Hormones and Behavior **37,** 1–14 (2000)
doi:10.1006/hbeh.1999.1552, available online at http://www.idealibrary.com on IDEAL®

Juvenile Hormone Paces Behavioral Development in the Adult Worker Honey Bee

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Received May 27, 1999; revised July 21, 1999; accepted August 12, 1999

Behavioral development in the adult worker honey bee (*Apis mellifera*), from performing tasks inside the hive to foraging, is associated with an increase in the blood titer of juvenile hormone III (JH), and hormone treatment results in precocious foraging. To study behavioral development in the absence of JH we removed its glandular source, the corpora allata, in 1-day-old adult bees. The age at onset of foraging for allatectomized bees in typical colonies was significantly older compared with that of sham-operated bees in 3 out of 4 colonies; this delay was eliminated by hormone replacement in 3 out of 3 colonies. To determine the effects of corpora allata removal on sensitivity to changes in conditions that influence the rate of behavioral development, we used "single-cohort" colonies (composed of only young bees) in which some colony members initiate foraging precociously. The age at onset of foraging for allatectomized bees was significantly older compared with that of sham-operated bees in 2 out of 3 colonies, and this delay was eliminated by hormone replacement. Allatectomized bees initiated foraging at significantly younger ages in single-cohort colonies than in typical colonies. These results demonstrate that JH influences the pace of behavioral development in honey bees, but is not essential for either foraging or altering behavioral development in response to changes in conditions. © 2000 Academic Press

Key Words: **allatectomy; behavioral development; corpora allata; foraging behavior; division of labor.**

Juvenile hormones regulate metamorphosis, reproduction, and behavioral development in insects (reviewed by Riddiford, 1994; Wyatt and Davey, 1996). The seven known juvenile hormones are sesquiterpenoid molecules synthesized by the corpora allata (CA) glands (reviewed in Gäde, Hoffman, and Spring, 1997). Juvenile hormone III (JH) is the only juvenile hormone present in *Apis mellifera,* the European honey bee (Hagenguth and Rembold, 1978; Huang, Robinson, Tobe, Yagi, Strambi, and Stay, 1991). In the honey bee, the JH titer in the blood is correlated with the rate of JH synthesis by the CA *in vitro,* which suggests that the amount of circulating JH is determined primarily by the rate of synthesis (Rachinsky and Hartfelder, 1990; Huang *et al.*, 1991). The brain regulates CA activity via neural and neuroendocrine signals (Fraser and Pipa, 1977; Stay, Tobe, and Bendena, 1994). The brain-CA system in insects, like the hypothalamic-pituitary axis in vertebrates, translates environmental stimuli and internal conditions into endocrine signals (Wyatt and Davey, 1996; Denver, 1997). Juvenile hormone in insects, like many hormones in vertebrates, affects key life history parameters such as growth rate and the timing of physiological and behavioral maturation (Roff, 1986; Moore, 1991; Silver, 1993; Nijhout, 1994; Clark and Galef, 1995; Bjornsson, 1997).

JH is involved in the regulation of age-related division of labor in honey bee colonies. Honey bees are social insects that live in colonies composed of one egg-laying queen, up to several thousand males that are involved solely in reproduction, and 20,000–40,000 adult female worker bees that perform all tasks related to colony growth and maintenance (Winston, 1987; Seeley, 1995). Division of labor is based on a pattern of behavioral development by adult worker bees in which they perform different tasks as they age. Bees work in the hive at tasks such as caring for brood (nursing) for the first 2 to 3 weeks of adult life and

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then forage for nectar and pollen outside the hive for 1 to 2 weeks until they die. The JH titer increases dramatically during honey bee behavioral development; it is low in nurse bees and high in foragers during the late spring and summer, the bees' active season (reviewed by Fahrbach, 1997; Robinson and Vargo, 1997). Foraging occurs sporadically during short periods of warmth in the winter and early spring in temperate climates. A recent finding that winter foragers have low JH titers suggested that high JH titers are not required for foraging (Huang and Robinson, 1995). However, treatment of 1-day-old bees with JH or the JH analog methoprene results in accelerated behavioral development and precocious foraging (Robinson, 1985, 1987a; Robinson, Page, Strambi, and Strambi, 1989; Sasagawa, Sasaki, and Okada, 1989).

Age-related division of labor shows great plasticity; bees can accelerate or delay behavioral development and can even revert from foraging to nursing (Robinson, 1992). For example, in "single-cohort" colonies initially composed of a few thousand young bees, some bees begin foraging when they are younger than 7 days of age while others show normal behavioral development and act as nurse bees (Robinson *et al.*, 1989). Plasticity in age-related division of labor is due, in part, to the sensitivity of bees to changing social conditions, particularly factors associated with colony age structure. Older bees inhibit the rate of behavioral development of younger bees via a process that requires direct social contact (Huang and Robinson, 1992, 1996; Huang, Plettner, and Robinson, 1998). When bees show plasticity in behavioral development, their JH titers change accordingly: precocious foragers have high titers and overage nurses and reverted nurses have low titers (Robinson *et al.*, 1989; Robinson, Page, Strambi, and Strambi, 1992). Older bees also inhibit JH production in younger bees (Huang and Robinson, 1992; Huang *et al.*, 1998). These results are consistent with the idea that plasticity in honey bee behavioral development is mediated by environmental effects on the JH system (Robinson, 1987a).

To further explore the role of JH in the regulation of honey bee behavioral development, we manipulated exposure to JH in adult worker bees and introduced them into typical colonies. We tested the hypothesis that JH affects behavioral development by surgically removing the CA (allatectomy) from young bees and treating some allatectomized (CA−) individuals with the JH analog methoprene. If a high JH titer is necessary for foraging, methoprene-treated CA− bees will forage and CA− bees will not. Alternatively, if the JH titer affects the pace of behavioral development, CA− bees will forage later in life than methoprene-treated CA− bees. To test whether behavioral development is sensitive to changes in colony age structure in the absence of JH, we determined the age at onset of foraging for CA− bees in single-cohort colonies.

METHODS

Bees

Honey bee colonies were maintained at the University of Illinois Bee Research Facility in Urbana, Illinois, according to standard beekeeping practices. To control for effects of genetic variation on the pace of behavioral development (Giray and Robinson, 1994; Guzmán-Novoa, Page, and Gary, 1994), bees in all treatment groups (focal bees) in each colony were daughters of a queen instrumentally inseminated (Laidlaw, 1977) with the semen of a single male. Focal bees within a colony thus had an average genetic relatedness of 0.75 due to haplodiploidy. A different, unrelated, queen was used as the source of focal bees for each colony within an experiment. Colonies headed by these queens are referred to as source colonies.

