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Physiological correlates of genetic variation for rate of behavioral development in the honeybee, *Apis mellifera*

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Abstract Two factors that influence age at onset of foraging in honeybees are juvenile hormone (JH) and colony age demography (older bees inhibit behavioral development of younger bees). We tested the hypothesis that genetic variation among bees for these factors influences genetic variation in behavioral development. Pairs of colonies showing genetic differences in rates of behavioral development were identified in a screening experiment and bees from these colonies were used for physiological and behavioral assays. Six pairs were assayed, three with European bees only and three with both European and Africanized bees. There was genetic variation for the following four components: (1) production of JH in four pairs (experiment 1); (2) sensitivity to JH in three pairs (experiment 2); (3) sensitivity to social inhibition in three pairs (experiment 3), and (4) potency of social inhibition in four pairs (experiment 4). Cross-fostering assays (experiment 5), which allowed all four components to be evaluated simultaneously, revealed genetic variation for production of JH, sensitivity to JH, or sensitivity to social inhibition in five of six pairs, and potency of social inhibition in five of six pairs. There was often evidence for genotypic differences in more than one component, and no consistent pattern of association among any of the components. Africanized bees had faster rates of behavioral development than European bees, but there were no racial differences in patterns of variation among the four components. These results indicate that there are at least several, apparently distinct, physiological processes associated with JH and colony age demography upon which natural selection can act to alter the rate of behavioral development in honeybees.

Key words *Apis mellifera* · Juvenile hormone · Africanized honeybee · European honeybee · Foraging

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Introduction

There is genetic variation for components of division of labor in some insect societies (reviewed by Page and Robinson 1991; see also Stuart and Page 1991; Carlin et al. 1993, Snyder 1993; O'Donnell 1996). In honeybee colonies, for example, there are genotypic differences in age-related division of labor. Bees of some genotypes show a faster rate of behavioral development and make the transition from working in the hive to foraging at a younger age than do workers of other genotypes (Winston and Katz 1982; Kolmes et al. 1989; Robinson et al. 1989; Page et al. 1992; Giray and Robinson 1994). The physiological correlates of genetic variation for rate of behavioral development in honeybees are unknown. This is the case for most instances of genetic variation for naturally occurring behavior (but see Arnold 1980; reviewed in Alcock 1998) even though this knowledge would increase our understanding of behavioral evolution.

One factor that influences age at onset of foraging in honeybees is juvenile hormone (JH) (reviewed in Fahrbach and Robinson 1996; Robinson and Vargo 1997). Low JH biosynthesis rates and blood titers are associated with hive duties during the first 2–3 weeks of adult life and high rates of JH biosynthesis and titers are associated with foraging (Huang et al. 1991, 1994; Huang and Robinson 1995). Treatments with JH, JH mimics, or JH analogs induce precocious foraging (re-

viewed in Fahrbach and Robinson 1996; Robinson and Vargo 1997). Removal of the corpora allata, the glands that produce JH, delays the onset of foraging, and this is eliminated with JH analog treatment (Sullivan et al., in press). In addition, bees are able to accelerate, delay, or even reverse their behavioral development in response to changing environmental conditions, and these changes are also associated with changes in JH (Robinson et al. 1989, 1992; Huang and Robinson 1996).

Colony age demography also influences age at onset of foraging in honeybees (Huang and Robinson 1992, 1996; Huang et al. 1998). Older bees inhibit the behavioral development of younger bees. For example, transplants of foragers from a typical colony to experimental single-cohort colonies, initially composed of only 1-day-old bees, inhibited precocious foraging in the resident bees even if transplanted foragers were not allowed to forage. This means that the resident bees likely sensed the foragers directly, rather than sensing changes in the hive environment such as the odor or actual amount of freshly collected food (Huang and Robinson 1992). The inhibition of precocious foraging requires physical contact, suggesting that it is mediated by a behavior, chemical, or both (Huang et al. 1998).

Huang and Robinson (1992) hypothesized that genetic variation for factors related to JH and colony age demography contributes to genetic variation in honeybee behavioral development. According to this hypothesis, genotypes of bees with relatively fast rates of behavioral development are expected to show the following: (1) higher production of JH; (2) greater sensitivity to the effects of JH; (3) less sensitivity to social inhibition (i.e., the inhibitory effects of older bees); (4) less inhibitory potency, or (5) some combination of these four components. Differences in sensitivity to social inhibition could either be behavioral or physiological, depending on the precise mechanism of inhibition, which is not known. We tested these four predictions, for both European and Africanized honeybees.

Behavioral differences between European and Africanized bees that are related to division of labor have been reported. European bees are a mixture of subspecies originally from Europe introduced repeatedly to North and South America over the past 300 years. Africanized bees are a subspecies of the European honeybee from south-central Africa, *Apis mellifera scutellata*, that was brought to Brazil in 1956 to cross with European subspecies for apicultural purposes. Africanized bees are typically more defensive (e.g., Guzmán-Novoa and Page 1993, 1994) and start foraging at younger ages than European bees (Winston and Katz 1982; Giray et al., in press). The increased defensiveness of Africanized bees is thought to be an adaptation to the higher vertebrate predation in Africa (Roubik 1989), but ecological correlates of differences in foraging age are not known. Working with both European and Africanized bees allowed us to begin to explore whether the control of behavioral development differs among bees that evolved in different environments.

General approach

We first identified six pairs of genotype groups (defined below) with strong differences in rates of behavioral development within each pair, one with a fast rate, the other with a slow rate. There were three pairs of genotype groups of European bees and three pairs with one genotype group of European bees (slow) and one of Africanized bees (fast). Using these pairs we looked for genetic variation in JH production (experiment 1), JH sensitivity (experiment 2), social inhibition sensitivity (experiment 3), and social inhibition potency (experiment 4). This was accomplished with behavioral and physiological assays that tested for genetic variation in a single component (experiments 1–4) and a cross-fostering experiment (experiment 5) that allowed all four components to be evaluated simultaneously.

Sources of bees

Experiments with European bees were performed in the summer of 1995 at the Bee Research Facility, University of Illinois, Urbana. Bees for experiments were obtained from 17 “source” colonies, each colony headed by a queen instrumentally inseminated with semen from a single, different drone.

Experiments with Africanized and European bees were performed in the fall (late wet season to early dry season) of 1994 at Miel Vita Real, a commercial beekeeping operation in Ixtapan de la Sal, Mexico (19° N, 99° W), 150 km southwest of Mexico City. Bees for experiments were obtained from seven European and eight Africanized source colonies, each colony headed by either an Africanized or a European queen that was instrumentally inseminated with semen from a single Africanized or a single European drone, respectively. Morphometric (Sylvester and Rinderer 1987) and molecular (Hall and Smith 1991) assays were used to verify that bees were truly European or Africanized (with samples of callow workers with fully expanded wings – clear not milky in appearance – collected on combs, n=20). Wing measurements were performed in the laboratory of E.G.-N. and mitochondrial DNA typing in the laboratory of R.E. Page, University of California, Davis.

