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Authors: Sousa, K. K. A., Catalani, G. C., Gianeti, T. M. R., Camargo, R. S., Caldato, N., et. al.

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A volatile semiochemical released by the fungus garden of leaf-cutting ants

K. K. A. Sousa¹, G. C. Catalani¹, T. M. R. Gianeti², R. S. Camargo^{3,*}, N. Caldato¹, V. M. Ramos³, and L. C. Forti¹

Abstract

The symbiosis between fungi and leaf-cutting ants (Hymenoptera: Formicidae) has aroused the interest of researchers about the mechanism used by ants to select plants. The nutritional needs of the fungus garden, and the absence of potentially deleterious substances from plants, are criteria for selection by foraging workers. This is supported by behavioral experiments using fungicide with baits (citrus pulp) or forage plants highly accepted by leaf-cutting ants. The fungus garden is hypothesized to emit a volatile semiochemical in response to a fungicide, which informs ants that a plant is unsuitable for its growth. The objectives of our study were to identify the volatile compounds released by the fungus garden of leaf-cutting ants in response to a fungicide, as well as to determine the behavioral response of workers to healthy and unhealthy fungus gardens. The results showed no difference in the proportion of volatile compounds released by either healthy or unhealthy fungus gardens. Analysis of the responses of ants to healthy or unhealthy fungus gardens in a dual-choice experiment revealed a strong attraction to the fungus garden, regardless of its health status. We therefore conclude that no volatile semiochemicals are emitted by the fungus garden due to the action of deleterious substances.

Key Words: Formicidae; *Atta*; symbiotic fungus

Resumen

La simbiosis entre los hongos y las hormigas cortadoras de hojas (Hymenoptera: Formicidae) ha despertado el interés de los investigadores en el mecanismo utilizado por las hormigas para seleccionar las plantas. Las necesidades nutricionales del jardín de los hongos y la ausencia de sustancias potencialmente dañinas de las plantas son criterios de selección de los trabajadores en búsqueda de alimento. Esto es respaldado por experimentos de comportamiento que usan fungicidas con cebos (pulpa de cítricos) o plantas de forraje altamente aceptadas por HCH. El jardín de hongos emite un semioquímico volátil en respuesta a un fungicida, que informa a las hormigas que una planta no es apta para su crecimiento. El objetivo de nuestro estudio fue identificar los compuestos volátiles liberados por el hongo jardín de HCH en respuesta a un fungicida, así como la respuesta conductual de los trabajadores hacia los jardines de hongos saludables y no saludables. Los resultados no mostraron diferencias en la proporción de compuestos liberados por jardines de hongos saludables y no saludables. El análisis de las respuestas de las hormigas a los jardines de hongos saludables y no saludables en un experimento de doble elección reveló una gran atracción para el jardín de los hongos, independientemente de su estado de salud. Por lo tanto, concluimos que no hay semioquímicos volátiles emitidos por el jardín de hongos bajo la acción de sustancias nocivas.

Palabras Clave: Formicidae; *Atta*; hongo simbiótico

Leaf-cutting ants (Hymenoptera: Formicidae) have a mutualism with the fungus *Leucoagaricus gongylophorus* (Heim) Moeller (Agaricaceae), the primary food source of the colony (Schultz et al. 2005; Hölldobler & Wilson 2009a). These ants select and cut several plant species for the cultivation of their symbiotic fungus (Weber 1972). Consequently, some species of ants are known principally as pests of crops in the Neotropical region (Della Lucia & Souza 2011). Plant selection involves the proper choice of plant species that are suitable for development of the symbiotic fungus (Hölldobler & Wilson 1990), although

the existence of an ant-fungus communication for the correct selection of plants is only hypothesized (Ridley et al. 1996; North et al. 1999; Green & Kooij 2018).

Studies investigating the existence of this communication have used a protein synthesis inhibitor, cycloheximide (CHX) (Obrig et al. 1971; Schneider-Poetsch et al. 2010). This substance, reported to be a fungicide, has been applied in behavioral studies on leaf-cutting ants. The effect of cycloheximide on the fungus garden was found to be responsible for changes in the foraging behavior of leaf-cutting ant workers, because

¹Universidade Estadual de São Paulo / UNESP, Departamento de Proteção Vegetal, Laboratório de Insetos Sociais-Praga, Botucatu, São Paulo, Brazil; E-mail: katiakaelly@gmail.com (K. K. A. S.); gchristalcatalani@yahoo.com.br (G. C. C.); nacbiol@gmail.com (N. C.); luizforti@fca.unesp.br (L.C. F.)

²Universidade Estadual de São Paulo / UNESP, Laboratório Central, Faculdade de Ciências Agronômicas UNESP, Botucatu, São Paulo, Brazil; E-mail: gianeti@gmail.com (T. M. R. G.)

³Laboratory of Agricultural Entomology, Agronomy Department, College of Agricultural Sciences, Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, São Paulo, Brazil; E-mail: vaniaramos@unoeste.br (V.M. R.)

