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Hydrocarbons on Harvester Ant (*Pogonomyrmex barbatus*) Middens Guide Foragers to the Nest

Shelby J. Sturgis · Michael J. Greene · Deborah M. Gordon

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Abstract Colony-specific cuticular hydrocarbons are used by social insects in nestmate recognition. Here, we showed that hydrocarbons found on the mound of *Pogonomyrmex barbatus* nests facilitate the return of foragers to the nest. Colony-specific hydrocarbons, which ants use to distinguish nestmates from non-nestmates, are found on the midden pebbles placed on the nest mound. Midden hydrocarbons occur in a concentration gradient, growing stronger near the nest entrance, which is in the center of a 1–2 m diameter nest mound. Foraging behavior was disrupted when the gradient of hydrocarbons was altered experimentally. When midden material was diluted with artificial pebbles lacking the colony-specific hydrocarbons, the speed of returning foragers decreased significantly. The chemical environment of the nest mound contributes to the regulation of foraging behavior in harvester ants.

Key Words *Pogonomyrmex barbatus* · Hydrocarbons · Homing · Chemical ecology · Foraging behavior

Introduction

Ant foragers use both individual and collective processes to find their way to and from the nest. The best-known example of navigation by foraging ants uses both. First, a scout navigating on its own searches for food, and when it finds food, returns to the nest while laying a chemical trail. The trail initiates a collective process that guides many other ants to the food source, while each ant lays a trail on the way back. In some species, this collective process regulates the number of foragers according to the abundance of food (Walsh et al., 1965). Other species use a different collective process that involves chemical trails. For example, workers of the polydomous Argentine ant *Linepithema humile* lay trail pheromone as they travel (Aron et al., 1989), thus leading all the ants to follow a shared trail system from one nest to another.

In the desert seed-eating ant, *Pogonomyrmex barbatus*, foragers do not use trail pheromone to recruit to specific locations (Greene and Gordon, 2007). Instead, foragers leave the nest together in a direction influenced by the patrollers, a small group of ants that scout the foraging area early in the morning, and a forager's memory of where it last collected food. The patrollers place a chemical cue from the Dufour's gland on about 20 cm of the nest mound, and this influences the directions foragers will travel up to 20 m from the nest (Greene and Gordon, 2007). The choice of direction allows colonies to avoid overlap with the foragers of neighboring conspecific colonies (Gordon, 1992; Gordon and Kulig, 1996). Each forager leaves the nest with a stream of other ants and searches for food. Once it finds food, it returns immediately to the nest. The colony uses another process, the rate of forager return, to regulate foraging activity; foragers are stimulated to leave the nest by the return of other foragers with food (Schafer et al., 2006; Gordon et al., 2008). The rate of forager return is a measure of the availability of food, because the more food is available, the more quickly foragers find it and return to the nest. Since foraging is regulated by the rate of return of

S. J. Sturgis (✉) · D. M. Gordon
Department of Biology, Stanford University,
371 Serra Mall Gilbert Building rm. 410,
Stanford, CA 94305–5020, USA
e-mail: ssturgis@stanford.edu

M. J. Greene
Department of Integrative Biology,
University of Colorado Denver,
PO Box 173364, Denver, CO 80217–3364, USA

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successful foragers, any process that facilitates the rapid return of foragers would promote the accuracy of this measure of food availability.

Olfactory cues on the nest mound or foraging area of ants play an important role in orientation and homing behavior (Cammaerts and Cammaerts, 1987; Wu, 1989; Jaffé et al., 1990). Cuticular hydrocarbons are vital chemical cues used by ants in discrimination between nestmates and non-nestmates (Singer, 1998; Wagner et al., 2000; d'Ettorre and Lenoir, 2010). Hydrocarbons (HCs) have been found in and on the nests of some ant species (Grasso et al., 2005; Lenoir et al., 2009). Hydrocarbons are transferrable, stable, species- and colony-specific compounds that make them ideal candidates for homing signals on the nest mounds of ants.

Nests of *P. barbatus* have large, flat, circular mounds about 1–2 m in diameter (Fig. 1a). Harvester ant nest mounds are generally, but not always, covered with small midden pebbles collected by the foragers (Fig. 1b; see Gordon, 1984a). The midden pebbles surround the nest entrance, which is located approximately in the center of the nest mound. Mounds with midden pebbles typically are cleared of vegetation by the ants.

