J Chem Ecol (2009) 35:1108–1116
DOI 10.1007/s10886-009-9683-8

Odorants that Induce Hygienic Behavior in Honeybees: Identification of Volatile Compounds in Chalkbrood-Infected Honeybee Larvae

Jodi A. I. Swanson · Baldwyn Torto · Stephen A. Kells · Karen A. Mesce · James H. Tumlinson · Marla Spivak

Received: 21 March 2009 / Revised: 21 July 2009 / Accepted: 11 August 2009 / Published online: 8 September 2009

Abstract Social insects that live in large colonies are vulnerable to disease transmission due to relatively high genetic relatedness among individuals and high rates of contact within and across generations. While individual insects rely on innate immune responses, groups of individuals also have evolved social immunity. Hygienic behavior, in which individual honeybees detect chemical stimuli from diseased larvae and subsequently remove the diseased brood from the nest, is one type of social immunity that reduces pathogen transmission. Three volatile compounds, collected from larvae infected with the fungal pathogen *Ascosphaera apis* and detected by adult honey bees, were identified by coupled gas chromatography-electroantennographic detection and gas chromatography-mass spectrometry. These three compounds, phenethyl acetate, 2-phenylethanol, and benzyl alcohol, were present in volatile collections from infected larvae but were absent from collections from healthy larvae. Two field bioassays revealed that one of the compounds, phenethyl acetate is a key compound associated with *Ascosphaera apis*-infected larvae that induces hygienic behavior.

J. A. I. Swanson · S. A. Kells · K. A. Mesce · M. Spivak (✉)
Department of Entomology, University of Minnesota,
219 Hodson Hall, 1980 Folwell Ave,
St. Paul, MN 55108, USA
e-mail: spiva001@umn.edu

B. Torto
International Centre of Insect Physiology and Ecology,
PO Box 30772, Nairobi, Kenya

J. H. Tumlinson
The Pennsylvania State University,
111 Chemical Ecology Lab,
University Park, PA 16802, USA

Keywords *Apis mellifera* · Hygienic behavior · Insect chemoreception · Social immunity · Chalkbrood · Gas chromatography-electroantennographic detection

Introduction

Many insect societies have evolved an array of defenses to combat the invasion and spread of pathogens and parasites in the nest (reviewed in Cremer et al., 2007; Wilson-Rich et al., 2009). Social insects that live in large colonies are vulnerable to disease transmission because of the relatively high genetic relatedness among individuals and high rates of contact within and across generations. While individual insects rely on innate immune responses, groups of individuals also have evolved collective immune defenses, called social immunity (Cremer et al., 2007). The social immune response of interest to this study is a behavioral defense, or antiseptic behavior (Wilson-Rich et al., 2009), that emerges at the colony level through the collective actions of individuals. Antiseptic behaviors decrease disease transmission; some examples include allo-grooming, undertaking (removal of diseased and dead adults from the nest), and hygienic behavior (the removal of diseased brood from the nest). Antiseptic behaviors are analogous to the cellular and humoral immune responses within an individual because they involve detection and recognition of the foreign pathogen or parasite, and subsequent initiation of an appropriate defensive response (Cremer and Sixt, 2009). In this study, we investigated the chemical stimuli that individual honeybees, *Apis mellifera* L. detect from diseased larvae and that initiate hygienic behavior. Hygienic behavior is specifically defined as the detection and removal of diseased larvae from the nest (Rothenbuhler 1964, reviewed in Wilson-Rich et al., 2009), and has been recognized since the late 1930's (Woodrow and

Holst, 1942) as an important natural mechanism of disease resistance in honeybee colonies. One of the most important aspects of hygienic behavior is that individual bees must detect and respond to appropriate stimuli from the diseased larvae early in the progression of the infection, before the pathogen becomes infectious. In this way, the quick and efficient detection and removal of diseased larvae by many bees prevents disease transmission throughout the colony.

Specifically, in this study, we investigated the hygienic response of honeybees to the fungal pathogen, *Ascosphaera apis*, which causes chalkbrood disease in larvae (Gilliam et al., 1983). Chalkbrood infection begins in the gut of a larva and grows slowly outward until it penetrates the cuticle. This typically occurs after a fifth instar has been capped, but before pupation begins. At this point, rapid growth and sporulation of the fungus occurs (Gilliam and Vandenberg, 1997). When adult bees remove infected larvae after the fungus has penetrated the cuticle, spores are ingested by adult bees and fed back to healthy larvae in the bee food. The healthy larvae ingest the spores, and the infection is perpetuated. Therefore, for hygienic behavior to be effective against chalkbrood, adult bees must remove the infected larva before the fungus has penetrated the cuticle.

Previous studies have suggested that the detection and subsequent removal of diseased larvae are guided by olfactory stimuli (Masterman et al., 2000, 2001; Gramacho and Spivak, 2003; Spivak et al., 2003). Differences in the degree of hygienic behavior at the colony level result from the relative speed and efficiency with which individual bees perform the behavior (Arathi et al., 2000, 2007; Arathi and Spivak, 2001). A response threshold model was postulated to describe the differences in colony-level expression of the behavior (Arathi and Spivak, 2001). In the laboratory, electrophysiological and behavioral conditioning of the proboscis-extension response (PER) were used to demonstrate that individual bees, from colonies selectively bred for hygienic behavior (see Methods), can detect larvae infected with chalkbrood, and discriminate between diseased and healthy brood at lower stimulus levels, compared to non-hygienic bees (Masterman et al., 2001; Spivak et al., 2003; for review, Fahrbach and Mesce, 2005). These studies, however, did not identify the specific volatile chemicals that bees use to detect diseased larvae, and did not test whether the application of such volatiles to healthy larvae could elicit an appropriate behavioral response in the field or not. In the present study we tested two hypotheses: 1) that hygienic behavior of honey bees can be elicited in field colonies by experimental application of appropriate olfactory stimuli; and 2) that hygienic behavior is based on a threshold response, in which colonies containing bees with the highest olfactory sensitivity to diseased brood initiate the hygienic response more quickly compared to colonies containing bees with a lower olfactory sensitivity.