Corpora Allata Removal

Frames of pupae were removed from a source colony and kept in an incubator (33°C, 80% RH). Adult bees were collected hourly from these frames so they could be allatectomized within 2 h after emerging from the cell. Bees were given access to honey and water *ad libitum* both before and after surgery. Bees were anesthetized on ice for 3 min before allatectomy. Previous studies have shown that cold anesthesia has no effect on the ontogeny of orientation and foraging flights or foraging activity (Ebadi, Gary, and Lorenzen, 1980; Robinson and Visscher, 1984). Allatectomy was performed on the stage of an Olympus SZH10 stereo microscope (70×). Bees were positioned and secured in molded plasticine (Fig. 1). A horizontal incision was made with a microscalpel in the back of the head and each corpus allatum was grasped and removed with forceps. The incision sealed as the cuticle resumed its original shape. Sham-allatectomized (sham) bees were treated identically but the CA were moved gently and not removed. Bees in the untreated group were anesthetized, but otherwise unmanipulated. Immediately after a bee was treated, it was

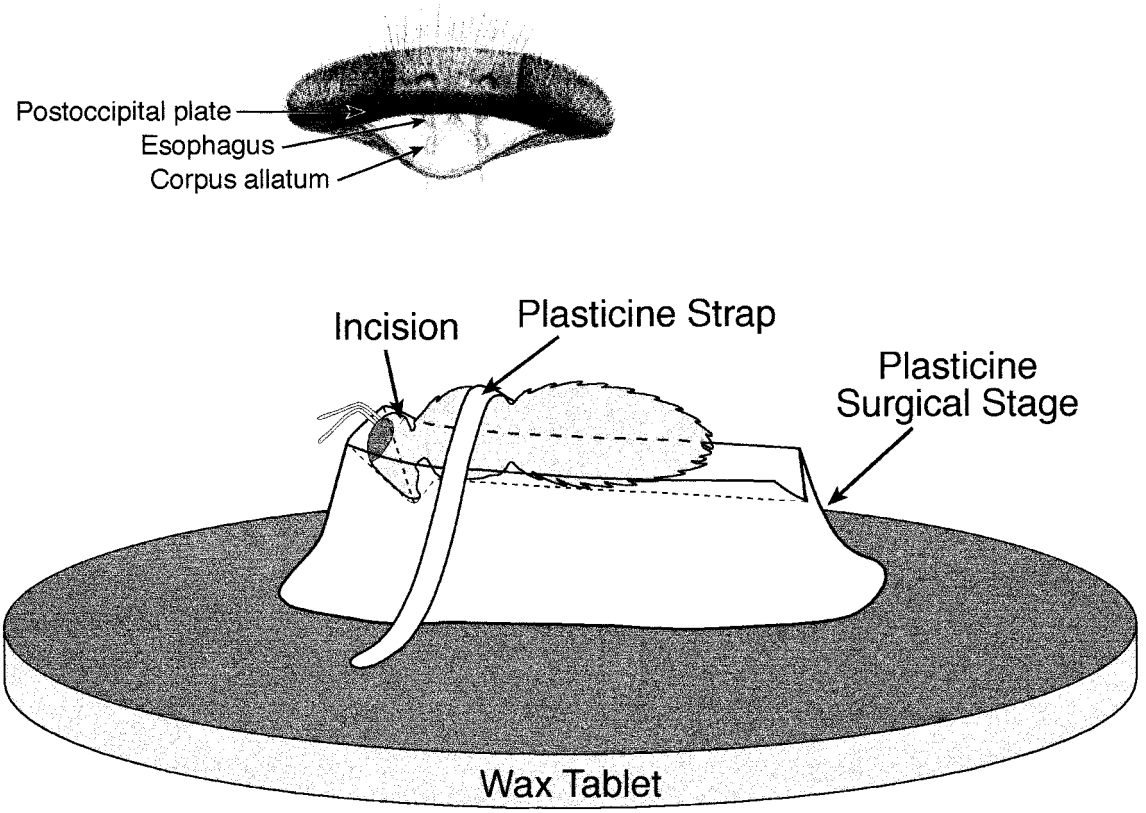


FIG. 1. A bee staged for allatectomy. Inset: A view through the incision has been drawn with the relevant glands enlarged. The corpora allata (CA) can be seen as hemispheres adjacent to the esophagus.

placed in a small holding cage with others of its treatment group and held in the incubator. To assess if the CA released JH during the allatectomy, we sampled blood (see below) from the incision before and after surgery in Experiment 1.

Bees were held in the incubator for an additional 12 h before being marked and introduced to a colony. During this period, 7% of the CA− bees ($n = 105$) and none of the sham ($n = 40$) or untreated bees ($n = 81$) died in Experiment 1. Fewer CA− bees died during this period in Experiments 2 and 3. Bees were chilled briefly before marking (to prevent their death by stinging the experimenter) and were marked on the dorsal thorax with either a dot of enamel paint (Testor's PLA) or a numbered plastic tag (Opalithplâttchen, Chr. Graze, KG, Endersbach, Germany). Marked bees were then introduced to a colony by attaching the holding cage to a hole at the top of the hive and allowing the bees to enter the hive on their own. Because it is well known that honey bee colonies reject bees that are in any way defective (Frisch, 1967), we were concerned about the fate of the CA− bees. We therefore observed the treatment of newly introduced focal bees in Experiment 1 by placing the colony in a glass-walled observation hive (see below). CA−, sham, and untreated bees were antennated and groomed by resident bees upon their entering the hive. Two days after introduction, there was no significant difference in the percentage of bees accepted: untreated, 87.7% ($n = 81$), sham, 85.0% ($n = 40$), and CA− bees, 82.7% ($n = 98$) [$\chi(2) = 0.07$, $P = 0.97$].

Juvenile Hormone Analog Treatment

In the typical colonies of Experiment 2 and single-cohort colonies of Experiment 3, 200 μg methoprene in 5 μl acetone was applied to the dorsal abdomen of a group of CA− bees (MCA−). This dose of methoprene consistently causes precocious behavioral development in honey bees (Robinson, 1985, 1987a; Robinson *et al.*, 1989; Withers, Fahrbach, and Robinson, 1995). Control bees were treated with acetone alone. No significant differences in exocrine gland physiology (Sasagawa *et al.*, 1989) or foraging behavior (Robinson,

TABLE 1
Effects of Allatectomy on Mean Blood Titers (±SEM) of Juvenile Hormone (ng/ml)

Colony	Untreated	Sham	CA−	MCA−
	Experiment 1: Observation Hive Colony			
1	265.0 ± 7.3 (27) a	333.9 ± 15.0 (15) a	31.5 ± 1.1 (23) b	—
	Experiment 2: Typical Colonies			
	Untreated	Sham	CA−	MCA−
1	175.6 ± 7.9 (10) a	342.6 ± 22.0 (12) b	14.0 ± 0.4 (22) c	8.6 ± 0.3 (21) c
2	200.0 ± 17.7 (9) a	262.9 ± 18.5 (9) a	12.9 ± 0.4 (42) b	28.3 ± 0.8 (34) c
	Experiment 3: Single-Cohort Colonies			
	Untreated	Sham	CA−	MCA−
1	478.9 ± 25.5 (10) a	436.7 ± 20.6 (10) a	15.5 ± 0.9 (41) b	64.4 ± 6.5 (21) b
2	438.2 ± 27.8 (9) a	320.9 ± 26.8 (9) a	17.3 ± 0.8 (29) b	13.1 ± 0.6 (39) b
3	584.5 ± 61.0 (4) a	467.0 ± 23.7 (6) a	15.2 ± 1.1 (39) b	3.3 ± 0.1 (36) b

Note. Means followed by the same letter within each colony are not significantly different (additional statistical analyses in text); sample sizes are in parentheses.

