

Kin discrimination by worker honey bees in genetically mixed groups

(kin recognition/*Apis mellifera*/sibling group/aggressive behavior)

MICHAEL D. BREED, LINDA BUTLER, AND TAMMY M. STILLER

Department of Environmental, Population, and Organismic Biology, Campus Box 334, The University of Colorado, Boulder, CO 80309

Communicated by Charles D. Michener, December 3, 1984

ABSTRACT We tested the hypothesis that in a genetically mixed assemblage of worker honey bees, individual workers would behave differently toward unfamiliar sisters than toward unfamiliar nonsisters. Groups of worker honey bees of mixed genetic composition were assembled by collecting pupae from separate colonies and placing the worker bees together on eclosion. A total of 10 workers, 5 from each of two kin groups, were used to form each group. When the workers were 5 days old, a worker of one of the two kin groups was introduced into the mixed group. This worker had previously been held in a group of its sisters, without contact with queen or nonsister bees. The interactions with the introduced bee indicate that in a mixed kin group, individual workers learn the composite identity of the group and do not attack unfamiliar bees differentially on the basis of kinship. However, kinship does influence the total number of interactions in which an introduced bee engages when placed in a genetically mixed group; bees interacted significantly more often with sisters than with nonsisters. There was a trend for bees to be involved in more feeding interactions with sisters. This finding indicates an ability of a bee to learn and use its own cues. In mixed groups, each bee maintains its genotypically correlated identity; the bees' odors do not come into a "group" or "gestalt" odor. The significance of these results is discussed in light of the genetic structure of natural colonies of honey bees.

Kin recognition has become a central issue in animal behavior. Of particular interest have been mechanisms that might facilitate differential responses to individuals on the basis of relationship. Honey bees (*Apis mellifera*) have the ability to discriminate sisters from nonsisters (1) and half sisters from full sisters (2), by using cues that are correlated with genotype. The purpose of this paper is to test for differential behavior when bees are maintained in genetically mixed groups.

The system used by honey bees to recognize nest mates has been the subject of many studies. Early reports, such as that of Kalmus and Ribbands (3), emphasized the importance of odor cues acquired from the environment in establishing differences between colonies that could be used by guard bees in discriminating between nest mates and non-nest mates. Similar findings were later presented concerning the recognition of queens (4) by nest mates. The finding that genotypically correlated cues could be used by sweat bees to identify nestmates (5) stimulated more refined tests for genotypically correlated recognition cues (recognition phenotypes) in the honey bee (1, 6, 7). In the absence of environmentally derived recognition signals, the honey bee queen and workers still possess recognition cues; these cues are more similar among bees that are closely related genetically. Buckle and Greenberg (8) provided an elegant model

of the process by which individual sweat bees learn the recognition characteristics of their nest mates. Their model provides a conceptual framework in which learning can be studied in the honey bee.

Central to the environmental odor hypothesis is the process of mixing of individual odors, presumably by contact among individuals, to produce a group odor. Such a process may also yield a colony "gestalt" of genotypically correlated odors (9, 10). We used genotypically mixed groups of *Apis mellifera* to test the hypothesis that a gestalt group odor is present.

The presence and use of genetically correlated (similar among relatives) cues by the honey bee presents something of a paradox when the mating system and sperm utilization of this species are considered. Honey bee queens are known to mate many times prior to onset of egg laying (11). Thus, from the standpoint of a worker bee, the colony is potentially a constantly changing mixture of full and half sibs. Under such circumstances, workers should have the capacity to learn the cues of surrounding bees, rather than relying on a comparison of their own cues with those of surrounding bees. However, under certain circumstances, such as the rearing of a new queen (12–14), the capacity to recognize full sisters within genetically mixed groups might become important. Until now, no study has specifically addressed the issue of whether such preferential treatment occurs in the context of nest-mate recognition. In fact, both mechanisms could coexist—learning of the surrounding individuals for use in certain contexts and use of self-identifying cues for others.

MATERIALS AND METHODS

The methods used in this experiment are similar to those used by Breed (1). A more detailed discussion of the behavioral categories used in this study is given by Breed (15).

