Journal of Insect Behavior, Vol. 13, No. 1, 2000

Extension Reflex Conditioning

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Accepted September 23, 1999; revised October 11, 1999

To understand the effect of abnormal brood odors on the initiation or control of hygienic behavior in honey bees, we employed the associative learning paradigm, proboscis extension reflex conditioning. Bees from two genetic lines(hygienic and non-hygienic) were able to discriminate between high concentrations of two floral odors equally well. Differential discrimination abilities were observed between the two lines when healthy and diseased brood odors were used, with the bees from the hygienic line discriminating between the pair of brood odors better than the non-hygienic bees. These results suggest that hygienic behavior in individual bees is associated with the bees' responses to olfactory stimuli emanating from diseased brood.

KEY WORDS: hygienic behavior; discrimination conditioning; honey bees; *Apis Mellifera;* olfaction.

INTRODUCTION

One of the classic studies in behavioral genetics involves nest cleaning, or hygienic behavior, by workers of the honey bee, *Apis mellifera* L. Early

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0892-7553/00/0100-0087$18.00/0

studies of the behavior revealed a genetic basis underlying the expression of this complex social trait (Rothenbuhler, 1964; reviewed in Spivak and Gilliam, 1998a, 1998b). However, the extent that the genetic composition of the colony influences the individual expression of this behavior has not been investigated. The study of social insects is fascinating because complex colony behaviors arise from a collection of simple, individual behaviors (Wilson, 1985). By studying the responses of individual bees, physiological and neural mechanisms underlying the expression of a complex behavior may be revealed and related to supraindividual levels of organization.

Hygienic behavior is a generalized behavioral response to the presence of pathogens and parasites in the nest, minimizing the adaptation of these pathogens to the genotypes of individual workers (Spivak and Gilliam, 1993). It is a behavioral mechanism of disease resistance (Woodrow and Holst, 1942; Rothenbuhler *et al.;* 1964, Gilliam *et al.,* 1983) and is one defense against the ectoparasitic mite *Varroa jacobsoni* Oudemans (Peng *et al.,* 1987; Boecking and Drescher, 1992). The mite is the most destructive pest of honey bees in the United States and Europe. Bees that perform hygienic behavior are able to detect, uncap, and remove honey bee pupae from the nest, which are diseased (Rothenbuhler, 1964; Gilliam *et al.,* 1983) or parasitized by *Varroa* mites (Boecking and Drescher, 1992; Spivak, 1996).

The behavior is postulated to be controlled by two, independently assorting, recessive loci (Rothenbuhler, 1964), or by other more complex patterns of inheritance (Moritz, 1988). Rothenbuhler's interpretation of the hygienic behavior data supported the existence of independent loci for the uncapping (opening the wax capping from a cell containing infected or infested brood) and removal (removing the brood) behavior. Moritz (1988) reanalyzed Rothenbuhler's data and agreed with the interpretation of the data for uncapping behavior, but suggested that there may be more than one locus for removal behavior.

Colony-level expression of hygienic behavior may depend on multiple factors related to the genetics and physiology of individual bees. First, the behavior may be performed as a result of genetic specialization of particular workers who have a lower response threshold to infested pupae. The presence of diseased or parasitized brood may trigger hygienic behavior (uncapping or removing) in these bees, but not in other bees with higher genetic response thresholds to these cues. The percentage of bees in the colony that are genetic specialists for hygienic behavior can influence the colony-level response (Trump *et al.,* 1967; Spivak and Gilliam, 1993). Second, the neurohormonal state of the bees in the colony may also be important in the initiation or control of this behavior. Levels of neuromodulators in honey bee brains change throughout the season, possibly as a result of changes in nutrition and population (Harris and Woodring, 1992). Changes

in the colony level expression of hygienic behavior might be correlated with the amine level changes that occur throughout the season. Third, environmental influences, including both the internal state of the colony (Spivak and Gilliam, 1993) and the availability of resources external to the colony (Thompson 1964; Momot and Rothenbuhler, 1971) can affect the degree to which hygienic behavior is performed in the colony. The effects of the environmental factors might be related to the neurohormonal state of the bee.