1985) have been found between untreated bees and bees treated with acetone. MCA− bees were incubated in a cage separate from the other treatment groups for 1 h to prevent the possible transfer of methoprene and then introduced to the colony with the other treatment groups.

Radioimmunoassay

Blood titers of JH were measured to confirm the efficacy of the allatectomies. We used a chiral-specific (Hunnicutt, Toong, and Borst, 1989) radioimmunoassay (RIA) specifically validated for the adult honey bee (Huang, Robinson, and Borst, 1994). Briefly, blood was collected in a calibrated capillary tube, mixed with acetonitrile, and stored at −20°C. Samples were extracted with hexane. Two aliquots of each sample were incubated for 2 h in tubes containing antiserum (1:28,000) and 10,000 dpm of [10-3H(N)] JH (NEN, 629 Gbq/mmol) mixture. Dextran-coated charcoal was added to absorb unbound JH and pelleted after 2.5 min. Radioactivity in the supernatant was quantified by liquid scintillation spectrometry (Packard Tri-Carb 2100TR). The standard curve and variance were calculated by the monotone function described in Straume, Johnson, and Veldhuis (1998). Inter- and intraassay coefficents of variance were 6 and 9%, respectively (n = 10).

Validation of Allatectomy

There was no significant difference between blood titer of JH (mean ng JH/ml blood ± SEM) before allatectomy (31.5 ± 1.1) and immediately afterward (31.2 ± 1.1, n = 23, $F(2, 44) = 0.52$; $P = 0.6$). JH titers were measured for 96% of the CA− and MCA− bees that were collected and bled; an insufficient volume of blood precluded RIA (JH measurement) for some bees. Analysis of variance revealed significant variation among groups in all sampled colonies: Experiment 1 [$F(3, 84) = 39.76$; $P < 0.0001$], Typical Colony 1 of Experiment 2 [$F(3, 61) = 27.22$; $P < 0.0001$], Typical Colony 2 of Experiment 2 [$F(3, 90) = 30.1$; $P < 0.0001$], Single-Cohort Colony 1 of Experiment 3 [$F(3, 71) = 33.41$; $P < 0.0001$], Single-Cohort Colony 2 of Experiment 3 [$F(3, 80) = 41.15$; $P < 0.0001$], and Single-Cohort Colony 3 of Experiment 3 [$F(3, 81) = 49.7$; $P < 0.0001$]. CA− and MCA− foragers had extremely low JH titers, that for the most part cannot be distinguished from zero (Table 1). Sham and untreated bees collected as foragers had high blood titers of JH, typical of foragers (e.g., Huang *et al.*, 1994). Microscopic inspection of (10 μm thick) brain sections (n = 26) for CA− bees collected 25 to 32 days after surgery in Experiment 1 revealed no partial CA or evidence for gland regeneration, or damage to adjacent tissues.

Experiment 1: Observation Hive Colony

CA−, sham, and untreated bees were introduced to a colony housed in an observation hive that contained a planar array of eight standard frames of honeycomb between glass walls. This was done to monitor the acceptance of CA− bees (above) and to permit limited observations of in-hive behavior to supplement the more detailed observations at the hive entrance (below). The colony was transferred to the observation hive in early April, and so it was well established, with a presumed typical age structure, by the time the focal bees were introduced in late May 1995.

Behavioral observations were made every other day for about 2 h between 1300 and 1700. Each frame was scanned from top to bottom by following a line of honeycomb cells back and forth across the comb. Behaviors were identified according to Robinson (1987a). We recorded the behavior a marked bee performed at the moment it was observed. Alternate sides of the hive were scanned until no additional marked individuals were identified. We observed a total of 3722 behavioral acts in >50 h of observation during 26 observation periods. Only the first behavior observed for a bee during each observation period (1911 behavioral acts) was used for analysis because of concerns about the nonindependence of subsequent observations.

We observed flight activity and behavior at the hive entrance to determine the age at onset of orientation behavior and foraging. Orientation flights are shorter flights, usually about 3 min long, taken by preforager bees beginning when they are 3–7 days old (Winston, 1987; Vollbehr, 1975). Orientation flights begin with a series of repeated figure-eight movements in front of the hive before the bee flies away; they function to allow the bee to learn the location of its hive and other prominent landmarks before it starts foraging (Frisch, 1967). Foraging flights are longer and do not begin with repeated movements in front of the hive. A ramp covered with glass was installed on the hive entrance to facilitate observation of focal bees. A thin film of petroleum jelly applied to the underside of the glass restricted bees to walking with their tags facing upward (Winston and Katz, 1982). Observations were made for ≥1 h twice daily between 900 and 1100 and 1500 and 1700. Flights <5 min long were classified as orientation flights. The following criteria were taken as strong evidence that a bee had initiated foraging: two sequential flights each >15 min long, two or more >15-min-long flights on 2 consecutive days, or the presence of pollen (easily seen in the "pollen baskets" located on the hind legs). A bee also was classified as a forager if it was observed performing a waggle dance (Frisch, 1967) during in-hive observations. Data from foraging flights in which we observed the bee leave and return were used for comparisons of flight duration among the treatment groups.

The 15-min criterion for classifying a flight as a foraging trip was conservative; many studies have classified flights ≥5 min as foraging trips (e.g., Ribbands, 1952; Winston and Katz, 1982; Robinson, 1987a). During preliminary experiments just before the onset of Experiment 1 we observed flights ≥5 min long, some taken by bees younger than 8 days of age, that appeared to be more like orientation flights than foraging flights, especially since they were often followed on subsequent days by shorter, more typical, orientation flights. The 15-min criterion may have overestimated the age at onset of foraging and underestimated the total number of bees that developed into foragers, but the criterion was applied equally to all treatment groups so our comparative analyses are valid. The same criteria for classifying orientation (<5 min) and foraging flights (>15 min) were used by Dukas and Visscher (1994).

To determine lifespan, the last day a focal bee was observed at the entrance or in the hive was taken as its last day of life. Lifespan was determined for only a subset of focal bees because many of them were collected after the onset of foraging for blood samples (above) and brain analyses (to be presented in a separate paper). Bees were collected with a modified vacuum device (Robinson and Page, 1988). Observations were stopped when <5% of the focal bees remained.

Experiment 2: Typical Colonies

The age at onset of foraging of focal bees was determined in three field colonies (adult populations of 20,000–50,000 bees and assumed typical age structures). The colonies were each housed in two standard Langstroth hive boxes. Focal bees from source colonies 45, 101, and 27 were introduced to typical colonies 1, 2, and 3, respectively, in July 1996 (sample sizes for untreated, sham, CA−, and MCA− groups: 281, 55, 60, 69, Typical Colony 1; 340, 55, 69, 60, Typical Colony 2; and 200, 58, 61, 60, Typical Colony 3). We installed a dead bee trap (Gary, 1984) on the hive entrance of each colony to monitor mortality because of the large number of CA− bees that apparently disappeared after initiating flight in the Observation Hive Colony of Experiment 1 (see Results). Upon termination of the experiment, the efficiency of the traps was estimated

at 96 and 84% (Typical Colony 1 and Typical Colony 2) based on the recovery of 50 freshly freeze-killed marked bees that were placed in each hive.