Combs containing worker pupae were removed from colonies A and B as needed and were incubated in the laboratory. In the following description, bees are referred to as coming from the same or different combs, designated A and B according to their colonies of origin. It is important to note that this means that bees from the same comb are part of the same genetic family (full sisters and half sisters), and bees from different combs are from different genetic families (nonsisters). (In *Results* and *Discussion* we sometimes refer to bees as being sisters or nonsisters.) As worker bees emerged, they were collected and placed in groups in 0.855-liter cardboard test containers with food [sugar candy with 5% pollen (wt/wt)] and distilled water ad lib. The term "group" refers to the bees in each single container.

All bees tested were 5 days old. Single bees from combs A or B were transferred to recipient groups. The transfer was made with forceps in a manner that minimized the disturbance of the transferred individual and of the recipient group. The interactions between the introduced individual and the bees in the recipient group were observed through

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

clear plastic lids on the cardboard containers for 10 min after the first contact between the introduced bee and a resident bee.

The categories of behavior used were (i) sustained biting or stinging of the introduced bee by a resident bee; (ii) brief bite of the introduced bee; (iii) antennation of the introduced bee; (iv) introduced bee solicits food from a resident and is fed; (v) resident bee solicits food from the introduced bee and is fed; (vi) resident refuses to feed the intruder; (vii) intruder refuses to feed the resident; (viii) the introduced bee offers food to the resident; (ix) the resident offers food to the intruder; and (x) contact behavior not involving antennation or feeding. Biting and stinging are clearly aggressive acts. They are referred to as "attack" in parts of the data analysis. Refusal to feed is indicative of a lack of cooperation and is correlated with aggressive acts (15). Feeding behavior is cooperative, and antennation may represent exploration and olfaction. The occurrence and frequency of each of these acts involving sisters and nonsisters of the intruder are analyzed, as indicated in *Results*.

Responses of Genetically Mixed Groups to Introduced Bees.

Hypothesis: A bee in a mixed group will respond differently to a nongroup sister than to a nongroup bee from the other comb.

Each set-up for this experiment consisted of 10 recipient groups and a source group. The source group consisted of 10 bees from a single comb and provided individuals to be transferred, one each, into mixed (A and B) recipient groups (Fig. 1).

A number of replicate set-ups were used. In some cases, mortality within a source container decreased the number of replicates obtained below the planned number. Consequently, the reported results are not exact multiples of the planned 10 recipient groups per source group.

Controls (Fig. 1) were used to verify that bees used in these experiments expressed the same levels of aggression toward bees from the same comb as were found by Breed (1). They also provide baseline data on the rate of nonag-

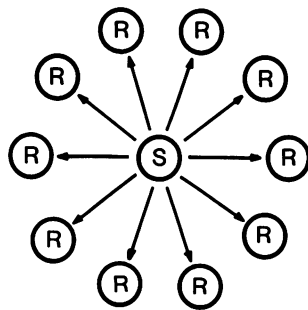


FIG. 1. Diagrammatic representation of the experimental design. In each experiment, individual bees were removed from the source group, S, in the center and transferred to the recipient group, R. Each bee from the source container was used only once, and each recipient group was used only once. Combs containing pupae from colonies A and B provided the bees. Experiments are designed to test for genetic differences between A and B. Specific experimental designs are as follows: (i) Behavior of bees in genetically mixed groups. Controls: The source group was 10 bees from comb A (colony A). Each recipient group was 10 bees from comb A. Experimentals: The source group was 10 bees from comb A. Each recipient group contained 5 bees from comb A and 5 from comb B. (ii) Gestalt odor experiment. This experiment was slightly different in that the source group was 20 bees, 10 from comb A and 10 from comb B. There were 20 recipient groups. One-half of the recipient groups contained 10 bees from comb A. The other half contained 10 bees from comb B. Transfers were done so that equal numbers of the possible combinations were made—i.e., 5 A bees were transferred to A groups, 5 A bees were transferred to B groups, and so forth.

gressive interactions between the resident bees and the introduced bee. This control was also used in comparisons in the gestalt experiment (see below). The controls repeat and corroborate previous work (1) (see Table 3).

Acquisition of Gestalt Odors in Genetically Mixed Groups.

Hypothesis: If bees from two combs are mixed, then each individual acquires an odor mix that results in it being treated as intermediate in recognition characteristics between bees from the two original combs.

The experimental approach was similar to that described above (Fig. 1). The specific design differs in the number of bees in the source group (20 in this case) and in the corresponding number of recipient groups (20 also). The source group was a mixture (50/50) of bees from combs A and B. One-half of the recipient groups contained only bees from comb A; the other half contained only bees from comb B. The transfers were designed to answer the following question: Does a comb A bee acquire odors from the comb B bees in the source group so that it is then either less acceptable to a group of comb A bees or more acceptable to a group of comb B bees?