The level of abnormal brood odors may exceed the response threshold of the bees within the colony, which would cause them to initiate hygienic behavior. To understand the effect of abnormal brood odors on the initiation or control of hygienic behavior in individual bees, we employed an associative learning paradigm (proboscis extension reflex [PER] conditioning) comparing individuals from a genetic line that perform hygienic behavior (hygienic bees) and bees from a different genetic line that do not perform hygienic behavior at a high rate (nonhygienic bees). We investigated whether the difference between the two lines might be based on their differential ability to detect and discriminate odors associated with diseased and healthy brood. It was hypothesized that individual bees from both lines could discriminate between floral odors equally well, but the hygienic bees would discriminate between the odors of healthy and diseased brood better than non-hygienic bees.

MATERIALS AND METHODS

Breeding

A breeding program for hygienic behavior was initiated at the University of Minnesota in 1993. Lines of hygienic and nonhygienic colonies used in this experiment were bred from Italian stock, *A. mellifera ligustica.* Hygienic behavior in the colonies was determined by a field assay in which the amount of time was recorded for bees to detect, uncap and remove freeze-killed brood from a 5×6 cm section of comb (Spivak and Downey, 1998). Colonies that removed the freeze-killed brood within 48 h were considered hygienic and colonies that took longer than 1 week to remove the dead brood were considered nonhygienic. To establish and maintain the lines, queen bees were raised from colonies that displayed the most rapid and least rapid removal rates. Each generation, the daughter hygienic and nonhygienic queens were instrumentally inseminated with a mixture of semen from drones from different hygienic and nonhygienic colonies, respectively (see Spivak and Gilliam, 1998b).

Observation Hives

In the summer of 1996, one hygienic and one nonhygienic colony were chosen from the third-generation colonies of selected lines to be the source of bees for two observation hives. Each observation hive was provided with a frame (comb within standard beekeeping equipment) of nectar and pollen, a frame of young larvae, and approximately 1000 unmarked bees of various ages from each parent colony to simulate a normal age structure in the colony. The inseminated queens from the parent colonies were introduced into the observation hives. Unmarked bees were prevented from eclosing in these colonies by replacing combs containing pre-eclosion brood with empty combs.

Two or 3 days before pupae were due to eclose in the parent colonies, combs were removed from each colony and placed in individual cages in an incubator (34° Celsius, 50% RH). Numbered honey bee tags were glued to the thorax of one day old hygienic and nonhygienic bees to identify them by age and line. The bees were added into their respective observation hives. Approximately 75 bees from each line were tagged and introduced to the observation hives every 3 days for 3 weeks.

The experiments began 4 weeks after the first tagged bees were added to the observation hive, when the oldest tagged hygienic and nonhygienic bees were 28 days old and the youngest tagged bees were 7 days old. Previous experiments indicated that bees performing hygienic behavior are approximately 15–17 days old (Arathi *et al.*, 2000). The youngest untagged bees would have been 25 days old. The learning trials were performed over 5 consecutive days at the end of August 1996. Each day, a comb section containing freeze-killed pupae was inserted into the observation hives to elicit the hygienic response. Two hours later, bees from the hygienic line were collected while uncapping or removing the freeze-killed brood by placing a wire screen cage over them and allowing them to walk up the side of the cage. Same-age tagged bees from the non-hygienic line were collected off of the comb section of freeze-killed pupae from the non-hygienic observation hive at the same time. However, these bees were not performing hygienic behavior.

After collection from the observation hives, the bees were directly transferred to the laboratory where they were cooled on ice until they became inactive. Immediately after cooling, the bees were harnessed in a restraining apparatus (Menzel and Bitterman 1983; http://IRIS.biosci.ohio-state.edu:80/honeybee). The restrained bees were able to move their antennae and proboscises freely. Fifteen minutes after the bees were harnessed, they were fed 0.4 μl of 2M sucrose solution. The conditioning trials began 2 h after they were harnessed and fed.