Source colonies were inspected frequently (once every 2 weeks) to make sure that each was headed by the same queen throughout the study period. Queens were labeled with colored, numbered tags (Opallitplatchen, Graze KG, Weinstadt, Germany) to facilitate these inspections. The right forewing of each queen was partially clipped after insemination to prevent mating flights (which are sometimes taken by instrumentally inseminated queens; see Kaftanoglu and Peng 1982).

Screening experiment: identification of genotype groups with differences in rates of behavioral development

Methods. Single-cohort colonies, initially composed of all 1-day-old bees, were used to screen for genotypic differences in rates of

behavioral development (Giray and Robinson 1994). The rate of behavioral development is accelerated in single-cohort colonies due to the absence of older bees (Huang and Robinson 1992, 1996); bees initiate foraging precociously, about 2 weeks earlier than in a typical colony. Genetic differences in rate of behavioral development observed in single-cohort colonies are also observed in colonies with more typical age structures (Giray and Robinson 1994).

In the Illinois study, seven single-cohort "composite" colonies were established, each with a population of 1500–3000 bees, composed of three to six "genotype groups" (500 bees from each). A genotype group refers to the offspring of a queen from one of the 17 source colonies described above. Using multiple genotype groups in each colony allowed us to screen more rapidly for genotypic differences. In the Mexico study, two genotype groups were used per colony (approximately 750 bees from each), one European and one Africanized, because we specifically wanted to compare these two types of bees.

Single-cohort composite colonies were created as follows. One-day-old bees were obtained by taking frames of sealed brood from each source colony and placing them overnight in an incubator (34°C). Emerging adults from each source colony were marked with a genotype-group-specific color of paint (Testor's PLA) on the thorax. Each single-cohort composite colony received one frame full of honey and pollen and one empty frame. A European queen, unrelated to any of the genotype-groups, was also introduced to the colony.

Observations of foraging behavior began when bees were 4 days old. When foraging began, usually on day 6, observations were made for 2 h daily, between 9000–1100 and 1500–1700 hours. Foragers were identified as bees with pollen loads on the hind legs (pollen foragers), or bees with distended abdomens (water or nectar foragers). Hive entrances were equipped with a small door to facilitate vacuum collection of returning foragers. Presumed nectar foragers were dissected to verify that they contained nectar in their crops. Only bees with clear fluid in the crop were counted as foragers. The number of presumed nectar foragers that were discarded according to this criterion was <5%. We collected the first 150 precocious foragers and determined to which genotype group they belonged by checking their paint marks. Sampled bees were not returned to their colony. In the Mexico study, only the first 50 precocious foragers were collected; previous studies showed that this was a sufficient sample to determine differences between two groups of bees (Robinson et al. 1989; Giray and Robinson 1994). Two-way *G*-tests were used to determine genotypic differences in the likelihood of becoming a precocious forager by comparing the representation of each genotype group in the precocious forager sample with its initial representation in each single-cohort composite colony.

Results. Genotype groups from 17 European source colonies in Illinois and 8 Africanized and 7 European source colonies in Mexico were screened. Results of the complete screening are part of another study (Giray et al., in press). Africanized bee genotype groups showed faster rates of behavioral development than European bee genotype groups (consistent with a previous report of faster behavioral development in Africanized bees; Winston and Katz 1982). Six pairs of genotype groups were selected for this study; the three pairs of European bees (pairs 1–3) and the three pairs of Africanized and European bees (pairs 4–6) with the biggest differences in likelihood of becoming a precocious forager (data not shown). For pairs 1–6, bees from the fast genotype groups represented 75–99% of the precocious forager sample even though they only accounted for 40–60% of the population of the entire colony. All experiments were performed with these six genotype group pairs; each pair is composed of a "fast" and a "slow" genotype group.

Workers did not experience a common environment prior to adult emergence in this screening and in most experiments in this study (except in part of experiment 1B). We do, however, interpret the observed behavioral differences between genotype groups as genotypic differences. This interpretation is supported by results from previous studies (Page and Erickson 1988; Robinson et al. 1989, 1990; Page et al. 1992). Because bees from these genotype groups have been used in another study (Giray et al., in press), we give the identity numbers for all source colonies: pair 1: 45 and 33 (fast and slow genotype groups, respectively); pair 2: 68 and 18; pair 3: 42 and 58; pair 4: TE-12 and 3-29; pair 5: TE-9 and 3-6; and pair 6: TE-6 and 7-28. Results of all six pairs are presented together for each experiment because there were no differences between genotype group pairs with both European bees (pairs 1–3) and pairs with Africanized and European bees (pairs 4–6).

Censuses

In experiments where individuals from groups were compared for their representation in the sample of foragers and initial experimental colonies (experiments 1B, 2A, 3, 4, and 5 and the screening experiment reported above), a census was performed after collecting the foragers, and all remaining marked individuals were counted. In no case was there a significant difference in mortality between groups during an experiment (data not shown). This means that observed differences in the likelihood of becoming a forager were not due to differential mortality across groups.

The following sections describe specific methods for each experiment and its results.

Experiment 1A: genotypic differences in JH titers

We tested the hypothesis that fast bees have a higher rate than slow bees of JH production (JHP) by measuring JH blood titers. It is much easier in the course of a field experiment to take blood samples for later laboratory determination of titers than to perform the intricate tissue culture assay needed to determine rates of JH biosynthesis immediately (Huang et al. 1991). Rates of JH biosynthesis are highly correlated with JH blood titers in adult worker honeybees under most conditions, including those of this experiment (Huang et al. 1991, 1998; Huang and Robinson 1992, 1995).