RESULTS

Total Interactions with Introduced Bees. The first question considered is whether sister bees interact more or less than nonsisters with the introduced bee. Sisters engaged in a mean of 4.6 (SD = 3.01; $n = 85$) behavioral acts (this includes all categories mentioned in *Materials and Methods*) toward the introduced bee, while nonsisters engaged in a mean of 3.7 (SD = 2.59; $n = 85$) such acts. These response rates are significantly different [paired comparison analysis of variance (ANOVA); $F = 5.59$; $P < 0.05$]. In the control groups (Fig. 1), the mean number of interactions with the introduced bee was 8.1 (SD = 5.34; $n = 100$); the total for both sisters and nonsisters in the experimental groups was 8.4 (SD = 4.8; $n = 85$). These are not significantly different (ANOVA; $F = 0.155$; not significant).

Aggressive Responses to Introduced Bees. In 17 (20%) of 85 experimental groups the introduced bee was the target of biting or stinging. This was not significantly different from the control groups, in which 20 of 100 (20%) introduced bees were attacked ($\chi^2 = 0$; not significant). In the test replicates, a total of 29 bees were involved in the biting and stinging directed toward the 17 introduced bees (more than one bee in each recipient group might attack). Of these 29 bees, 19 were sisters of the introduced bee and 10 were nonsisters ($\chi^2 = 2.79$; not significant). Ten introduced bees were attacked only by sisters and 4 bees were attacked only by nonsisters; the remainder were attacked by both classes of bees ($\chi^2 = 2.57$; not significant). Since the initial attack might attract other bees or synergize the activity of potential attackers, the first attacking individual was analyzed separately. In 12 cases, the first attacking bee was a sister to the introduced bee, and in 5 cases she was a nonsister ($\chi^2 = 2.88$; not significant).

Feeding Behavior. Feeding behavior involves solicitation, feeding in response to solicitation, refusal to feed in response to solicitation, and offering food when not previously solicited. The simplest question was to ask whether sisters or nonsisters were involved in more feeding interactions with the introduced bee. Perhaps food flow within the group is structured around genetic groups. In each replicate, then, the total number of soliciting and feeding interactions (behaviors d, e, h, and i) was calculated for each replicate and a paired comparisons *t* test was performed to compare interactions of the introduced bee with sisters and nonsisters. Although the resident sisters tended to be involved in more total feeding interactions with the introduced bee than the nonsisters, no statistically significant difference was

found (Table 1). A trend in the same direction exists for solicitation by the resident bees (Table 1), but in this case it is statistically significant. However, there was no significant difference between solicitations by the introduced bees. Finally, refusal to feed in response to solicitations by the introduced bee yielded no significant difference between sisters and nonsisters.

Introduced bees offered food significantly more often (27 times) than resident bees (14 times) ($\chi^2 = 4.12$; $P < 0.05$). Offering food could serve as an appeasement act to decrease the chances of being attacked. Twenty-seven introduced bees offered food, 15 to sisters and 12 to nonsisters (this difference is not significant, $\chi^2 = 0.33$).

Gestalt Odors. When bees maintained in genetically mixed groups (combs A and B) were introduced into a group consisting of bees from only one of the combs, there was a clear difference in behavior depending on whether the introduced bee was from the same comb as the recipient bees (Table 2). In introductions in which the recipient bees were sisters to the introduced bee, 28% of the introduced bees were attacked ($n = 75$). In cases in which the introduced bee was a nonsister, 55% of the introduced bees were attacked ($n = 74$). There is a significant difference between these frequencies of attack ($\chi^2 = 11.1$; $P < 0.005$).

These data are more meaningful when compared to the control data and to data obtained in other studies (Table 3). There is no apparent effect of maintenance condition on attack rate (whether the source group contained bees from the same comb or from two combs). When the bee from a group of sisters only is transferred into a group of sisters, the probability of attack is between 0.13 and 0.30. If the odors of bees from the other comb in the mixed group rubbed onto these bees, then an increased rate of attack would be expected; this is not the case, as the rate was 0.28. When the bee is transferred into a group of nonsisters, the probability of attack in controls was 0.56 and 0.69. A decrease would be expected if odors were mingled. The probability obtained, 0.55, is not different from the expected values, indicating no mingling of odors.