Learning Trials

In separate experiments, two floral or two brood odors were used for the conditioned stimuli (CS) to test the ability of bees from each line to discriminate between the pairs of odors. In each experiment, one odor (CS+) was paired with a sucrose reward (unconditioned stimulus, US+) and the other odor (CS−) was paired with a salt punishment (US−). It was recorded whether or not the bees extended their proboscises to the presentation of the CS before the application of the US. Bees that learned to discriminate between odors would elicit the proboscis extension conditioned response (CR) in the presence of the CS+ before the application of the US+ and withhold their proboscis as the CR when presented with the CS− before the application of the US−.

For the conditioning trials, bees were positioned in an open Plexiglas box that was attached to an exhaust system. This set-up facilitated removal of the odors from the box where the bees were trained. The bees were positioned in the box and allowed to adjust to the surroundings for 30 sec before each trial began. The odors used as the conditioned stimuli were kept in separate syringe barrels placed 1.5 cm from the head of the bee. The tip of the syringe facing away from the bee was fitted with tubing for the delivery of air through the barrel. Airflow was provided by an aquarium pump controlled by a computer. Air containing the odor (either CS+ or CS−) was blown through the syringe barrel and over the bee for 4 sec. Three seconds after the beginning of the odor exposure, the computer signaled for the presentation of the unconditioned stimulus. The appetitive US+ (0.4 μl of 2 M sucrose) was presented to the bee after the CS+ by first touching a drop to the antenna and then to the proboscis for two seconds of feeding. The aversive US− (0.4 μl of 3 M NaCl) was touched to the antenna of each bee, regardless of their response to the odor of the CS−. In most conditioning sessions, eight bees were trained at a time, four from the hygienic line and four from the non-hygienic line. Conditioning sessions were repeated with new bees on successive days until the sample size was sufficient. There was an 8-min inter-trial interval for each bee.

The bees were exposed to each odor in the pair for 8 trials, for a total of 16 trials in an experiment. In the first trial, the CS+ was paired with the US+. In the second trial, the CS− was paired with the US−. Thereafter the odors were presented in a pseudorandom sequence of the CS+ and CS−. Bees that extended their proboscises (spontaneous responders) to the presentation of the CS+ in the first trial were excluded from the experiments because it could not be determined if their future responses to the CS+ and CS− were a result of conditioning.

Floral Odor Discrimination Experiment

In the first experiment, a pair of floral odors, geraniol (Sigma, approx. 98% pure) and 1-hexanol (Sigma, approx. 98% pure), were used as the conditioned stimuli (either CS+ or CS−). A 1cc glass syringe barrel was used for odor delivery. A small piece of filter paper containing 3.0 μl of the odor was put into the syringe. One floral odor was paired with the US+ and the other with the US−. Later the same day, new bees were trained to the reversed odors, with the odor formerly paired with the US+ being paired with the US−.

Brood Odor Experiment

In the second experiment, a pair of brood odors were used as the conditioned stimuli. We used the odor of healthy pupae and that of diseased pupae infected with the fungal disease, chalkbrood, caused by *Ascophaera apis.* Two to 3 days after pupation, live pupae with light pink-purple eyes (Jay, 1962) were removed from their capped cells carefully to prevent injury. White and black chalkbrood mummies were collected from a frame of brood and kept at 4°C. The mummies and healthy pupae were placed into separate 12-cc plastic syringe barrels. Four live pupae were used for one of the CS and four chalkbrood mummies (two white and two black) were used for the other CS in the discrimination experiment. As before, the odor used as the CS+ was used as the CS− later in the same day to test a new set of bees.

Statistical Analysis

In the discrimination experiments, a positive response (proboscis extension) to the presentation of either the CS+ or CS− was scored as 1, a negative response (no proboscis extension) was scored as 0. The number of positive responses was then summed for each bee. There were seven possible positive responses to the rewarded odor (the first trial had to be 0) and eight possible positive responses to the punished odor. The probability of proboscis extension to each odor over the 16 trials was plotted in graphs. Because the dependent variable was binary, logistic regression was used to analyze the data using the CATMOD procedure (SAS Institute Inc., 1989). This analysis showed the probability of the bees proboscis response in relation to their genetic line and odor (CS+ or CS−) presented to them.