Methods. JH titers were determined for bees that were reared in isolation in the laboratory. This was done to avoid the possibility that genotypic differences in sensitivity to social inhibition contribute to differences in JH titers. Bees reared in isolation show precocious behavioral development and have forager-like JH titers and rates of JH biosynthesis (Huang and Robinson 1992; Huang et al. 1998). We reared bees in social isolation using the following method (Huang et al. 1998). Newly emerged bees from each genotype group pair were obtained from the incubator (as described above) and placed individually in plastic cages (40×25×10 mm,

Table 1 Results of two-way ANOVA for genotypic differences in juvenile hormone (JH) titer of fast and slow bees reared in isolation. Data in Fig. 1 (significant results are *italicized*)

Pair	Genotype	Age	Interaction
1	0.72	*0.0001*	0.28
2	0.14	*0.0001*	*0.03*
3	0.31	*0.0001*	*0.02*
4	*0.0001*	*0.0001*	0.08
5	0.85	*0.0001*	0.10
6	0.31	*0.0001*	0.08

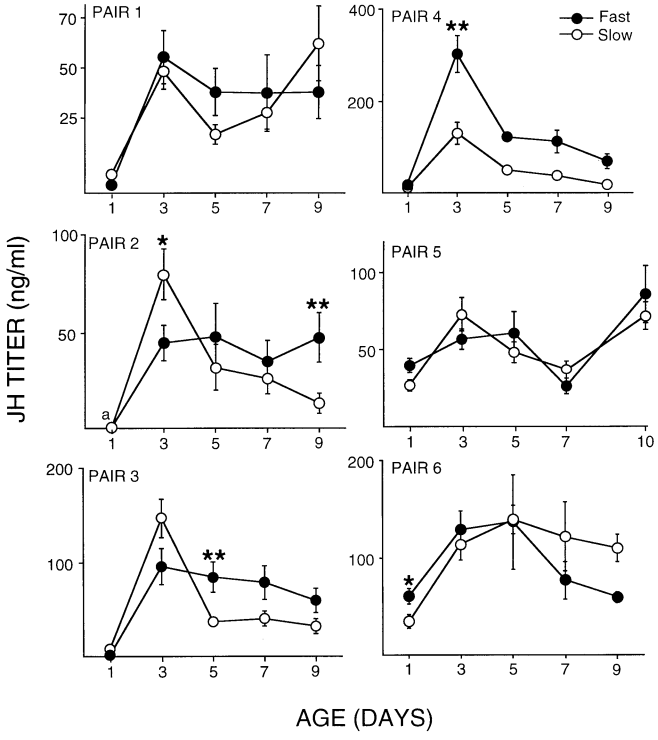

Fig. 1 Genotypic differences in juvenile hormone (JH) titers (mean±SE). *n*=10 individual bees per data point (except *n*=9: pair 1, fast bees, day 5; pair 3, fast and slow bees, day 5; pair 6, days 5–9; *n*=8: pair 2, slow bees, day 3). Significant differences between fast and slow bees for each day are indicated by asterisks (**P*<0.05, ***P*<0.01). For additional statistical analyses see Table 1

JZ^{s} BZ^{s}, California) used by beekeepers to ship queens. The cages were then placed in a wooden frame that itself was enclosed in a metal screen cage (46×24×8 cm) before being placed into a typical colony (with an adult population of 30,000 individuals of all ages). The metal screen cage prevented the bees from physically contacting colony members. Each cage was provisioned with about 400 mg of sugar candy (a standard 1:1 mixture of confectionery sugar and honey), more than enough food to sustain a bee for the 9-day isolation period (average consumption per bee: 11 mg/day, Z.-Y. Huang, unpublished observation). A drop of water was given to each caged bee daily to prevent dessication of the candy.

Blood samples were collected from caged bees on days 1, 3, 5, 7, and 9 (*n*=10 bees per day per genotype group). Blood samples (0.8–8 µl) were obtained by piercing the intersegmental membrane between the second and the third abdominal segments with an insect pin and applying a calibrated capillary tube to the wound. The sample was measured to the nearest 0.1 µl and then expelled into a culture tube containing 500 µl acetonitrile (to precipitate blood proteins, including enzymes that degrade JH).

JH titers of individual bees were measured using a chiral-specific radioimmunoassay (RIA) specifically validated for adult worker honeybees (Huang et al. 1994). JH III is the only JH homolog found in honeybees (Hagenguth and Rembold 1978; Robinson et al. 1991; Huang et al. 1994). Previous results (Huang et al. 1994; Huang and Robinson 1995) indicate that values from this RIA agree with two other RIAs, both of which have been validated with gas chromatography/mass spectroscopy (de Kort et al. 1985; Goodman et al. 1990). The sensitivity of the RIA is about 5 pg R(–) JH III per sample and typical inter- and intra-assay variation for JH determinations is 9.2% and 10.6%, respectively (Huang and Robinson 1996). A detailed description of this assay can be found in Huang et al. (1994).

JH titers were analyzed with two-way ANOVA on log(*x*+1)-transformed data, with age and genotype as independent variables. A significant age by genotype effect indicates a genotypic difference in the timing of an age-related change in JH. ANOVA was followed by *t*-tests for each sampling day to determine on which days there were differences in JH titers between fast and slow genotype groups.

Results. JH titers varied significantly with age in six of six pairs (Table 1). Fast bees had significantly higher JH titers than slow bees at some point during the 9-day isolation period in four out of six pairs (Fig. 1). In contrast, slow bees had significantly higher JH titers than fast bees on just 1 day in one out of six pairs. Fast bees in pairs 2, 3, and 4 reached their peak titer at about the same age as slow bees but they maintained a higher titer for longer periods. Fast bees in pair 6 already showed a higher JH titer on the first day of adult life.

To determine whether there was a consistent genotype by age interaction effect on JH titers over the whole experiment, we combined the probabilities for all genotype by age interactions from the six pairs (Sokal and Rohlf 1995, pp. 794–797). The combined probability was significant ($P<0.005$, $\chi^2=32.2$, $df=12$).

Experiment 1B: genotypic differences in rate of behavioral development in association with differences in JH titers

Methods. Socially isolated bees with precociously high JH titers also show precocious behavioral development (Huang and Robinson 1992; Huang et al. 1998). To verify that this also occurred under our experimental conditions, fast and slow bees reared in social isolation for 3 days (*n*=50 per group) were introduced to a single-cohort colony composed of 3-day-old bees unrelated to either genotype group. A control group was also added: 3-day-old bees from the same fast and slow genotype groups (*n*=50 per group), but reared in a typical colony rather than in social isolation. All 200 focal bees were individually tagged with numbered, colored plastic tags in such a way as to allow for blind observations. Observations of foraging behavior were made as above and the first circa 50 precocious foragers were collected. We hypothesized that both groups of socially isolated bees are more likely to become precocious foragers than control bees, and that fast and slow genotype groups that show differences in JH titers (in experiment 1A) would also show differences in the likelihood of becoming precocious foragers. Bees in pairs 1–3 were reared from eggs in a common nursery colony before placing them in experimental colonies; bees in pairs 4–6 were reared in their natal colonies from egg to pupae as in the other experiments described in this paper.