Observations suggest that midden material is coated with HCs. Harvester ants of a related species, *Pogonomyrmex badius,* collect bits of charcoal, which absorb organic compounds, for their nest middens. The charcoal middens prevent incursion on the mound by ants of other species (Gordon, 1984b), which suggests that midden material may contain species-specific HCs or other discriminatory olfactory cues. Hölldobler, (1971) found that secretions from the Dufour's gland act as homing signals for *P. badius* foragers locating the nest. Dufour's gland secretions in several *Messor* harvester ant species contain HCs (Brand and Mpuru, 1993; Co et al., 2003; Tullio et al., 2003).

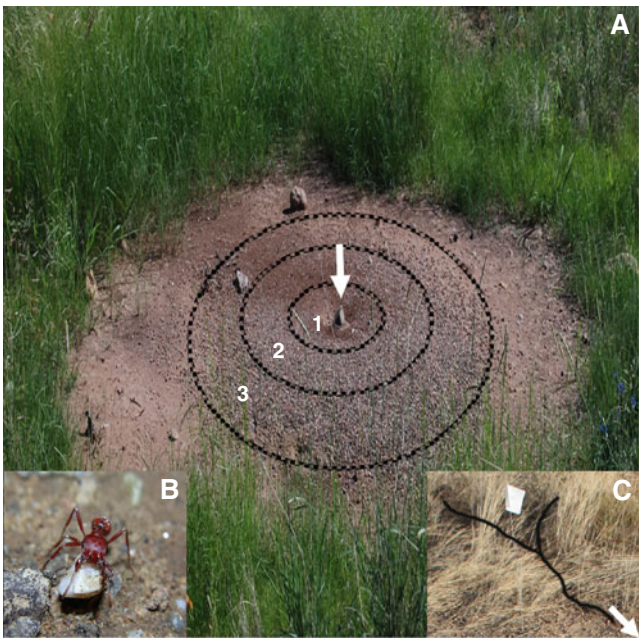

Fig. 1 **a** Anatomy of a *Pogonomyrmex barbatus* nest mound. Midden zones are indicated by dashed circles; 1 = center, 2 = middle, and 3 = outer zone. The white arrows indicate the nest entrance (center of midden zone 1). **b** Photograph in the lower left corner shows a *P. barbatus* forager carrying a midden pebble. **c** Photograph located in lower right corner shows an established foraging trail at the edge of the nest mound. The black line marks the trail. The white arrow points towards the nest entrance

Midden workers of *P. barbatus* arrange midden pebbles on the nest mound (Gordon, 1984a), and have been observed manipulating midden pebbles with their mouthparts (Gordon and Mehdiabadi, 1999). Hydrocarbons can be passively distributed through contact with the ant cuticle (Soroker et al., 1995; Vienne et al., 1995) or secreted onto objects from glands inside the ant (Soroker et al., 1995, 2003). It is unclear whether ants are actively or passively marking midden pebbles. Returning foragers can be seen attennating midden pebbles on their way back to the nest entrance (SJS personal observation), which suggests that there may be a spatial pattern in the concentration of midden HCs.

Here, we examined how HCs on *P. barbatus* nest mounds influence the speed at which foragers travel from the edge of the nest mound to the nest entrance. (1) We tested for the presence of HCs in *P. barbatus* midden pebbles. (2) We tested whether ants can discriminate between their own midden HCs and foreign midden HCs. (3) We examined the spatial pattern of HC abundance in midden pebbles on the nest mound. (4) To determine the effects of nest mound chemical cues on foraging behavior, we experimentally manipulated the distribution of midden material.

Methods and Materials

Study Site. All experiments were conducted with *P. barbatus* colonies at the site of a long-term field study near Rodeo, NM, USA, in which colonies have been censused since 1985 so that colony ages are known (census methods in Gordon and Kulig, 1996).