The age at onset of foraging was determined by blocking the hive entrance periodically throughout the morning and afternoon with a No. 8 hardware cloth for 5 min each time. Marked bees attempting to enter the hive with either loads of pollen on their hind legs or a distended abdomen were identified as foragers and collected. The entrance was then opened to allow unmarked foragers to reenter. Blood samples were taken from foragers in Typical Colonies 1 and 2.

Observations were stopped when <5% of the focal bees remained. This was determined by conducting two censuses (midway and terminal) at dusk, a time with sufficient light and minimal foraging activity. Each frame was lifted out of the hive, inspected by two observers for marked bees, and then placed in a second hive box. Census data also were used to calculate the percentage of bees from each group that were missing (not observed foraging and not captured in the dead bee trap).

Experiment 3: Single-Cohort Colonies

To test whether bees are able to show plasticity in behavioral development in response to changes in colony age structure in the absence of JH, we studied the behavioral development of CA− bees in single-cohort colonies. A single-cohort colony was made by placing a queen, 200 one-day-old unmarked bees and the focal bees into a small hive box with one empty frame and a second frame containing a small amount of pollen and nectar. Three single-cohort colonies were made in May–June 1996, with focal bees from source colonies 45, 101, and 110, respectively (sample sizes for untreated, sham, CA−, and MCA− groups: 51, 51, 58, 42, Single-Cohort Colony 1; 50, 50, 50, 65, Single-Cohort Colony 2; and 355, 60, 60, 57, Single-Cohort Colony 3). We added 1000 one-day-old bees to each single-cohort colony daily for 4 consecutive days beginning when focal bees were 3 days old to increase the likelihood of foraging in the focal bees (Page, Robinson, Britton, and Fondrk, 1992). Colonies were kept inside the Bee Research Facility with their hive entrances connected to holes in the wall to allow free flight outside. Observations were as in Experiment 2, except all foragers observed, both marked and unmarked foragers, were collected. Blood samples were taken from foragers in single-cohort colonies 1, 2, and 3. Observations were stopped when <5% of the focal bees remained.

Statistical Analysis

SAS (SAS Institute Inc., Cary, NC) and SPSS (SPSS, Inc., Chicago, IL) programs were used. Kaplan-Meier survival analysis, which yields the Breslow (B) statistic, was used on the age at onset of foraging. The Mann-Whitney U test was used to compare age at onset of foraging for each group between the first two pairs of typical and single-cohort colonies that used the same source colonies. (Typical Colony 1 of Experiment 2 and Single-Cohort Colony 1 of Experiment 3 used source colony 45; Typical Colony 2 of Experiment 2 and Single-Cohort Colony 2 of Experiment 3 used source colony 101). To determine whether allatectomy and hormone replacement caused consistent effects in all three experiments, we calculated the relative age at onset of foraging for each treatment group in a colony by normalizing with the untreated group in that colony. The relative age at onset of foraging for a given treatment group (sham, CA−, or MCA−) was calculated by dividing its mean age at onset of foraging by the mean age at onset of foraging for the untreated group in that colony. When appropriate, data were log-transformed (Zar, 1996). Analysis of covariance was performed on flight duration with the period (days) the bee flew as a covariate. The Tukey test or post hoc tests modeled on the Tukey test (Zar, 1996) were used to make multiple comparisons ($P < 0.05$) between groups for JH titer, relative age at onset of foraging, and proportion of dead and missing bees after a test for equal proportions among groups (Zar, 1996). Other statistical tests are identified with the results.

RESULTS

Results are presented for anesthetized (untreated), sham-allatectomized (sham), and allatectomized (CA−) bees in an observation hive colony in Experiment 1 and these groups plus methoprene-treated allatectomized (MCA−) bees in the typical colonies (Experiment 2) and the single-cohort colonies (Experiment 3).

Effects of Allatectomy on Age at Onset of Foraging

There was significant variation in the age at onset of foraging in the Observation Hive Colony [$B(2) = 26.46$; $P < 0.00001$] and in Typical Colony 1 [$B(3) = 31.56$; $P < 0.00001$], 2 [$B(3) = 62.63$; $P < 0.00001$],

and 3 [$B(3) = 140.69$; $P < 0.00001$]. Sham bees initiated foraging significantly earlier in life than untreated bees in 3 out of 4 colonies. We decided to focus on comparisons of CA− and sham bees. CA− bees from all colonies in Experiments 1 and 2 foraged, but they initiated foraging significantly later in life than sham bees in 3 out of 4 colonies (Fig. 2). MCA− bees began foraging at significantly younger ages than CA− bees in 3 out of 3 colonies in Experiment 2 (there were no MCA− bees in Experiment 1). When the Observation Hive Colony was terminated on Day 35, significantly fewer CA− bees had started foraging compared with untreated and sham bees [log-likelihood test; $G(2) = 28.50$; $P < 0.0001$]. When Experiment 2 was terminated, no focal bees were present in the typical colonies.

There also was significant variation in the age at onset of foraging in Single-Cohort Colony 1 [$B(3) = 27.66$; $P < 0.00001$], 2 [$B(3) = 26.89$; $P < 0.00001$], and 3 [$B(3) = 50.15$; $P < 0.00001$]. Sham bees again foraged at significantly younger ages than untreated bees in 2 out of 3 colonies. CA− bees began to forage at significantly older ages than sham bees in 2 out of 3 colonies (Fig. 3). MCA− bees foraged at significantly younger ages than CA− bees in 3 out of 3 colonies. Upon termination of Experiment 3, significantly fewer CA− bees in Single-Cohort Colony 1 had started foraging by 15 days of age [$G(3) = 14.12$; $P < 0.01$]. No significant difference in the proportion of bees initiating foraging was found between the treatment groups in Single-Cohort Colony 2 [$G(3) = 6.57$; $P = 0.09$]. No focal bees were present in Single-Cohort Colony 3 upon termination.

Calculation of relative ages at the onset of foraging revealed consistent effects across all three experiments (Fig. 4). There was significant variation among the groups [Kruskal-Wallis test: $H_C(2) = 14.59$; $P < 0.001$]. The age at onset of foraging was about 14% greater for CA− bees than sham bees and 23% greater than MCA− bees. However, for the two source colonies used in both Experiments 2 and 3 each treatment group foraged at significantly younger ages in single-cohort colonies relative to typical colonies [Source Colony 45, used in colonies 1 of Experiments 2 and 3: Untreated: $\chi(1) = 98.97$, $P < 0.0001$; Sham: $\chi(1) = 46.93$, $P < 0.0001$; CA−: $\chi(1) = 41.47$, $P < 0.0001$; MCA−: $\chi(1) = 31.18$, $P < 0.0001$; Source Colony 101, used in colonies 2 of Experiment 2 and 3: Untreated: $\chi(1) = 112.62$, $P < 0.0001$; Sham: $\chi(1) = 63.859$, $P = 0.0001$; CA−: $\chi(1) = 48.44$, $P = 0.0001$; MCA−: $\chi(1) = 56.85$, $P = 0.0001$].