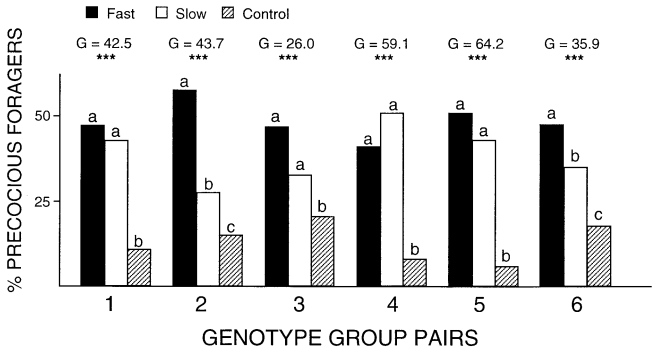

Fig. 2 Genotypic differences in rate of behavioral development for bees reared in social isolation. Control bees were from the same fast and slow genotype groups. Results of 3×2 *G*-tests (performed on actual frequencies of precocious foragers vs nonforagers) are indicated above each group of bars (***P<0.001). Pairwise differences between groups (2×2 *G*-tests) are indicated by different letters (P<0.05). n=100 bees for each control group; n=50 per genotype group in pair 1, 40 in pair 2, 50 in pair 3, 48 in pair 4, 48 for the fast and 50 for the slow genotype group in pair 5, and 35 for the fast and 45 for the slow genotype group in pair 6

Results. Isolated bees showed an increased likelihood of becoming precocious foragers. The proportion of each group of isolated bees observed as precocious foragers was significantly higher than for colony-reared control bees in all six colonies (Fig. 2). Results of behavioral assays were consistent with JH measurements in four of six pairs (Figs. 1, 2). In pairs 1 and 5, there were no significant differences between fast and slow bees in either JH titers or the likelihood of becoming precocious foragers. In pairs 2 and 6, there were significant differences between fast and slow bees in both JH titers and the likelihood of becoming precocious foragers. In pairs 3 and 4, there were significant differences between fast and slow bees in JH titers but not in the likelihood of becoming precocious foragers.

An experiment-wide analysis was conducted to determine whether the results of the behavioral isolation experiment were consistent with those from the initial behavioral screening. Bees from genotype groups determined to be fast in the screening were significantly over-represented in precocious forager samples in the isolation assay (P<0.05, t=2, n=6, one-tailed Wilcoxon signed-rank test; Ott 1988), indicating general agreement between results of the inital screening and the isolation assay, despite the discrepancies noted above.

There were no significant differences in the results from pairs 1–3 compared to those from pairs 4–6 (P>0.1, t=12, n_1, n_2=3, Wilcoxon rank sum test). This result suggests that similar differences in rates of behavioral development were detected between genotype groups regardless of whether they were in the same colony from egg through adulthood (pairs 1–3) or only during the adult stage (pairs 4–6).

Experiment 2: genotypic differences in sensitivity to JH

We tested the hypothesis that fast bees are more sensitive to JH than slow bees. Since JH receptors have not been identified definitively from any insect species (see Jones and Sharp 1997; Ashok et al. 1998), this was accomplished with two indirect assays.

Experiment 2A: sensitivity to methoprene treatment

Methods. In pairs 1–3, we measured differences in sensitivity to methoprene, a JH analog known to cause premature behavioral development in honeybees (Robinson 1985, 1987; Withers et al. 1995). The rationale for this approach is based on the fact that methoprene has shown JH-like effects at the behavioral, physiological, and molecular levels in many insect species, including honeybees (reviewed by Robinson and Vargo 1997). Particularly compelling is the finding that insensitivity to methoprene in *Drosophila melanogaster* is due to a mutation affecting a cytoplasmic JH-binding protein (Shemshedini and Wilson 1990; Ashok et al. 1998). Newly emerged fast and slow bees (n=50 per group) were tagged and topically treated with 100 or 150 µg methoprene, dissolved in 5 µl acetone (as in Robinson 1985, 1987). These doses have been shown to be active but less potent than higher doses (Robinson 1987); they were thus selected to increase the probability of detecting genotypic differences in sensitivity. Control bees (from the same genotype groups) were treated with acetone alone (earlier studies showed no effects of acetone; see Robinson 1985).

Treated and control bees from each pair of genotype groups were introduced to a typical colony that was equipped with a glass-topped entrance ramp to facilitate behavioral observations. The glass was covered with a thin film of petroleum jelly to prevent bees from walking tag-side down during observations (Winston and Katz 1982). A census was conducted 2 days after bees were introduced to the colony to check mortality. The census was performed by removing and visually scanning each comb twice and recording on audiotape the color and tag number for each marked bee.

Behavioral observations began on day 7; there were two, 1-h observation periods per day, between 0900–1000 and 1600–1700 hours. During each observation period, we recorded the time that each tagged bee left or entered the hive, and whether it was clearly foraging for pollen or nectar (water). Bees making round trips of ≥5 min in duration were also classified as foragers (Winston and Katz 1982; Robinson 1987). Observations were recorded and later transcribed to determine the age at onset of foraging. Observations were made until 100 foragers were observed or focal bees reached 23 days of age, whichever came first. Three-way *G*-tests were used to determine effects of genotype and methoprene dose on the proportion of bees from each group that initiated foraging. Effects of each dose on each genotype group were also examined with Mantel-Cox survival analysis (O'Donnell and Jeanne 1995).

Results. Significant dose-dependent effects of methoprene on age at onset of foraging were detected in three of three colonies (Fig. 3; pair 1: P<0.01, G=12.7; pair 2: P<0.001, G=55.9; pair 3: P<0.001, G=47.9). Significant genotype effects were detected in two of three colonies (pair 1: P<0.001, G=32.6; pair 3: P<0.001, G=12.4). Significant dose by genotype interactions were detected in two of three colonies (pair 1: P<0.001, G=19.1; pair 3: P<0.05, G=6.6). Bees from the fast genotype groups showed a strong response to the 100-µg dose, while bees from the slow genotype responded strongly only to the 150-µg dose. Mantel-Cox survival analysis tests also showed that fast bees responded significantly more strongly to the 100-µg dose than did slow bees in pairs 1 and 3 (Fig. 3).