Two additional tests were used to analyze the variability among the bees' responses to the odors. Bees from different lines could have significantly different numbers of positive responses (proboscis extensions), but the difference between the sums of the responses to each odor could be the same. Therefore, a discrimination index was used to analyze the differences between the hygienic and nonhygienic bees' responses to each odor. The discrimination index was calculated by subtracting the sum of the responses to the punished odor from the sum of the responses of the rewarded odor. A high positive index would indicate that the bees extended their proboscises to the rewarded odor, but withheld them for the punished odor. The results of the discrimination index could range from a high of seven (seven positive responses to the CS+ and 0 positive responses to the CS−) to a low of -8 (0 positive responses to the CS+ and eight positive responses to the CS−). The indices for each line were compared using Mann-Whitney *U*-tests.

In addition, the total number of times the bees extended their proboscises in an experiment was compared. The sum of proboscis extension responses to the CS+ and CS− were added for each bee. The range of the total proboscis extension index could range from 0 to 15. A high total would indicate that the bees tended to extend their proboscises to (one or) both odors, while a low total would indicate a more conservative response to the odors. The total number of proboscis extension responses for the bees in each line were analyzed using Mann-Whitney U- tests.

RESULTS

Figures 1a and b show the probability of response from the hygienic and non-hygienic bees to the two floral odors over the sixteen learning trials (eight for each odor). The results of the statistical analyses are shown in Table I. The two lines were able to discriminate between floral odors irrespective of which odor was rewarded. The logistic regression analysis indicated there were significant differences between the responses to the two odors, but the response levels did not differ between the genetic lines of bees. The interaction terms were also not significant, which indicates that the relative response patterns to the CS+ and CS− were the same for the two lines. In addition, there were no significant differences between the hygienic and nonhygienic bees in either the discrimination index or the total proboscis extension response index.

Figures 1c and 1d show the probability of the conditioned response over the sixteen trials for brood odor discrimination. The logistic regression of the pupae (CS+)/chalkbrood (CS−) odor pair revealed significant line