*Corresponding author; E-mail: camargobotucatu@yahoo.com.br

cycloheximide is not identified as a harmful compound for the colony in the first contact with the workers (Ridley et al. 1996). North et al. (1999) hypothesized that a semiochemical emitted by the fungus garden regulates the selection of plants by leaf-cutting ant foragers, and is interpreted as communication between the symbiotic fungus and leaf-cutting ant workers. The authors showed that the ants learn to reject plant material that contains chemicals injurious to the fungus. After an initial period of acceptance, ants from laboratory nests stopped harvesting granular bait containing a fungicidal agent (cycloheximide) and orange peel. Subsequently, many behavioral studies have used cycloheximide to understand the effects on the long-term olfactory memory of leaf-cutting ants. Some of these studies support the existence of an ant-fungus communication through a semiochemical volatile compound that has not yet been identified and whose presence is only hypothesized (Cazin et al. 1989; Ridley et al. 1996; North et al. 1999; Herz et al. 2008; Saverschek et al. 2010; Saverschek & Roces 2011; Thiele et al. 2014; Falibene et al. 2015; Arenas & Roces 2016a, b, 2017).

The objective of our study was to test the hypothesis proposed by North et al. (1999). This hypothesis suggests that a volatile semiochemical emitted by the fungus garden regulates the selection of plants by leaf-cutting ant foragers, which is interpreted as a communication between the symbiotic fungus and leaf-cutting ant workers. Specifically, we sought to identify the volatile compounds released by the fungus garden of leaf-cutting ants and to determine the behavioral response of workers to healthy and unhealthy fungus gardens.

Materials and Methods

COLONIES

Colonies of *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae), known as *saúvas*, were used for the experiments, which were kept in the Laboratory of Social Insects, College of Agricultural Sciences, São Paulo State University-UNESP, Botucatu, São Paulo, Brazil. The queen-right colonies, collected in Mar 2016, were 2 yr old at the start of the experiments. Each colony has a container (15 cm × 15 cm × 15 cm) with a fungus garden, and was fed *Acalypha* spp. and *Ligustrum* spp. leaves and stems. The fungus garden container was connected to 2 additional chambers, 1 for foraging of the supplied plants and another for waste deposition. The colonies were kept at a temperature of 24 ± 2 °C, relative humidity of 80%, and a 14:10 h (L:D) photoperiod in the laboratory.

PREPARATION OF ORANGE PEEL PELLETS

The pellets consisted of small granules made of orange peels, which were dehydrated for 72 h at 50 °C. The orange peels were from organic crops. The dehydrated peels were ground into a powder in a milling process. Next, 1.8 g of this orange peel powder was homogeneously mixed with 0.2 g carboxymethyl cellulose, and 0.025 g cycloheximide (Sigma-Aldrich, St. Louis, Missouri, USA) dissolved in 2.5 mL water was added to produce the orange granules with cycloheximide. The matrix was transferred to a 20 mL syringe to produce the granules, which were allowed to dry for 24 h at 25 °C. The same method was used to produce pellets without the fungicide and without cycloheximide. The pellets were cut into equal-size pieces and stored in a freezer inside plastic pots until the time of use (method adapted from Ridley et al. 1996, and Sousa et al. 2017).

VOLATILES RELEASED BY THE FUNGUS GARDEN

For assessment of the volatiles released by the fungus garden, 2 experimental groups were compared: (a) colonies receiving cycloheximide

pellets for 7 d, followed by cycloheximide-free pellets for an additional 7 d, totalling 14 d (N = 6); (b) colonies receiving cycloheximide-free pellets for 14 d (N = 6). Fungus garden samples (0.75 g) were collected on different days from the 2 groups: d 1 (beginning of the experiment), d 7, and d 14. According to Sousa et al. (2017), the decline of the fungus garden and its reduction in volume indicate that it is unhealthy, due to cycloheximide effects (Fig. 1). The collected fungus garden samples (healthy and unhealthy) were submitted to chromatography analysis.

CHROMATOGRAPHY ANALYSIS

The chemical study of the fungus garden was conducted at the Central Laboratory, Department of Agriculture, College of Agricultural Sciences, São Paulo State University-UNESP, Botucatu, São Paulo, Brazil. A Perkin Elmer TurboMatrix™ Headspace (Perkin Elmer, Wellesley, Massachusetts, USA) without a trap, connected to a Perkin Elmer SQ8 T GC/MS (Perkin Elmer, Wellesley, Massachusetts, USA), operating in the headspace mode without the trap, was used for this experiment. The Headspace conditions were: vial equilibration at 80 °C for 5 min, needle temperature of 90 °C, transfer line temperature of 125 °C, and helium as carrier gas at 20 psi. The mass spectrometer conditions were: scan range of 30 to 300 Daltons, scan time of 0.1 s, source temperature of 180 °C, and inlet temperature of 200 °C. A slightly polar Elite 5 column (5% phenyl-silicone, 30 m × 0.25 mm × 0.25 μm) (Perkin Elmer, Shelton, Connecticut, USA) was used. This thick-film column provided sufficient retention to separate the early-eluting, most volatile components, and provided the dynamic range necessary to separate both high-level and low-level components present in the matrix.

