Hemolymph Juvenile Hormone Titers in Worker Honey Bees under Normal and Preswarming Conditions

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ABSTRACT Swarming is an important mechanism by which honey bee, *Apis mellifera* L., colonies reproduce, yet very little is known about the physiological changes in workers that are preparing to swarm. In this study, we determined the endocrine status of worker honey bees in preswarming colonies and in normal (nonswarming) colonies. Juvenile hormone (JH) titers in worker bees were similar in both groups before queen cells were present, but they became significantly lower in preswarming colonies compared with normal colonies when queen cells occurred in preswarming colonies. The lower JH titers in the preswarming colonies suggest that behavioral development is delayed in these colonies, consistent with previous reports that preswarming colonies have reduced foraging activities. Understanding the endocrine status of bees preparing for swarming will help us to better understand the biology of swarming.

KEY WORDS *Apis mellifera*, juvenile hormone, swarming, behavioral development

SWARMING IS A PROCESS of colony fission whereby about one-half of the colony workers, with the old queen, leave their old nest site in search of a new one, thus accomplishing reproduction at the colony level. The process of swarming is not only a biologically interesting phenomenon but also economically important because beekeepers lose about one-half of their bees to each successful swarming. Different hypotheses have been proposed to explain what triggers swarming. These include a surplus of young bees resulting in too much brood food, crowding of adult workers and limited space for brood, and reduced transmission of queen mandibular pheromone among workers (Winston 1987, Naumann et al. 1993). These factors, as well as colony size and worker age distribution, all play roles in stimulating swarming preparation; however, none of them alone consistently induces swarming (Winston 1987).

We believe that behavioral development in colonies preparing for swarming might be delayed (see below). Workers typically change their jobs as they age in a colony with a stable age demography, progressing from cell cleaning and brood rearing during the first 2 wk, to nectar processing and comb building in the third week, and finally to foraging when they are ≈25–30 d old (Seeley 1982). This progression of tasks, a form of behavioral development, is correlated with levels of juvenile hormone (JH). JH titers in blood or rates of biosynthesis typically increase with age; they are low in bees that perform in-hive tasks such as nursing, comb building, and other activities, and high in foragers (Rutz et al. 1976; Fluri et al. 1982; Robinson et al. 1987, 1989; Huang et al. 1991, 1994; Huang and Robinson 1995). Applying JH, JH analog, or JH mimic to bees causes workers to forage earlier (Jaycox et al. 1974; Jaycox 1976; Robinson et al. 1987, 1989; Robinson and Ratnieks 1987; Sasagawa et al. 1989). These results suggest that JH not only is correlated with behavioral development in honey bees but also plays a key role in modulating the pace of behavioral transition in workers.

One recent study showed that workers with their corpora allata (the sole source of JH) removed still commenced foraging (Sullivan et al. 2000), suggesting that JH is not needed at all for normal foraging. This has lead to the conclusion that JH might not be the regulator for behavioral development (Page and Peng 2001; but see Sullivan et al. 2001). However, a very recent study showed that a suite of genes that characterize foragers can be turned on directly by applying a JH analog (Whitfield et al. 2003). Together, these results suggest that JH plays a major role in the nurse to forager transition, although another redundant pathway(s) might exist. We suggest that JH still remains as the best indicator for behavioral development for workers in a colony: one can randomly sample a bee from the hive, measure its JH titers, and infer what stage it is at in the developmental trajectory.

Even when worker bees are under manipulated colony conditions resulting in precocious or reverted behavioral development, JH titers of worker bees still accurately reflect their behavioral status. For example, worker bees foraging precocious show an early rise in

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JH titers (Jassim et al. 2000), whereas foragers "reverting" into nurses show a drop in JH titers (Huang and Robinson 1996). The modulation of behavioral development in honey bee workers can be explained via a "social inhibition" model whereby a lack of sufficient inhibition from enough foragers causes precocious development and an overabundance of the inhibition causes delayed or reversed development (Huang and Robinson 1992, 1996; Leoncini et al. 2004). Recent studies showed that both brood pheromone and the queen mandibular pheromone can delay behavioral development by suppressing JH production (Pankiw et al. 1998, Le Conte et al. 2001), the later accomplishes this by regulating gene expression in the brain (Grozinger et al. 2003). These studies again show that JH is a reliable indicator of behavioral development.