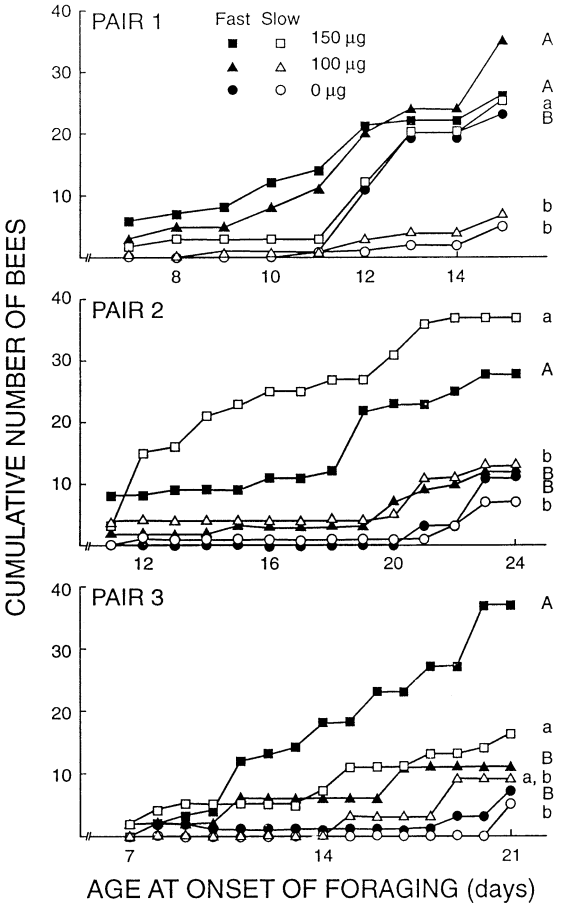

Fig. 3 Genotypic differences in sensitivity to the JH analog methoprene. Results of three-way G-tests in text. Different letters (*upper case* fast genotype; *lower case* slow genotype) indicate significant differences in methoprene sensitivity within each genotype group ($P<0.05$, Mantel-Cox survival tests)

Experiment 2B: JH titers at the onset of foraging

Methods. In pairs 1, 3, 4, 5, and 6, we measured JH titers (as in experiment 1A) in bees just returning from their first foraging flight. We reasoned that bees that are more sensitive to JH should have lower titers of JH at the onset of foraging. Bees collected for these analyses were foragers from colonies used in experiment 5 (see below). Results were analyzed with *t*-tests.

Results. JH titers of foragers varied widely from colony to colony (Fig. 4); the reason for this large variance is not known but has been seen previously in every study of foragers (e.g., Huang and Robinson 1995). Fast bees on their first foraging flight had significantly lower JH titers than did slow bees in two out of five pairs (3 and 5), which included one of the two pairs that showed genotypic differences in methoprene sensitivity. An experiment-wise analysis was conducted by combining the probabilities from the individual *t*-tests; the combined probability was significant ($P<0.05$, $\chi^2=19.03$, $df=10$). Results from experiments 2A and 2B are consistent with the prediction that genotypic differences in sensitivity to JH can be important in determining genotypic differences in rates of behavioral development.

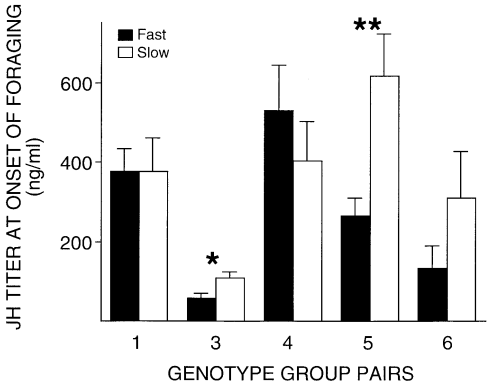

Fig. 4 Genotypic differences in JH titers (mean±SE) of new foragers. *t*-tests conducted on $n=12$ for genotype groups in pairs 1 and 3, $n=15$ for genotype groups in pairs 4–6 (*$P<0.05$, **$P<0.01$)

Experiment 3: genotypic differences in social inhibition sensitivity

We tested the hypothesis that fast bees are less sensitive than slow bees to social inhibition by using the "transplant" assay (Huang and Robinson 1992), in which foragers transplanted into a single-cohort colony inhibit precocious foraging of resident bees.

Methods. Foragers from the same colony were transplanted into two single-cohort colonies (each composed of 1000 bees), one with fast and the other with slow bees. The transplants were from a colony unrelated to any of the genotype groups used in this study. Preliminary studies conducted in Illinois and Mexico indicated that a transplant size of 15 foragers produced a circa 50% inhibition of precocious foraging (Fig. 5), so this transplant size was used throughout experiment 3. Colonies were closed for 6 days to allow transplants to interact with resident bees (Huang and Robinson 1992). Transplant survival was monitored daily and additional transplants added to maintain a constant number of 15. Colonies were housed in glass-walled observation hives for easy monitoring of transplant survival (hives were covered with panels of insulation at all other times).

After the 6-day confinement period, transplants were removed and colony entrances opened. Both colonies were observed simultaneously for equal amounts of time, twice daily (0900–1100 and 1600–1800 hours). Observations continued until resident bees were 12 days of age or a total of 50 precocious foragers were collected from both colonies, whichever came first. The numbers of precocious foragers from the two colonies were compared with *G*-tests; a greater number of precocious foragers was interpreted as reduced sensitivity to social inhibition.

Results. There were significant differences in the number of precocious foragers observed in three of six pairs of colonies (Fig. 6). In all three cases, it was the colony composed of fast bees that produced more foragers, suggesting that they were less sensitive to the inhibitory effects of the transplanted foragers. An experiment-wise analysis was conducted by combining the probabilities from the *G*-tests for all six pairs. The combined probability was significant ($P<0.001$, $\chi^2=56.75$, $df=12$). This result is consistent with the prediction that genotypic differences in sensitivity to social inhibition are important in determining genotypic differences in rates of behavioral development.

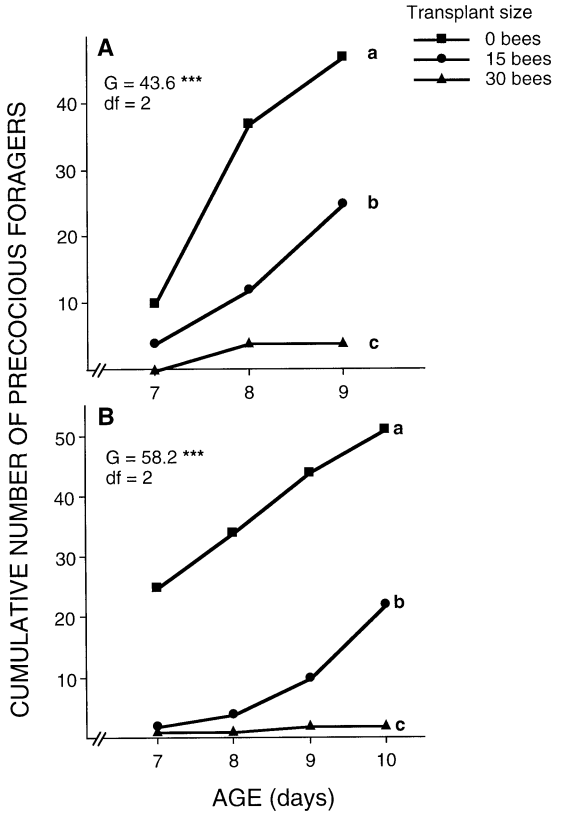

Fig. 5 Effect of transplant size on rate of behavioral development in single-cohort colonies in trials conducted in Illinois with European bees (**A**), and in Mexico with Africanized and European bees (**B**). *G*-tests (***P<0.001) on actual frequencies of precocious foragers and non-foragers; colonies with significantly different numbers of precocious foragers are indicated with different letters (P<0.05)