CHOICE BIOASSAYS

Ant responses to healthy and unhealthy fungus gardens: Y-shaped choice system

To determine whether leaf-cutting ant workers are attracted to healthy and unhealthy fungus gardens, we monitored the worker choice in 6 experimental bioassays, 12 times per bioassay: (1) d 1, healthy fungus vs. healthy fungus; (2) d 1, healthy fungus vs. no fungus; (3) d 7, unhealthy fungus vs. healthy fungus; (4) d 7, unhealthy fungus vs. no fungus; (5) d 14, unhealthy fungus vs. healthy fungus; (6) d 14, unhealthy fungus vs. no fungus.

A simple Y-shaped choice system made of transparent glass was used, which consisted of branches of equal length (40 cm) and width (3 cm), with the upper branches arranged at an angle of 60° (Fig. 2). The worker container was coupled to the base branch of the Y trail. A plastic tray was installed on each upper branch where the choices were provided randomly in a plastic container.

Fifty ants with a mean head width of approximately 2.2 mm were introduced into the working container to allow bioassay choices containing 0.5 g (500 mg) of clean fungus, after removing ants, eggs, larvae, and pupae from the fungus. The number of workers was counted at 5 min intervals for 30 min, corresponding to the worker choice in each container. Each bioassay was repeated 12 times.

Ant responses to extracts of healthy and unhealthy fungus: simultaneous choice

The fungus extracts were obtained using pieces of the fungus from the colonies that received cycloheximide pellets and cycloheximide-free pellets as described above. The fungus pieces were cleaned with needles, removing all brood and workers, and about 500 mg of fungus was used (Viana et al. 2001). These fungus gardens were immersed in 5 mL dichloromethane for 2 h, filtered with filter paper (Qualy®, São

