

VARIABILITY OF CHEMOSENSORY STIMULI WITHIN  
HONEYBEE (*Apis mellifera*) COLONIES:  
Differential Conditioning Assay for Discrimination Cues

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**Abstract**—Differential training of honeybee workers using the proboscis extension reflex is applied to the problem of evaluating compounds that may potentially provide cues for kin recognition in the honeybee *Apis mellifera*. These cues were obtained by contaminating glass rods and steel needles with different materials found in the hive. In particular it is shown that workers discriminate between: cuticular waxes from different adult workers; eggs from the same and different hives; similar aged larvae within the same hive; and needles contaminated with the Nasonov gland secretions of different adult workers. It appears that some of these differences are due to phenotypic variation among individuals that cannot be directly attributed to environmental factors.

**Key Words**—Chemosensory cues, olfaction, kin recognition, honeybees, *Apis mellifera*, Hymenoptera, Apidae, differential conditioning, proboscis extension reflex, learning.

INTRODUCTION

Chemicals play a role in mediating kinship interactions in several social insect species (for reviews, see: Breed and Bennett, 1987; Gadagkar, 1985; Gamboa et al., 1986a). Honeybees, for example, are polyandrous and can discriminate within the hive between those individuals that are their full sisters and those that are their maternal half sisters (Frumhoff and Schneider, 1987). The recognition cues appear to have a genetic component (Breed et al., 1985; Getz and

Smith, 1983, 1986). It has also been shown that there is sufficient genetic variability in the volatile odors emanating from adult workers so that, using the proboscis extension reflex, workers can be differentially trained to discriminate between groups of adult workers from different patriline within the same hive (Getz et al., 1986). Preferential rearing results, obtained by Noonan (1985) and Page and Erickson (1984), suggest that nurse bees can assess the relatedness of larvae to themselves. There is even evidence that workers are able to assess the relatedness of eggs or, at least, very young larvae to themselves (Visscher, 1986).

In several other species of social insects, kin and nestmate recognition, mediated by olfactory cues, is also a well-established phenomenon. Questions relating to the origin of these cues—specifically, the relative contributions of environmental sources, gestalt sources (chemicals transferred between individuals), and individual genetic variation—have been addressed in carpenter ants (Carlin and Hölldobler, 1986, 1987; Carlin et al., 1987), fire ants (Obin, 1986), paper wasps (Gamboa et al., 1986a, b), and sweat bees (Buckle and Greenberg, 1981; Smith and Wenzel, 1987). Although we have previously demonstrated that volatile odors contain information that potentially could be used by workers to assess their kinship to other workers (Getz et al., 1986), cues sensed through contact chemoreception appear to be more likely as candidates for recognition of kin (for a discussion of this see Obin, 1986). Inside a crowded hive, volatiles are constantly mingling, and the origin of a particular odor may be masked. On the other hand, it is possible that a worker can readily determine the relative fraction of certain chemicals present in the epicuticle of another individual by placing its antennae on that individual. Evidence suggests that genetic variation in the composition of cuticular waxes of worker honeybees exists (Carlson and Bolten, 1984). In other species of social insects, especially several species of ants (Clement et al., 1987; Morel and Vander Meer, 1987; Obin, 1986), the existence of variation in the cuticular wax components of individuals is well established.

It has been reported that workers use “the colony odour, which adheres to their body” to recognize individuals that belong to the same honeybee colony (Renner, 1960). It has also been demonstrated, using differential conditioning of the proboscis extension reflex, that the olfactory system of worker honeybees is sensitive enough to discriminate between mixtures containing different proportions of the following compounds occurring in the epicuticle of workers: the two fatty acids, un- and dodecanoic acids; and mixtures containing different proportions of the two *n*-alkanes, tri- and pentacosane (Getz and Smith, 1987). Many other chemicals may be implicated in recognition including secretions from the Nasonov and mandibular glands (Breed, 1981; Crewe, 1982; Free and Winder, 1983). Thus the picture is complex, and the role of recognition cues could be context-dependent.

A first step towards understanding the problem of kin recognition within

honeybee colonies is to identify sources of variation in the chemosensory labels of individuals. In the experiments described here, we show how the proboscis extension reflex in honeybees can be used to identify and assay the discrimination of such chemosensory cues. Exactly how an individual can use these cues to assess its relatedness to other individuals, however, is a question that involves some understanding of the honeybee olfactory system and, more generally, how olfactory signals are stored and processed by individuals (for further discussion on this point, see Getz and Chapman, 1986).

