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ORIGINAL ARTICLE

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Differences in olfactory sensitivity and behavioral responses among honey bees bred for hygienic behavior

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Abstract Honey bees, *Apis mellifera* L., bred for hygienic behavior uncap and remove diseased and mite-infested brood. We hypothesized that within a colony bred for hygienic behavior, there would be differences in olfactory sensitivity among bees of the same age. We predicted that bees that initiate the behavior by perforating and uncapping brood would have greater olfactory sensitivity to the odor of the diseased brood, and would be better able to discriminate between odors of healthy and diseased brood, compared to bees that complete the behavior by removing the uncapped brood from the cells. Electroantennogram recordings of 15- to 21-day-old bees from three colonies demonstrated that bees collected while uncapping dead brood had significantly greater olfactory sensitivity to all concentrations of diseased brood odor compared to bees collected while removing brood. Proboscis-extension reflex discrimination conditioning demonstrated that 15- to 21-day-old bees collected while uncapping discriminated significantly better and generalized significantly less between the odors of diseased and healthy brood compared to bees collected while removing, when the odor of diseased brood was rewarded, but not when the odor of healthy brood was rewarded. Bees collected while uncapping brood that had been pierced with a pin had significantly less olfactory sensitivity than bees collected while uncapping freeze-killed brood, most likely because the pierced brood had greater stimulus intensity. Initiation of hygienic behavior depends on the olfactory sensitivity of the bee and stimulus intensity of the abnormal brood. Differential olfactory sensitivity and responsiveness among hygienic bees could lead to the apparent partitioning of the behavior into uncapping and removing components.

Keywords *Apis mellifera* · Hygienic behavior · Electroantennogram · Proboscis-extension response conditioning · Task partitioning

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Introduction

Hygienic behavior of honey bees *Apis mellifera* L., has been described as a two-step process: bees uncap wax-covered cells containing diseased brood (fifth-instar larvae and pupae) and then remove the brood (Rothenbuhler 1964a). Hygienic behavior is a mechanism of disease resistance if bees are able to remove brood from the nest before the pathogen becomes infectious. Hygienic colonies are resistant to American foulbrood (a bacterial disease; Rothenbuhler 1964a, 1964b; Spivak 1996) and chalkbrood (a fungal disease; Gilliam et al. 1983). Hygienic behavior is also one mechanism of resistance to the parasitic mite *Varroa destructor* (Anderson and Trueman2000), because bees are able to uncap and remove mite-infested pupae (Peng et al. 1987; Boecking and Drescher 1991; Spivak 1996). Such removal interrupts the reproductive cycle of the mite, thereby limiting the number of mite offspring produced.

The number of genes controlling hygienic behavior has been evaluated in several ways. From experimental crosses between disease-resistant (hygienic) and disease-susceptible (non-hygienic) lines of bees Rothenbuhler (1964b) determined that hygienic behavior was a recessive trait, and proposed a Mendelian two-locus model for its expression: one locus controlled uncapping behavior (u), and another controlled removal behavior (r). Moritz (1988) re-evaluated Rothenbuhler's original data, and suggested that a three locus model (u, r_1 and r_2) better fit the data. Based on experimental observations of the

removal of mite-infested brood by hygienic bees, Gramacho (1999) emphasized that the first step in the hygienic process is the perforation of the wax capping over the cell that contains the infested brood. She proposed a model based on three recessive loci: two that control perforation and uncapping behavior (u_1 and u_2) and one (r) controlling removal. Current molecular techniques and quantitative trait loci (QTL) mapping have revealed seven putative loci that may influence hygienic behavior (Lapidge et al. 2002). The latter study suggests that the behavior is inherited in a quantitative, rather than Mendelian manner. That hygienic behavior might be a quantitative trait is consistent with our observations that the variation in expression of the behavior is continuously distributed among bees. All honey bees can and will uncap and remove dead or diseased brood, but at variable rates. Selective breeding for the behavior accentuates the differences in the rate of its expression, such that individual bees within a colony bred for hygienic behavior initiate uncapping and removal behaviors very quickly, while bees within a non-hygienic colony initiate uncapping and removal very slowly. It is likely that the neural networks that regulate the motor programs underlying uncapping and removing are the same between bees bred for hygienic and non-hygienic behavior (Spivak et al. 2003). The difference lies probably in the ease with which these programs are released.

Our working hypothesis is that olfactory cues trigger detection of abnormal brood within a wax-capped cell, which elicits perforation and uncapping of the cell, and the removal of the cell's contents. We have found that hygienic bees can detect the odor of diseased brood at lower stimulus levels than non-hygienic bees (Masterman et al. 2001). In addition, hygienic bees can discriminate between the odors of healthy and diseased brood at a lower stimulus level than non-hygienic bees.