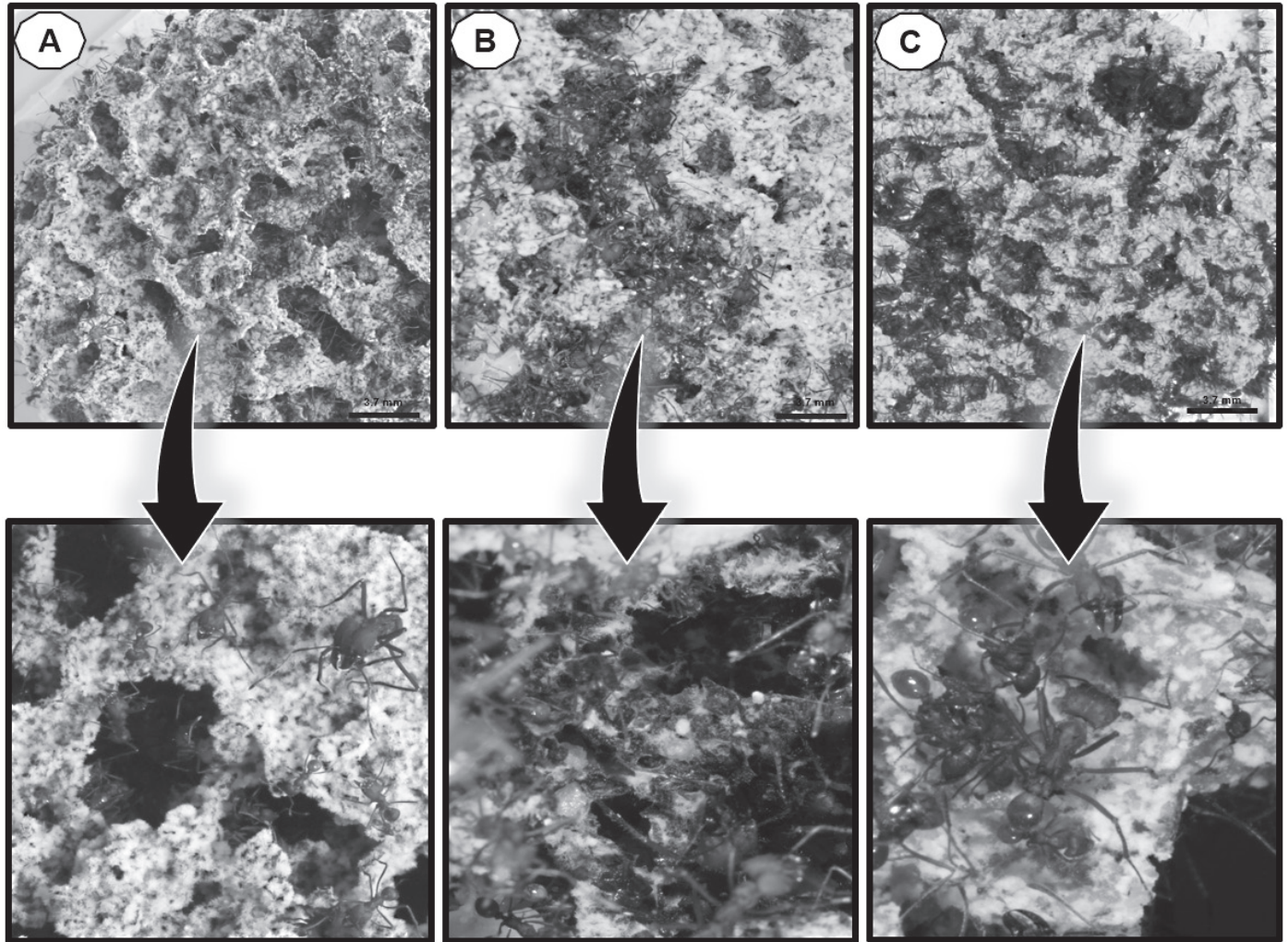


Fig. 1. Fungus garden. A: Fungus healthy; B: fungus with incorporation of pellets with cycloheximide on d 7 of the experiment; and C: fungus on d 14 of the experiment.

José dos Pinhas, Paraná, Brazil), and kept at low temperature. These extracts were used in the bioassays.

To determine whether leaf-cutting ant workers are attracted to healthy and unhealthy fungus extracts, filter paper disks (Qualy®, São

José dos Pinhas, Paraná, Brazil) (0.6 cm diam) with the extract were offered in the foraging arena in 5 treatments: (1) 10 disks with healthy fungus extract; (2) 10 disks with unhealthy fungus extract from d 7; (3) 10 disks with unhealthy fungus extract from d 14; (4) 10 disks with dichloromethane; and (5) 10 disks without dichloromethane and extracts as control.

Four colonies were used and 7 trials were conducted per colony, totaling 350 disks offered per colony. The bioassay was terminated when the treatment was completely transported by foragers into the colony. The total number of transported disks was counted for each treatment.

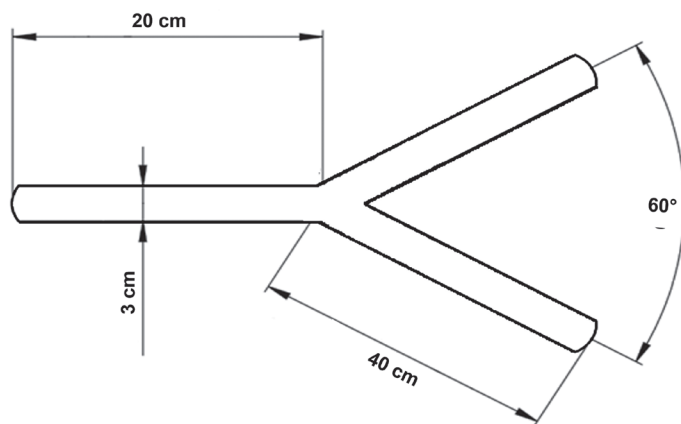


Fig. 2. Olfactometer model used in the experiment of ant response to healthy or unhealthy fungus in the Y-shaped choice system.

STATISTICAL ANALYSIS

First, the Shapiro-Wilk test was applied to determine whether the data were normally distributed or not. The proportion of aldehyde compounds on d 1, 7, and 14 was compared between colonies that received cycloheximide pellets and those that received pellets without cycloheximide, using the Mann-Whitney test for independent samples. The same test was used for comparison of the data of the Y-shaped choice system. The total number of transported disks was submitted to the Kruskal-Wallis test and Student-Newman-Keuls test as a post-test. A level of significance of 1% was adopted for all tests. The analyses were done through the program BioEstat 5.0 (Manaus, Amazônia, Brazil).

Results

The fungus garden emitted 4 volatile substances, 2-methylpropanal, 3-methylbutanal, pentanal, and hexanal, all of which are aldehydes (Fig. 3). The proportion of aldehydes was similar in the 4 colonies that received cycloheximide pellets and the colonies that received cycloheximide-free pellets (Fig. 4). We found no significant differences in the proportion of 2-methylpropanal [d 1 ($U = 1.29$; $n =$

4; $P > 0.01$), d 7 ($U = 2.16$; $n = 4$; $P > 0.01$), d 14 ($U = 1.87$; $n = 4$; $P > 0.01$)]; 3-methylbutanal [d 1 ($U = 1.01$; $n = 4$; $P > 0.01$), d 7 ($U = 2.02$; $n = 4$; $P > 0.01$), d 14 ($U = 1.88$; $n = 4$; $P > 0.01$)]; pentanal [d 1 ($U = 2.30$; $n = 4$; $P > 0.01$), d 7 ($U = 0.86$; $n = 4$; $P > 0.01$), d 14 ($U = 0.86$; $n = 4$; $P > 0.01$)]; or hexanal [d 1 ($U = 0.57$; $n = 4$; $P > 0.01$), d 7 ($U = 0.10$; $n = 4$; $P > 0.01$), d 14 ($U = 1.29$; $n = 4$; $P > 0.01$)] between colonies that received cycloheximide pellets and those that received pellets without cycloheximide.