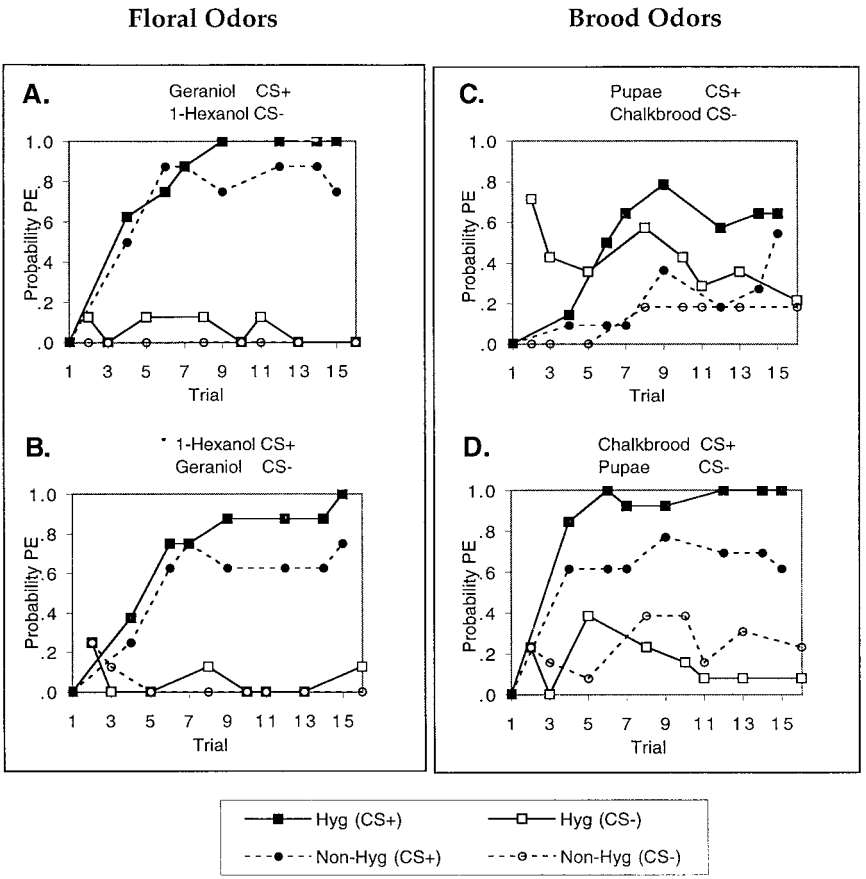

Fig. 1. Probability of proboscis extension response (PER) over 16 trials (8 trials for each odor in a pair) by hygienic (solid lines) and non-hygienic (dashed lines) bees. 1A) Geraniol was rewarded odor (CS+) and 1-hexanol was punished odor (CS−). 1B) Floral odors reversed: 1-hexanol was CS+ and geraniol CS−. 1C) Live pupae was rewarded odor (CS+) and chalkbrood was punished odor (CS−). 1D) Brood odors reversed: chalkbrood was CS+ and live pupae was punished odor (CS−).

and odor effects (Table I). Both lines displayed a tendency to respond to the brood odors. However, the hygienic line had a higher probability of response to both odors than the non-hygienic line. The hygienic line displayed a response pattern typical for discrimination conditioning. There was little spontaneous response to the CS+, which was always the first trial. The hygienic bees tended to generalize to the CS− on the second trial, but the discrimination gradually improved across trials as the hygienic

Table I. Analysis of PER Discrimination Training in Hygienic and Nonhygienic Bees

Corresponding figure	CS^+	Hygienic n =	Non-hygienic n =	Logistic regression		Discrimination index	Total PE index
1a.	Geraniol	8	8	Odor	$\chi^2 = 104.56$, df = 1; $P < 0.0001$[a]	$U_s = 30.00$	$U_s = 41.50$
				Line	$\chi^2 = 3.07$, df = 1; $P < 0.08$	$P = 0.82$	$P = 0.28$
				Interaction	$\chi^2 = 0.21$, df = 1; $P < 0.65$		
1b.	1-hexanol	8	8	Odor	$\chi^2 = 68.38$, df = 1; $P < 0.0001$[a]	$U_s = 39.50$	$U_s = 39.00$
				Line	$\chi^2 = 0.85$, df = 1; $P < 0.36$	$P = 0.42$	$P = 0.45$
				Interaction	$\chi^2 = 0.85$, df = 1; $P < 0.36$		
1c.	Pupae	14	11	Odor	$\chi^2 = 7.84$, df = 1; $P < 0.01$[a]	$U_s = 81.00$	$U_s = 124.00$
				Line	$\chi^2 = 37.98$, df = 1; $P < 0.001$[a]	$P = 0.82$	$P = 0.01$[a]
				Interaction	$\chi^2 = 0.33$, df = 1; $P < 0.56$		
1d.	Chalkbrood	13	13	Odor	$\chi^2 = 100.86$, df = 1; $P < 0.0001$[a]	$U_s = 144.00$	$U_s = 101.00$
				Line	$\chi^2 = 7.53$, df = 1; $P < 0.01$[a]	$P < 0.01$[a]	$P = 0.39$
				Interaction	$\chi^2 = 19.53$, df = 1; $P < 0.001$[a]		

[a]Denotes statistical significance.

bees learned the pattern of reinforcement. Discrimination was revealed by the significant odor effect, which arose because the response to the CS+ was higher than the response to the CS−. Although the probability of response for the hygienic bees was significantly different from that of the nonhygienic bees, the pattern of discrimination of the CS+ from the CS− was the same for both lines, which was revealed by the lack of a significant interaction term.