There has been abundant evidence showing that foraging activity is much reduced in preswarming colonies (Demuth 1921, Ribbands 1953, Butler 1954, Caron 1970). Reduced foraging should cause delayed increase of JH titers resulting in delayed behavioral development, due to increased exposure of younger bees to foragers, which results in a higher efficiency of inhibitor transfer (Huang and Robinson 1996, Leoncini et al. 2004). We therefore hypothesize that workers in preswarming colonies should have lower hemolymph JH titers compared with those in normal colonies.

In this study, we determined the JH titers in honey bee workers that were reared under normal and preswarming colony conditions.

Materials and Methods

Honey Bee Colonies. Experiments were conducted during summer 2003 at the Michigan State University Bee Biology Building, East Lansing (42.44° N, 84.29° W), MI. Bees were from colonies maintained according to standard techniques. We selected paired colonies that were similar in strength and in proximity and then randomly assigned one colony to be in the preswarming group and one to the control group. Colonies in the preswarming group were manipulated to increase the likelihood of swarming initiation, by removing supers and compacting the colony down into 10 frames, whereas those in the control group were given extra supers to provide bees with room for brood rearing and food storage. Eleven days after the manipulation, newly emerged bees (<24 h after emergence and designated as 1-d-old bees) from three unrelated source colonies were used to provide experimental bees (focal bees) for both preswarming and control colonies. One unrelated source colony was used for each pair of control and preswarming colonies. Using this third source colony, rather than using one of the experimental colonies as the source of focal bees, created symmetry for both treatments because bees were foreign to both the preswarming and control colonies. This way, any (possible) treatment effect is not confound with whether focal bees were nestmates or non-nestmates to the host colonies. Bees from the third source colony were obtained by incubating brood at 35°C and 60% RH. They were then divided into two groups (≈658 per group), marked with two different colors and introduced into the preswarming and control colonies on the same day. On the day of emergence and every 4 d thereafter, 8–10 color-marked focal bees were sampled to collect their hemolymph for JH determinations. The experiment was repeated three times, i.e., three pairs of colonies were used.

Hemolymph Collection. Ten focal bees per colony were sampled for bees aged 1 and 4 d, and eight focal bees for at other ages (8, 12, 16, and 20 d). Sampled bees were immediately placed on ice for anesthetization, and their hemolymph was collected as quickly as possible (within 15–30 min from being removed from the colony) to ensure that the JH titers did not increase due to experimental stress (Lin et al. 2004). Hemolymph was obtained by placing individual bees under a microscope, pricking a hole in the intersegmental membrane between the second and third abdominal segment, and collecting the hemolymph with a capillary tube. Hemolymph volume (1.5–9.0 μl per bee) was measured to the nearest 0.1 μl and immediately mixed with 500 μl of acetonitrile to denature enzymes that could affect JH. Samples were immediately placed on ice and then stored at −20°C for later JH analysis. In total, 282 hemolymph samples were collected.

Determination of Juvenile Hormone Titers. JH III, the only form of JH found in honey bees (Hagenguth and Rembold 1978), was measured in individual bees using a chiral-specific radioimmunoassay (Hunnicutt et al. 1989). This assay was specifically validated for adult worker honey bees (Huang et al. 1994) and yielded comparative JH titers to two other radioimmunoassays that were verified with gas chromatography-mass spectrometry (de Kort et al. 1985, Goodman et al. 1990). We followed the standard radioimmunoassay procedure described previously (Huang et al. 1994; Huang and Robinson 1995, 1996), with one modification. Sample JH was redissolved in 100 μl of methanol, and an aliquot (usually 10 μl methanol) was removed and added into duplicate assay tubes and the methanol dried under vacuum. Incubation was started by adding 200 μl of mix of JH antiserum and radiolabeled JH to these dried tubes containing sample JH. In previous studies, 2.5 μl of methanol containing JH was added directly to 200 μl of mix of JH antiserum and radiolabeled JH. This modification is similar to that of Jassim et al. (2000) and increased the sensitivity of the assay slightly due to the elimination of methanol, which interferes slightly with JH-antibody binding. We analyzed equal numbers of hemolymph samples from the preswarming and control colony on the same day. Intra- and interassay variations were 10.3 and 9.2%, respectively (Lin et al. 2004).