Methods

Breeding The breeding program for colonies that display hygienic behavior began in 1993 at the University of Minnesota. Rapid-hygienic and slow-hygienic lines of bees (*sensu* Wilson-Rich et al., 2009) were selected from colonies derived from the Italian subspecies *Apis mellifera ligustica*. Colonies were selected initially based on their abilities to survive winter in Minnesota, to increase the population rapidly in spring, to produce large amounts of honey, and to be gentle (not sting) during normal management. If the colonies displayed the above set of traits, they were tested for hygienic behavior using a freeze-killed brood assay (Spivak and Reuter, 1998), which is an indirect measure of a colony's hygienic behavior. The removal of freeze-killed brood is correlated with the removal rates of diseased brood; colonies that uncap and remove >95% of freeze-killed pupae within 24 h will tend to remove diseased brood rapidly. Colonies that exhibited the most rapid removal rates were chosen to raise queens for the next generation. Daughter queens then were instrumentally inseminated with semen from drones of other rapid-hygienic colonies. The criterion for choosing rapid-hygienic colonies for breeding is an arbitrary cutoff established at 95% removal of freeze-killed brood within 24 h. Freeze-killed brood tests of many colonies yield a continuum of values, with colonies that remove a higher proportion of freeze-killed brood having higher degrees of hygienic behavior.

Volatile Collection Volatiles were collected from bees for two reasons. The first was to identify the changes between healthy and diseased brood. Three types of larvae were used: a) healthy fifth instars, b) fifth instars in the early stage of chalkbrood infection, and c) larvae fully overcome with fungal mycelia (mummies). Early stage chalkbrood-infected larvae were identified under a dissecting scope by a subtle discoloration of the larval cuticle, with no evidence of visible sporulation. Each type of larva was removed from the comb with sterile forceps and placed in quick-fit glass aeration chambers (46-cm-long × 19-cm-wide, RodaViss chambers, ARS, Gainesville, FL, USA). The disease was confirmed by fungal growth through the larva after the volatile collections were completed. Collections were determined to be from healthy larvae if no larvae showed clinical symptoms of disease at the conclusion of the volatile collection period. Each collection consisted of: a) 25 healthy fifth instars, b) 25 early-stage infection larvae, or c) 75 mummies. The second reason for collecting volatiles was for use in the gas chromatography-electroantennogram (GC-EAD) experiment. For this, volatiles were collected from a large quantity of healthy larvae or from diseased larvae placed in a larger aeration chamber (80-cm-long × 40-cm-wide, RodaViss chambers, ARS, Gainesville, FL, USA).

For each experiment, the volatile collection apparatus was constructed as follows: charcoal-filtered and humidified air at 0.5 Lmin^{-1} was passed over the larvae and then through pre-conditioned Super-Q adsorbent (30 mg, Grace, *nee* Alltech, Deerfield, IL, USA) traps for 24 h at room temperature. Each trap was eluted with 150 μl methylene chloride forced through the absorbent with filtered nitrogen gas. Elutions were stored at -70°C until used.

Chemical Analysis To identify the disease-related volatiles, chromatograms from the healthy, early-stage infected and fully diseased brood were compared. For the purposes of identification, 174 ng of butyl butyrate, as an internal standard, were added to 40 μl of extract, with 1μl samples of extract analyzed by gas chromatography-mass spectrometry (GC-MS) on an HP-6890 (Agilent Inc., Palo Alto, CA, USA) gas chromatograph linked to an HP 5973 mass spectrometer in the electron impact mode (70 eV, Agilent, Palo Alto). An HP-1 column (30 mm × 0.25 mm ID × 0.25 μm, J & W Scientific, Folsom, CA, USA), with helium as carrier gas, was used for the analyses; injection was splitless. The oven temperature was held at 50ºC for 5 min, then increased at 5ºC min^{-1} to 110°C, then 25°C min^{-1} to 230°C and held at this temperature for 10 min. Volatile compounds were confirmed by comparison of their chromatographic retention times and mass spectra with commercially available standards analyzed on the same instrument.

To isolate compounds perceived by bees, GC-EAD was used for the diseased-brood samples, the healthy larvae samples, and the standards. Aliquots (4 μl) of extracts were analyzed with an Agilent 6890 N gas chromatograph equipped with a TR-Wax column (30 m × 0.32 mm ID × 0.25 μm; Thermo Electron Corp, Belefonte PA, USA). This column permitted better separation of the compounds of interest than the HP1 column. Injection was splitless with helium as carrier gas. The oven temperature was held at 50°C for 5 min, then increased by 5°C min^{-1} to 110°C, then by 25°C min^{-1} to 200°C, and held at this temperature for 5 min. The effluent was split (SGE splitter, SGE Incorp., Austin, TX, USA) to allow simultaneous recording of the flame ionization detector (FID) and the EAD responses. Nitrogen was used as a make-up gas for the FID and as an auxiliary make-up gas (~15 mL min^{-1}) to carry the effluent to the EAD. GC-EAD Pro (Syntech, Hilversum, Netherlands) was used for simultaneous recording of the FID and EAD signals.

EAD recordings were obtained from excised antennae of bees that were 15–20 days old, as this is the age of bee that performs hygienic behavior (Arathi et al., 2000). In 2007, bees of known age were obtained by placing frames with brood ready to emerge in a cage in an incubator for 24 h. Emerged bees were marked with enamel paint on the thorax and returned to the source colonies for collection 15–20 days later. Three source colonies used for the GC-EAD tests were from the University of Minnesota breeding program and were deemed greater than 95% hygienic based on freeze-killed brood tests. The antennae were held between stainless steel electrodes (Syntech), and connections to the electrodes were made with conductivity gel (Spectra Gel® 360, Parker Laboratories, Fairfield, NJ, USA). A humidified air stream, containing the GC effluent, was delivered at 20 ml min^{-1} through a 44-cm glass tube to the antenna.

