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Behavioral Responses of *Apis mellifera* Adult Workers to Odors from Healthy Brood and Diseased Brood

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Abstract

Studies of adult workers' responses to infected brood were undertaken to isolate discrete volatile compounds that elicited honeybee hygienic behavior. Using a freeze-killed brood assay, we determined that in healthy colonies adult workers recognized and emptied infected cells with a 95% clearance rate. SPME-GC-MS results emptied >95% infected cells indicated differences in the composition and relative content of volatile compounds released by healthy and diseased brood. Additionally, we determined that the main volatile compound released from the pathogen *Ascosphaera apis* was phenethyl alcohol. The Y-tube olfactometer indicated that 10- to 20-day-old workers of healthy colonies, but only 15-day-old workers of diseased colonies, were significantly sensitive to differences in characteristic volatile compounds. This information could facilitate honey bee selection based on mechanisms that contribute to chalkbrood disease tolerance.

Introduction

Chalkbrood disease is an invasive mycosis in *Apis mellifera ligustica* (*A. mellifera*) produced by *Ascosphaera apis* (*A. apis*), which mainly affects larvae (Aronstein & Murray, 2010). Some colonies survive in areas that are seriously damaged by *A. apis*, which indicates the existence of tolerance in some genotypes of honey bees (Wallner, 1999). Hygienic behavior is the natural mechanism of disease resistance in honey bee colonies. One important aspect of hygienic behavior is the ability of individual bees to detect and respond to stimuli from diseased larvae early in the progression of the infection. The quick and efficient detection and removal of diseased larvae by these bees prevents the transmission of the disease throughout the colony. Unhealthy brood emit disease-related odorants, which trigger hygienic bees to recognize and remove diseased brood (Masterman et al., 1999; Spivak et al., 2003).

The key factor in the tolerance of *A. mellifera* strains to chalkbrood disease is the honey bee's sensitive olfactory perception that triggers the hygienic behavior (Francesco et al., 2004). In a dark hive, diseased brood emitted special odors, which appeared to be the most important source of recognition cues. Volatile compounds mediated the capability of adult workers to recognize infested cells and empty them. Nazzi et al. (2002) and Nazzi and Della (2004) indicated that infected brood emitted short-chain unsaturated hydrocarbons to induce honey bee hygienic behavior. Masterman et al. (2001) suggested that the characteristic compounds of diseased brood inspired the behavioral response of adult workers. Swanson et al. (2009) detected three volatile compounds from larvae infected with *A. apis* that were absent from healthy larvae. Thus, we used the SPME-GC-MS (Solid phase microextraction- Gas chromatography and Mass spectrometry) method to measure these differences of volatile compounds (Swanson et al., 2009).

Previously, Kraus (1990) indicated that adult workers produced an alarm pheromone repellent to *Varroa destructor* (*V. destructor*). Kraus (1993, 1994) used a Y-tube olfactometer to measure mite behavior, which is triggered by the odors of capped-cells over those of other life stages. The odors of capped-cells

belong to Volatile compounds produced by larvae. Swanson et al. (2009) indicated that chalkbrood-infected bee larvae emitted characteristic volatile compounds, which contained phenethyl acetate, 2-phenylethanol, and benzyl alcohol. The Y-tube olfactometer bioassay was used to test the orientation behavior of adult workers to volatile compounds. Our research aimed to measure the sensitivity of the workers olfactory perception to the characteristic volatile compounds of diseased brood.

Here, we tested the ability of individual hygienic and non-hygienic bees to discriminate healthy brood from diseased brood. Colonies displaying diseased or healthy traits were tested for hygienic behavior using a freeze-killed brood assay (Spivak & Reuter, 1998a, 1998b), which is an indirect measure of a colony's hygienic behavior. The number of dead pupae that were in the process of being removed (uncapped and/or partially removed), and the number completely removed from the cells were recorded every 24 h for the trial colonies. In the current study, only those colonies that had uncapped and removed more than 95% of the freeze-killed brood within 48 h were considered as hygienic behavior. We also used SPME-GC-MS to measure the volatile compound differences of diseased brood and healthy brood. Then, we used Y-tube olfactometer to measure the olfactory responses of non-hygienic bees and hygienic bees.