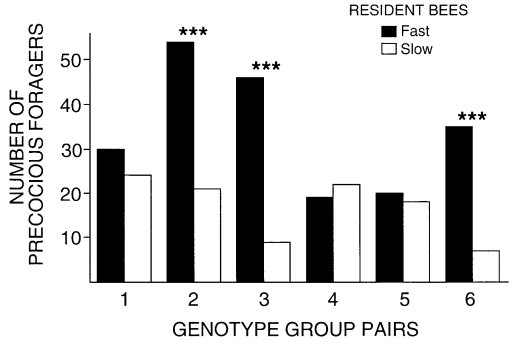

Fig. 6 Genotypic differences in sensitivity to social inhibition. A greater number of precocious foragers indicates lower sensitivity of resident bees to social inhibition. (2×2 *G*-tests, pair 2: G=15.6; pair 3: G=27.9; pair 6: G=21.0; ***P<0.001)

Experiment 4: genotypic differences in potency of social inhibition

We tested the hypothesis that fast bees have lower inhibitory potency than slow bees, again with the transplant assay. But for this experiment the *resident* bees for the two single-cohort colonies came from a colony unrelated to any genotype group pair, and the *transplants* were 15 foragers from a fast or slow genotype group. Otherwise the experiment was performed identically to experiment 3.

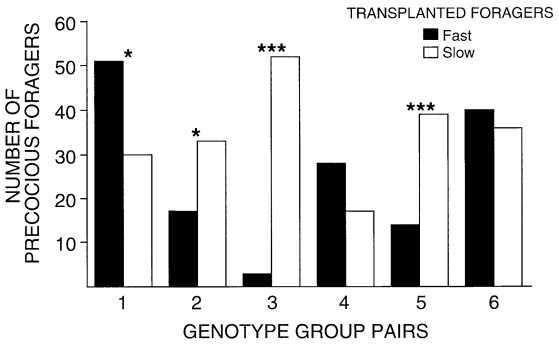

Fig. 7 Genotypic differences in potency of social inhibition. A greater number of precocious foragers indicates lower social inhibition potency by the transplanted bees. Analyses as in Fig. 5 (pair 1: G=5.7; pair 2: G=5.3; pair 3: G=54.2; pair 5: G=12.6; *P<0.05, ***P<0.001)

Methods. Since it is known that some foragers "drift" into colonies other than their own, the following precautions were taken to ensure that foragers selected as transplants truly belonged to the source colony from which they were collected. In the Illinois study, drifting was minimized by locating each source colony more than 20 m away from the nearest colony and facing it in a different direction. In the Mexico study, source colonies had to be located closer together, so samples of 50 foragers from each source colony were collected for verification purposes. The wing lengths of these foragers were measured (Sylvester and Rinderer 1987) and compared with those of callow workers (>1 day old) collected from frames in the same colony. There were no significant differences for any of the six source colonies (data not shown). Approximately 2–4% of the foragers had wing length measurements that were outside the range for the callow bees; if this reflects the composition of the transplant groups, <1 in each group of 15 transplanted foragers might have originated from a colony other than the source colony from which it was sampled.

A greater number of precocious foragers in one colony was taken to mean that the transplant in that colony (composed of either fast or slow bees) exhibited reduced social inhibition potency.

Results. The number of precocious foragers observed between colonies was significantly different in four of six pairs (Fig. 7). However, there was no consistent difference between fast and slow genotype groups. In pair 1, the colony that received the forager transplant from the fast genotype group produced significantly more precocious foragers, indicating that the fast genotype group had lower social inhibition potency. The opposite result was obtained in pairs 2, 3, and 5. A combined probability analysis showed higher social inhibition potency overall for fast bees (P<0.005, χ^2=27.58, df=12). Our hypothesis was not supported; we predicted *lower* social inhibition potency for fast bees.

Experiment 5: genotypic differences in rate of behavioral development under cross-fostered conditions

Experiments 1–4 were each designed to probe for genotypic differences in a single physiological component

thought to be related to behavioral development. Experiment 5 was designed to study genotypic differences under more natural conditions, where all four components can act simultaneously. This allowed us to determine whether some components play a stronger role in governing the rate of behavioral development than others. The four components can be grouped into two types of effects, those occurring at the individual level and those occurring at the colony level. A difference in JH production, JH sensitivity, or social inhibition sensitivity of an individual bee affects only the rate of behavioral development of that particular individual. In contrast, a difference in the social inhibition potency of an individual bee exerts a colony effect – it affects other individuals in the colony.

Methods. We cross-fostered 100 1-day-old fast and slow bees in pairs of triple-cohort colonies. Each triple-cohort colony was entirely composed of either the fast or the slow genotype group. Triple-cohort colonies were each composed of 500 1-day-old bees, 500 nurses, and 500 foragers (Giray and Robinson 1994). The colonies thus had a somewhat more natural age structure than single-cohort colonies, but still differed from the continuous age distribution found in typical colonies. Using triple-cohort colonies allowed us to control age demography and population size precisely, facilitating inter-colony comparisons.

Nurses and foragers were vacuum collected from a source colony directly into a small beehive. Nurses were identified as bees with their heads in cells with larvae (e.g., Robinson 1987). Foragers were identified as described above and collected at the hive entrance by blocking it intermittently with a wire-mesh screen. One-day-old bees were obtained as described in the General approach; they were marked on the thorax with paint and served as the focal bees in each colony. Additional groups of 500 1-day-old bees were added on days 3 and 5 of the experiment. Despite the relatively small size of these colonies (2500 bees), the age at which foraging begins has been shown to be the same as in colonies with more typical population sizes (Giray and Robinson 1994).

Colonies with cross-fostered bees were studied in pairs. Both colonies were set up at the same time, in the same apiary, and were observed for the same amount of time and at the same times of the day (by one observer in alternating fashion). Entrance observations of paint-marked focal bees began on day 10 (as described above). Observations continued until about 50 foragers were collected from both colonies or until day 28, whichever came first.