Field Bioassay I. Topical Applications The topical application assay tested the hypothesis that hygienic behavior of honey bees can be elicited in field colonies by experimental application of appropriate olfactory stimuli to healthy larvae. The assays were conducted in August 2007. Twelve colonies were chosen for the bioassay based on the freeze-kill removal method (one of the source colonies for the GC-EAD analyses also was used in this assay). A comb containing larvae was removed from each of the twelve colonies, and the position of 25 fifth instars on the comb was marked on a transparency overlaying the comb. A Picospritzer II (General Valve Corporation) was used to dispense 0.5 μl of each treatment onto healthy larvae. Five treatments were applied to separate groups of 25 healthy larvae: benzyl alcohol (Sigma-Aldrich, St Louis, MO, USA), 2-phenylethanol (Fluka, Steinheim, Germany), phenethyl acetate (Sigma-Aldrich, St. Louis, MO, USA), deionized water, and a mixture of equal parts benzyl alcohol, 2-phenylethanol, and phenethyl acetate. The compounds were drawn up by capillary action into a 5 μl capillary tube, and a 3 msec pulse of air at 80 psi was used to puff the compound onto a given larva. Neighboring larvae were not treated. The comb then was returned to the colony from which it was taken. At 4 h and 24 h, the transparency was replaced on the frame, and the number of larvae that the adult bees had removed was recorded.

Field Bioassay II. Paraffin Larval Dummies The assay utilizing paraffin larval dummies tested the hypothesis that hygienic behavior is based on a threshold response in which bees with the highest olfactory sensitivity to diseased brood initiate the hygienic response more quickly compared to bees with a lower olfactory sensitivity. Paraffin larval dummies were made by melting paraffin wax at 60–65°C with a 10^{-2} dilution of methyl linolenate (Acros Organics, NJ, USA). Methyl linolenate is one of several compounds present in brood pheromone of fifth instar larvae. When this compound is added to a paraffin wax dummy and placed in a larval cell, adult bees cap the cell containing the dummy (Le Conte et al., 1990). The addition of chalkbrood volatile compounds to wax dummies that contain brood pheromone

should result in a decrease in capping of these dummies, if adult bees detect and respond to the disease-related compounds. Bees are unable physically to remove paraffin dummies from the cell at nest temperatures (32–34°C), because the paraffin is soft and sticky. Therefore, in this assay, when brood pheromone was added to the paraffin dummy, and bees placed a wax capping over the dummy, it was an indication that the adult bees accepted the 5th instar as normal. When both brood pheromone and disease volatile(s) were added to the paraffin, and the bees did not cap the dummies with wax, it was assumed that bees detected the disease volatiles and exhibited hygienic behavior.

The response threshold model was tested by measuring the differential abilities of adult bees to detect chalkbrood compounds at differing concentrations. Using the mixture of wax and methyl linolenate, a total of eight treatments was formulated: phenethyl acetate at 10^{-2} (v/v) and 10^{-9}, 2-phenylethanol at 10^{-2} and 10^{-9}, benzyl alcohol at 10^{-2} and 10^{-9}, and a mixture of the previous three compounds at 10^{-2} and 10^{-9}. The concentrations were chosen based on differences in the frequency of capping observed in the preliminary development of this assay. Additional treatments consisted of methyl linolenate (in wax) alone and of paraffin wax without the addition of methyl linolenate or disease-associated compounds. The liquid wax containing each treatment was poured into plastic drinking straws (0.5 cm diam) and allowed to cool and harden. The straw then was cut away from the wax, and the wax was cut into 25 mm sections to create larval dummies.

The assays were conducted in August 2008. Six colonies that displayed varying degrees of hygienic behavior, ranging from 39% to 99%, as determined through freeze-killed brood assays (see Fig. 3), were chosen for this bioassay. A comb containing fifth instars was removed from the colony. Combs with fifth instars were chosen so that the larval dummies would be in context with actual fifth instars that were about to be capped with wax by adult bees. Twenty larval dummies of each treatment were placed into empty cells, and their location was marked on a transparency. No more than two treatments were tested on a single comb at a time. The combs were then returned to the colony and the number of dummies the bees capped with wax was recorded after 24 h.

Statistical Analysis Binomial regressions were performed to compare the proportion of healthy topically treated larvae removed or paraffin dummies left uncapped in response to each treatment, with the degree of hygienic behavior of the colony as a covariate. The delta method was used to calculate the student's t value for contrasts between treatments with differences declared at $\alpha=0.05$. Analyses were done with *Arc* (Cook and Weisberg, 1999).

Results

Chemical Analysis Mass chromatograms of volatiles collected from healthy fifth instars and larvae in early stages of chalkbrood infection are shown in Figure 1. Three peaks associated with diseased brood were non-detectable in collections from the healthy brood; these peaks appeared in the early stage diseased larvae and increased in quantity in those fully diseased. GC-MS analyses identified these compounds as phenethyl acetate, 2-phenylethanol, and benzyl alcohol. GC-EAD analyses of the volatiles of larvae in the early stages of chalkbrood infection showed that bee antennae responded consistently to these compounds (Fig. 2).

Field Bioassay I. Topical applications Colonies with higher degrees of hygienic behavior removed more treated larvae at 4 h ($t=4.844$, $df=54$, $P<0.001$) and at 24 h ($t=8.917$, $df=54$; $P<0.001$) compared to colonies that displayed slower hygienic behavior. There were no significant differences in the slopes of the regression lines among treatments; all colonies responded similarly to all treatments 4 h and 24 h after treatment (interaction between treatment and hygienic behavior for all; $P>0.05$).

At 4 h, most larvae treated with 2-phenylethanol, benzyl alcohol, the mixture, and water were not removed. However, more larvae treated with phenethyl acetate were removed compared to all other treatments: 2-phenylethanol ($t=4.485$, df=54, $P<0.001$), benzyl alcohol ($t=4.483$, df=54, $P<0.001$), the mixture ($t=6.181$, df=54, $P<0.001$), and water ($t=3.557$, df=54, $P<0.001$). Phenethyl acetate removal rates ranged from 0 to 84%.