The paper aim to veritify behavioral responses of *Apis mellifera* adult workers to odors from healthy brood and diseased brood.

Materials and methods

Biological materials

Experiments were conducted at Fujian Agriculture and Forestry University during spring of the years 2011 and 2012. The six colonies of *A. mellifera* was divided into three infected -chalkbrood colonies and three healthy-larvae colonies.

Experiments

Freeze-killed brood assay measured the hygienic behavior

We chose a sealed brood of white-eyed pupae containing approximately 300 cells on each side (5 × 6 cm) from a frame and froze it for 5 min in liquid nitrogen. We recorded the number of sealed cells and put the frame back in the colony. The tests were repeated on the same colony at least twice.

Collection of volatiles released by infested-brood and healthy-brood cells

Semiochemicals released by infested and uninfected worker brood cells were studied by means of SPME-GC-MS. We collected chalkbrood-infected larvae. Four types of *A. mellifera* larvae were used: a) five healthy fifth instars; b) five chalkbrood-infected larvae; c) five chalkbrood-infected larvae fed an artificial diet with fungal mycelia; and d) fungal mycelia (mummies) from potato dextrose agar (PDA) medium of *A. apis*. First, we prepared a larval diet that consisted of 50% royal jelly (Guangdong entomological institute bee industry co., Ltd, China), 6% D-glucose (Amresco, USA), 6% D-fructose, 37% double distilled water, and 1% yeast extract by volume (Aupinel et al., 2005). Prior to adding the diet to each cell, using 24-well tissue culture plates (BD Falcon™, USA) (Evans, 2004; Aupinel et al., 2005; Gregorc & Ellis, 2011), we homogeneously mixed and pre-warmed the food in an incubator 35ºC. On consecutive days, we transferred larvae to a clean culture plate containing fresh diet. We fed the larvae 350 µL of the diet daily (Aupinel et al., 2007). For the diseased brood we maintained the 350 µL of larvae food in individual wells, but added 1.0×10^8/mL *A. apis* spores (Vandenberg & Shimanuki, 1987). *A. apis* gradually infected the larvae, reaching from the abdomen to the head by 5 d. The pathogenic fungus of *A. apis* was cultured on PDA medium for 5 days at 35ºC in darkness. PDA medium contains potato 200 g, anhydrous dextrose glucose 20 g, agar 20 g, distilled water 1,000 mL (Reynald et al., 2004).

Healthy brood and diseased brood were removed from the 24-well plate with sterile forceps and placed in flasks (50 mL) that were then sealed with foil. Additionally, we analyzed fungal mycelia (mummies) from PDA medium of *A. apis*.

Compounds released from healthy and diseased brood were studied by means of SPME-GC-MS. The 75µm CAR/PDMS SPME fiber coating was exposed to a headspace gas phase for 30 min in the incubator at 35°C before immediate desorption into the GC-MS apparatus. Analytes were separated and analyzed by GC-MS using a Varian 450 gas chromatograph with a mass selective detector (CP-Sil 8 CB-MS 30 m×0.25 mm column, USA). The helium flow rate through the column was 1 mL/min in constant flow mode. The injector (270ºC worked in splitless mode. The initial column temperature was 40°C for 2 min, increasing from 40°C to 120ºC by 3ºC/min, and then rising to 250ºC at 5ºC/min. The detector was held at 250ºC The electron-ionization mass spectra were obtained at 70 eV of ionization energy. Detection was performed in a full scan mode from 29 to 600 a.m.u. After integration, the fraction of each component in the total ion current was calculated. The above process was repeated three times to measure the volatile compounds of larvae.

Y-tube olfactometer bioassay

We tested the olfactory responses of different ages (1-,10-,15-, 20-, 25-, 30-, day-old workers) of honey bees to volatile compounds using a Y-tube olfactometer bioassay. The olfactometer apparatus was situated inside an incubator (RT 30ºC, RH 70%, dark room). The mix of volatile compounds were placed in one of the sample holding chambers of the olfactometer while the other sample holding chamber remained empty as a clean air "blank". All the different aged bees tested remained *in situ* for 20 min, after which time the sample

holding chambers were reversed to avoid any positional bias. Clean holding containers were used for next bee samples.