DISCUSSION

In the behavioral context of nest-mate recognition, worker bees that have matured in genetically mixed groups do not use cues related to kinship to modify their aggressive behavior. An intruder is as likely to be attacked by a sister as by a nonsister if the two classes of bees have lived together. There are intriguing differences in behavior between sister and nonsister interactions, however, that lead to overall higher levels of interaction between sisters; this is due in part

Table 1. Mean number of total feeding interactions and of selected feeding acts during 10-min observation periods

	Sister	<i>F</i>		Nonsister
Total feeding interactions of introduced bee	1.3 ± 1.44	1.01	NS	1.1 ± 1.50
Introduced bee solicits food	0.31 ± 0.574	1.70	NS	0.46 ± 0.952
Resident bee solicits food from introduced bee	0.68 ± 1.008	4.23	*	0.45 ± 0.760
Introduced bee refuses to feed	0.27 ± 0.581	0.27	NS	0.31 ± 0.686
Resident bee refuses to feed introduced bee	0.26 ± 0.556	0.69	NS	0.20 ± 0.455

Means are shown ± SD. Sample size is 85 in each case. NS, not significant; *, $P < 0.05$. All comparisons are by analysis of variance.

Table 2. Results from gestalt odor experiment

Set-up	Type of transfer			
	A to A	B to B	A to B	B to A
1	2 (5)	3 (5)	1 (5)	1 (5)
2	0 (5)	0 (5)	1 (5)	0 (5)
3	0 (5)	0 (5)	3 (5)	2 (5)
4	3 (4)	1 (4)	3 (4)	2 (4)
5	1 (5)	0 (4)	2 (4)	2 (4)
6	5 (5)	1 (4)	2 (4)	5 (5)
7	2 (5)	0 (4)	4 (5)	5 (5)
8	0 (5)	3 (5)	5 (5)	4 (5)
Subtotal	13 (39)	8 (36)	21 (37)	20 (37)
	Total	21 (75)		41 (74)

Data from each set-up are given separately. Number of bees attacked is shown, with the total number transferred in parentheses. A and B denote different combs. The type of transfer (see Fig. 1) is indicated.

to a trend for intruders to be involved in more feeding interactions with sister bees.

The result with aggressive behavior supports the theory that bees learn a composite of available recognition cues for use in discrimination of nest mates from non-nest mates, and consequently the colony is able to adjust to changing patterns of paternity. The present study does not answer the question of how the colony adjusts if a cohort of workers emerges that has different paternity than any existing cohort in the colony. Further studies will also be required to fully explain how such mechanisms affect the interactions between guard bees of one colony and robbing foragers of another colony.

Perhaps more surprising than the result with aggressive behavior were the results with feeding behavior. In this case, workers seem to discriminate their own genotype. This means that they have the capacity to learn the recognition cues they produce, independent of those produced by surrounding individuals, or that they have a genetic program (recognition alleles) that carries this information internally (16). In general, in social insect colonies food is collected and brought to the colony by workers. It is passed among the workers, but normally a significant proportion of the collected food ultimately goes to the reproductives and developing larvae in the colony. Direction of food flow toward full sister groups might result in some ultimate selective advantage to a given sibship (e.g., a buildup of one full sister group at the expense of others might allow the first to control the genotype of the larvae selected for queen rearing). In the artificial context of the experiments presented here, there is

Table 3. Comparison of attack rates (%) on bees kept in different types of groups

Maintained with	Transferred to groups of		
	Sisters only	Nonsisters only	Sisters and nonsisters
Sisters only	20 (100) 30.6 (108)* 13 (69)*	68.7 (99)* 56.4 (39)*	20 (85)
Sisters and nonsisters	28 (75)	55 (74)	NA

Sisters are from the same comb; nonsisters are from different combs. Sample size is in parentheses. χ^2 test for heterogeneity in the sisters only column was not significant ($\chi^2 = 8.69$; 7 degrees of freedom); the nonsisters only column also yielded a result that was not significant ($\chi^2 = 3.6$; 5 degrees of freedom). NA, not available. *From ref. 1. The sisters only to nonsisters only transfers were performed in two separate experiments in ref. 1.

some evidence for such direction, but further experiments will be required to elucidate such differential interactions.