Statistical Analyses. The dependent variables, JH titers, were transformed (logarithmic) to meet the requirements of parametric analysis. All analyses were performed using the general linear models (PROC GLM, SAS Institute 2000). Differences in JH titers

for bees in the preswarming and control colonies (referred to as "treatment" effect) at different time intervals were first analyzed as a three-way analysis of variance (ANOVA) (PROC GLM; age × treatment × trial). Each trial was then analyzed as a two-way ANOVA (age × treatment). Data from the first day was not included in any analysis because bees on the first day did not belong to a particular treatment. Means (without transformation) and standard errors (SE) are used in the figures and throughout the text.

Results

The colony manipulations to induce swarming were effective; however, the three preswarming colonies did not behave exactly the same. The preswarming colony in trial 1 swarmed when the focal bees were 3 d old, whereas the preswarming colonies in trials 2 and 3 had queen cells when focal bees were 11–20 d old, and the two colonies were prevented from swarming by removing all queen cells when focal bees were 25–27 d old.

There was a significant trial by treatment interaction in JH titers ($F = 3.0$; df = 3, 222; $P = 0.03$); therefore, further analysis was done on each individual trial. When all age groups were included, the three trials showed no significant difference in JH titers between preswarming and control colonies (F tests, $P > 0.05$ in all trials), although significant differences did exist among JH titers in different aged bees (F tests, $P < 0.01$ in all trials); the interactions between treatment and age were not significant in any of the three trials ($P > 0.05$ in all trials). Because in two trials (trials 2 and 3) queen cells occurred in the preswarming colonies when the focal bees were 11–20 d old, we did further analysis using only the last two age groups (16- and 20-d-old bees). The difference between preswarming and control colony was not significant for trial 1 ($F = 3.98$; df = 1, 28; $P = 0.056$), but significant for both trial 2 ($F = 5.10$; df = 1, 28; $P = 0.03$) and trial 3 ($F = 10.7$; df = 1, 28; $P = 0.003$). In other words, JH titers are significantly lower in workers from preswarming colonies compared with the control colonies, in both trials 2 and 3 (Fig. 1) when workers were 16 d and older.