Statistical analysis of data

Frequencies of honey bees responding to treatment vs. control stimuli were compared using the χ^2 tests for equal proportions. "*" indicates significantly different at α=0.05 ($0.01<P<0.05$).

Results

Liquid nitrogen frozen brood

We tested the ability of individual hygienic and non-hygienic colonies to discriminate healthy from diseased brood. The removal of freeze-killed brood was correlated with the removal rates of diseased brood, and colonies that uncapped and removed >95% of freeze-killed brood within 48 h tended to remove diseased brood rapidly. Thus, we measured the clearance ratio of adult workers to remove the diseased brood. The frozen brood insert into the colonies to assess bee hygienic behavior after 48 h. Three healthy-larvae colonies uncapped and removed more than 95% of the killed brood by 48 h after insertion into the colony (Fig 1). However, in the same time period three diseased-brood colonies only uncapped and removed 45% of the killed brood.

Volatile compounds of diseased brood and healthy brood

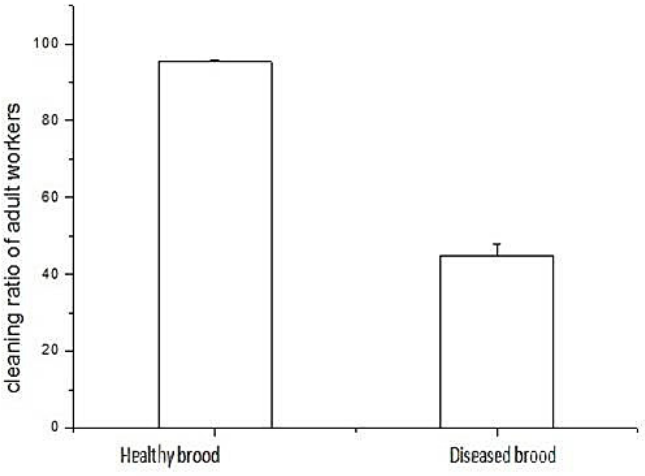

Fig 1. Hygienic behavior of honey bee, *Apis mellifera*, adult workers in the healthy brood and diseased brood.