The probability of response over sixteen trials when the brood odor pair was reversed (chalkbrood CS+/live pupae CS−) is shown in Fig. 1d. Both lines of bees showed typical discrimination training response patterns. The hygienic bees had a higher level of response to the CS+ than the non-hygienic bees. Both lines of bees displayed a low level of generalization to the CS− on its first presentation with the probability of response of 0.23 for both lines of bees. The responses of the hygienic bees to the CS− decreased over trials, while the nonhygienic bees maintained a higher level of response than the hygienic bees. There was a significant interaction term between the effects of odor and line of bees, revealing the ability of the hygienic bees to discriminate between the brood odors was better than the nonhygienic bees.

Box plots of the discrimination and total proboscis extension response indices for the diseased and healthy brood odor discrimination experiments are shown in Figs. 2a–d. When the odor of healthy pupae was the CS+ and chalkbrood odor was CS− (Fig. 2a), the discrimination indices between the hygienic and nonhygienic bees were not significantly different (Table I). However, the total proboscis extension response between the bees from each line were significantly different (Fig. 2b). When chalkbrood mummy odor was the CS+ and live pupae odor was the CS−, the reverse was observed. The discrimination indices for the hygienic and nonhygienic line were significantly different (Fig. 2c), but the total number of proboscis extension responses were not significantly different (Fig. 2d).

DISCUSSION

Bees from colonies selected for hygienic and non-hygienic behaviors were able to discriminate between two floral odors equally well. The floral odor experiment suggests that bees from each line are able to discriminate between two odors if the conditioned and unconditioned stimuli are salient enough for the bees to learn the association between them. It is possible that a significant difference could be found between the lines if less floral odorant is used. If so, it would indicate that there are inherent differences between the lines in their ability to discriminate odors in general.