1 Are there Hydrocarbons (HCs) in the Midden?

Hydrocarbon Extraction and Analysis To determine whether midden pebbles contain HCs, midden pebbles were collected from 10 mature *P. barbatus* colonies (ages 6–18 year). HCs were extracted from 5 g of midden pebbles by immersing the pebbles in 5 ml of 100% pentane (Sigma, St. Louis, MO, USA) for 30 min. For comparison, ant cuticular HCs were extracted from individual ants by immersing the ants in 0.5 ml of 100% pentane (Sigma) for 7 min. Suspensions of

both midden pebbles and ants were lightly shaken during the first and last minute of the soak period. The suspension solution was eluted from a 1-cm silica gel (28–200 mesh, Sigma) column with a 1 ml wash of 100% pentane. The eluant was dried overnight, and the samples were redissolved in 100 μl of pentane for midden pebble HCs or 25 μl of pentane for ant cuticular HCs, including an internal standard (27 ng per μl of triatriacontane,$C_{33}H_{68}$; Fluka Analytical, Buchs, Switzerland). The amounts of midden HCs were quantified by comparing their peak areas with that of the internal standard. Samples were analyzed by gas chromatography (GC). GC analyses were performed on a Varian 3900 GC with a CP-8400/8410 auto sampler and a flame ionization detector (Tissot et al., 2001; Greene and Gordon, 2003). Aliquots of 8 μl were introduced by split-less injection onto a capillary column (DB-1, 30 m, 0.25 mm ID, 0.25-μm film thickness; Agilent J&W Scientific, Santa Clara, CA, USA), with helium as the carrier gas flowing at 1 ml/min. The injector temperature was 300°C. The temperature was 170°C during injection, and was then increased to 220°C at 25°C/min, 220°C–310°C at 3°C/min. Retention times were verified on the GC for 6 *n*-alkanes using a standard mixture containing *n*-tricosane, *n*-tetracosane, *n*-pentacosane, *n*-heptacosane, *n*-hexacosane, and *n*-nonacosane (Sigma) interspersed among runs.

There were no readily available standards for methyl alkane and alkene identification. Tentative methyl alkane and alkene identifications were based on analyses of a subset of midden pebble (N=3), and *P. barbatus* worker (N=3) HC samples by coupled gas chromatography/mass spectrometry (GC/MS), and by cross reference with previously published work (Wagner et al., 1998; Tissot et al., 2001). GC/MS was conducted at the Department of Environmental Sciences, Policy and Management at the University of California Berkeley, using a 7890A Agilent GC (Agilent Technologies, Santa Clara, CA, USA) coupled with a 5975 C Agilent Mass Selective Detector (electron impact ionization at 70 eV). Aliquots of 1 μl were injected by using an Agilent 7683B automatic injector operated in split mode (5:1) onto a capillary column (DB-5, 30 m, 0.32 mm ID, 0.25-μm film thickness; Agilent J&W Scientific), with helium as the carrier gas flowing at 0.7 ml/min. Mass Selective Detector (MSD) scanning parameters ranged from 50 to 550 amu, with a sampling rate of 2.91 scans/sec and a threshold detection of 150 counts.

Statistical Analysis Nest mounds were divided into three zones; center, middle, and outer, for all colonies (Fig. 1a; 3 zones per colony, 10 colonies, N=10 replicates per midden zone). The average concentrations in nanograms (SD) of each HC and HC class present in the midden pebble samples were calculated for each midden zone. Nonparametric Kruskal Wallis one-way ANOVAs were used to compare the average individual and average total HC concentrations of the 3 midden zones. To compare the concentrations of HCs on midden pebbles and on forager cuticles, the average concentration in ng (SD) of each HC and HC class was calculated for 50 *P. barbatus* foragers.

2 Can *P. barbatus* Workers Discriminate between their own Midden and that of Another Nest?

Bioassay We tested whether harvester ants recognize chemical cues on midden pebbles, and HCs extracted from the surface of midden pebbles, by measuring the ants' agonistic response to midden pebbles and objects coated with a chemical stimulus (Wagner et al., 2000). Stimulus objects, either a pile of midden pebbles or a 5×5×10 mm glass block (Wagner et al., 2000) coated with a chemical stimulus, were placed on the nest mound approximately 10 cm from the nest entrance, but never directly along a foraging trail. After placement of the stimulus objects, we recorded every 30 sec for 5 min the number of ants biting or stinging the objects, and the total number of ants in contact with the objects, either with antennae, abdomen, legs, or any other body part.

Trials were conducted on 4–6 August 2001 and 8–17 May 2002. We measured the proportion of ants that exhibited agonistic behavior, biting, and stinging. The first experiment tested the response of workers to midden pebbles of their own and of another nest. About 20 midden pebbles were placed in a small pile on the nest mound. On a single day, colonies (ages 3–16 year; N=15 colonies) were offered midden pebbles from its own nest, and from another nest, once in a randomized sequence. The second experiment tested the response of workers to midden HCs and worker cuticular HCs of their own and of another colony. On a single day, colonies (ages 3–16 year; N=8 colonies) were offered one 5×5×10 mm glass block either coated with nestmate cuticular HCs from same-colony workers, non-nestmate cuticular HCs from foreign-colony workers, HCs from same-colony midden pebbles, HCs from foreign-colony midden pebbles, and pentane (solvent control), once in a randomized sequence.