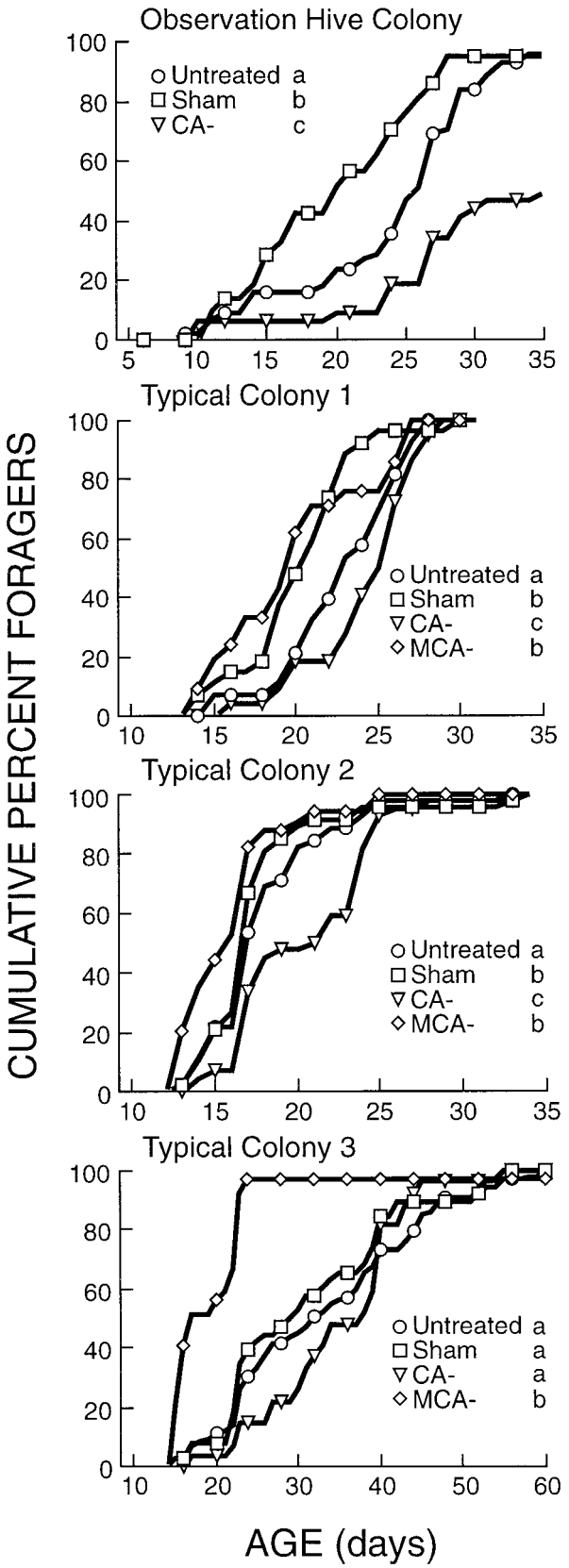
Effects of Allatectomy on Other Behavior

In the Observation Hive Colony, there were no significant differences between CA−, sham, and untreated bees in the age at onset of orientation flights [ANOVA: $F(2, 78) = 0.4$; $P = 0.67$] (Fig. 5A). CA− bees also showed apparently normal foraging behavior once they initiated it (Fig. 5B); there was no significant difference in the duration of their foraging flights compared with untreated bees [ANCOVA: $F(2, 1, 92) = 3.14$; $P < 0.05$]. Sham bees took significantly longer flights than untreated bees. These measures were not taken in Experiments 2 and 3.

CA− bees showed no gross differences in in-hive behavior, based on limited observations in the Observation Hive Colony. CA− bees performed the same range of behaviors as untreated and sham bees (Table 2). The frequency of six of the most frequently observed behaviors, *cleaning cells*, *smoothing the comb*, *walking*, and *hygienic* behaviors did not differ among the groups. Sham bees were observed *caring for brood* less often than untreated and CA− bees. CA− bees were observed *standing* more often than untreated bees. The limited number of observations per bee over the duration of the experiment only allows us to conclude that there were no extreme aberrations of hive behavior for CA− bees.

Missing Bees

The percentage of bees missing from the Observation Hive Colony after being observed to take at least one flight varied significantly among the groups [$Q(2) = 12.64$, $P < 0.005$]. Fifty-five percent of the CA− bees were missing (Fig. 6). This percentage was strikingly higher than for untreated and sham bees. Regression analysis of bees that never initiated foraging revealed that the age at first orientation flight was a significant determinant of lifespan for CA− bees [$n = 23$, $F(1, 21) = 4.4$; $P < 0.05$] but not for untreated bees [$n = 9$, $F(1, 7) = 0.08$; $P = 0.79$] or sham bees [$n = 4$, $F(1, 2) = 0.02$; $P = 0.91$]. Some CA− bees went missing after taking orientation flights. (Analysis performed only for bees that were not arbitrarily collected for blood or brain sampling.) In contrast, the age at onset of foraging, a known determinant of honey bee longevity (Winston and Katz, 1981; Dukas and Visscher, 1994), was a significant determinant of lifespan for foragers in all groups: untreated [$n = 13$, $F(1, 11) = 161.5$; $P < 0.0001$], sham [$n = 4$, $F(1, 2) = 21.68$; $P < 0.05$], and CA− bees [$n = 8$, $F(1, 6) = 665.0$; $P < 0.0001$]. Age at first orientation flight was not a significant determinant of



longevity in these same foragers: untreated [$n = 13$, $F(1, 11) = 2.0$; $P = 0.19$], sham [$n = 4$, $F(1, 2) = 4.85$; $P = 0.16$], and CA− bees [$n = 8$, $F(1, 6) = 0.01$; $P = 0.93$]. The age at onset of foraging but not the age at onset of orientation flight reliably indicated the lifespan of foragers from all groups.

Results from Experiment 1 led us to install dead bee traps on the hive entrances of the typical colonies in Experiment 2. This enabled us to determine whether CA− bees perish after they leave the hive for a flight, as suggested by the results of Experiment 1, or instead die in the hive and are carried out by corpse removal bees (Visscher, 1983). In Experiment 2, a large percentage of CA− bees again were missing from their colonies. Differences in the percentage missing bees among the groups were significant in all separate analyses of the colonies [Typical Colony 1: $Q(3) = 26.06$, $P < 0.001$; Typical Colony 2: $Q(3) = 15.23$, $P < 0.005$; Typical Colony 3: $Q(3) = 13.85$, $P < 0.005$]. The percentage of missing CA− bees was significantly higher than for untreated bees in 3 out of 3 colonies, sham bees in 2 out of 3 colonies, and MCA− bees in 1 out of 3 colonies (Fig. 7). Data from the dead bee traps revealed that the high percentage of missing CA− bees was not due to mortality within the hive; there were no significant differences between surgically treated groups in the percentage of dead bees recovered in dead bee traps in any colony [Colony 1: $Q(3) = 5.86$, NS; Colony 2: $Q(3) = 8.54$, $P < 0.05$; Colony 3: $Q(3) = 4.24$, NS] (Fig. 7). Pooling the data across all three colonies, the CA− and MCA− groups had a similar percentage of bees missing, higher than that of the sham group, and all three of these groups had significantly higher percentage of missing bees than that of the untreated group [Cochran-Mantel-Haenszel Test: dead; $Q_{MH}(3) = 9.33$, $P < 0.05$; missing; $Q_{MH}(3) = 46.61$, $P < 0.0001$].