At 24 h, there were significant differences in removal among all treatments (Table 1 and Fig. 3). Larvae treated with phenethyl acetate were removed in greatest numbers followed by larvae treated with the mixture, 2-phenylethanol, benzyl alcohol, and water (Fig. 3).

Field Bioassay II. Paraffin Larval Dummies None of the colonies capped paraffin dummies that lacked brood pheromone. The addition of the brood pheromone component, methyl linolenate, to the dummies resulted in a significant increase in capping. Capping of brood pheromone dummies ranged from 70% to 95%.

As predicted, when dummies were treated with both brood pheromone and one or a mixture of the disease volatiles, the bees in all colonies capped significantly fewer dummies treated with higher concentrations (10^{-2}) of the diseased larval compounds, as compared to dummies treated with lower concentrations (10^{-9}). This was true for all compounds: phenethyl acetate ($t=4.437$, $df=9$, $P<0.001$), 2-phenylethanol ($t=9.778$, $df=9$, $P<0.001$), benzyl alcohol ($t=7.388$, $df=9$, $P<0.001$), and the mixture

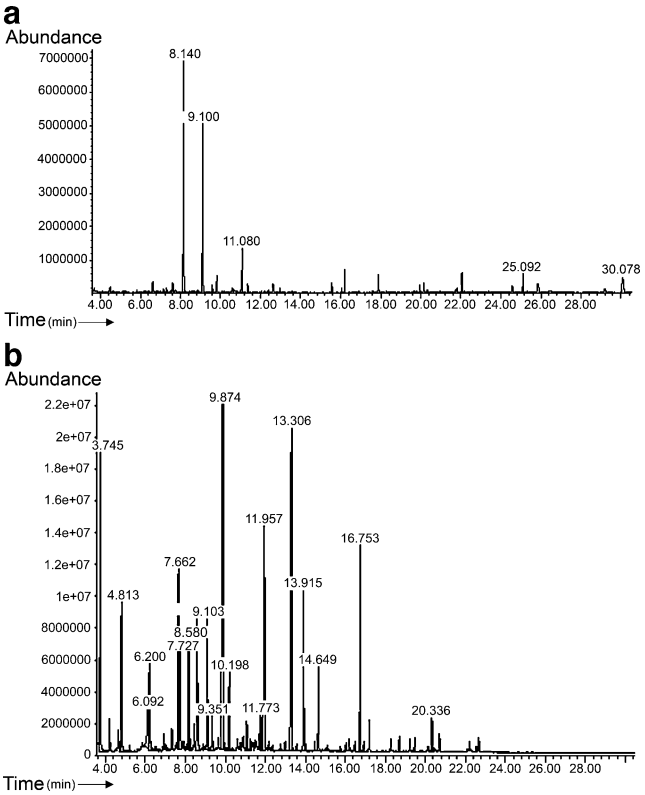

Fig. 1 Representative total ion mass chromatograms of volatile chemicals released by a) healthy 5th instar honeybee larvae and b) 5th instar larvae in early stages of chalkbrood infection. Retention time (min) of compounds of note: 8.14- butyl butyrate (IS), 8.58- benzyl alcohol, 9.1- (*E*)-β-ocimene, 9.874- 2-phenylethanol, 11.957- phenethyl acetate

(t=3.138, df=9, P=0.001) (Fig. 4). Colonies with higher degrees of hygienic behavior capped significantly fewer larval dummies (P<0.001). There were no significant differences in the slopes of the regression lines among treatments in the paraffin experiment, and the interaction between treatment and hygienic behavior, for all treatments, was not significant (P>0.05).

At the higher concentration (10^{-2}), there was no significant difference among the number of dummies capped for the treatments phenethyl acetate, the mixture, and paraffin alone. Dummies treated with phenethyl acetate, the mixture, and paraffin were capped significantly less than those treated with 2-phenylethanol and benzyl alcohol. All dummies treated with compounds at the 10^{-2} concentration were capped significantly less than those treated with the brood pheromone component alone, methyl linolenate (Table 2).

At the lower concentration (10^{-9}), dummies treated with phenethyl acetate and the mixture were capped significantly less frequently than dummies treated with methyl linolenate, 2-phenylethanol or benzyl alcohol. There was no difference in the numbers of dummies capped between the treatments methyl linolenate alone and 2-phenylethanol, methyl linolenate alone and benzyl alcohol, and between 2-phenylethanol and benzyl alcohol. There was no significant difference in the removal of dummies treated with phenethyl acetate or with the mixture (Table 3).

Discussion

This is the first identification of volatile compounds associated with diseased honeybee larvae that elicit hygienic behavior in adult honeybees. Three volatile compounds collected from larvae infected with the fungal pathogen, *Ascosphaera apis*, were not detected in healthy brood, and elicited electroantennogram responses from adult honeybees. Our bioassays showed that of the three compounds, phenethyl acetate elicited the greatest hygienic responses from bees in field colonies.