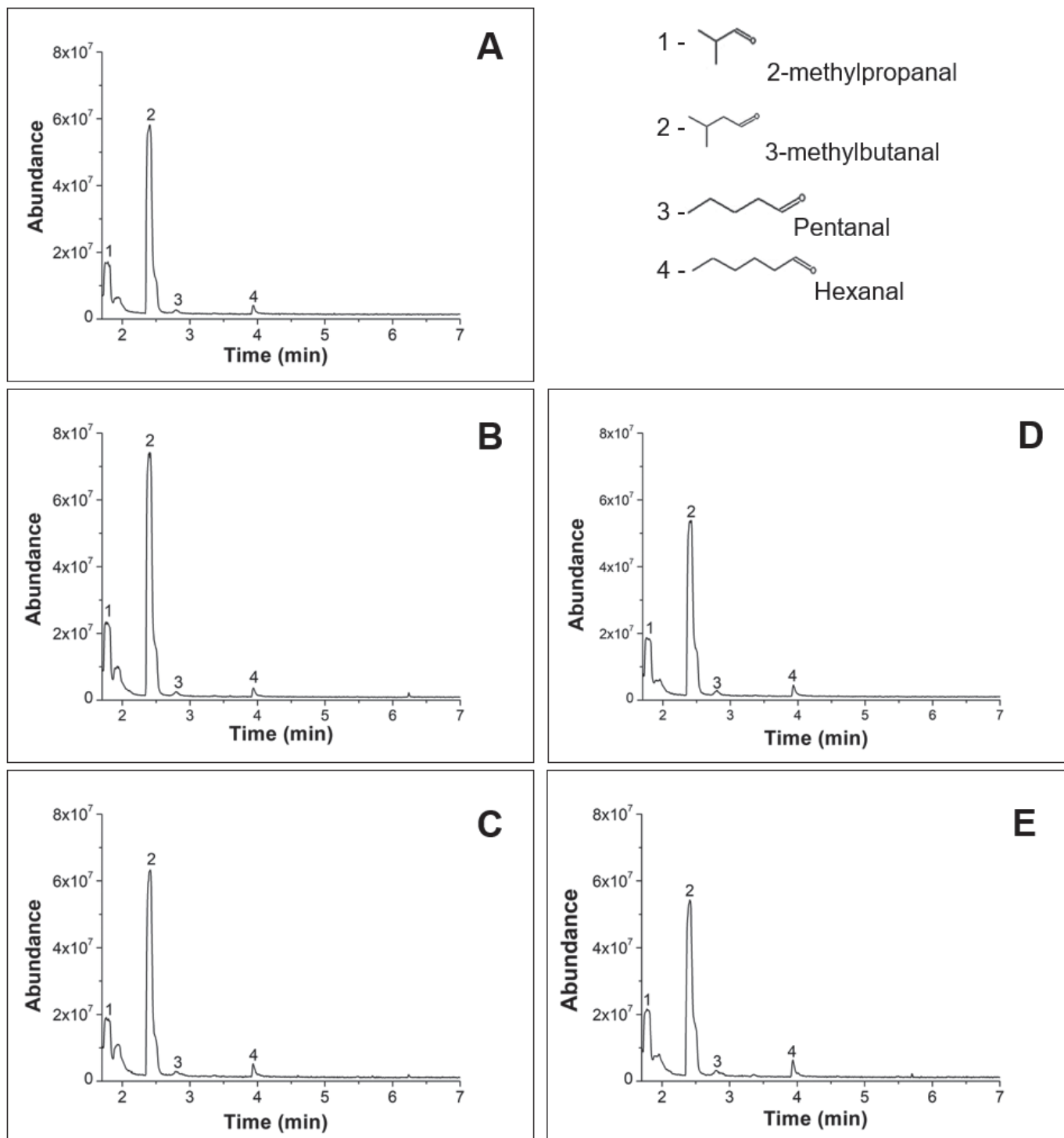


Fig. 3. Volatiles emitted by the fungus. A: Healthy fungus; B: fungus without cycloheximide for 7 d; C: fungus without cycloheximide for 14 d; D: fungus with cycloheximide for 7 d; E: fungus with cycloheximide for 14 d.