DISCUSSION

The principal significance of these results is that they demonstrate that JH influences the pace of be-

FIG. 2. Effect of allatectomy and methoprene replacement on the age at onset of foraging in typical colonies. Groups followed by the same letter in the legends are not significantly different. Additional statistical analyses are in the text. Sample sizes for untreated, sham, CA−, and MCA− groups are as follows: 45, 21, 32, Observation Hive Colony; 204, 27, 22, 21, Typical Colony 1; 303, 48, 44, 34, Typical Colony 2; and 149, 38, 27, 38, Typical Colony 3. The age (days) range for Typical Colony 3 differs from the other colonies.

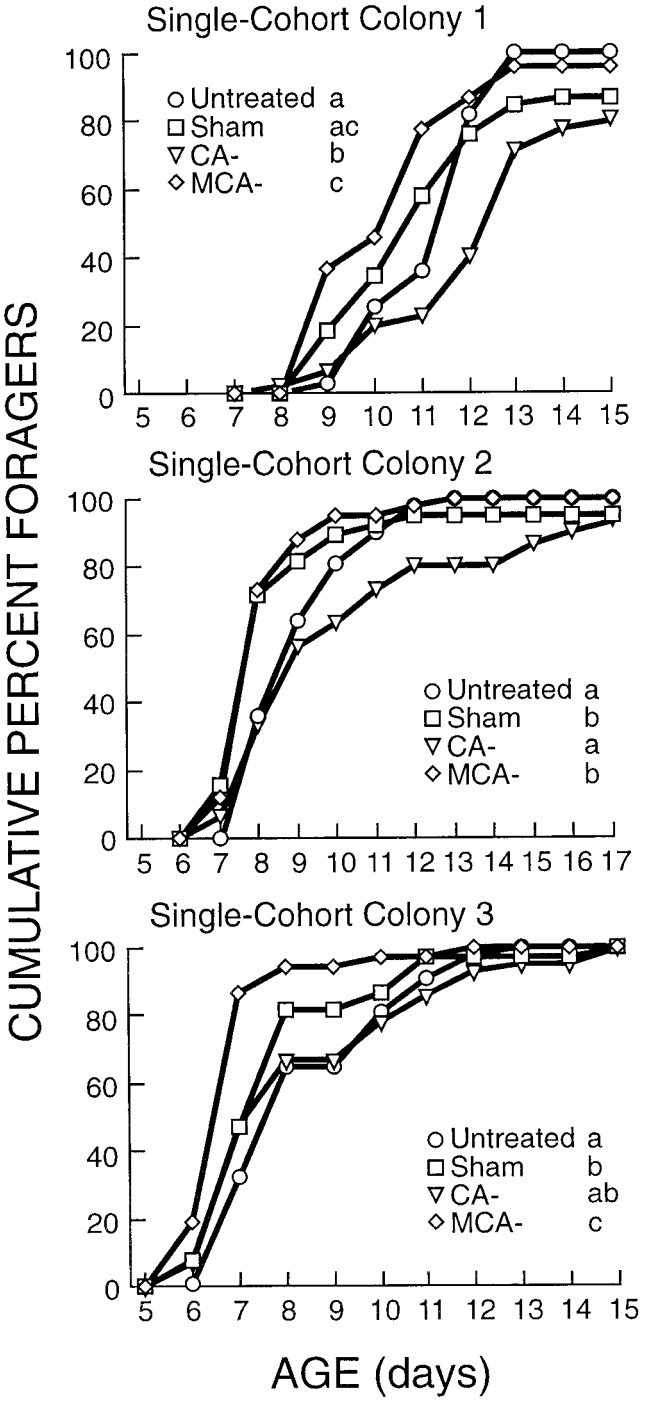


FIG. 3. Effect of allatectomy and methoprene replacement on the age at onset of foraging in single-cohort colonies. Groups followed by the same letter in the legends are not significantly different. Additional statistical analyses are in the text. Sample sizes for untreated, sham, CA−, and MCA− groups are as follows: 39, 38, 45, 22, Single-Cohort Colony 1; 42, 37, 30, 41, Single-Cohort Colony 2; and 246, 38, 42, 37, Single-Cohort Colony 3.

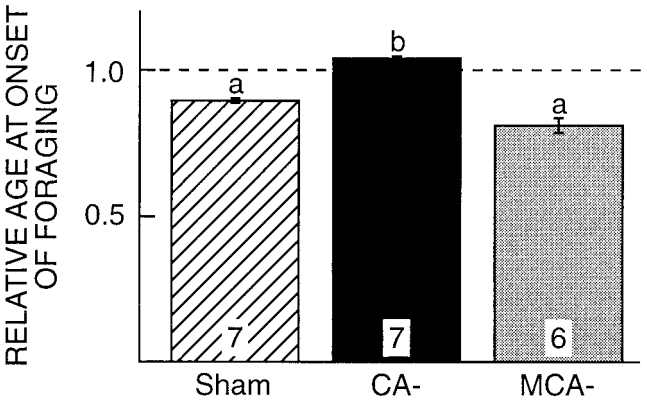


FIG. 4. Mean ± SEM relative age at onset of foraging of sham, CA−, and MCA− groups in all seven colonies of Experiments 1, 2, and 3 (sample sizes in bars). A value >1 indicates that the group foraged later in life than the untreated group (see Methods for details). Means with different letters are significantly different from each other (Dunn post hoc test).

havioral development in adult worker honey bees, but is not necessary for behavioral development to occur. JH does not activate foraging behavior in honey bees but rather influences when it will begin. These conclusions are based on the assumption that removal of the CA, the only known source of JH for bees, results in an absence of JH in the bee brain, the putative site of JH action for behavioral development (Robinson, 1987b). Even if the bee brain did produce JH, analogous to the songbird brain producing gonadal steroids (reviewed by Schlinger, 1994), results from songbirds suggest that we should have been able to detect the presence of brain-synthesized JH in our blood samples. Analysis of blood samples revealed negligible levels of JH in CA− forager bees.