In the controls, a moderate level of rejection of introduced bees is expected because of the divergence between groups caused by genetic sampling and environmental differences outside the investigators' control. The lack of a significant difference between the experimental and control groups in levels of aggression to introduced bees indicates that changing the genetic diversity of the recipient group did not affect those parameters.

The slight difference in interaction rate between the experimental and control groups may be a result of higher levels of genetic variation in the test groups; perhaps, when such levels are high, bees require more interaction to make discriminations.

The offering of food, in the form of a visible droplet, is clearly more often from the introduced bee to a resident bee, rather than the reverse. This may decrease the likelihood of attack. Ribbands (17) came to a similar conclusion from observations of bees attempting to enter hives other than their own.

In summary, our results indicate that honey bee recognition cues used in discrimination of nest mates are learned as a composite that includes information from surrounding individuals. This composite may include both information produced by the individuals themselves and information gained from the environment (i.e., the comb) prior to adult emergence. Previous results (1, 2) support the hypothesis that cues are produced by the bees themselves. Honey bees may also use information concerning kinship in other contexts, such as feeding.

One drawback to the experiment as designed in this study is that the workers were the offspring of multiply inseminated queens. Artificial insemination could be used to decrease the amount of genetic variation among the sister bees, which include a mixture of half and full sibs. Such reduced variation might amplify the differences between the experimentals and controls.

A more appropriate experiment to test the model developed by Buckle and Greenberg (8) would be to construct mixed groups with nine bees from one genetic source and one bee from another source. Unfortunately, in honey bees the identity of the guarding (primed for aggression) individuals cannot be controlled as easily as in *Lasioglossum zephyrum*. Thus, an unfeasibly large number of set-ups would be needed to obtain the required number in which the "odd bee" was aggressive. The experiments reported here,

however, do indicate that bees use information other than the simple composite identity of the other bees in the group.

The significant difference between sister and nonsister introductions in the gestalt experiment indicates that odor mixing due to physical contact among the bees is not a factor in modifying recognition characteristics used in nest defense. Sisters returned to groups of sisters in this experiment were attacked with approximately the same frequency as in transfers from sister groups done by Breed (1). This leads to the hypothesis that bees either learn individual odors of surrounding bees and then use an internal process to generate a template that can be used to approximately discriminate previously unmet nestmates, or that bees somehow learn the recognition characteristics of several different genetic groups in the colony simultaneously. More data will be required to refine these models for nestmate recognition.

This research was supported by Grant BNS 82-16787 from the National Science Foundation.

1. Breed, M. D. (1983) *Anim. Behav.* **31**, 86-91.
2. Getz, W. M. & Smith, K. B. (1983) *Nature (London)* **302**, 147-148.
3. Kalmus, H. & Ribbands, C. R. (1952) *Proc. R. Soc. London Ser. B* **140**, 50-59.
4. Boch, R. & Morse, R. A. (1981) *Ann. Entomol. Soc. Am.* **74**, 66-67.
5. Kukuk, P. F., Breed, M. D., Sobti, A. & Bell, W. J. (1977) *Behav. Ecol. Sociobiol.* **2**, 319-327.
6. Breed, M. D. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2635-2637.
7. Boch, R. & Morse, R. A. (1982) *Ann. Entomol. Soc. Am.* **75**, 654-656.
8. Buckle, G. R. & Greenberg, L. (1981) *Anim. Behav.* **29**, 802-809.
9. Carlin, N. F. & Holldobler, B. (1983) *Science* **222**, 1027-1029.
10. Crozier, R. H. & Dix, M. W. (1979) *Behav. Ecol. Sociobiol.* **4**, 217-224.
11. Page, R. E. (1980) *Genetics* **96**, 263-273.
12. Getz, W. M., Bruckner, D. & Parisian, T. R. (1982) *Behav. Ecol. Sociobiol.* **10**, 265-270.
13. Page, R. E. & Erickson, E. (1984) *Ann. Entomol. Soc. Am.* **77**, 578-580.
14. Breed, M. D., Velthuis, H. H. W. & Robinson, G. (1984) *Ann. Entomol. Soc. Am.* **77**, 737-739.
15. Breed, M. D. (1983) *Insectes Soc.* **30**, 482-495.
16. Blaustein, A. R. (1983) *Am. Nat.* **121**, 749-754.
17. Ribbands, C. R. (1954) *Proc. R. Soc. London Ser. B* **142**, 514-524.