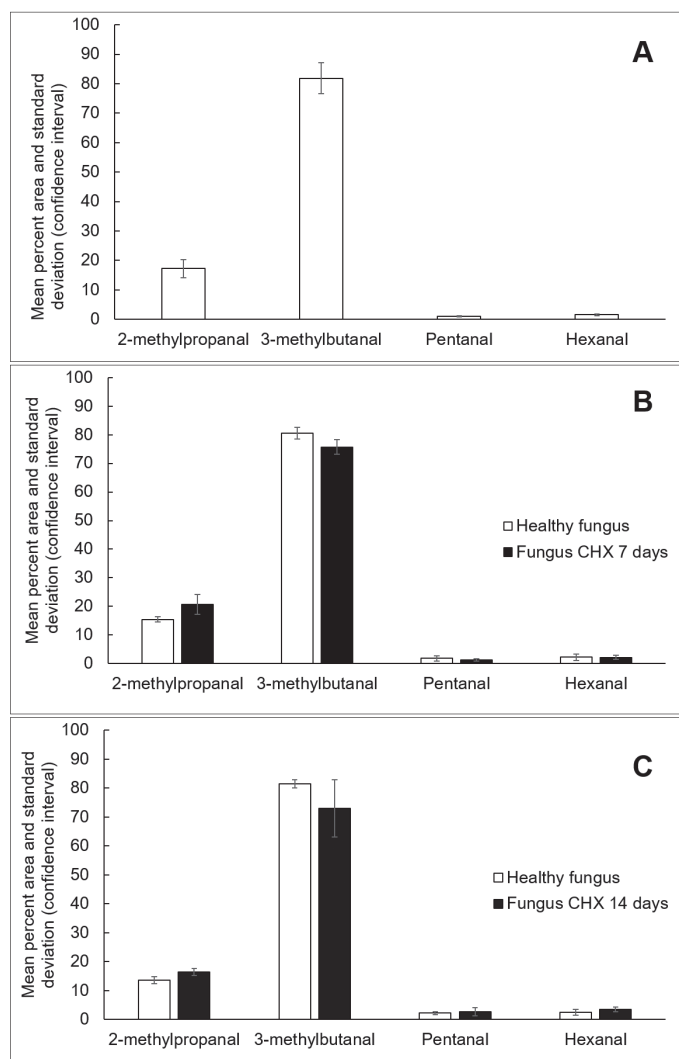


Fig. 4. A: Mean percent area and standard deviation (confidence interval) of volatiles emitted by the fungus; B: healthy fungus and fungus with cycloheximide for 7 d; C: healthy fungus and fungus with cycloheximide for 14 d.

Analysis of ant responses to healthy and unhealthy fungus gardens using a Y-shaped choice system (Fig. 2) revealed a strong attraction to the fungus garden, regardless of its health status (Fig. 5). We observed significant differences between healthy fungus vs. no fungus on d 1 ($U = 42$; $n = 12$; $P < 0.01$), between unhealthy fungus vs. no fungus on d 7 ($U = 28.8$; $n = 12$; $P < 0.01$), and between unhealthy fungus vs. no fungus on d 14 ($U = 41.5$; $n = 12$; $P < 0.01$). On the other hand, there were no significant differences between healthy fungus vs. healthy fungus on d 1 ($U = 0.54$; $n = 12$; $P > 0.01$), between unhealthy fungus vs. healthy fungus on d 7 ($U = 24.8$; $n = 12$; $P > 0.01$), or between unhealthy fungus vs. healthy fungus on d 14 ($U = 0.054$; $n = 12$; $P > 0.01$).

As expected, a significant difference was detected among treatments with healthy and unhealthy fungus extracts (Fig. 6) (Kruskal-Wallis test, $H = 27.15$; $df = 4$; $P < 0.001$). The disks with healthy fungus extract were highly transported by foragers (175 disks), with a significant difference compared to disks with unhealthy fungus extract on d 7 (59 disks, $P < 0.001$) and disks with unhealthy fungus extract on d 14 (89 disks, $P < 0.001$). However, the number of transported disks with healthy fungus extract was not significantly dif-

ferent from the number of disks with dichloromethane (155 disks, $P = 0.5542$), or of disks without dichloromethane and extracts (182 disks, $P = 0.7858$).

Discussion

One of the major findings in this study is that the aldehyde compounds released by the fungus garden were the same in colonies that received cycloheximide pellets and colonies that received cycloheximide-free pellets on d 1, 7, and 14 (Fig. 3). This result does not corroborate the study suggested by North et al. (1999) that a volatile semiochemical emitted by the fungus garden regulates the selection of plants by leaf-cutting ant foragers, which is interpreted as a communication between the symbiotic fungus and leaf-cutting ant workers. They showed that the ants learn to reject plants containing chemicals that are harmful to the fungus. After an initial period of acceptance, ants from laboratory nests stopped harvesting granular bait containing a fungicidal agent (cycloheximide) and orange peel. In contrast, studies show that cycloheximide causes mortality to leaf-cutting ant and fungal workers, and thus may promote pellet rejection due to worker mortality and fungus garden breakage (Sousa et al. 2017).

Approximately 250 volatile organic compounds have been identified in 129 fungal species (126 Macromycetes and 3 Micromycetes pathogenic for plants: *Puccinia*), where they occur as aldehydes, ketones, alcohols, and phenols, among many others (Chiron & Michelot 2005). However, in our study, the fungus garden emitted 4 volatile substances, 2-methylpropanal, 3-methylbutanal, pentanal, and hexanal, which are aldehydes. The flowers, vegetative parts, and roots of plants are known to release more than 1,000 volatile organic compounds, principally 6-carbon aldehydes, alcohols, esters, and various terpenoids (Pichersky et al. 2006). In some cases, plants release volatile compounds when damaged by herbivores (Tumlinson et al. 1999). In the case of leaf-cutting ants, the leaves of plants are cut, processed into a pulp, and incorporated into the fungus garden surface by workers (Camargo et al. 2007; Garrett et al. 2016). It is likely that these leaf fragments in the fungus garden emit volatile substances, as observed in mechanically damaged leaves of poplar cuttings (*Populus simonii* Carrière and *P. pyramidalis* Salisb.) (Salicaceae) (Hu et al. 2008). These authors identified 16 aldehydes: acetaldehyde, butanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal, undecanal, dodecanal, tetradecanal, (Z)-3-hexenal, (E)-2-hexenal, (E)-2-nonenal, benzaldehyde, and furfural. Interestingly, most of these substances were released 24 h after wounding (Hu et al. 2008), suggesting that the same may occur in the fragmented leaves of the fungus garden of leaf-cutting ants.