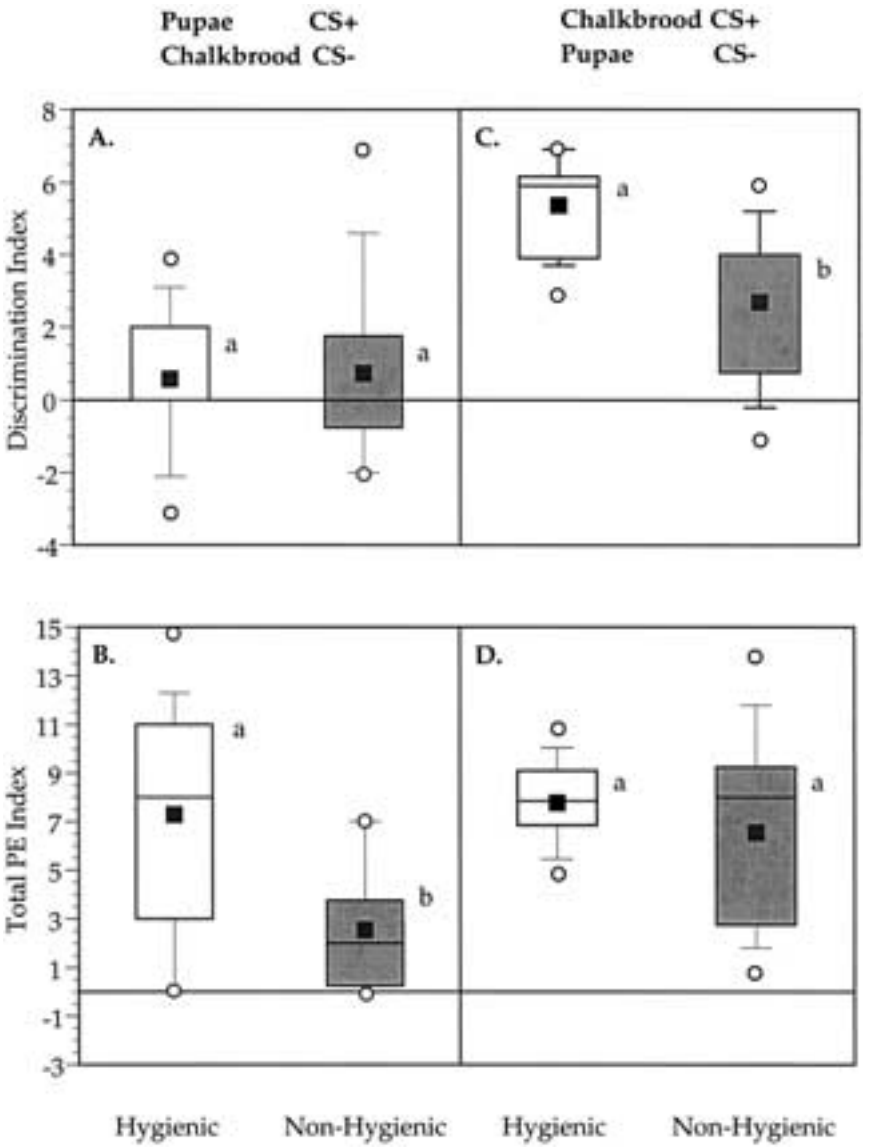

Fig. 2. The Discrimination Index (sum of responses to the CS+ minus the sum of responses to the CS−) and the Total Proboscis Extension Index (sum of responses to the CS+ plus the sum of responses to the CS−) are shown using box plots. Indices for the bees from the hygienic line are shown in the open boxes and indices for the nonhygienic bees are shown in the shaded boxes. Box plots show the quartiles of a data set. The ends of each box are drawn at the upper and lower quartiles, with the median designated by the line through the box, or a bold end of the box if the median is the same as the upper or lower quartile. Data points that are located within 1.5 of the distance between the upper and lower quartile, the interquartile range, are shown using lines drawn from the ends of the boxes. The means of each data set are represented by the black rectangles within each box. Extreme data points are indicated by an open circle. Fig. 2A, B show results from trials where live pupae odor is the CS+ and chalkbrood mummy odor is the CS−. Figures 2C, D show results from trials where brood odors are reversed.

When the odors used for the conditioned stimuli were changed from floral to brood odors (and the unconditioned stimuli remained constant), differential discrimination abilities were observed between the two lines of bees. Regardless of the way the odors were paired, the bees from the hygienic line discriminated between the pair of brood odors better than the nonhygienic bees. These results suggest that hygienic behavior in individual bees is associated with the bees' responses to olfactory stimuli emanating from diseased brood. Bees that perform hygienic behavior might have a lower stimulus response threshold to odors associated with diseased brood odors, thus their olfactory capabilities translate into better performance in laboratory discrimination experiments.

There was a difference in the total number of proboscis extensions between the hygienic and nonhygienic bees when the CS+ was live pupae (Fig. 2.). The bees from the hygienic line extended their proboscises more than the bees from the nonhygienic line. A number of the bees from the nonhygienic line did not respond to the training. However, when the nonhygienic bees were tested with the floral odors, this phenomenon was not observed. Therefore, their response may be based on their lack of ability to detect the levels of brood odors that were used. Benatar *et al.* (1995) suggested that the poor discrimination abilities found in their low performance line was the result of the bees' inability to associate the CS's to the correct US's. The results of our study suggest that the bees from the nonhygienic line can learn to associate the CS and US if they are both salient to the bee. The floral odors that were used were sufficiently salient to allow the nonhygienic bees to learn the associations between the different conditioned and unconditioned stimuli. The brood odors that we used were not salient enough for the nonhygienic bees to associate the stimuli correctly. This interpretation is consistent with the hypothesis that the hygienic and nonhygienic lines have different response thresholds to the brood odors.

We observed asymmetrical abilities to discriminate odors within the two lines depending on the way the conditioned and unconditioned stimuli were paired. We observed better discrimination between the brood odor pair when the chalkbrood mummies were paired with the reward and the live pupae were paired with the punishment. An asymmetrical response suggests that the two odors were not equally salient to the bees. This asymmetrical response was not observed in the floral odor discrimination experiments at the odor levels used. Although, with decreasing floral odorant level, an asymmetrical response might be detected as the bees reach the lower limits of response to one odor and not the other. Here we might expect that the non-hygienic bees would demonstrate an asymmetrical response before the hygienic bees as our brood odor data suggest that their olfactory sensitivity may be lower than that of hygienic bees.

