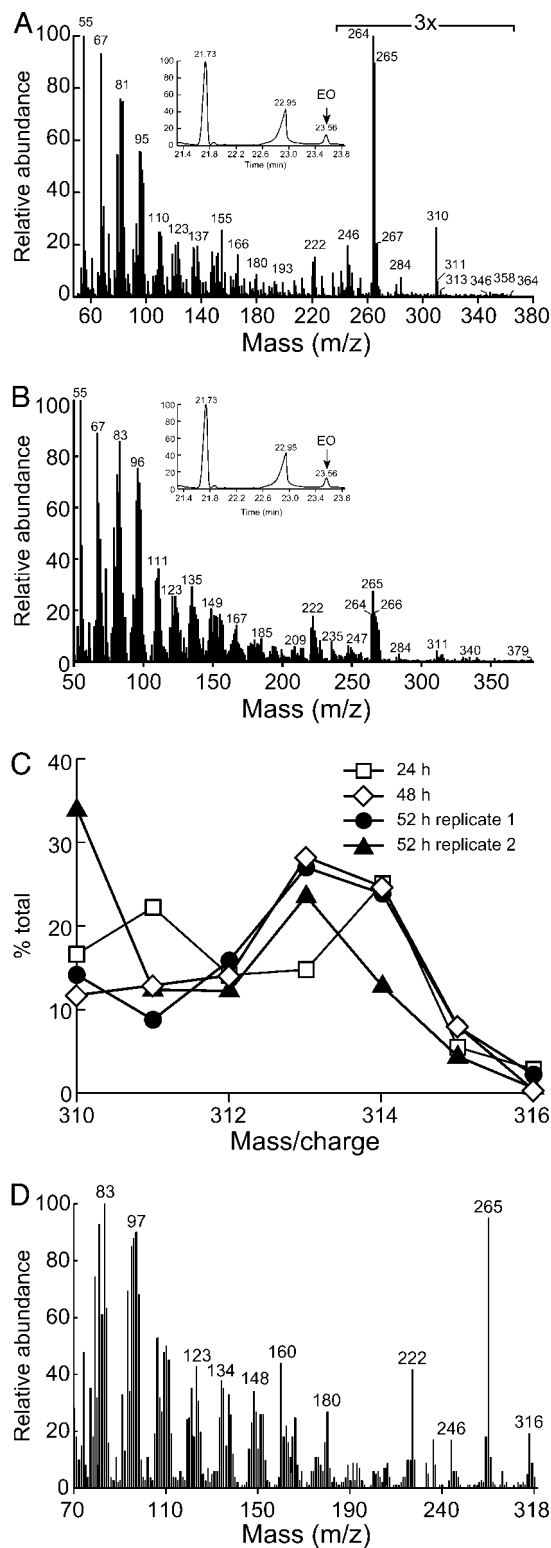


Fig. 3. *De novo* biosynthesis of EO by honey bees. (A and B) Mass spectrum of EO extracted from the honey crop of foragers fed unlabeled glucose (A) or 1-¹³C glucose (B). Arrows denote EO. (A and B Insets) GC results that show similar quantities of EO in both samples. (C) Incorporation of ¹³C label from 1-¹³C glucose into EO over time, as measured by the isotope composition of the molecular ion (*m/z* 310 for unlabeled EO, 311 for a single ¹³C, 312 for two ¹³C, etc.). Percentages of incorporation were estimated by correcting for natural isotope abundance (see *Materials and Methods*). Foragers were fed for 24, 48, or 52 h (two replicates). (D) Mass spectrum of EO extracted from isolated honey crops perfused with 8% D₆ ethanol.

actions is well established, and the effects are seen independent of the presence or absence of brood (4–6, 8). Second, worker-produced EO provides a chemical signal that effectively tracks the changes in the age structure of the adult worker force itself; EO is present at much higher levels in foragers than nurses. Although QMP and BP also delay the age at onset of foraging, it is likely that these factors provide a tonic, “baseline” inhibition; the most plausible candidate for dynamic modulation based on changes in colony age structure is a worker-produced substance. Third, as discussed in the next paragraph, EO in adult workers is localized just where it would need to be to act as a pheromone, on the cuticle and in the crop. A cuticular extract from foragers that delays the onset of foraging (13) contains EO, but an extract from nurse bees that accelerates the onset of foraging does not (T. Pankiw, personal communication). Definitive confirmation awaits experiments that track the flow of labeled worker-produced EO, especially from foragers (directly or indirectly) to younger individuals. These experiments would also shed light on key aspects of this communication system, namely the frequencies of transfer, the quantities involved, and the speed with which changes in colony age structure are reflected in changes in EO flow through the colony.