It is known that unhealthy plant parts, mechanical damage, pathogen infections, and herbivore injuries can induce the rapid synthesis of volatile compounds such as hexenal and hexanal (Arimura et al. 2000). Analogously, the symbiotic fungus degrades leaves as a substrate for its colonization, and may induce the release of some aldehydes. *Leucoagaricus gongylophorus* is a basidiomycetous fungus that produces specialized hyphae (gongylidia) for feeding ants and larvae (Hölldobler & Wilson 2009), as well as enzymes for lignocellulose degradation (Boyd & Martin 1975; Martin et al. 1975; Schiøtt et al. 2008, 2010; Aylward et al. 2013; Grell et al. 2013; Kooij et al. 2014, 2016). The degradation of lignin by basidiomycetes produces a variety of extracellular aromatic metabolites (Jong et al. 1994), especially aldehydes (Gallois et al. 1990) such as hexanal, heptanal, 2-butenal, 2-methyl-2-butenal, 4-nonenal, and 2,4-decadienal. These findings support the production of some aldehydes by the fungus garden during lignocellulose degradation.

The worker responses to healthy and unhealthy fungus gardens indicated an attraction to the fungus garden (Fig. 5). This attraction might be attributed to the presence of ant hydrocarbons and large quantities of n-alkanes in the fungus garden that constitute a signal for leaf-cutting ant workers (Viana et al. 2001). This fact also may explain why the workers did not distinguish between healthy and unhealthy fungus gardens (Fig. 5), although the worker in a colony can take the unhealthy

fungus to the waste chamber (Sousa et al. 2017). This observation was corroborated when the healthy and unhealthy fungal extracts were tested, suggesting that workers cannot distinguish the health status of the fungus (Fig. 6). It is likely that behavioral results reinforce the lack of a volatile semiochemical emitted by the fungus garden, as suggested by North et al. (1999), because workers must avoid breakdown products from unhealthy or dead fungi. Besides, the choice of these