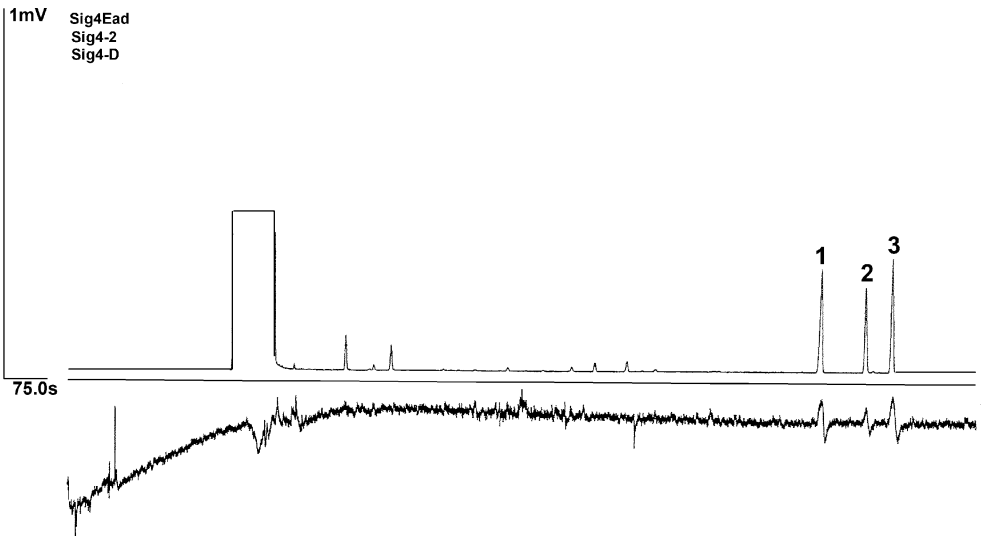

Fig. 2 Representative coupled gas chromatographic-electroantennographic detection (GC-EAD; flame ionization detection response shown on top; EAD response shown on bottom) traces of compounds collected from early stage chalkbrood-infected larvae, using antennae of 15–20 day-old honeybees. Numbered peaks correspond to compounds that elicited consistent EAD responses: 1) phenethyl acetate, 2) 2-phenylethanol, and 3) benzyl alcohol

Table 1 Student's *t* and *p* values of delta contrasts between odorant treatments for proportion of topically treated larvae removed by adult bees at 24 hours. Larvae were treated with 0.05 µl of an individual chemical or a mixture of the three chemicals

$\alpha = 0.05$, $df = 54$.

	Water		Mixture		Benzyl alcohol		Phenyl Ethanol	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*
Phenethyl Acetate	8.245	**< 0.001**	5.920	**< 0.001**	11.642	**< 0.001**	8.870	**< 0.001**
2-Phenylethanol	5.549	**< 0.001**	3.280	**0.002**	3.454	**0.001**		
Benzyl alcohol	4.473	**< 0.001**	6.530	**< 0.001**				
Mixture	6.496	**< 0.001**						

Colony-level behavioral responses to parasites and pathogens are an integral part of social immunity, but reports of social animals detecting and responding to diseased conspecifics are not common. Such behavioral responses generally involve avoidance or engagement (e.g., grooming or removal) of conspecifics that are infected, which likely involves the detection of disease-related chemical stimuli. For example, gregarious Caribbean spiny lobsters *Panulirus argus*, which occupy communal dens, exhibit avoidance behavior of lobsters infected with a lethal virus, even during early periods when the lobsters are not yet infectious (Behringer et al., 2006). Leaf cutting ants, *Acromyrmex echinatior,* use self- and allo-grooming behaviors in defense of the fungal disease *Metarhizium anisophae* (Hughes et al., 2002). Ants detect oleic and linoleic acids, emitted from dead adult conspecifics in their nests, and exhibit necrophoric or undertaking behavior, which favor colony health by reducing contact with potential pathogens (Wilson et al 1958; Akino and Yamaoka, 1996; Howard and Tschinkel, 1976). Honeybees also display necrophoric behavior, although the chemical cues have not been identified (Visscher, 1983). Our study demonstrated that disease-related odorants are important because the early detection and efficient removal of the source of the pathogen reduce disease transmission among individuals within the nest.

Our field bioassays confirmed two hypotheses, previously tested only in laboratory assays (Masterman et al., 2001; Spivak et al., 2003): 1) hygienic behavior of honey bees is elicited by olfactory stimuli; and 2) the expression of hygienic behavior depends on the olfactory response threshold of individual bees within the colony. Colonies that contain a majority of bees with high olfactory sensitivity respond quickly to low concentration stimuli associated with diseased brood, while colonies of bees with lower sensitivity take longer to respond, allowing transmission of spores and expression of clinical symptoms.

The compounds that elicited consistent GC-EAD responses, phenethyl acetate, 2-phenylethanol, and benzyl alcohol, were present both in larvae in the early stages of infection and in mummies, but were absent from healthy larvae. At this time, it is unclear whether the chemicals are produced by the larvae (pheromonal) or by the fungus (kairomonal). Laboratory inoculation with chalkbrood of potato dextrose agar, pollen and homogenized and sterilized brood failed to produce emissions of these volatiles, suggesting that the compounds are produced only in the presence of live brood (Baldwyn Torto, USDA, unpublished data).

When 0.5 µl of synthetic disease-associated volatile chemicals were applied topically to healthy larvae, adult bees responded to the olfactory stimulus and removed healthy larvae as though they were diseased. The degree of hygienic behavior, as indicated by the amount of freeze-killed brood removed within 24 h, was affected by the colony level response. The more hygienic a colony, the more likely the removal of larvae treated topically with disease-associated compounds. Bees removed up to 100% of healthy larvae treated with phenethyl acetate within 24 h, which is roughly the same time it takes bees from a rapid-hygienic colony to remove freeze-killed brood. We have not determined the odor of freeze-killed brood for comparison.

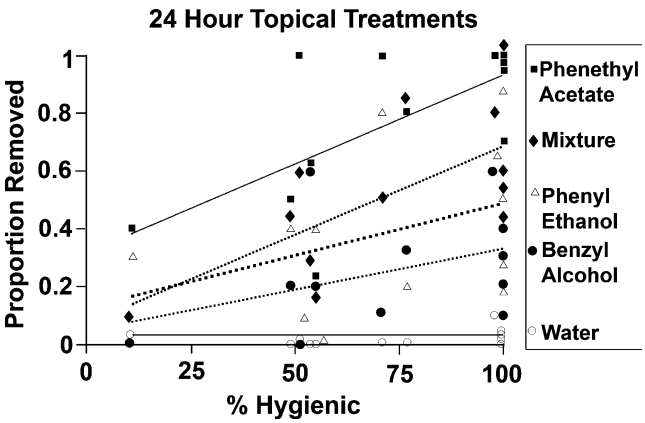

Fig. 3 Regression lines comparing the proportion of healthy larvae removed by adult bees, for each treatment at 24 h (y-axis), to the degree of hygienic behavior [based on the percent of dead brood removed in the freeze-killed brood assay (x-axis)]. Each point corresponds to the results from one of the twelve colonies treated. Treatments applied topically to larvae included de-ionized water (N=10 larvae per colony), phenethyl acetate, 2-phenylethanol, benzyl alcohol, or a mixture of the three chemicals (N=25 larvae per colony for each chemical treatment). Regression lines correspond to the compound listed to the right of each line. Significantly different proportions of larvae were removed for each treatment (Table 1; Student's *t,* a= *0.05, df= 54; P*<0.002)