#### METHODS AND MATERIALS

In all experiments the proboscis extension reflex in honeybees was used to differentially condition workers to discriminate between two glass rods or two stainless-steel needles contaminated with various worker secretions and hive products. A total of three hives was used. Each hive was obtained by instrumentally inseminating a dark queen with semen from one dark and one light drone to yield two visually distinguishable worker patrines: a light patrine and a dark patrine (for more details see Getz et al., 1986). The differential conditioning methodology is modified from Bitterman et al. (1983) and reported in detail in Getz et al. (1986). For the sake of completeness, we summarize the methodology below.

In the late afternoon on the day preceding an experiment, 40–50 workers (primarily foragers) were removed from the hive and harnessed in small brass tubes with their mouthparts, antennae, and legs free to move. They were then fed a 1.5 M sucrose solution until satiated and left overnight in the dark, at room temperature (around 18°C). The following morning, one half to one hour prior to training, each bee was fed several droplets of sucrose solution.

As elucidated in the experiments discussed below, pairs of 50-mm-long, 1-mm-OD, hollow glass rods or pairs of thin steel dissecting needles were used in the differential training experiments. These rods and needles were prepared within an hour of the start of each replicate of an experiment. Depending on the experiment, glass rods were contaminated by rubbing them on various parts of workers of different ages and life stages or areas of comb. Also, the tips of some glass rods were dipped in albumen which then acted as a ‘glue’ to extract eggs from cells. The needles were used in experiments involving Nasonov gland secretions. Each needle was contaminated by rotating its tip in the area of the opening to the Nasonov gland located between the 6th and 7th abdominal tergites of an adult worker. This area was exposed by stretching the abdomen with forceps (for more details see Pickett et al., 1980). With the exception of experiment 7 described below, care was taken that the needles did not touch any part of the bee other than Nasonov gland area.

Individual bees were differentially conditioned to respond (by extending their proboscides; see Bitterman et al., 1983) to one of two contaminated rods

or needles. Stimulation was achieved by touching a rod to both antennae. The positive conditioned stimulus (CS+) was rewarded with 1.5 M sucrose solution as a positive unconditioned stimulus (US+). The latter was given as a droplet to the bee to drink immediately after a 3-sec application of the CS+ (if the proboscis had not been extended, extension was elicited by touching the antenna with the same solution). The negative conditioned stimulus (CS-) was unrewarded and, if the proboscis was extended upon stimulation, a drop of 1.0 M solution of sodium chloride was placed on the proboscis as a negative unconditioned stimulus (US-) (for more details see Getz et al., 1986).

In each experiment, approximately 30 bees were differentially conditioned to two different rods by presenting one or other of these rods in a sequence of 16 trials. This conditioning was carried out by placing each test bee in turn on a platform below an air exhaust system approximately every 10 min and stimulating it with the CS+ (using the appropriate rod or needle) and the associated US+ or the CS- and, when necessary, the associated US-. The stimuli were presented in the following order (the indicated division into two eight-trial groups is only for the purposes of data analysis):

16 trial sequence

$$= \left\{ \begin{array}{l} \text{Trial (training):} \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8 \\ \text{CS:} \quad \quad \quad \quad + \quad - \quad - \quad + \quad - \quad + \quad + \quad - \\ \\ \text{Trial (evaluation):} \quad 9 \quad 10 \quad 11 \quad 12 \quad 13 \quad 14 \quad 15 \quad 16 \\ \text{CS:} \quad \quad \quad \quad + \quad - \quad - \quad + \quad - \quad + \quad + \quad - \end{array} \right.$$

Data were obtained by scoring the number of errors that an individual bee made during trials 9-16 (evaluation sequence). Thus, responses (extension of the proboscis) to any of trials 10, 11, 13, or 16, or nonresponses to any of trials 9, 12, 14, or 15 were scored as errors. Each individual could thus make 0-8 errors and an error histogram and average error  $\bar{e}$  could be calculated for each group. The distribution of errors in these histograms can be compared using an  $n \times 2$  chi-squared contingency table analysis, where the value of  $n$  depends on whether the tail categories of the histograms need to be combined to meet minimum expected frequency criteria (for more details see Getz et al., 1986).

The following experiments were conducted using pairs of glass rods. The number of times each experiment was repeated is noted in parentheses.