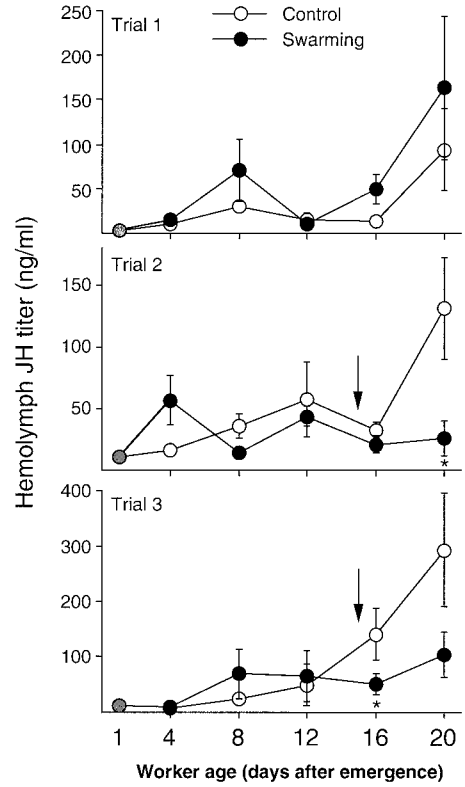

Fig. 1. Hemolymph juvenile hormone titers (mean ± SE) of worker honey bees reared in normal colonies (open circle) and preswarming colonies (closed circle), for trials 1–3. $n = 10$ bees per data point for bees aged 1 and 4 d, and $n = 8$ for others. Data of newly emerged bees are shown as gray circles. Arrows indicate the times sealed queen cells were observed in trials 1 and 3. In trial 1, the experimental colony swarmed on day 3. An asterisk (*) on the *x*-axis on a particular age indicates that the JH titers on that date are significantly different between the workers in the two colonies (contrasts, $P < 0.05$).

Discussion

Our results show that JH titers are lower in worker honey bees from preswarming colonies than those in normal colonies, when queen cells are present in the preswarming colonies. This suggests that behavioral development is delayed in workers from preswarming colonies compared with those in normal colonies, because JH titers are usually higher in foragers than bees performing in-hive duties. Although this conclusion is drawn from results in trials 2 and 3, results from trial 1 are also consistent. This is because in trial 1, the preswarming colony actually accomplished swarming when the focal bees were only 3 d old, perhaps because queen cells were present and we failed to detect them during colony manipulations. Presumably this reduced the worker population significantly and removed most, if not all, stimuli for swarming, thereby making the treated colony the same as the control colony. As a result we did not see a significant difference in JH titers between the two colonies. In trials 2 and 3, suppression of JH levels did not start early and only became significant when queen cells occurred in the manipulated colonies (when focal bees were 16 and 20 d old). There was a trend for increasing JH titers with age with the control colonies of all trials, whereas the preswarming colonies in trials 2 and 3 had consistently low JH titers (Fig. 1). There was considerable variation in the JH titers of 20-d-old bees in different colonies. This type of intercolony variation, approximately two- to three-fold, has been observed previously in foragers from different colonies (Huang and Robinson 1995, Jassim et al. 2000) and was postulated to be due to genetic variation.

Even though workers do not require high JH levels to perform foraging behavior (Huang and Robinson 1995, Sullivan et al. 2000), juvenile hormone levels in general correlate tightly with the status of behavioral development, even when bees are under extreme conditions leading to accelerated or reversed development (Robinson et al. 1987; Huang and Robinson 1992, 1996). Furthermore, two other primer pheromones (queen mandibular pheromone and brood pheromone) have been shown to delay behavioral development through affecting JH titers (Pankiw et al. 1998, Le Conte et al. 2001). Our results here, therefore, suggest that the age of first foraging is delayed in workers from preswarming colonies because of their lower hemolymph JH titers.

Although many studies had been conducted on preswarming colonies, it was not clear whether young bees delay or accelerate their behavioral development. The social inhibition model (Huang and Robinson 1992, 1996; Leoncini et al. 2004) predicts that bees in preswarming colonies should delay their behavioral development, because there is overwhelming evidence for reduced foraging levels in preswarming colonies (see citations in Introduction). This makes good ecological sense because physiologically young bees in a new swarm would be more beneficial than physiologically old bees—at the new nest site, most workers have to be preoccupied with secreting wax to build new combs and feeding young larvae, because newly emerged workers will not occur until 21 d after eggs are laid by the queen at the new location. Indeed, swarms are composed of more young bees than old bees (Gilley 1998). Another piece of evidence supporting delayed behavioral development in preswarming colonies is that the brood food (hypopharyngeal) glands of workers are more developed in workers in preswarming colonies (Butler 1954) than normal colonies. The larger glands indicate that these workers are physiologically younger (Huang and Otis 1989, Huang and Robinson 1996).

In summary, JH titers showed a consistent pattern in responding to colony conditions leading to swarming. Specifically, JH titers were lower in workers when sealed queen cells were present in preswarming colonies. Further behavioral observations are needed to confirm the suggestion that young workers in preswarming colonies delay their age of foraging. To minimize colony swarming, we must first understand the mechanism of this complicated biological process.

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