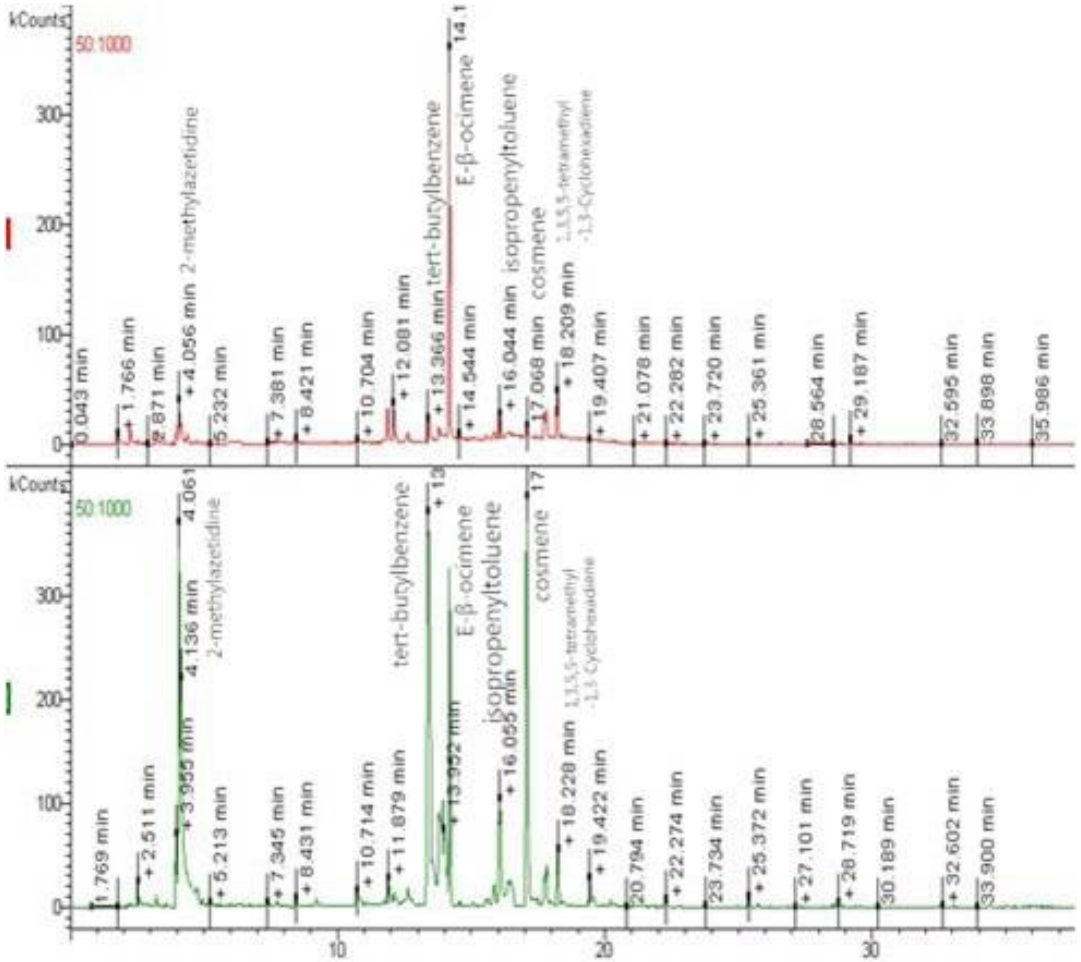

Fig 2. Gas chromatography–mass spectrometry (GCMS) total ion chromatography of healthy and diseasedhoney bee, *Apis mellifera*, broods by solid phase microextraction (SPME) method. A: GCMS total ion chromatography of healthy brood by SPME method. B: GCMS total ion chromatography of diseased brood by SPME method.

Differences in the amount of compounds released by healthy and diseased broods were assessed by comparing the size of the corresponding peaks in SPME-GC-MS analysis performed under the same conditions. Both the chalkbrood-infected brood in the healthy colonies and the chalkbrood-infected brood from an artificial diet with fungal mycelia emitted compounds with some similar ingredients.

The results indicated that the differences between volatile compounds of healthy brood and diseased brood are in composition and relative content, especially that of tert-butylbenzene, E-*β*-ocimene, cosmene, 2-menthene, 2-isopropenyltoluene, and 1,3,5,5-tetramethyl-3-cyclohexadiene (Fig 2). E-*β*-ocimene was the main volatile compound of healthy brood phase compared with diseased brood. E-*β*-ocimene made up 1/5th of the relative volatile content from the healthy brood phase, but was only1/10th of the relative content from the diseased brood phase. The amount of tert-butylbenzene emitted by diseased brood was approximately five times more than the amount emitted by healthy brood, and 2-isopropenyltoluene was three times higher from the healthy brood phase than diseased brood phase. The relative contents of 2-menthene, cosmene, and 1,3,5,5-tetramethyl-3-cyclohexadiene, also showed some differences.

Laboratory inoculation of PDA with chalkbrood disease causing *A. apis* fungal hyphae, released γ-Decalactone,2,5,5-Trimethyl-1-hexenbenzylalcohol, 2-(1,1-dimethylethyl)-3-methyl-Oxirane, phenethyl alcohol, phenethyl acetate, and benzyl alcohol (Fig 3).