A two-way ANOVA [on log(x)-transformed data] was used to compare the age at onset of foraging for focal bees from the fast and slow genotype groups in fast and slow host colonies. Post hoc t-tests (Sokal and Rohlf 1995) also were used to determine whether there were significant worker genotype differences within each host colony. We examined effects of host colony, worker genotype, and their interaction on age at onset of foraging. With four factors potentially contributing to the behavioral variation, the results of such a study can be interpreted in several different ways. We have attempted to provide the simplest and broadest interpretation of these results. A significant effect of worker genotype is interpreted as evidence for variation in one or more of the individual-level components (JH production, JH sensitivity, or social inhibition sensitivity). A significant effect of host colony is interpreted as evidence for variation in the colony-level component, social inhibition potency. An interaction effect indicates differences in both individual and colony-level components.

Results. Mean ages at onset of foraging are presented in Fig. 8; statistical analyses are given in Fig. 8 and Table 2. The following summarizes the results for each pair.

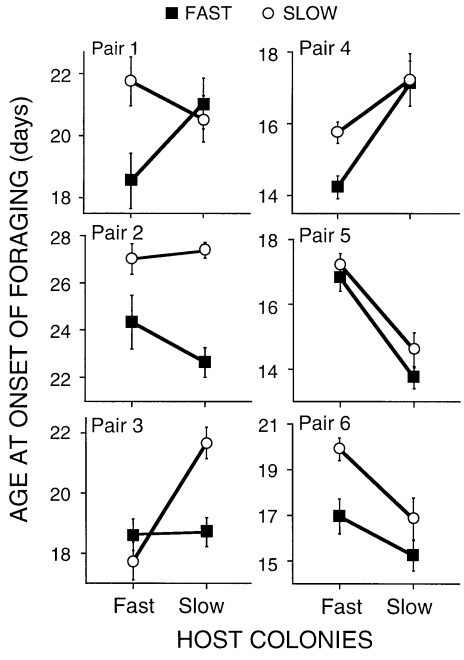

Fig. 8 Genotypic differences in age at onset of foraging (mean±SE) under cross-fostered conditions. Two-way ANOVA results in Table 2

Table 2 Results of two-way ANOVA (P-values; *italicized* results are significant) for differences in age at onset of foraging in a cross-fostering experiment. Data in Fig. 8 [for pair 4, worker genotype differences in the fast host colony (see Fig. 8) were detected by post hoc t-test comparison (t=– 3.67, df=72, P<0.0005)]

Pair	Worker genotype	Host colony	Interaction
1	0.11	0.37	*0.03*
2	*0.0004*	0.50	0.32
3	0.16	*0.01*	*0.02*
4*	0.07	*0.0001*	0.09
5	0.13	*0.0001*	0.56
6	*0.01*	*0.01*	0.41

Pair 1. There was a significant interaction between worker genotype and host colony on age at onset of foraging. Focal bees initiated foraging at younger ages in the fast host colony. The simplest explanation for this result is that fast bees had both relatively lower inhibitor potency and differed from slow bees in one or more of the individual-level components.

Pair 2. There was a significant effect of worker genotype on age at onset of foraging. This suggests that fast bees had relatively higher JH production, higher JH sensitivity, lower social inhibition sensitivity, or some combination of these three individual-level components. Absence of a host colony effect suggests that there were no differences in inhibitor potency between fast and slow bees.

Pair 3. There were significant effects of host colony and the interaction of worker genotype and host colony on age at onset of foraging. Focal bees initiated foraging at younger ages in the fast host colony. These results

Table 3 Comparison of results from single-component assays (experiments 1–4) and cross-fostering experiments (experiment 5). The relative differences between the six different pairs of fast (*F*) and slow (*S*) genotype groups are summarized for experiments 1–4. Experiments 1–3 measure individual-level components: JH production (*JHP*), JH sensitivity (*JHS*), and social inhibition sensitivity (*SIS*). Experiment 4 measures the colony-level component: social inhibition potency (*SIP*). The agreement for comparisons of F and S genotype groups in experiment 5 vs experiments 1–3 and experiment 4 are indicated by *Yes* or *No* in the individual-level and colony-level component columns, respectively

Pair	Experiment 1 JHP	Experiment 2 JHS[a]	Experiment 3 SIS	Experiment 4 SIP	Experiment 5	
					Individual-level components	Colony-level component
1	F=S	F>S	F=S	F<S	Yes	Yes (F<S)
2	F>S	F=S	F<S	F>S	Yes	No (F=S)
3	F>S	F>S	F<S	F>S	Yes	No (F<S)
4	F>S	F=S	F=S	F=S	Yes	No (F<S)
5	F=S	F>S	F=S	F>S	No	Yes (F>S)
6	F>S	F=S	F<S	F=S	Yes	No (F>S)

[a] Results from methoprene treatment assay only for pairs 1–3

most likely indicate that fast bees had lower inhibitor potency, and differed from slow bees in one or more of the individual-level components.

Pair 4. There was a significant effect of host colony on age at onset of foraging. Focal bees initiated foraging at younger ages in the fast host colony. This result most likely indicates that fast bees had lower inhibitor potency. The effect of worker genotype was almost significant, and a post hoc comparison of fast and slow bees in the fast host colony revealed a significant difference for age at onset of foraging. These results suggest that there was also a difference in one or more individual-level components for the genotype groups in this pair.

Pair 5. There were significant effects of host colony on age at onset of foraging. Focal bees initiated foraging later in the fast host colony. This result most likely indicates that fast bees had higher inhibitor potency.

Pair 6. There were significant effects of worker genotype and host colony. These results most likely indicate genotypic differences in both the colony-level and individual-level components. Focal bees initiated foraging at older ages in the fast host colony, indicating that fast bees had higher inhibitor potency.

Table 3 summarizes the results from the single-component assays (experiments 1–4) and the inferences drawn from cross-fostering experiments (experiment 5) for each pair of genotype groups. Single-component assays revealed genetic variation for JH production in four of six pairs, sensitivity to JH in three of six pairs, sensitivity to social inhibition in three of six pairs, and potency of social inhibition in four of six pairs. Cross-fostering experiments revealed genetic variation for individual-level components in five of six pairs, and potency of social inhibition also in five of six pairs.