Based on results from our previous studies (Masterman et al. 2000, 2001), we hypothesize here that olfactory sensitivity and discrimination abilities vary among bees within a hygienic colony bred to be homozygous for the alleles governing the behavior. The inherent variation in olfactory sensitivities and responses among same-aged bees may result in the tendency for some bees to spend a greater proportion of their time uncapping, and others to spend an equivalent amount of time uncapping and removing (Arathi and Spivak 2001). Specifically, we tested whether bees that tend to initiate the behavior by perforating and uncapping a cell containing dead brood (here collectively referred to as 'uncappers') have greater olfactory sensitivity to the odor of diseased brood, and can discriminate between the odors of healthy and diseased brood better than same aged bees that are collected while removing the uncapped brood from the cell (referred to as 'removers'). We also presented hygienic colonies with wax-capped, dead brood of different stimulus intensities to test whether an increased stimulus would trigger the behavior in bees with less olfactory sensitivity.

As in previous experiments, we used a combination of proboscis-extension response (PER) conditioning, to test whether bees can discriminate between healthy and diseased brood odors, and electroantennograms (EAG), to determine the differences in olfactory sensitivity among bees when presented the odor of diseased brood at different concentrations. EAG recordings provide a measure of the summed potentials of all chemosensory neurons of the antennae, and so measure overall olfactory sensitivity. PER discrimination conditioning is a complex task in that the bees must learn to associate one odor with a reward, and a second odor with an aversive stimulus. It provides a measure of chemical detection and motor response to olfactory stimuli, and reveals differences in learning abilities among bees (Hammer 1993; Hammer and Menzel 1995). The ability to discriminate between the brood odors is an important component of hygienic behavior because it ensures that the bees uncap and remove from the nest only diseased brood, and not healthy brood. The PER data complement the EAG data by demonstrating that the olfactory sensitivity is coupled with an appropriate response.

Methods

Breeding

The breeding program for hygienic behavior was initiated in 1993 by selecting colonies of Italian-derived, *A. mellifera* bees using a freeze-killed brood assay described in Spivak and Downey (1998). Colonies that uncapped and removed freeze-killed brood within 48 h were considered hygienic, those that took over 6 days to perform the same task were considered non-hygienic. To establish and maintain discrete lines, queen bees were raised from colonies that displayed the most rapid and least rapid removal rates. For each generation, the daughter hygienic queens were instrumentally inseminated with a mixture of semen from drones from different hygienic colonies. Similarly, daughters from the most non-hygienic queens were inseminated with sperm of drones from the most non-hygienic colonies.

Collection of uncappers and removers

In the year 2000, one hygienic colony was chosen from the seventh generation of selected colonies to be the source of bees (parental colony) for the experiments that year. In 2001, two different hygienic colonies were chosen from the eighth generation.

For the experiments, 1-day-old bees from each hygienic colony, that emerged in cages within an incubator (34°C and 50% RH), were marked with enamel paint on the thorax to identify them by age. They were then introduced into observation hives containing approximately 1,000 unmarked bees of various ages from a hygienic colony and a queen. Every 3 days for 3 weeks, 100–200 marked bees were added to each observation hive. The trials began 3 weeks after the first marked bees were added to the observation hive.

In our previous experiments, extensive focal sampling was conducted on individual bees to determine if they tended to perform uncapping behavior more frequently than removing behavior (Arathi et al. 2000; Arathi and Spivak 2001). Here, we developed a quick but repeatable dual-choice method to collect uncappers and removers from the observation hives. Using a comb section containing approximately 50 cells of freeze-killed sealed brood, we manually uncapped 25 of the cells containing purple-eyed pupae

on each side using a forceps, and left the remaining cells intact. The comb section was then inserted into a full comb containing healthy brood within each observation hive. The comb section was accessible through a portal in the glass walls of the observation hive. Smoke was gently blown through the portal so that bees dispersed from the comb section. After the smoke dissipated, the bees slowly returned to the comb section. If a marked bee began poking a hole through a capping of a cell that was intact or enlarged the perforated hole made by another bee, she was considered an "uncapper" and collected. If she began removing pupa from a cell that was manually uncapped, she was considered a "remover." Only bees that were observed tugging on, and removing an intact pupa were collected. Although some bees tend to consume the hemolymph from freeze-killed pupae, thereby removing the pupae, these bees were not considered removers in this study. All collected bees were between 15 and 21 days old, the age range at which bees normally perform hygienic behavior (Arathi et al. 2000).