Our results also indicate that JH is not necessary for bees to show plasticity in the age at onset of foraging in response to altered conditions. The onset of foraging in CA− bees was consistently delayed, but the delay was proportional to the age at onset of foraging of the control groups in each colony. Thus, even though CA− bees in single-cohort colonies showed a delay in the age at onset of foraging relative to control groups, they nevertheless showed precocious foraging relative to bees in the more typical colonies of Experiments 1 and 2. These interexperiment differences in the age at onset of foraging for CA− bees cannot be attributed to genotypic effects because the analyses were limited to genotypes used in both experiments. Robinson (1987a) proposed a model in which JH plays a central role in mediating the response to environmental change. Our results demonstrate that JH is not

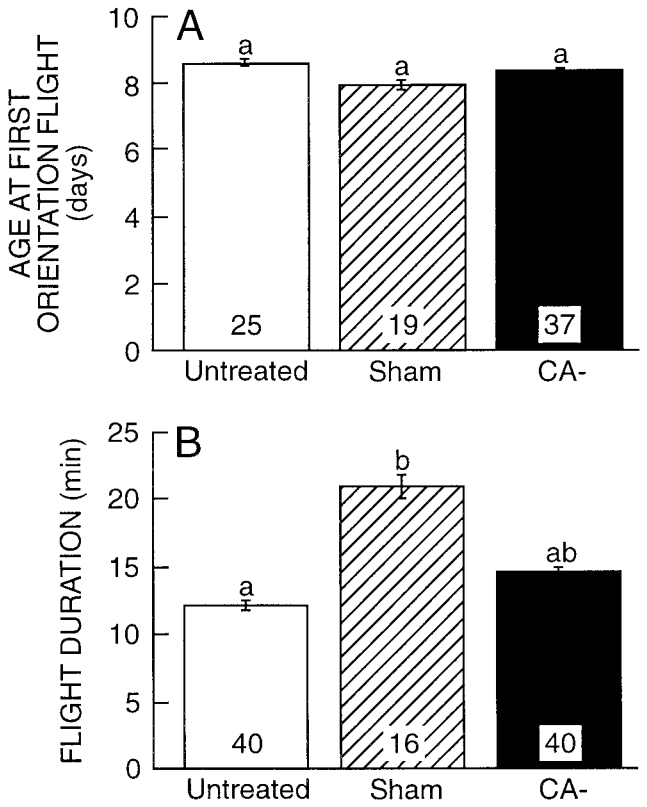


FIG. 5. Effect of allatectomy on the age at onset of orientation behavior (A) and duration of foraging flights (B) (sample sizes in bars). Means with different letters are significantly different. Additional statistical analyses are in the text.

an essential intermediary between perception of colony conditions and altered behavioral ontogeny. Huang and Robinson (1992) subsequently proposed a model in which behavioral development in younger bees is caused by an age-related intrinsic increase in JH and inhibited by older bees. Further studies have not only revealed the inhibitory effects of older bees (Huang *et al.,* 1998) but also identified inhibition by pheromones produced by the queen (Pankiw *et al.,* 1998) and brood (Y. Le Conte and G. E. Robinson, unpublished observations). Taken together, these results suggest that multiple factors regulate the onset of foraging.

Limited observations failed to detect extreme aberrations in in-hive behavior for CA− bees. However, more observations per bee are necessary before definitive conclusions are possible, especially for brood care, one of the main tasks performed by bees before they begin to forage. Bees performing brood care have low titers of JH, and social manipulations that cause bees to "revert" from foraging to brood care result in

TABLE 2

Mean (±SEM) Number of Observed Behavioral Acts of the Most Common Behaviors

Behavior	Untreated (n = 71)	Sham (n = 34)	CA− (n = 81)
Walking	2.97 ± 0.03 a	2.12 ± 0.05 a	2.49 ± 0.02 a
Smoothing Substrate	1.96 ± 0.02 a	1.47 ± 0.05 a	1.51 ± 0.02 a
Hygienic	1.41 ± 0.02 a	1.53 ± 0.04 a	1.44 ± 0.02 a
Standing	0.85 ± 0.01 a	0.91 ± 0.03 ab	1.09 ± 0.01 b
Brood Care	0.61 ± 0.01 a	0.18 ± 0.01 b	0.38 ± 0.01 a
Cleaning Cells	0.54 ± 0.01 a	0.44 ± 0.02 a	0.63 ± 0.01 a

Note. Means followed by the same letter within each behavior are not significantly different. The number of behavioral acts, an occurrence of a behavior, was summed for each bee over its lifespan. Analysis of covariance was used with the total number of observations of each individual bee as a covariate; walking [$F(2, 1, 182) = 0.63$; $P = 0.63$]; smoothing substrate [$F(2, 1, 182) = 0.02$; $P = 0.98$]; hygienic [$F(2, 1, 182) = 1.45$; $P = 0.24$]; standing [$F(2, 1, 182) = 3.29$; $P < 0.05$]; brood care [$F(2, 1, 182) = 4.55$; $P < 0.05$]; cleaning cells [$F(2, 1, 182) = 1.16$; $P = 0.31$].

a drop in JH (Robinson *et al.,* 1992; Huang and Robinson, 1996). These results suggest that low titers of JH are required for brood care, perhaps to coordinate behavior with exocrine gland activity (Robinson 1987a). Determination of the role of the corpora allata in behavioral reversion may be a fruitful topic for investigation.

Treatment with methoprene eliminated the delay in the onset of foraging in allatectomized bees. Methoprene is widely used as a JH analog because it is more resistant to enzymatic degradation and thus more persistent than the native hormone (Staal, 1975). Recent

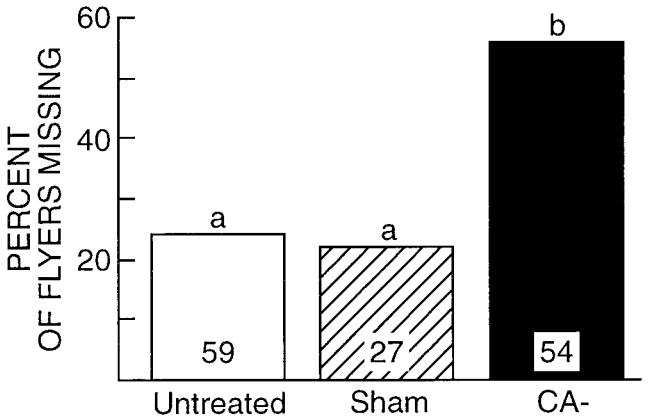


FIG. 6. Effect of allatectomy on disappearance after taking at least one orientation flight (sample sizes in bars). Groups with the same letter are not significantly different. Additional statistical analyses are in the text.