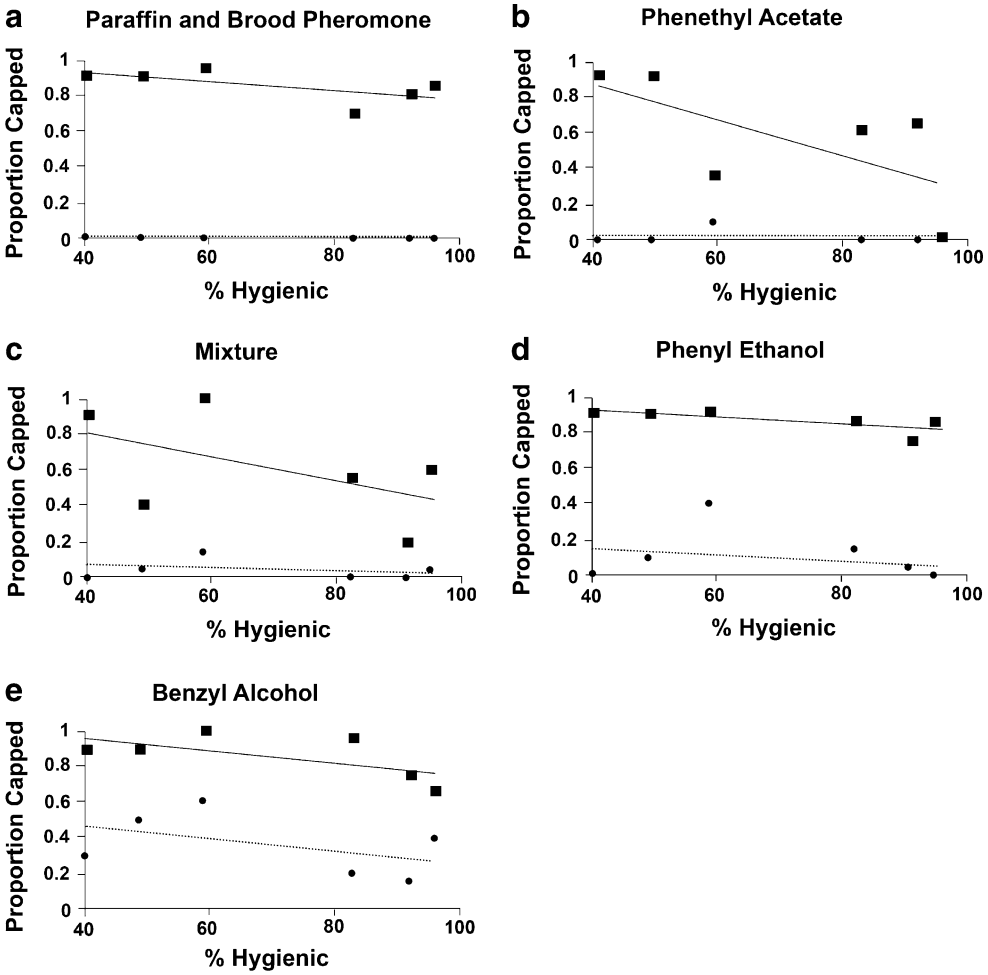

Fig. 4 Regression lines comparing the proportion of paraffin larval dummies capped with wax by adult bees to the degree of hygienic behavior (based on the percent of dead brood removed in the freeze-killed brood assay). Bees are unable to remove paraffin dummies, thus a lower proportion of capping was an indication of rejection of the cell's contents by adult bees; i.e., hygienic behavior. A) Rejection of dummies treated with brood pheromone (squares, top line) and paraffin dummies (circle, bottom line). B – E) Rejection of dummies treated with each compound or a mixture of the compounds, at high concentrations (10^{-2}; circles, bottom lines) and low concentrations (10^{-9}; squares, top lines). Each point corresponds to the results from one of the six colonies treated. Treatments (20 per treatment per colony): paraffin wax only, paraffin wax plus brood pheromone (methyl linolenate), paraffin wax plus brood pheromone plus one of phenethyl acetate, 2- phenylethanol, benzyl alcohol, or a mixture of the three

Bees are very sensitive to abnormal stimuli from larvae. For example, when larvae are touched with a paintbrush, larvae are removed within 5 h (Free and Winder, 1983). Our original intent was to test the response-threshold hypothesis by diluting disease-related compounds in a solvent. However, we observed that application of hexane or pentane alone induced very rapid removal, within 4 h, indicating that solvents elicited an unnatural and abnormally fast removal response. Although bees did not remove larvae treated with water (control in one assay), water was not a suitable solvent for phenethyl acetate, 2-phenylethanol, or benzyl alcohol. Thus we used an indirect bioassay to test

Table 2 Student's *t* and *p* values of delta contrasts between various treatments of paraffin larval dummies capped with wax by adult bees at 24 hours. Paraffin dummies were treated with one component of brood pheromone (methyl linolenate) alone at a 10^{-2} concentration (v/v), or brood pheromone (10^{-2}) plus one additional chemical or a mixture of three chemicals at a 10^{-2} concentration (v/v)