Collection of bees uncapping pin-killed brood

In 2001, we also compared the olfactory sensitivity of 15- to 21-day-old uncappers and removers from two colonies when the bees were presented with pin-killed brood to elicit hygienic behavior. We presumed that pin-killed brood might have higher olfactory stimulus intensity than freeze-killed brood (see Discussion), and would elicit uncapping by bees with less olfactory sensitivity than bees that uncapped freeze-killed brood. An insect pin was used to pierce 25 sealed brood cells containing purple-eyed pupae in the two hygienic colonies housed in observation hives, described above. The cells were pierced through the center of the cell cap, penetrating the body of the brood until the pin reached the base of the cell (Newton and Ostasiewski 1986; Gramacho and Gonçalves 1994; Spivak and Downey 1998). The pierced cells were accessible through the portals in the observation hives. As before, smoke was gently blown through the portal so that bees dispersed, then slowly returned to the comb section containing pin-killed brood. The first bees that began uncapping (enlarging the small hole made in the cell capping by the pin) were collected, and used in EAG experiments only.

PER discrimination conditioning

After collection from the observation hives, bees for all PER experiments were directly transferred to the laboratory where they were cooled on ice until they became inactive. Immediately after cooling, bees were harnessed in plastic tubes (Bitterman et al. 1983). Restrained bees were able to move their antennae and proboscises freely. Fifteen min after the bees were harnessed, they were fed 0.4 μl of 2 M sucrose to ensure they could extend the proboscis to feed, and then were starved for 2 h before the conditioning trials began.

The odors used as conditioned stimuli (CS) were live, healthy pupae and pupae infected with chalkbrood (a fungal disease caused by *Ascosphaera apis).* Live, healthy pupae with light pink-purple eyes were removed from their capped cells carefully to prevent injury. Chalkbrood infected pupae were collected from a diseased colony in the field and kept in a refrigerator (4 °C) in a covered petri dish. The intact diseased and healthy pupae were placed into separate 12 cc plastic syringe barrels through which air was passed. Two healthy pupae and two chalkbrood-infected pupae were used as the CS. This stimulus level was chosen because in previous experiments it was found that most hygienic bees could discriminate between the odors, but there was significant variability among bees, possibly because of differences in their detection and response thresholds for that stimulus intensity (Masterman et al. 2001). In addition, at this stimulus level, bees bred for non-hygienic behavior were not able to discriminate well between the odors. New diseased and healthy pupae were placed in new plastic syringes for each set of bees tested.

For PER conditioning, a restrained bee was placed in the conditioning arena and left to acclimate for approximately 30 s. One of the odors was then blown across the bee for 4 s in an airstream of 0.5 m/s. A computer controlled the duration of the airstream. Three s after the presentation of the odor, the computer signaled for the experimenter to touch the antenna of the bee with 0.4 µl of 2 M sucrose (an appetitive unconditioned stimulus, US+) to elicit the proboscis extension response. The bee was then fed sucrose for 1 s. The computer again signaled to end the feeding period. After repeated trials, if the bee learned to associate the odor (the CS+) with the sucrose reward, she extended her proboscis upon presentation of the odor alone, in anticipation of the reward. In discrimination conditioning, a second odor (the CS−) was forward paired with an aversive US (0.4 µl of 3 M NaCl touched to the bee's antenna). After repeated trials, if the bee learned to associate this odor with the aversive stimulus, she would withhold proboscis extension upon presentation of the second odor. An aversive stimulus was used for consistency with our previous experiments (Masterman et al. 2001), although discrimination conditioning is also effective when the second odor is simply unrewarded (Smith et al. 1991; Sandoz et al. 2001).

In a first set of trials, the CS+ was diseased pupae and the CS− was healthy pupae. In a second set of trials, the CS were reversed. Bees were exposed to each odor eight times (trials). In all cases, the rewarded odor (the CS+) was presented first, followed by the CS−. The order presentation of the CS+ and CS− was presented in a pseudorandom sequence repeated twice (CS+ CS—CS− CS+ CS− CS+ CS+ CS−) following Smith et al. (1991). In most conditioning sessions, 10–12 bees were trained at a time, 5–6 uncappers and 5–6 removers. There was an 8-min inter-trial interval between each CS for each bee. Bees that extended their proboscises (spontaneous responders) to the presentation of the CS+ in the first trial (less than 10% of both uncappers and removers) 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. Conditioning sessions were repeated with new bees on successive days until an adequate sample size was reached.