Y-tube olfactometer bioassay

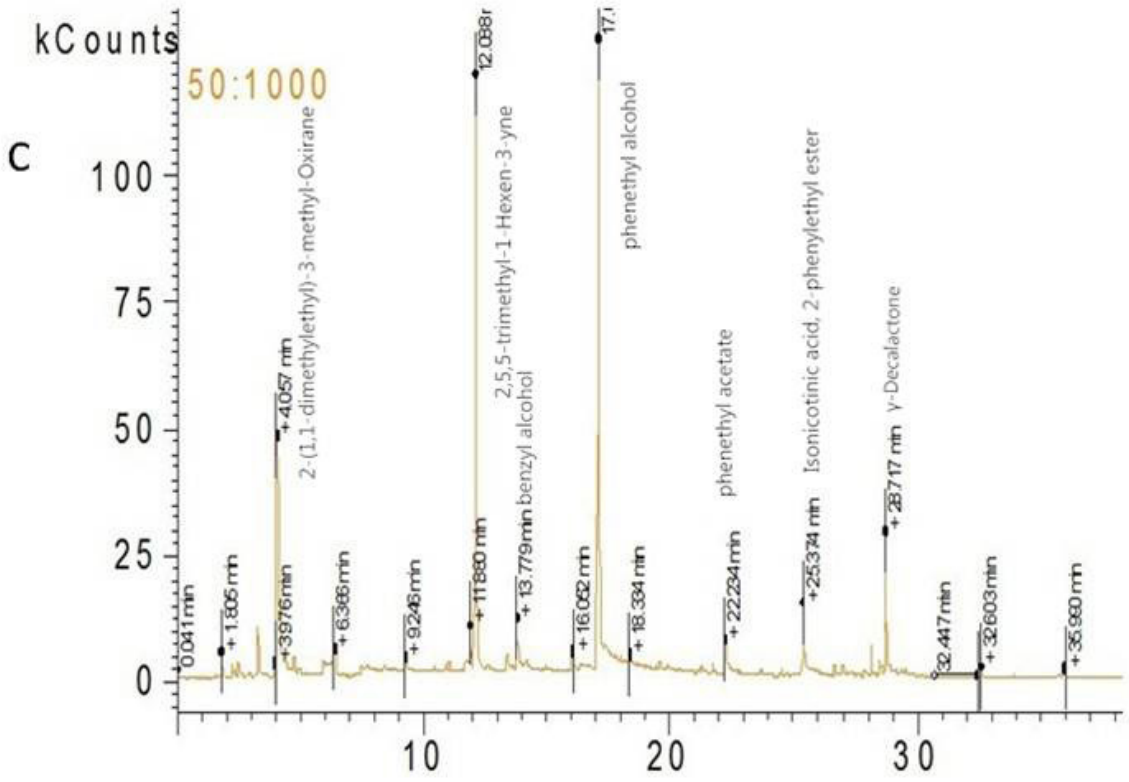

Fig 3. Gas chromatography–mass spectrometry (GCMS) total ion chromatography of fungal mycelia (mummies) from PDA medium of *Ascosphaeraapis* by solid phase microextraction (SPME) method.

Compared with blank coloies, the 10- to 20-day-old workers of healthy colonies are significantly more sensitive to differences in characteristic volatiles compounds than workers of other ages, while only the 15-day-old workers of diseased colonies display a significantly heightened sensitivity to differences in characteristic volatiles compounds (Fig 4). Newly emerged workers have an immature olfactory sense that leads to a weaker sensitivity.

Honey bees take less than 3 min to reach the junction of a Y-tube olfactometer. Even with the variation observed, the open Y-tube olfactometer is an efficient bioassay that showed workers could discriminate between the healthy larvae and diseased larvae.

Discussion

The honey bee is a model to observe the olfactory system of insects and it was observed that some colonies perform hygienic behavior to eliminate diseased brood. The stimuli triggering hygienic was thought to be olfactory cues emanating from cells containing diseased brood (Boecking & Spivak, 1999), but the identities of these cues were still unknown.