The asymmetrical results are similar to those reported by Bhagavan and Smith (1997) when bees were asked to discriminate between an odor (1-hexanol or geraniol) diluted in a solvent (hexane) and the solvent alone. The bees were able to discriminate between the odor and solvent better when the odor was the CS+ than when the solvent was the CS+, suggesting that the 1-hexanol and geraniol odors were more salient conditioned stimuli than hexane. In the results of the discrimination training reported here, the bees were able to discriminate between the pupae and chalkbrood odors better when the chalkbrood mummies were the CS+. When the odors were reversed and live pupae were the CS+, both lines of bees had more difficulty discriminating between the two odors, with bees from the nonhygienic line showing a very low probability of response to both odors, similar to the results of Bhagavan and Smith (1997) when solvent was the CS+ and odor was the CS−. Therefore, chalkbrood odor might be a more salient conditioned stimulus than pupae odor.

When the CS+ was live pupae (Fig. 1c) the hygienic bees tended to generalize to the odor of chalkbrood (CS−) the first time that it was presented (Fig. 1c, trial 2). This generalization could be a result of the low stimulus intensity of the live pupae. Bhagavan and Smith (1997) reported less generalization when bees were trained to high concentrations of stimuli. Future experiments designed to examine whether a stimulus response threshold exists for each line of bees to different levels of the normal and diseased brood odor will show whether consistent generalization to the first presentation of the CS− occurs at a low stimulus level.

The present study examined how the behavior of individual bees may comprise the colony level response. The discrimination and total proboscis extension indices shown in the box plots demonstrate the individual variability in the bees' responses to the two brood odors. It is not known whether there are differences among bees within the hygienic line in their abilities to discriminate between odors based upon their tendency to perform hygienic behavior in a field colony. Bees that tend to uncap or remove diseased, parasitized or dead brood might be specialists (Arathi *et al.*, 2000). The variability seen among individual hygienic bees in the discrimination and total PE index for the pupae CS+/chalkbrood CS− experiment (Fig. 2a-b) could be a result of having two different types of task specialists. The uncappers might be better at odor discrimination tasks because they initially identify the abnormal pupa underneath the wax capping. The poorest performers in the discrimination learning might be the removers because their task is more general and does not require as specific odor discrimination abilities.

The presence of abnormal olfactory cues from brood might induce nonassociative learning effects, sensitization and habituation, on the bees

that perform hygienic behavior. These bees could become sensitized or habituated to olfactory cues that are associated with diseased or parasitized brood. Sensitization would increase the probability or frequency of a behavioral response to the abnormal olfactory cues emanating from the brood; whereas, habituation would decrease the probability of a behavioral response.

Past performance of hygienic behavior may influence odor discrimination, however, our results indicate that differences in the ability to discriminate between brood odors is genetically based (unpublished data). Although determined by genetics, environmental factors, experience and ultimately, changes in neuromodulators, could bias the activation, form and intensity of hygienic behavior (Kravitz, 1983). In addition, the composition of genetic specialists and non-specialists in the colony, and incoming resources, might affect the level of hygienic behavior in the colony. The experiments reported here demonstrate that these factors can be examined at the individual level using PER discrimination conditioning.

ACKNOWLEDGMENTS

We are grateful to G. Reuter for his technical assistance. We thank Dr. Mesce for providing direction in this study and for her helpful comments on improving this manuscript and Dr. Venette for his assistance with SAS. This work is in partial fulfillment of the Ph.D. degree for R. M. at the University of Minnesota. We acknowledge the Louise T. Dosdall Fellowship, Alexander P. and Lydia Anderson and University of Minnesota Department of Entomology Fellowships for support of R. M., and the National Science Foundation IBN-9722416 awarded to M. S. This paper is contribution #99-1-17-0008 from the Minnesota Agriculture Experiment Station.

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