PER statistical analysis

In the PER 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. A discrimination index (DI) was used to analyze the differences between the uncappers' and removers' ability to discriminate between each odor pair. The DI was calculated by subtracting the sum of the responses to the CS− from the sum of the responses to the CS+ for each bee. The DI could range from a high of 7 to a low of −8 (0 positive responses to the CS+ and 8 positive responses to the CS−). The DI scores were compared using Kruskal-Wallis tests (SAS 1989).

A generalization index (GI) was calculated to compare the degree that the uncappers and removers generalized between two the odors, following Sandoz et al. (2001). The GI score for each bee was calculated by dividing the DI score by the sum of the responses to both the CS+ and CS−, and subtracting the quotient from 1. The GI values could range from 0 to 2. A value of 0 would indicate that the bees responded only to the CS+ and did not generalize between the odors; a value of 1 would indicate the bees responded equally to the CS+ and the CS−, thus generalizing completely between them; a value over 1 would indicate the bees responded more to the CS− than the CS+. The GI indices were compared using Kruskal-Wallis tests.

EAG recordings

For the EAG experiments, bees were collected from the observation hives as described. Different bees were collected from the same colonies for the EAG recordings that were used in PER conditioning. The electrophysiological methods used for EAG recordings are

the same as those in Masterman et al. (2001). Intact bees were chilled briefly and harnessed as for PER. A ground electrode was inserted into the haemolymph of the posterior region of the head capsule, just above the occipital foramen. The distal tip of one antenna was removed, and the recording electrode (glass micro-capillary tube containing chloridized silver wire and physiological saline) was advanced slightly into the tip opening until electrical continuity with ground was achieved. EAG responses were amplified and recorded using a Cornerstone IX2-700 intracellular DC pre-amplifier (Dagan, Minneapolis, Minn.) and a Maclab digital acquisition system using the Chart program.

A constant flow (125 ml/min) of charcoal-filtered humidified air was passed continuously over the bee during recording sessions. Hexane [saturated vapor pressure (SVP) of 0.68] was used as the control odorant at 1/23 of SVP (370 ppm) (Patte et al. 1989). Odorant preparations consisted of chalkbrood extract dissolved in hexane. One chalkbrood equivalent (CBE) was defined as one diseased pupa dissolved in 2 ml of control strength hexane. Subsequent dilutions were made by addition of hexane. A 5-μl aliquot of odorant was placed onto a filter strip inserted into glass tubes. Tubes were sealed until use and discarded after each trial presentation. Stimuli were presented to bees by shunting odorant into the constant air stream. For all presentations, the duration of stimulus delivery was 2 s, and inter-stimulus intervals equaled 2 min. Order of presentation was as follows: control, 0.1 CBE, control, 0.5 CBE, control, 1.0 CBE. Peak amplitude of the response was measured using Maclab software. Subtracting the response to hexane (control) from the response to each CBE allowed for standardized data.

EAG statistical analysis

The data was distributed normally, so two-way ANOVAs were used to analyze the EAG responses of the bees at each odor concentration separately (0.1, 0.5 and 1.0 CBE) (Proc ANOVA, SAS 1989). Behavioral group (uncappers or removers) and colony were modeled as main effects.

Results

PER discrimination conditioning

Table 1 shows the discrimination and generalization indices, comparing the responses of bees collected while uncapping or removing freeze-killed brood for each colony. There was no significant colony level effect, so the data was pooled within behavioral groups for each CS odor pairing (Fig. 1). A comparison of the DI indicated that the uncappers discriminated significantly better than the removers when the CS+ was the odor of chalkbrood ($\chi2$=7.56, *df*=1, *P*=0.006) (Fig. 1a). A comparison of the GI indicated that the removers generalized between the odors in this pairing significantly more than the uncappers; i.e., they were less able to tell the odors apart ($\chi2$=18.93, df=1, *P*<0.001) (Fig. 1b).

When the CS+ was the odor of healthy pupae, there was no significant difference between the DI of the behavioral groups; the uncappers and removers discriminated equally well between the odors ($\chi2$=0.77, *df*=1, *P*=0.380) (Fig. 1c). They also did not differ significantly in the degree of generalization between the odors ($\chi2$=1.486, *df*=1, *P*=0.223) (Fig. 1d).

To analyze this asymmetrical response to the two CS+ in more detail, we compared the discrimination indices between the two odor pairings for all bees combined. All bees (pooled responses of uncappers and removers across all colonies) had significantly higher DI when the CS+ was chalkbrood compared to when the CS+ was healthy pupae ($\chi2$=4.357, *df*=1, *P*=0.037).