Our findings suggest that EO is transmitted via trophallaxis, a form of food exchange widely thought to be a prominent communication channel in insect societies (14). EO was found in highest concentrations in the crop, a specialized foregut for temporary storage of nectar and honey, not digestion. Food in the crop is circulated throughout the colony by trophallaxis (24). This is also true for honey bee QMP (25) and substances produced by the postpharyngeal gland in the head of *Cataglyphis niger* ants that mediate nestmate recognition (26). Both the bee and ant substances are also found on the cuticle, as was EO, suggesting a communication system that involves trophallaxis, grooming, and licking. Reminiscent of the situation in *C. niger*, preliminary results also indicate substantial amounts of EO in the honey bee labial glands (Y.L.C. and E.P., unpublished results).

We detected no evidence of dose dependence for EO in this study. In many trials either the low dose was more effective than the high dose or vice versa. One interpretation of these results is that there is genotypic variation in sensitivity to EO; genotypic variation in sensitivity to social inhibition has been shown in behavioral studies (27). There also may have been interindividual variation in dose caused by differences in food consumption. In addition, a low dose of BP accelerates age at onset of foraging, whereas a high dose delays it (12, 28). It is not known whether the BP results are directly attributable to EO, but they show that the regulation of age at onset of foraging is a complex and multifactorial process that involves social inhibition and likely other forms of social modulation as well.

EO is clearly synthesized *de novo* by honey bees but the process is not yet completely elucidated. Our results suggest that coupling of an acyl unit to the ethyl portion of the ester likely occurs in honey crop tissue. However, it is not clear yet whether it is a fatty acyl CoA or some other activated acyl equivalent. The incorporation of the CD₃CD₂O- unit from ethanol is expected to give D₅ EO, which was observed. The reaction occurred in isolated perfused honey crops, which implies that the honey crop should contain an activated acyl unit (such as a CoA ester) and an enzyme that can catalyze the coupling of ethanol with the activated acyl unit. Taken together, the current observations with ¹³C glucose and labeled ethanol provide evidence that the foragers biosynthesize EO *de novo*, with at least the late part of the biosynthesis occurring in the honey crop. Pheromone production in the digestive tract of other insect species has been reported (29). Additional studies are required to elucidate the precise location within the bee where biosynthesis occurs, the

nature of the activated acyl unit, and the biosynthetic enzymes involved.

There was an age-related increase in EO levels. Foragers had more EO than nurses and 14-day-old bees had more than 7-day-old bees. These results suggest that EO production is related to honey bee behavioral maturation. Because behavioral maturation is socially regulated, it is likely that the activities of at least some EO synthesis enzymes are under social control. Social regulation of pheromone biosynthetic enzymes has been reported for QMP (30).

Removal of mandibular glands renders older bees incapable of inhibiting the maturation of younger individuals (6), but we showed that worker mandibular gland compounds failed to cause consistent inhibition. Because the mandibular glands open into the buccal cavity (31), perhaps gland removal interferes with trophallaxis. This idea can be tested after development of a procedure to study the transfer of labeled worker-produced EO from bee to bee.

How might EO function to affect age at onset of foraging? The transition from working in the hive to foraging in honey bees is associated with neuroendocrine and structural changes in the brain and extensive changes in brain gene expression (3, 32, 33). We suggest that EO influences behavioral maturation by affecting one or more of the pathways involved in mediating the transition from hive work to foraging.

Speculation on the functioning of EO is based on studies on the effects of QMP on behavior, endocrine function, and gene expression. An increase in circulating levels of juvenile hormone is associated with the onset of foraging in honey bees (3); bees that lack social contact have precociously high rates of hormone biosynthesis and are more likely to become precocious foragers (4, 6). Bees from colonies treated with supplemental doses of QMP have depressed titers of juvenile hormone and show a delay in the age at onset of foraging (6). QMP also influences brain structure (34) and expression of thousands of genes in the brain (35); it activates genes associated with nursing and re-

presses genes associated with foraging. Identification of genes regulated by pheromones from the queen, brood, and workers will provide leads to understanding the neural basis for pheromone action.

The transition from working in the hive to foraging in honey bees also is associated with changes in responsiveness to various foraging-related stimuli, including sucrose (36) and light (37). For example, sucrose responsiveness increases with age and is highest in foragers. The finding that a hexane extract from foragers delays the onset of foraging and also depresses responsiveness to sucrose (13) provides further evidence that pheromones influence division of labor via effects on physiological processes that underlie behavioral maturation.

Pheromone inhibition is a prominent characteristic of mammalian and insect societies (1, 14). In both cases there is inhibition of sexual maturation, whereas in insect societies there also are pheromone-mediated inhibitory processes that regulate the ratios of various types of physically or behaviorally specialized (nonreproductive) workers, as in this study. With three sources of pheromones identified that regulate rate of behavioral maturation in honey bee colonies, this system should prove to be a useful model for understanding the mechanistic and evolutionary bases of pheromone inhibition in animal societies.

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