*Experiment 1.* (A) Each rod was rubbed on the cuticle of the dorsal surface of the thorax of a different forager from the same hive (not repeated). (B) As a no-discrimination control for the above experiment, each rod was rubbed on the cuticle of the dorsal surface of the thorax of the same forager (two replicates). (C) As a discrimination control for the above experiment, one rod was uncontaminated and the other rod was rubbed on the cuticle of the dorsal surface of the thorax of a forager (not repeated).

*Experiment 2.* (A) Each rod was rubbed on the cuticle of the dorsal surface of the thorax of a different newly eclosed worker (both workers were removed from cells just prior to eclosion—see Getz and Smith, 1986 for details) (two replicates). (B) As a control for the above experiment, each rod was rubbed around the inner surface of a different cell (two replicates).

*Experiment 3.* (A) Each rod was pushed into the center of a different cell containing a curled 5-day-old larva (avoiding any type of injury to the larva) (two replicates). (B) As a control for the above experiment, each rod was dipped into a different cell containing a globule of food provisioned by the workers (two replicates).

*Experiment 4.* (A) Each rod was tipped with wetted albumen (as a glue) and was used to remove an egg from a cell, where both eggs came from the same hive (four replicates). Note that, as far as was possible, eggs were selected from contiguous cells to increase the probability that they were of similar age. (B) As a control for the above experiment, the same procedure was repeated except the eggs were from different hives (three replicates).

*Experiment 5.* To obtain a baseline error level for the above discrimination experiments, one rod was contaminated with paraffin wax and the other with beeswax (not repeated).

The workers and material used to contaminate rods in the above experiments all came from the same hive, except for one of the rods in each replicate of experiment 4B which required the use of a second hive. A third hive set up from the same genetic stock as the first two provided bees for the following Nasonov gland secretion experiments which used pairs of steel needles rather than glass rods.

*Experiment 6.* (A) Each needle was rotated in the exposed Nasonov gland area of a different individual forager, where both individuals were from the same hive (two replicates). (B) As a discrimination control for the above experiment, one needle was contaminated with Nasonov gland secretion while the other was not (cf. experiment 1C) (not repeated).

*Experiment 7.* As in 6A, each needle was first rotated in the exposed Nasonov gland area of a different individual, but then was also rubbed on the upper thorax of that same worker. Both individuals were from the same hive (cf. experiment 1A) (not repeated).

Note that the designations FS and HS are used in the Results section below to indicate whether a particular replicate of one of the experiments 1A, 2A or 6A involved full or half sisters, respectively.

## RESULTS

The sample size and average error over the eight-trial evaluation sequence (see Methods and Materials) are given in Table 1 for each replicate of each experiment. The largest values for the average error  $\bar{e}$  were obtained for the no-

TABLE 1. SUMMARY OF RESULTS<sup>a</sup>

Expt. No.	Brief description	Rep. No.	CS+	CS-	Test group size (N)	Average number of errors ( $\bar{e}$ )
1A	Different foragers	i	Thorax (FS <sup>b</sup> )	Thorax	25	3.2
		ii	Thorax (HS <sup>c</sup> )	Thorax	32	3.4
B	Same forager	i		Thorax	29	4.6
		ii		Thorax	29	4.4
C	Control	i	Clean rod	Thorax	16	2.1
		ii	Thorax	Clean rod	17	1.5
2A	Different enclosing workers	i	Thorax (HS)	Thorax	35	3.5
		ii	Thorax (FS)	Thorax	31	3.9
B	Different cells	i	Cell 1	Cell 2	35	4.2
		ii	Cell 1	Cell 2	39	4.1
3A	Different larvae	i	Larva 1	Larva 2	35	1.9
		ii	Larva 1	Larva 2	32	2.8

B	Different larval food	i ii	Cell 1 Cell 1	Cell 2 Cell 2	35 38	3.9 3.9
4A	Eggs from same hive	i ii iii iv	Egg 1 Egg 1 Egg 1 Egg 1	Egg 2 Egg 2 Egg 2 Egg 2	22 21 24 21	3.1 3.0 4.0 3.9
B	Eggs from different hives	i ii iii	Egg 1 Egg 1 Egg 1	Egg 2 Egg 2 Egg 2	26 23 19	3.8 3.6 3.4
5	Control <sup>c</sup>	i ii	Paraffin Beeswax	Beeswax Paraffin	12 13	1.7 0.4
6A	Different workers	i ii	Nasonov (HS) Nasonov (HS)	Nasonov Nasonov	29 30	4.2 3.7
B	Control		Nasonov	Clean needle	26	0.5
7	Combination		Nasonov plus thorax (HS)	Nasonov plus thorax	30	3.5

<sup>a</sup>The significance of these results is discussed in the text.