In sum, all bees discriminated between the odors of chalkbrood and healthy pupae significantly better when the CS+ was chalkbrood compared to when the CS+ was healthy pupae. Within this odor pairing (CS+ chalkbrood), bees collected while uncapping discriminated better and generalized less compared to bees collected

Table 1 Results of proboscis-extension discrimination conditioning. Median discrimination indices (*DI*) and generalization indices (*GI*), and their ranges, of bees from three colonies collected while uncapping or removing freeze-killed brood. (*A*) *DI* and *GI* when the CS+ was the odor of chalkbrood (diseased brood) and the CS− was the odor of healthy pupae. (*B*) *DI* and *GI* when the odors were reversed (CS+ healthy pupae, CS− chalkbrood). There were no significant differences in the responses of uncappers or of removers among the three colonies, based on Kruskal-Wallis tests (*P* values for comparison among colonies shown at bottom of columns for each CS+); thus, further analysis was performed on pooled data

Colony—Year	Sample size: uncappers, removers	DI Median (range)		GI Median (range)	
		uncapper	remover	uncapper	remover
A. CS+ Chalkbrood					
1 – 2000	20,20	4.5 (1–6)	3.0 (0–7)	0.44 (0–0.8)	0.47 (0–1.0)
2 – 2001	20,17	4.0 (1–5)	2.0 (1–7)	0.00 (0–0.8)	0.73 (0–0.9)
3 – 2001	18,20	3.0 (0–5)	2.0 (-2–5)	0.14 (0–1.0)	0.67 (0–1.3)
		P = 0.052	*P* = 0.266	*P* = 0.196	*P* = 0.412
B. CS+ Healthy Pupae					
1–2000	22,22	3.5 (1–5)	3.0 (-1–4)	0.54 (0–0.9)	0.54 (0–1.1)
2–2001	16,25	2.5 (-1–6)	3.0 (-2–6)	0.31 (0–1.0)	0.67 (0–1.5)
3–2001	20,19	2.5 (-2–5)	2.0 (-1–6)	0.49 (0–1.3)	0.50 (0–1.1)
		P = 0.056	*P* = 0.334	*P* = 0.616	*P* = 0.705

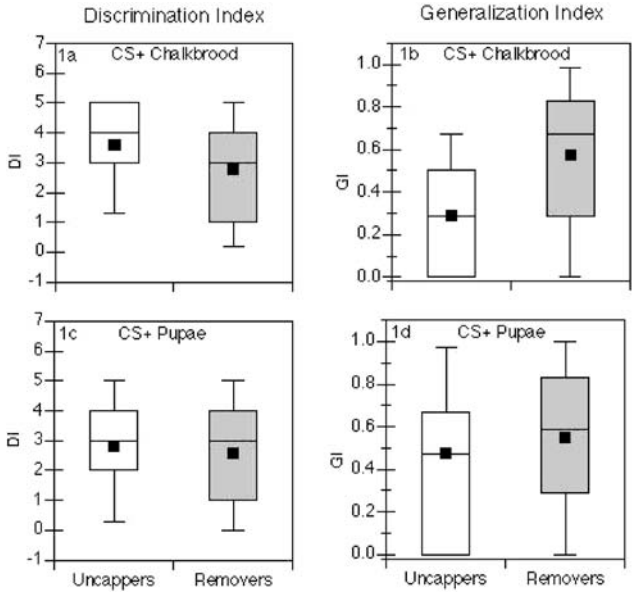

Fig. 1a–d Discrimination indices (*DI*) and generalization indices (*GI*) generated from proboscis-extension discrimination conditioning. Box plots show quartiles and interquartile range (*lines from ends of boxes*) of the data set. The median value for each plot is designated by a *line* through the box, and the mean by a *black square*. Responses of bees within each behavioral group (*uncapper* or *remover*) from three hygienic colonies are pooled. All bees were collected while uncapping or removing freeze-killed brood. **a** Bees collected while uncapping discriminated between the odors of diseased brood (chalkbrood) and healthy brood significantly better than bees collected while removing when the CS+ was chalkbrood and the CS− was healthy pupae. **b** Removers generalized between the odors significantly more when the CS+ was chalkbrood. **c, d** When the odor pair was reversed and the CS+ was healthy pupae, there were no significant differences in *DI* or *GI* between the behavioral groups

while removing, indicating that they were better able to tell the odors apart.