	Brood Pheromone (methyl linolenate)10^{-2}		Paraffin wax		Brood Pheromone+ Mixture 10^{-2}		Brood Pheromone+ Benzyl alcohol 10^{-2}		Brood Pheromone+ 2- Phenylethanol 10^{-2}	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*	*t*	*P*
Brood Pheromone+Phenethyl Acetate 10^{-2}	7.753	**< 0.001**	0.700	0.483	1.110	0.278	4.780	**< 0.001**	2.690	**0.013**
Brood Pheromone+2- Phenylethanol 10^{-2}	9.851	**< 0.001**	3.629	**< 0.001**	1.110	**0.049**	4.248	**< 0.001**		
Brood Pheromone+Benzyl alcohol 10^{-2}	7.308	**< 0.001**	5.772	**< 0.001**	5.199	**< 0.001**				
Brood Pheromone+Mixture 10^{-2}	9.359	**< 0.001**	1.957	**0.050**						
Paraffin wax	8.735	**< 0.001**								

$\alpha = 0.05$, $df = 29$.

Table 3 Student's *t* and *p* values of delta contrasts between various treatments of paraffin larval dummies capped with wax by adult bees at 24 hours. Paraffin dummies were treated with one component of brood pheromone (methyl linolenate) alone at a 10^{-2} concentration (v/v), or brood pheromone (10^{-2}) plus one additional chemical or a mixture of the three chemicals at a 10^{-9} concentration

	Brood Pheromone (methyl linolenate) 10^{-2}		Paraffin wax		Brood Pheromone+ Mixture 10^{-9}		Brood Pheromone+ Benzyl alcohol 10^{-9}		Brood Pheromone+ 2-Phenylethanol 10^{-9}	
	t	*P*	*t*	*P*	*t*	*P*	*t*	*P*	*t*	*P*
Brood Pheromone+Phenethyl Acetate 10^{-9}	4.826	< **0.001**	5.160	< **0.001**	0.688	0.497	4.965	< **0.001**	4.965	< **0.001**
Brood Pheromone+2-Phenylethanol 10^{-9}	0.187	0.852	6.650	< **0.001**	4.382	< **0.001**	0.000	1.000		
Brood Pheromone+Benzyl alcohol 10^{-9}	0.187	0.852	6.770	< **0.001**	4.383	< **0.001**				
Brood Pheromone+Mixture 10^{-9}	4.235	< **0.001**	5.353	< **0.001**						
Paraffin wax	6.613	< **0.001**								

*$\alpha = 0.05$, $df = 29$.

the response-threshold hypothesis by combining brood pheromone and disease volatiles at two different concentrations in paraffin larval dummies. When one component of brood pheromone, methyl linolenate, was added to a paraffin dummy placed within a cell, bees capped that dummy with wax as though it were a 5th instar ready to pupate. However, when both brood pheromone and disease-related volatile(s) were added to the paraffin, bees from rapid-hygienic colonies did not cap the dummies with wax; apparently they detected the disease-associated volatiles over the brood pheromone and behaved accordingly. We observed that bees with less olfactory sensitivity (slow-hygienic colonies) were not able to detect the disease-associated volatiles over the brood pheromone, and initiated the capping of the dummy. We determined that there was a significant correlation between the level of hygienic behavior, as indicated by the freeze-killed brood assay and the lack of capping of the treated paraffin dummies. Thus, the lack of capping in this assay was an indirect, but sensitive, measure of the level of hygienic behavior of the tested colonies.

We expected that a mixture of phenethyl acetate, 2-phenylethanol, and benzyl alcohol would elicit an increased response from honeybees over any of the compounds tested singly. However, our two bioassays showed no difference between responses to the mixture and that of phenethyl acetate alone, suggesting a lack of mixture-specific synergistic effects. It should be noted that only one ratio of components was tested in our bioassays; it is possible that a different ratio may give enhanced stimulation.

The results of this study have a practical application for beekeepers and important implications for the health of honey bees. It is critical that the beekeeping industry reduce its reliance on chemical treatments for diseases and parasitic mites due to the risk of contaminating hive products with residue, and the development of resistance to the treatments by the pathogens and parasites (Milani, 1999; Wallner, 1999). The most sustainable solution is to breed bees selectively for resistance to diseases and mites. To date, hygienic behavior is one of the few resistance mechanisms that has a simple field assay that beekeepers can employ. Colonies that rapidly remove freeze-killed brood tend to be behaviorally resistant to two brood pathogens, chalkbrood, *A. apis*, and American foulbrood, *Paenibacullus larvae* (Spivak and Reuter, 2001a). Colonies that rapidly remove freeze-killed brood also tend to detect and remove pupae infested with the parasitic mite, *Varroa destructor* (Spivak, 1996). However, the correspondence between the removal of freeze-killed brood and removal of diseased or parasitized brood is not perfect. For example, when 18 rapid-hygienic colonies, as determined by the freeze-killed brood assay, were challenged with American foulbrood pathogen, two colonies developed persistent clinical symptoms of the disease (Spivak and Reuter, 2001a). Also, colonies selected for hygienic behavior still require treatments to control *V. destructor*, as the bees are unable to detect and remove sufficient infested pupae during high mite infestations (Spivak and Reuter, 2001b; Ibrahim et al, 2007). While hygienic behavior is a generalized response to the presence of diseased, parasitized, and abnormal pupae, it is necessary to have a more specific assay to breed colonies selectively for resistance to a particular pathogen or parasite. The experiments performed here could be modified to focus on the specific stimuli that elicit detection and removal of larvae infected with the American foulbrood pathogen and pupae infested with *V. destructor*, thus furthering efforts to breed bees for resistance to these economically important problems.

Acknowledgements

We thank Gary S. Reuter, Kathleen A. Klukas, Peter Teal, and Steve Willms for technical support and valuable input. Constructive comments by the anonymous reviewers were also appreciated. This study was funded by NSF-IBN 031991, awarded to M. Spivak and K. A. Mesce.

References

AKINO, T., and YAMAOKA, R. 1996. Origin of oleic acid corpse recognition signal in the ant, *Formica japonica* Motschlsky (Hymenoptera: Formicidae). *Jap. J. Appl. Entomol. Zool.* 40: 265–271.