Healthy colonies removed 95% frozen brood. However,

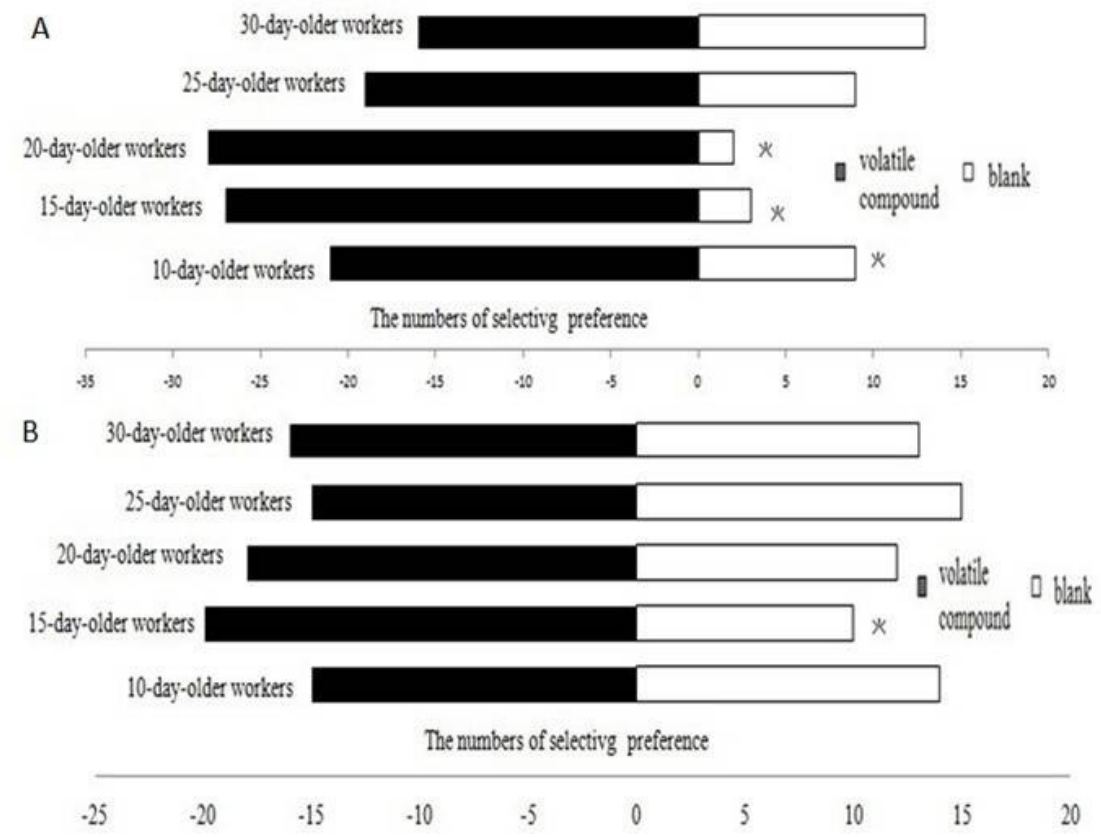

Fig 4. Olfactory responses of honey bees,*Apis mellifera*,to odors from the characteristic volatile compounds and a blank.
A: Olfactory responses of different ages of workers in the healthy colony. B: Olfactory responses of different ages of workers in the diseased colony. Note: * indicated significant different, Significant at α=0.05, (0.01<P<0.05).

the diseased colonies only removed 45% frozen brood, and they did not display significant hygienic behavior. Hygienic behavior is specifically defined as the detection and removal of 95% diseased larvae from the colony (Rothenbuhler, 1964; Wilson-Rich et al., 2009). Colonies that uncap and remove >95% of freeze-killed pupae within 48 h will rapidly remove diseased brood (Swanson et al., 2009). Olfactory stimuli guided the detection and removal of diseased larvae (Masterman et al., 2001; Gramacho & Spivak, 2003; Spivak et al., 2003).

Volatile compounds mediate olfactory stimuli. The diseased brood and healthy brood of *A. mellifera* released different volatile compounds that were distinguished by adult workers, and these differences affected the workers' behavior. In the healthy colony, the detection of the diseased brood's odor would trigger workers with even the lowest detection sensitivities, and they would rapidly begin uncapping and removing the diseased brood (Palacio et al., 2010). In a darkened hive, odors emitted from diseased brood appeared to be the most important source of recognition cues. Spivak and Boecking (2001) indicated that olfactory cues mediated the tolerance of *A. mellifera* strains by triggering the hygienic behavior to clean mites, and that the chemical compounds induced the ability of the bees to recognize infested cells and empty them (Spivak & Downey, 1999). Cells infested with mite emitted different volatile compounds than uninfected cells. Martin et al. (2001) suggested that the honey bee's hygienic behavior was triggered by the polar compounds of the mite cuticle. Nazzi et al. (2001, 2004) indicated that there might be slight differences in the amount of some short-chain unsaturated hydrocarbons. Martin et al. (2001) showed that mites emitted volatile compounds to induce bee hygienic behavior. Masterman et al. (2001) suggested that olfactory cues from the diseased brood itself stimulates the behavioral response of bees. The body fluid of brood was very important as a stimulus for adult workers to remove treated brood (Aumeier et al., 2002). In our experiment, we used the SPME-GC-MS method to analyze the volatile compounds of healthy brood and diseased brood, which was infected with *A. apis*. Our results indicated a difference in volatile compound composition and relative contents, especially of tert-butylbenzene and E-β-ocimene. This was the first report on tert-butylbenzene. E-β-ocimene had previously been shown to be emitted during the queens mating and to play a role in regulating worker ovary development (Gilley et al., 2006). Additionally, larvae emit E-β-ocimene, which acts as a hunger signal. The 1- to 2-day-old larvae have a higher need for food than 3- to 4-day-old larvae, so younger larvae produce more E-β-ocimene than older larvae (Maisonnasse et al., 2009, 2010). Although diseased brood and healthy brood released similar volatile compounds, they had significant differences, and they especially emitted different amounts of E-β-ocimene. We propose that the young infected brood needed little food and did