A comparison of results from experiments 1–4 and experiment 5 is presented in Table 3. Individual-level components differed significantly and in the predicted direction in both single-component assays (experiments 1–3) and cross-fostering experiments in five of six pairs. The colony-level component, social inhibition potency, differed significantly and in the predicted direction in both single-component assays (experiment 4) and cross-fostering experiments in two of six pairs. In addition, bees from genotype groups that were identified to be developing fast in the initial screen showed faster rates of behavioral development in cross-fostering assays. Fast bees were faster than slow bees in 7 of 12 colonies and not different from slow bees in the remaining 5 colonies (P=0.039, χ^2=6.5, df=2); fast bees were never slower than slow bees in a cross-foster colony.

Discussion

The principal significance of these results is their demonstration of genetic variation for physiological components that are hypothesized to play a role in determining genetic differences in the rate of behavioral development in honeybees. In all six cases, genotype groups with fast and slow rates of behavioral development differed in at least one component in a manner consistent with the observed behavioral differences. Fast and slow bees often differed in more than one component, with no consistent pattern of association among any of the components. With data from only six comparisons, these findings cannot be used to conclude that some components are more important than others. Rather, what emerges most clearly from this study is that natural selection can potentially act on at least several, apparently distinct, physiological processes associated with worker JH and age demography to alter the rate of honeybee behavioral development.

Methodological issues

Some of the results of this study depend on highly artificial and indirect assays. We now discuss some of the limitations associated with these assays.

Results of the JH production assay revealed that fast bees tend to have higher JH titers than slow bees, but the patterns of these differences varied. This variation may

have been an artifact of maintaining the bees in social isolation during this experiment. Social isolation was necessary, however, to avoid the potentially confounding effects of genotypic variation in social inhibition sensitivity and potency.

We relied upon two indirect assays to probe for differences in JH sensitivity because no JH receptor has yet been characterized. The rationale for the methoprene sensitivity assay is discussed above. The rationale for measurements of JH in new foragers assumes that the JH titer of a new forager is in some way directly related to its new occupation, with more sensitive genotypes requiring a lower threshold dose to initate foraging. However, recent results, obtained after this study was performed, demonstrate that bees with their corpora allata removed can develop into foragers, albeit at later ages (Sullivan et al., in press). In our study, the assay based on measurements of JH in new foragers was less successful in detecting genotypic differences than the first approach, perhaps because the above-mentioned assumption is not met in honeybees. Until it is possible to work directly with a JH receptor, we suggest that any further work on genotypic differences in JH sensitivity use the assay from experiment 2A, methoprene treatment.

In some cases, a component shown to vary between genotype groups in a single-component assay (experiments 1–4) was not found to vary in the cross-fostering experiment (experiment 5), and vice versa. The biggest discrepancies involved experiments 4 and 5. Results of experiment 4 showed differences in four of six pairs, but only two of these pairs differed in this way in experiment 5. This is surprising given that the assay used in experiment 4 was a "mirror image" of that used in experiment 3 and the results of this experiment showed better agreement with the results of experiment 5. In retrospect, there is an important difference in methodology between the assays used in experiments 3 and 4. In experiment 3, the transplanted foragers were from a pre-tested colony; we showed that a transplant of 15 foragers from that very colony was sufficient to cause a 50% inhibition of precocious foraging (Fig. 5). In contrast, the colony used to supply the resident bees in experiment 4 was not pretested. If these resident bees were highly sensitive to social inhibition then it would be harder to detect differences in social inhibition potency of foragers from fast and slow genotype groups.

Activator-inhibitor model

Our results are consistent with the model of Huang and Robinson (1992, 1999). This descriptive model seeks to explain how JH and colony age demography can influence the rate of behavioral development in honeybees. According to this model, JH acts as one internal activator: as levels of this hormone increase, the likelihood of initiating foraging increases. Older bees are hypothesized to inhibit the rate of behavioral development, presumably via an inhibitory effect on the rate of JH increase. Empirical support for this model has been published (Huang and Robinson 1992, 1996; Huang et al. 1998), but a specific worker inhibitor has not yet been identified. Our results are consistent with the activator-inhibitor model because they demonstrate that genetic variation for the four components identified in this model are associated with genetic differences in rate of behavioral development. Another result consistent with the activator-inhibitor model is the finding that a transplant of 30 foragers exerted a greater inhibitory effect on the rate of behavioral development than one of 15 foragers (Fig. 5). This finding, though limited to just two replicates, supports the suggestion that social inhibition occurs in a "dose"-dependent manner.

Huang and Robinson (1992) suggested that levels of JH and social inhibitor potency increase together during behavioral development. We did not test this hypothesis on individual bees, but our results show that social inhibition potency is not consistently associated with either fast or slow rates of behavioral development. This suggests that, while there may be an ontogenetic linkage between levels of JH and social inhibition potency, there is no linkage between genetic variation in JH production and social inhibition potency.

Colony life history analysis

Giray and Robinson (1994) proposed that colony age demography is one factor that influences norms of reaction (Stearns 1989) for the rate of behavioral development in honeybees. Our results suggest that colony genetic structure is another factor that can influence these norms of reaction. In paired colonies with the same age structure, differences in rates of behavioral development were detected in one genetic background and not in the other. Calderone and Page (1992) suggested that colony genetic structure can influence the norms of reaction for another honeybee trait associated with division of labor, the tendency to collect either nectar or pollen.

This study confirms that Africanized bees begin foraging at younger ages than European bees, as first reported by Winston and Katz (1982). Using a similar cross-fostering experimental design, Winston and Katz (1982) found, as we did, strong host colony effects on age at first foraging, again suggesting the importance of social inhibition (or other factors that were not studied). Although Africanized bees in general show faster rates of behavioral development than European bees, they showed no obvious differences in the prominence of any one of the four components studied here. This conclusion is limited by the fact that we only tested three Africanized bee genotype groups, but it is consistent with our findings in general. Our data do not allow us to infer that nature has favored a single mechanism for producing fast or slow rates of behavioral development in Africanized and European honeybees despite the different environments in which they evolved. This tentative conclusion should be examined more rigorously by study-

ing pure races of honeybees from different parts of the Old World, rather than the more "mongrel" Africanized and European bees available for study in the New World.

At least two of the four components associated with genetic variation in rate of behavioral development in honeybees involve the JH system. Genetic variation for traits related to JH has been found in other insect species (e.g., Shemshedini and Wilson 1990; Muszynska-Pytel et al. 1992; Zera and Zeisset 1996). Recent studies have revealed the importance of hormonally regulated traits in life history evolution, emphasizing that hormones exert "pleiotropic" effects on suites of characters (reviewed in Finch and Rose 1995; e.g., Gu and Zera 1996; Ketterson et al. 1996). Future studies that explore more broadly the phenotypic consequences of genetic variation for the four components studied here may help us understand the evolution of physiological mechanisms regulating behavior and their role in providing for plasticity in age-related division of labor, an adaptive feature of colony design.

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