ARATHI, H.S., BURNS, I., and SPIVAK, M. 2000. Ethology of hygienic behavior in the honey bee *Apis mellifera* L. (Hymenoptera: Apidae): behavioral repertoire of hygienic bees. *Ethology* 106: 365–379.

ARATHI, H. S., and SPIVAK, M. 2001. Influence of colony genotypic composition on the performance of hygienic behavior in the honey bee (*Apis mellifera* L.) *Anim. Behav.* 62:57–66.

ARATHI, H. S., HO, G., and SPIVAK, M. 2007. Inefficient task partitioning among nonhygienic honeybees, *Apis mellifera* L., and implications for disease transmission. *Anim. Behav.* 72:431–438.

BEHRINGER, D. C., BUTLER, M. J., and SHIELDS, J. D. 2006. Avoidance of disease by social lobsters. *Nature* 25:421.

COOK, R.D., and WEISBERG, S. 1999. Applied Regression Including Computing and Graphics, John Wiley & Sons, New York, NY. 593 pp. (Arc software available from http://www.stat.umn.edu.floyd.lib.umn.edu/arc/software.html, downloaded on 3 September 2008).

CREMER, S., ARMITAGE, S.A.O., and SCHMID-HEMPEL, P. 2007. Social immunity. *Curr. Biol*. 17: R693–R702.

CREMER, S., and SIXT, M. 2009. Analogies in the evolution of individual and social immunity. *Phil. Trans. R. Soc. B* 364: 129–142.

FAHRBACH, S.E., and MESCE, K.A. 2005. "Neuroethoendocrinology": integration of field and laboratory studies in insect neuroendocrinology. *Horm. Behav.* 48: 352–359.

FREE, J. B., and WINDER, M. E. 1983. Brood recognition by honeybee (*Apis mellifera*) workers. *Anim. Behav.* 31: 539–545.

GILLIAM, M., TABER, S. III, and RICHARDSON, G. 1983. Hygienic behavior of honey bees in relation to chalkbrood disease. *Apidologie* 14: 29–39.

GILLIAM, M., and VANDENBERG, J.D. 1997. Fungi, pp. 81–110, in R. A. Morse and K. Flottum (eds.). Honey Bee Pests, Predators and Diseases. Third Edition. A. I. Root Co, Medina, Ohio

GRAMACHO, K. P., and SPIVAK, M. 2003. Differences in olfactory sensitivity and behavioral responses among honey bees bred for hygienic behavior. *Behav. Ecol. Sociobiol.* 54: 472–479.

HOWARD, D.F., and TSCHINKEL, W. R. 1976. Aspects of necrophoric behavior in the red imported fire ant, *Solenpsis invicta*. *Behaviour* 56: 157–178

HUGHES, W. O. H., EILENBERG, J., and BOOMSMA, J. J. 2002. Trade-offs in group living: transmission and disease resistance in leaf-cutting ants. *Proc. Biol. Sci.* 269: 1811–1819.

IBRAHIM, A., REUTER, G. S., and SPIVAK, M. 2007. Field trial of honey bee colonies bred for mechanisms of resistance against *Varroa destructor*. *Apidologie* 38: 67–76.

LE CONTE, Y., ARNOLD, G., TROUILLER, J., MASSON, C., and CHAPPE, B. 1990. Identification of a brood pheromone in honey bees. *Naturwissenschaften* 77: 334–336

MASTERMAN, R., ROSS, R., MESCE, K. A., and SPIVAK, M. 2001. Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L.). *J. Comp. Physiol*. A 187: 441–452.

MASTERMAN, R., SMITH, B. H., and SPIVAK, M. 2000. Brood odor discrimination abilities in hygienic honey bees (*Apis mellifera* L.) using proboscis extension reflex conditioning. *J. Insect Behav.* 13: 87–101

MILANI, N. 1999. The resistance of *Varroa jacobsoni* Oud. to acaracides. *Apidologie* 30: 229–234.

ROTHENBUHLER, W. C. 1964. Behavior genetics of nest cleaning in honey bees. I. Responses of four inbred lines to disease-killed brood. *Anim. Behav* 12: 578–583.

SPIVAK, M. 1996. Honey bee hygienic behavior and defense against *Varroa jacobsoni*. *Apidologie* 27: 245–260.

SPIVAK, M., and REUTER, G. S. 1998. Performance of hygienic honey bee colonies in a commercial apiary. *Apidologie* 29: 291–302.

SPIVAK, M., and REUTER, G. S. 2001a. Resistance to American foulbrood disease by honey bee colonies, *Apis mellifera*, bred for hygienic behavior. *Apidologie* 32: 555–565.

SPIVAK, M., and REUTER, G. S. 2001b. *Varroa jacobsoni* infestation in untreated honey bee (Hymenoptera: Apidae) colonies selected for hygienic behavior. *J. Econ. Entomol*. 94: 326–331.

SPIVAK, M., MASTERMAN, R., ROSS, R., and MESCE, K. A. 2003. Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. *J. Neurobiol*. 55: 341–354.

VISSCHER P. K. 1983. The honeybee way of death: necrophoric behavior in *Apis mellifera* colonies. *Anim. Behav.* 31:1790–1801.

WALLNER A. 1999. Varroacides and their determination in bee products. *Apidologie* 30: 235– 248

WILSON, E. O., DURLACH, N. I., and ROTH, L. M. 1958. Chemical releasers of necrophoric behavior in ants. *Psyche.* 65: 108–114

WILSON-RICH, N., SPIVAK, M., FEFFERMAN, N. H., and STARKS, P. T. 2009. Genetic, individual, and group facilitation of disease resistance in insect societies. *Annu. Rev. Entomol*. 54: 405–423

WOODROW, A. W., and HOLST, E. C. 1942. The mechanism of colony resistance to American foulbrood. *J. Econ. Entomol.* 35: 327–330.