EAG responses

Uncappers versus removers of freeze-killed brood

The mean EAG responses of the bees from each colony to the odor of chalkbrood at three concentrations are given in Table 2. At each odor level (CBE), bees collected while uncapping freeze-killed brood had significantly higher EAG responses (greater olfactory sensitivity) to the odor of chalkbrood than the same age bees collected while removing freeze-killed brood (0.1 CBE: F=107.9, df=1,118, P<0.001; 0.5 CBE: F=64.24, df=1,118, P<0.001; 1.0 CBE: F=10.70, df=1,118, P=0.0014). At the lowest odor level, 0.1 CBE, there was a significant interaction between behavior and colony (F=6.11, df=2,118, P=0.003); the differences in mean responses of the uncappers and of the removers were not consistent among colonies at that stimulus level (Table 2). However, there were no significant interaction effects, or significant differences among colonies at the other two odor concentrations (P>0.10 for all). Figure 2 shows the data pooled across all three colonies. Overall, the results demonstrate that bees that uncapped freeze-killed brood had greater olfactory sensitivity to the odor of diseased brood than bees that removed freeze-killed brood.

Table 2 Mean EAG response (± SD) of bees from the three colonies to odor of diseased brood at three concentrations (0.1, 0.5, 1.0 chalkbrood equivalents, *CBE*). In the first two columns, the bees were collected while uncapping or removing freeze-killed brood, and each bee was tested at all three concentrations. In the last column, the bees were collected while uncapping brood that had been killed with a pin. Data on bees uncapping pin-killed brood was not collected for colony 1 in 2000. Sample sizes (uncappers freeze-killed, removers freeze-killed, uncappers pin-killed): Colony 1: (28, 28, 0); Colony 2: (17, 17, 21); Colony 3: (17, 17, 21) for each odor concentration

	Uncappers Freeze-killed brood	Removers Freeze-killed brood	Uncappers Pin-killed brood
0.1 CBE			
Colony 1	0.197 ± 0.042	0.151 ± 0.044	–
Colony 2	0.231 ± 0.044	0.132 ± 0.047	0.109 ± 0.043
Colony 3	0.212 ± 0.042	0.111 ± 0.034	0.129 ± 0.044
0.5 CBE			
Colony 1	0.289 ± 0.047	0.232 ± 0.057	–
Colony 2	0.293 ± 0.053	0.215 ± 0.063	0.197 ± 0.061
Colony 3	0.302 ± 0.030	0.217 ± 0.031	0.239 ± 0.048
1.0 CBE			
Colony 1	0.383 ± 0.053	0.346 ± 0.058	–
Colony 2	0.391 ± 0.059	0.359 ± 0.064	0.317 ± 0.072
Colony 3	0.395 ± 0.035	0.365 ± 0.046	0.366 ± 0.058

Uncappers of pin-killed versus freeze-killed brood

The mean EAG responses of the bees collected while uncapping brood that had been pin-killed, from colonies 2 and 3 in 2001, are also given in Table 2. As no bees were collected while removing pin-killed brood from colony 1 in 2000, separate two-way ANOVAs were used to compare the responses of bees from colonies 2 and 3 among three behavioral groups: uncappers of pin-killed brood, uncappers of freeze-killed brood, and removers of freeze-killed brood. At each odor concentration, there was a significant effect of behavior on the responses (0.1 CBE: F=63.1, df=2,103, P<0.001; 0.5 CBE: F=29.55, df=2,103, P<0.001; 1.0 CBE: F=7.26, df=2,103, P=0.001). There were no significant effects of colony, or significant interactions between behavior and colony (P>0.07 for all). Subsequent Tukey's HSD tests on the effect of behavior indicated that bees collected while uncapping freeze-killed brood had significantly higher EAG responses (greater olfactory sensitivity) to the odor of chalkbrood than the same age bees from the same colony collected while uncapping pin-killed brood (P<0.05). The Tukey's tests also revealed no significant differences between bees collected while uncapping pin-

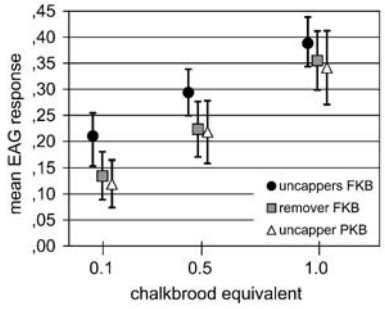

Fig. 2 Mean electronantennogram (EAG) response (±SD) by bees collected while uncapping or removing freeze-killed brood (*FKB*) (*black circles* and *gray squares*), and by bees collected while uncapping pin-killed brood (*PKB*) (*white triangles*). In this figure, data were pooled among the three colonies from which bees were collected uncapping and removing FKB, and between the two colonies from which bees were collected uncapping PKB, although statistical analysis was performed on unpooled data (see text). Each bee was presented with the odor of diseased brood (chalkbrood) at three concentrations, *0.1, 0.5* and *1.0* chalkbrood equivalents (CBE). One CBE=one diseased pupa dissolved in 2 ml of hexane. The response to hexane was subtracted from the total response for each CBE to standardize the data

killed brood and bees collected while removing freeze-killed brood at all three odor concentrations *(P*>0.5). Figure 2 shows the pooled data from colonies 2 and 3 for the bees collected while uncapping pin-killed brood, and illustrates that bees that uncapped pin-killed brood had the same olfactory sensitivity as removers of freeze-killed brood.