<sup>b</sup>FS and HS denote whether the two individuals are full sisters or maternal half sisters, respectively.

<sup>c</sup>Both replicates are actually the same run of an experiment in which the test bees have been split in two groups, each receiving the mirror image treatment with regard to the CS+ and CS- stimuli.

discrimination control experiment (1B), where the error combined across both replicates is  $\bar{e} = 4.5$  ( $n = 58$ ). The lowest values for  $\bar{e}$  were obtained for the discrimination control experiments (1C, 5, 6B), especially for the runs beeswax vs. paraffin ( $\bar{e} = 0.4$ ) and Nasonov vs. clean needle ( $\bar{e} = 0.5$ ). Note from experiments 1C and 5 that there appears to be an asymmetry in the results based on which of the stimuli are used as the CS+ and CS-. In experiment 1C, discrimination is stronger (that is,  $\bar{e}$  is smaller) when the contaminated rather than the blank rod is used as the CS+ ( $P < 0.01$ ,  $2 \times 2$  chi-squared analysis). In experiment 5, discrimination is stronger when the more natural beeswax stimulus is used as the CS+ ( $P < 0.05$ ,  $2 \times 2$  chi-squared analysis). Thus, in conjunction with our experience in using odor stimuli (unpublished results), it appears that workers are more easily trained to discriminate between two chemosensory stimuli if they are positively conditioned to the stimulus that is either stronger or contextually the more natural of the two than vice versa.

In experiments 1A and 1B, the results from replicates within experiments are very similar, but across experiments the differences between any two replicates are significant, at least at the  $P < 0.01$  level ( $2 \times 4$  chi-squared analysis). Furthermore, the results obtained from either replicate of experiment 1A are significantly different from either replicate of experiment 1C, at least at the  $P < 0.05$  level ( $2 \times 3$  chi-squared analysis). Thus it follows that workers can perceive differences between rods rubbed on the backs of different foragers but discrimination is not as strong as exhibited by the discrimination control.

Since the replicates in experiment 1B are so similar ( $P > 0.95$ ,  $4 \times 2$  chi-squared analysis), they can be combined and used as a no-discrimination control for experiment 2A, as well as for the other experiments discussed below. In this case, both replicates exhibit that discrimination has taken place (for example, replicate ii of experiment 2A is significantly different from the no-discrimination control at the level  $P < 0.01$ ,  $3 \times 2$  chi-squared analysis), albeit not as strongly as obtained in the different foragers experiment 1A. Only the results from replicates i of experiment 1A and ii of experiment 2A are significantly different (in this case  $P < 0.01$ ,  $3 \times 2$  chi-squared analysis). The results from experiment 2B can be used to assess whether differences in the cuticular waxes of eclosing workers could be due to contaminants picked up from the inside surfaces of cells. Only replicate ii of this experiment differed significantly from the combined no-learning control ( $P < 0.05$ ,  $4 \times 2$  chi-squared analysis), while only replicate i of experiment 2A differed significantly from both replicates of experiment 2B (in both cases  $P < 0.01$ ,  $4 \times 2$  chi-squared analysis).

Among all experiments, the level of discrimination between pairs of natural cues was strongest in the different larvae experiment ( $P < 0.001$ ,  $4 \times 2$  chi-squared analysis for both replicates of experiment 3A when compared with the no-learning control). The results of experiments 3B can be used to assess whether food contaminants influence the larval discrimination results. Although

both replicates of experiment 3B indicate that some level of discrimination is evident with respect to the no-learning control experiment 1B ( $P < 0.05$  for replicate i and  $P < 0.01$  for replicate ii,  $4 \times 2$  chi-squared analysis), the level of discrimination is much weaker than in the larval experiment 3A (for any two replicates between experiments 3A and 3B, we have  $P < 0.001$ ,  $4 \times 2$  chi-squared analysis).