not nurse, while the healthy brood needed more food and care. Thus, E-*ß*-ocimene was emitted as a characteristic compound, which could be used to discriminate between a healthy brood and diseased brood. Stimuli coming from both the diseased brood and the fungal pathogen, *A. apis*, were considered in our test. To discriminate which volatile compounds came from diseased brood, and were not emitted by *A. apis*, we measured the volatile compounds of *A. apis* in the artificial PDA culture medium. Completely different compounds were emitted. *A. apis* produced benzyl alcohol, phenethyl alcohol, and phenethyl acetate. These three compounds were reportedly emitted by diseased brood (Swanson et al., 2009). The field bioassay of topical applications and paraffin larval dummies revealed that phenethyl acetate induce hygienic behavior (Swanson et al., 2009). In our test, we detected phenethyl alcohol as the main volatile compound of *A. apis*, accounting for 1/4th of the relevant volatile compounds. However, the relative amount of benzyl alcohol and phenethyl acetate were <5%. In our test, we detected phenethyl alcohol and phenethyl acetate from diseased brood, but we could not detected benzyl alcohol from diseased brood.

We found that diseased brood and healthy brood have significantly different volatile compounds, and the relative content of the main volatile compounds were also significantly different.

To confirm that honey bees are able to recognize brood cells infested by *A. apis*, we used a Y-tube olfactometer to test the olfactory responses of workers. We verified the existence different chemical compounds of healthy brood and diseased brood, and then identified, the chemical stimuli that induces the hygienic behavior of bees towards *A. apis* infested cells. Actually, we chose five volatile compounds and analyzed their effects on the olfactory behavior of honey bees. The 10- to 20-day-old workers showed significant olfactory stimulated behavior in the healthy colony. It has been reported that 15-day-old workers performed hygienic activities (Invernizzi & Corbella 1999, Arathi et al., 2000). Moreover, 15-day-old workers rapidly initiated uncapping behaviors depending on the stimulus from a dead brood (Arathi, 2000). Middle-aged bees (12- to 21-day-old) built and maintained the hive, and received and processed nectar (Johnson, 2003, 2008a). They did not engage in nursing behavior (Ben-Shahar et al., 2002; Ben-Shahar, 2005). Workers displayed age-related division of labor and natural behavioral plasticity (LeConte & Hefetz, 2008; Ben-Shahar, 2005). The change from cell cleaning to nurse and other in-hive tasks were performed by 21-day-old workers (Whitfield, 1987). Our research showed that 10- to 20-day-old workers in hygienic colonies had the ability to discriminate characteristic odors. Only the 15-day-old workers in the non-hygienic colony had sensitive enough olfactory perception to differentiate preferential odors. Thus, 15-day-old workers showed a sensitive olfactory response, which correlated with the workers age. As a social insect, adult workers perform different tasks in keeping with temporal polyethism. The 10- to 20-day-old workers of the healthy colonies could select five volatile compounds by Y-tube olfactometer. These volatile compounds both detected in healthy brood and diseased brood, but they have the different relative contents. Thus, we proposed adult workers of some colonies have olfactory perception sensitive enough to detect diseased brood. This information could facilitate the selection of honey bees with pronounced hygienic behavior based on mechanisms that contribute to chalkbrood disease tolerance.

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