Discussion

The results support our hypothesis that 15- to 21-day-old bees within a colony bred for hygienic behavior, and thus presumably homozygous for the alleles that regulate the behavior, display variation in their olfactory sensitivity to the odor of diseased brood and in their ability to discriminate between the odors of diseased and healthy brood. When the stimulus to elicit hygienic behavior was freeze-killed brood, bees that initiated the behavior by perforating and uncapping the brood had significantly greater olfactory sensitivity compared to bees that completed the behavior by removing the brood from the cell, as measured by EAG recordings. The uncappers discriminated between the odors of chalkbrood-diseased brood and healthy brood significantly better than the removers when the CS+ was diseased brood, as measured by PER conditioning. The uncappers also generalized between the odors significantly less in this odor pairing, which is another measure of their ability to distinguish and respond appropriately to the two odors. When the odor pair was reversed so that the CS+ was healthy pupae, there was no difference in the bees' discrimination or generalization between the odors. Asymmetrical responses to these two-odor pairings were observed in each of our previous studies (Masterman et al. 2000, 2001), and as in this study, bees discriminated better when chalkbrood was the CS+ and healthy pupae was the CS−. It is possible that the odor of chalkbrood has stronger stimulus intensity than healthy pupae, making it easier for both uncappers and removers to associate the odor of chalkbrood with the sucrose reward (Bhagavan and Smith 1997; Pelz et al. 1997). The significant differences in EAG responses between uncappers and removers support the PER data and demonstrate that there are inherent differences in olfactory sensitivity among hygienic bees at the level of the peripheral nervous system. Overall, the results emphasize the variability in detection, perception, and responses to olfactory stimuli among bees of the same genotype.

We compared the olfactory sensitivity of bees collected while uncapping freeze-killed brood with bees uncapping pin-killed brood as an indirect way to measure the influence of olfactory stimulus intensity on the response of the bees. A common assay in breeding programs, when selecting for hygienic behavior in the field, involves determining how long it takes a colony to uncap and remove comb sections containing wax-capped pupae that have been either freeze-killed or pin-killed (Spivak and Downey 1998; Gramacho et al. 1999). The relative rate at which the bees remove dead brood is correlated with the removal of diseased brood, and hence disease resistance (Jones and Rothenbuhler 1964; Gilliam et al. 1983; Spivak and Reuter 2001). Bees from all colonies (bred for hygienic behavior or not) remove pin-killed brood much faster than freeze-killed brood (Spivak and Downey 1998) presumably because the pin-hole made in the cell capping combined with the piercing of the pupa increases the olfactory stimulus relative to intact frozen brood (Gramacho et al. 1999). We predicted that bees that uncap freeze-killed brood would have greater olfactory sensitivity than bees that uncap pin-killed brood. This prediction was confirmed by the EAG data: bees collected while uncapping pin-killed brood had significantly less olfactory sensitivity to the odor of diseased brood than bees collected while uncapping freeze-killed brood. Although we did not directly measure the relative stimulus intensities of the freeze-killed and pin-killed brood, our results indirectly suggest that pin-killed brood elicited a behavioral response (uncapping) by bees with less olfactory sensitivity.

Our findings, couched in terms of a response threshold model, could explain the variation in the rate at which individual bees initiate hygienic behavior, and the apparent subsequent partitioning of the behavior into uncapping and removal components. Response threshold models, used to explain aspects of the division of labor within a social insect colony, suggest that individuals encounter different cues and those with lower detection thresholds for such cues initiate tasks specifically associated with those stimuli (reviewed in Bonabeau and Theraulaz 1999; Beshers and Fewell 2001). All bees can perform the motor components of the behavior (perforation, uncapping, removal), but only bees that are able to detect abnormal brood odors at a low stimulus level, and

accurately discriminate between normal and abnormal brood, may perforate and uncap the cell first, exposing the stimulus within the cell. These bees may also continue removing the contents of the cell (Arathi et al. 2000). Other bees, with less olfactory sensitivity, may initiate uncapping and/or removal only when the stimulus level is higher. In this experiment, we did not differentiate between bees that perforated the cell and bees that continued enlarging the perforation; we considered both as 'uncappers.' It is possible that bees that make the first puncture hole in the cell capping have the highest olfactory sensitivity among hygienic bees.