The results across replicates of the discrimination between eggs experiments 4A and 4B are quite variable. Furthermore, there is no evidence for increased discrimination in the case of eggs obtained from different hives compared with the case of eggs obtained from the same hive. In replicates iii and iv of experiment 4A, discrimination is weak but evident when compared with no-learning control experiment 1B ( $P < 0.05$  for both replicates,  $3 \times 2$  chi-squared analysis). However, replicates i and ii of experiment 4A exhibit stronger levels of discrimination than do replicates iii and iv (for example, the results of replicates i and iv are significantly different at  $P < 0.05$  while the results of replicates ii and iii are significantly different at  $P < 0.001$ ,  $2 \times 2$  chi-squared analysis).

From experiments 6A and 6B it is evident that, although honeybee workers experience no difficulties learning to discriminate the Nasonov gland secretion, they do experience some difficulties discriminating between secretions from half sisters in the same hive. A stronger level of discrimination is exhibited in replicate ii when compared with replicate i ( $P < 0.05$ ,  $3 \times 2$  chi-squared analysis) but, interestingly, little improvement in the level of discrimination is obtained through the addition of cues from the thorax in experiment 7. Note that it is difficult to compare the results from experiments 6A and 7 with 1A since the bees are from different hives, which may affect the level of cue variability among individuals (even though the hives are from the same genetic stock).

#### DISCUSSION

The results indicate that workers can discriminate between glass rods rubbed over the epicuticle of different foragers irrespective of whether the individuals are full or half sisters. They appear to be able to do the same if the individuals are newly eclosed half sister workers, but possibly to a lesser extent if the newly eclosed workers are full sisters. The difference between cues in the cuticle of eclosing individuals cannot be attributed to contaminants picked up in the cells. One could speculate that newly eclosed full sisters are more similar than half sisters in terms of recognition cues, but they become more distinct through environmental odors acquired as adults. However, the results presented here are too cursory to come to any firm conclusions. A definitive study of this and some of the other questions discussed below would involve testing several

hives, preferably set up with specific genealogies. It would also involve more replicates per hive and perhaps the presentation of stimuli obtained from groups containing different numbers of individuals, as was done in our volatile odor recognition cue study (Getz et al., 1986).

The Nasonov gland secretion also exhibits some variability between individuals, and this variability may be enhanced through additional cuticular wax discrimination. As mentioned in the previous section, the results of experiments 7 and 1A cannot be compared since different hives were used in these experiments. Thus one cannot come to any conclusions relating to additive or synergistic effects of cues. Again, a definitive study involves an extensive amount of experimentation and is required before any concrete conclusions can be reached.

The relatively strong differences in cues produced by different larvae raises some interesting questions. First, these differences cannot be attributed to contamination from the food provisioned for those larvae. The cues, however, may have nothing to do with individual or kin recognition, but may reflect the cues (pheromones) that larvae use to communicate their physiologic state (for example, need for food) to the nurse bees (see Jaycox, 1970; Free and Winder, 1983).

Prior to the experiment, we had anticipated that if workers could discriminate between eggs, then the level of discrimination would be stronger if the eggs come from different hives rather than the same hive. This would be the case, as discussed by Visscher (1986) in interpreting the results of his experiments, if queen pheromone or cues from an environmental source played an important role in labeling eggs. Although discrimination is evident in all replicates of within- and between-hive experiments, the strongest level came from the within-hive replicates. Thus it would appear that some other factors, such as the age of the egg (developmental stage), provide the strongest cues for discrimination. That is not to say that other cues do not exist or, if cues do exist, that they cannot be perceived by the nurse bees.

This raises the general question of what we are able to infer about an individual's ability to discriminate cues by observing its behavior. In the context of discrimination training, failure to observe discrimination does not imply that the test individuals are not detecting differences between two stimuli. This includes the proboscis extension reflex; individuals that are particularly hungry, regardless of any other considerations, may extend their proboscides in the hope of being fed, or some individuals may be able to detect differences but are poor learners. Also, it may be easier to train bees using the proboscis extension reflex if the cues occur naturally in a nectar-gathering context, for example certain floral odors. Finally, if one conducted experiments, such as 7, to assess the combined effects of two sets of cues, then the occurrence of thresholds, saturation effects, hierarchical ordering of information (see Carlin and Hölldobler, 1986), to mention just a few, would obscure what the insect is actually sensing.

## CONCLUSION

From the results presented here, it is clear that the proboscis extension reflex can be used to assess the potential of different secretions as a source of labels for kin recognition. The situation is not always straightforward since time-dependent differences in the physiology of individuals, especially in preadult stages, may influence the results. Once a promising system, such as cuticular waxes in adults, has been identified, then more extensive studies, including the heritability aspects of the labeling system, need to be undertaken.

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