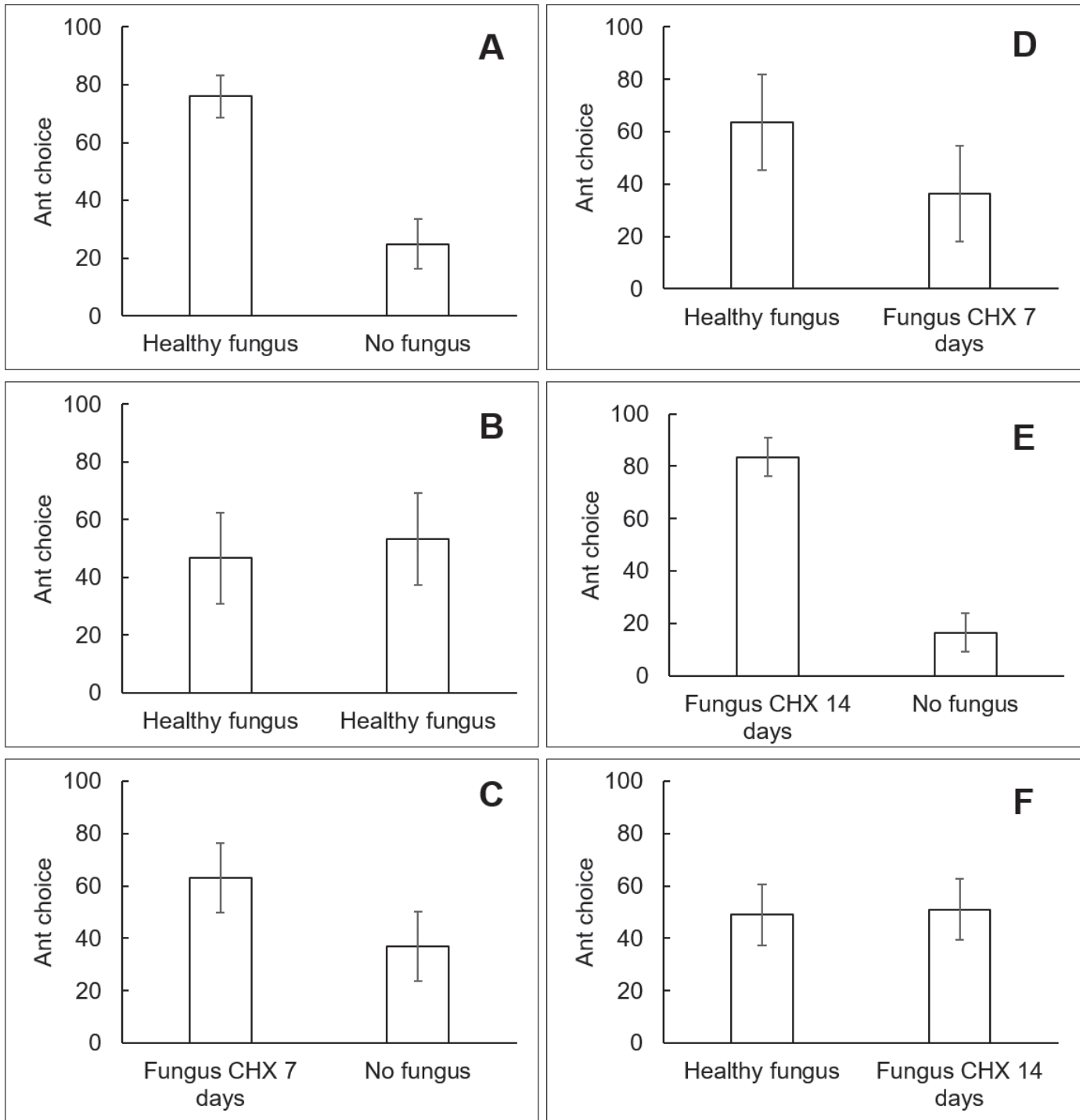


Fig. 5. Percent and confidence interval of ant choice in the Y-shaped choice system. A: Chamber with healthy fungus and chamber without fungus; B: chamber with healthy fungus and chamber without healthy fungus; C: chamber containing fungus with cycloheximide for 7 d and chamber without fungus; D: chamber with healthy fungus and chamber containing fungus with cycloheximide for 7 d; E: chamber containing fungus with cycloheximide for 14 d and chamber without fungus; F: chamber with healthy fungus and chamber containing fungus with cycloheximide for 14 d.

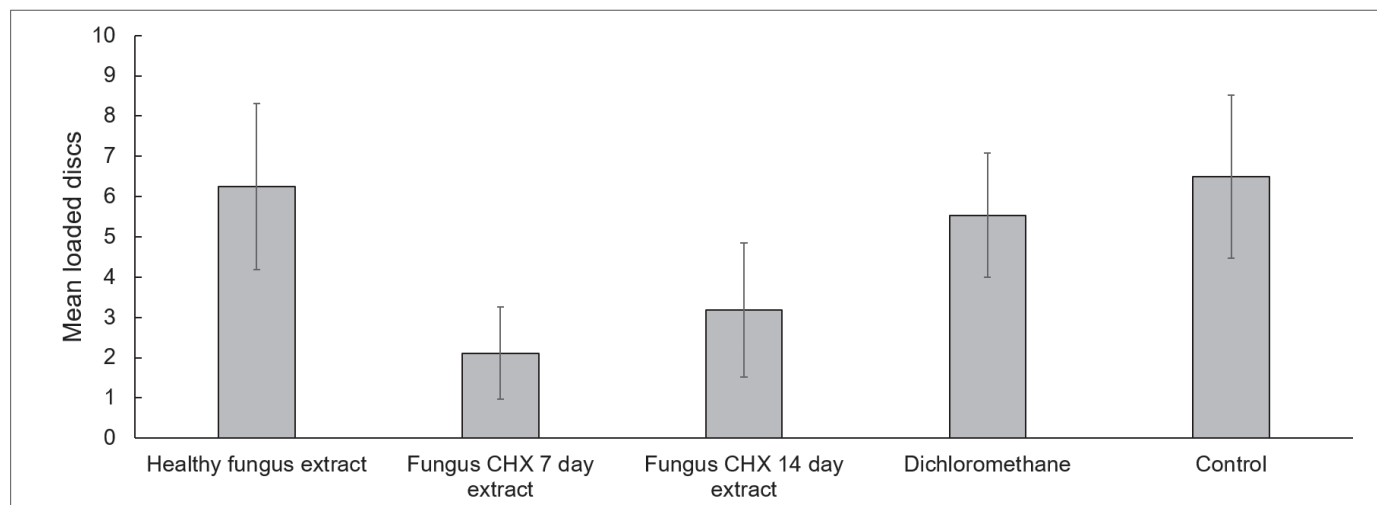


Fig. 6. Means and confidence interval of disks transported containing healthy and unhealthy fungus extract into the colony.

ants could have been affected by many other factors, such as the type of workers, the way the extracts were produced, the way the healthy and unhealthy parts were selected, etc.

Performing behavioral experiments, the study of North et al. (1999) concluded that highly volatile compounds emitted by the fungus garden directly affect the foraging behavior of leaf-cutting ant workers. The major flaw in their study is that they did not identify any volatile compounds from unhealthy or dead fungi because of the effect of cycloheximide. At high concentrations, as used by Ridley et al. (1996), cycloheximide causes the death of workers and the fungus garden (Sousa et al. 2017, 2018). Foraging behavior is probably affected by the toxicity of cycloheximide to workers and the fungus garden, and is not a behavioral response to the volatile semiochemical.

In conclusion, we observed that healthy and unhealthy fungus gardens emitted 4 volatile substances at the same proportion, and that workers did not discriminate between them. Thus, we hypothesize that non-volatile semiochemicals may be involved.

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