It is important to compare these results with previous studies by Robinson and Page (1988) and Frumhoff and Baker (1988) that examined the effect of genetics on task performance and division of labor within honey bee colonies. In those landmark studies, worker bees that guarded the nest or removed corpses (Robinson and Page 1988), and that engaged in social grooming or trophallaxis (Frumhoff and Baker 1988) were from genetically distinct patrilines, or subfamilies, within the colony. These studies reported for the first time that genotype influenced the probability of task performance in social insects. Prior to that, it was assumed that variation in behavioral repertoires among nestmates with similar behavioral competencies arose solely through environmental differences during development or age-based (temporal ontogentic) differences among adults (Oster and Wilson 1978; Winston 1987).

Hygienic behavior fits the general model described above, in that a bee's genotype strongly influences the probability that it will initiate hygienic behavior when the stimulus level is low. In the broad sense, the response threshold model used to explain guarding, undertaking, grooming and trophallaxis in honey bees also fits for the performance of hygienic behavior: genetically "distinct" individuals (hygienic and non-hygienic bees) differ in task performance because they have different distributions of behavioral response thresholds for the stimuli eliciting a task (Robinson and Page 1988). However, our present study demonstrates that bees within a colony bred for the hygienic trait, which are not genetically distinct, still exhibit variability in rate of behavioral expression among individual bees. The observed differences in olfactory sensitivity among hygienic bees were not due to age-based differences in developmental acuity, as all bees were middle-aged, 15–21 days old. More likely, the differences stem from variability in the facility with which the behaviors are expressed, an effect that may be associated with modulation of the nervous system. We have evidence that nueromodulation plays an important role in the expression of hygienic behavior (Spivak et al. 2003). Subtle differences in the internal or external environment of the bee may influence the actions of these modulators, thus contributing to the differences in olfactory sensitivity and behaviors we observed in the hygienic line (Mesce 2002).

Our behavioral observations on hygienic behavior have revealed that an individual bee does not usually perforate, uncap and remove the contents of an entire cell from start to finish (Gramacho 1999; Arathi et al. 2000). One bee may perforate the cell's capping and not return to continue working on that cell. Other bees may enlarge the hole made in the cell's capping, and still others may participate in removing the abnormal pupae from the cell. In previous studies with colonies containing all hygienic bees, and in colonies containing both hygienic and non-hygienic bees in equal proportions, there was little or no persistence of bees performing hygienic behavior, defined as the probability that a bee would be observed performing the behavior more than once (Arathi and Spivak 2001). In addition, colonies that consisted of all hygienic bees were found to be highly efficient in removing dead brood from the nest as compared with colonies that had a mixture of hygienic and non-hygienic bees (Arathi and Spivak 2001). When there were sufficient numbers of bees of the hygienic genotype, with high olfactory sensitivities and discrimination abilities, they organized themselves into a class that performed uncapping at higher frequency, and a class that performed both uncapping and removing at equal frequency. In colonies with very few hygienic bees, bees with the greatest olfactory sensitivity were more persistent in their attempts to uncap and remove the abnormal brood, and a group of bees that performed uncapping with higher frequency was not observed.

In summary, the uncapping and removal components of hygienic behavior are not discrete motor programs. All bees can both uncap and remove diseased or dead brood. Breeding for hygienic behavior most likely selects for bees at one end of the continuum of the quantitative trait, such that the range in rate of expression of the trait among bees within a hygienic colony is relatively narrow. Hygienic colonies contain bees with low, but not uniform, response thresholds that are able to rapidly and efficiently detect and remove diseased brood before it becomes infectious. Our findings suggest that bees with the greatest olfactory sensitivity may tend to initiate hygienic behavior first because they are able to detect and accurately discriminate between abnormal and normal brood at low stimulus intensity. If the cells containing diseased, parasitized or dead brood are capped with wax, these bees would initiate hygienic behavior by perforating the cell. If the abnormal brood is in the larval stage and hence still unsealed (e.g., in the early stages of infection with American foulbrood or chalkbrood disease), it is not necessary to perforate and uncap the cell, and bees with the greatest olfactory sensitivity might initiate hygienic behavior by removing the larva (Rothenbuhler 1964a, Brødsgaard et al. 2000). Thus, the facility with which the hygienic motor program is released is biased both by the genotype of the bee which influences its olfactory sensitivity and behavioral responses, and the intensity of the olfactory stimulus.

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