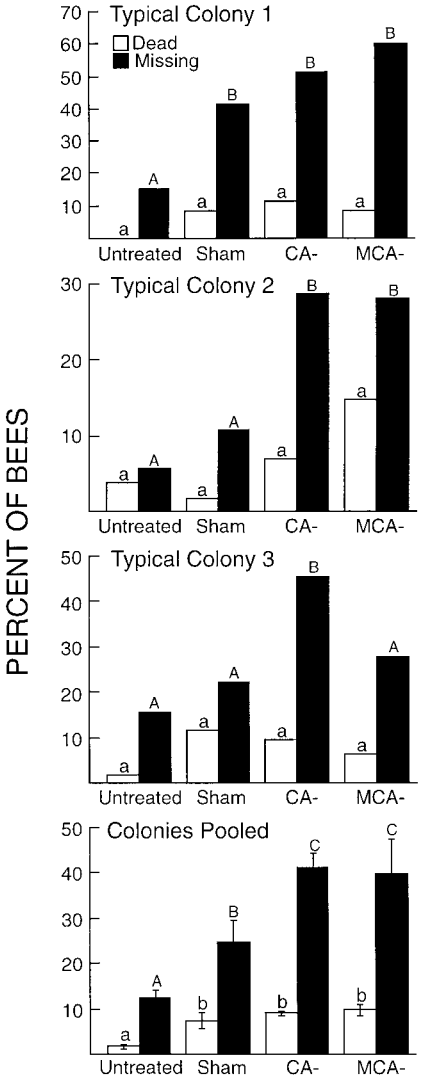


FIG. 7. Effect of allatectomy on mortality in the hive and disappearance in Typical Colony 1 (A), Typical Colony 2 (B), and Typical Colony 3 (C). Mean ± SEM percentage dead and missing bees in typical colonies (D). Groups with the same letter are not significantly different (upper case for dead; lower case for missing). Additional statistical analyses are in the text. Sample sizes are the number of bees introduced for each group in Experiment 2 (see Methods for details).

results in *Drosophila melanogaster* suggest JH and methoprene activate common molecular mechanisms to regulate gene expression (Noriega, Shah, and Wells, 1997; Ashok, Turner, and Wilson, 1998; Restifo and Wilson, 1998).

Muller and Hepburn (1994) reported no effect of either allatectomy or methoprene treatment on the timing of wax secretion in the Cape honey bee (*Apis mellifera* capensis), and on the basis of these results questioned the role of JH in honey bee division of labor in general. Robinson and Vargo (1997) pointed out that it is not surprising that there is less (if any) influence of JH on wax secretion because the timing of this process is not regulated as tightly as the onset of foraging. Wax secretion begins at 3 days of age, increases gradually, and wanes as a bee begins to forage (King, 1933; Seeley, 1982; Muller and Hepburn, 1992, 1994; Pratt, 1998). Muller and Hepburn (1994) also suggested that methoprene treatment can cause toxic effects on honey bees, based on their finding of inhibitory effects of methoprene on the quantity of wax produced. However, the methoprene treatment used in our study has been shown previously to exert no effects on flight duration and number of flights per hour taken by foragers (Robinson, 1985). Foraging is the most metabolically demanding task for bees (Winston, 1987). In addition, treatment of young honey bees with JH or methoprene results in a similar acceleration in behavioral development (reviewed in Fahrbach, 1997). We conclude that our method of methoprene treatment served as reliable hormone replacement therapy. We do not know, however, how well our topical treatment of methoprene mimicked the normal profile of age-related increase in JH that is experienced during normal behavioral development. In the fire ant, *Solenopsis invicta*, 80% of a topical treatment of radiolabeled methoprene was excreted within 24 h after application (Bigley and Vinson, 1979). If methoprene is degraded and excreted similarly in the honey bee, then a peak of methoprene may have occurred between 2 and 3 days of age in the MCA− bees (treatment was on Day 2), and this peak may have been responsible for accelerating behavioral development. A small peak in rate of JH biosynthesis *in vitro* (Kaatz, Hildebrandt, and Engels, 1992) and JH blood titer (O. Jassim, Z.-Y. Huang, and G. E. Robinson, unpublished observations) occurs in worker honey bees between 2 and 3 days of age. JH may also act later in life to accelerate behavioral development. Guards and corpse removers have higher JH titers than other middle-age bees; they also start to forage at younger ages (Trumbo, Huang, and Robinson, 1997). Allatectomy and hormone replacement at different ages could be used to determine how JH affects the temporal dynamics of honey bee behavioral development.

JH influences a wide range of physiological and behavioral processes in insects that have a developmental component (reviewed by Wyatt and Davey, 1996). In adult insects, JH is best known for its effects on the coordination of physiological and behavioral

aspects of reproductive maturation. For example, in crickets, JH plays a key role in vitellogenesis and egg maturation and also influences the development of female sensitivity to the mating calls of males. In both of these contexts, treatment with JH or JH analogs hastens the onset of the process, and allatectomy decreases but does not eliminate the process completely (Hoffmann and Sorge, 1996; Stout, Hao, Kim, Mbungu, Bronsert, Slikkers, Maier, Kim, Bacchus, and Atkins, 1998). These results are very similar to those that we report and suggest a common molecular mechanism by which JH potentiates diverse maturational processes. Research on the molecular basis of JH action has been hampered by a lack of characterized JH receptors, a situation that may change in the near future (Jones and Sharp, 1997; Ashok *et al.*, 1998).

Sham bees in our study foraged at a younger age than untreated bees. This is why the effects of allatectomy were considered by comparing CA− and sham bees. Surgery may have activated a stress mechanism that accelerates behavioral development. Such a stress effect could be mediated by JH, as this hormone is known to be sensitive to stress in a variety of insect species (Jankovic-Hladni, 1991). Other neuroendocrine factors could be involved, such as the biogenic amine octopamine (Hirashima, Takeya, Taniguchi, and Eto, 1995; Hirashima, Hirokado, Tojikubo, Takeya, Taniguchi, and Eto, 1998). Grabbing a bee with forceps and holding its legs in an alligator clamp for several minutes triggers a transient increase in octopamine levels in the bee brain (Harris and Woodring, 1992), and high brain octopamine levels are associated with foraging (Harris and Woodring, 1992; Wagener-Hulme, Kuehn, Schulz, and Robinson, 1999; Schulz and Robinson, 1999). If the sham effect involved factors other than JH, then the effect of allatectomy relative to untreated bees was probably underestimated in this study. A better understanding of this stress effect may provide new insights into the regulation of honey bee behavioral development.

An unanticipated and striking result was that about half of the allatectomized bees disappeared from the study colonies, but not because they died inside the hive. Limited observations of the performance aspects of foraging and of in-hive behavior suggest that CA− did not show lower levels of overall activity. Rather, it appears that CA− bees did not return to their natal colony after embarking on one of their initial orientation flights. We do not know whether they perished in the field or were able to gain acceptance into an unrelated colony in the vicinity. Equal numbers of missing CA− and MCA− bees indicate that the dose and timing of methoprene treatment we employed did not affect the ability of CA− bees to return to their natal colony. It is tempting to speculate that developmental changes in endogenous JH titers are in some way necessary for proper orientation capability, due to influences on metabolism, sensory perception, or learning and memory. These issues are currently being examined with hormone removal and replacement experiments.

In conclusion, JH influences the pace of behavioral development in honey bees, in a manner analogous to the role of vertebrate steroid hormones (Short and Adams, 1988; Hayes, 1997).

ACKNOWLEDGMENTS

We thank J. C. Kuehn for technical assistance; T. Hallam and J. Drew for assistance with observations, Z.-Y. Huang and T. Giray for advice on the RIA, and M. Straume for advice and software for analysis of hormone data. Methoprene was a generous gift of Sandoz Agro, Inc., Palo Alto, California. The comments of T. Giray, J. Nardi, and members of the Robinson and Fahrbach labs improved this paper. Supported by an NSF Award to S.E.F. and G.E.R., an NSF REU Site Award to S.E.F. and G.E.R., and an NIDCD Award to G.E.R..

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