Statistical Analysis For both experiments, we calculated the angular transformation (Zar, 1999) of the proportion of ants that displayed either biting or stinging, out of the total number of ants that made contact with the pile of midden pebbles or the glass blocks coated with a chemical stimulus. For the first experiment, the angular transformed data were compared for the two midden pebble treatments (same-colony and foreign-colony midden pebbles), using a *t*-test. For the second experiment, all 5 treatments (same-colony worker cuticular HCs, foreign-colony worker cuticular

HCs, same-colony midden pebble HCs, foreign-colony midden pebble HCs, and solvent control) were compared using an ANOVA with Fisher's PLSD for *post hoc* analysis.

3 Is there a Spatial Pattern of Hydrocarbon Abundance in Midden?

Midden Collection Midden pebbles were collected from 10 mature *P. barbatus* colonies (ages 6–18 year) in August 2008. Pebble cover on mounds, estimated by sight, ranged from 40–100% of the total mound surface area, with the remainder of the nest mound as bare soil. Nest mounds were defined as the entire area around the nest entrance of a colony extending either to the edge of the vegetation or of the pebble cover. Nest mounds were divided into 3 zones of approximately similar radii: center (closest to nest entrance), middle, and outer (Fig. 1a). From each zone, we gathered ~10 g of midden pebbles using nylon gloves, and placed the midden pebbles into separate plastic bags. Analysis of samples stored in the bags indicated that there was no detectable contamination from the plastic. In each of the three zones, on five nest mounds, ~2 g of soil were collected from the surface beneath the midden pebbles. Samples were stored at −20°C. All samples were transferred to 16×100 mm glass culture tubes (VWR Scientific Products, West Chester, PA, USA) before HC extraction. Five grams of midden pebbles and 2 g of surface soil were extracted by immersing the pebbles or soil in 5 ml of 100% pentane (Sigma) for 30 min. Hydrocarbon extraction and analysis of both midden pebbles and surface soil were performed as above. Samples were redissolved in 100 μl of pentane before GC analysis.

Statistical Analysis We calculated the mean total HC concentration in ng (SD) for midden pebbles and surface soil from each zone. Nonparametric Kruskal Wallis one-way ANOVAS were used to test for differences between midden zones. Dunn's Multiple Comparison tests were used for post-hoc analysis.

4 Does the Hydrocarbon Gradient in the Harvester Ant Midden Influence Foraging Behavior?

Colony Selection Twenty colonies, all at least 5 year old, were selected in August 2009. Nest mound size ranged from 2.62 m^2 to 10.50 m^2, and pebble cover ranged from 50–99%. Each colony was located at least 10 m away from the nearest neighboring colony. All nest mounds were approximately circular. The area of each nest mound was calculated using the longest chord of the irregularly-shaped circular nest mounds.

Data Collection before Experimental Manipulation We measured the time foragers took to reach the nest entrance from the edge of the mound. In all 20 colonies, measurements were made during peak foraging hours, from 7 am to 10:45 am daily, for 5 day from 12–16 August 2009. Because foraging activity changes in the course of the activity period (Gordon, 2002), the sequence of colonies in which foraging was observed was reversed from 1 day to the next. For each colony we chose a foraging trail cleared of vegetation that had been used by foragers for at least two consecutive days (Fig. 1c). A flag was placed at the perimeter of the nest mound to mark the beginning of the trail. Distances from the flag to the nest entrance ranged from 0.84 m to 2.03 m. We recorded the time it took individual foragers to return to the nest entrance from the flag, and calculated forager speed as the return time divided by the distance from the flag to the nest entrance. We counted only returning foragers, which could be distinguished from other workers because the returning forager carried a food item in its mandibles. For each colony, and each of the 5 day of observation before treatment, we measured the speeds of 3 foragers. In a total of 60 observations from 10 of the 20 colonies, there was no foraging activity, and these 60 observations were excluded. A total of 240 observations from 20 colonies were used in the analysis.

Experimental Manipulations To determine the effect of chemical cues on the return of foragers, we either (a) diluted nest middens by adding an inert substance to the nest mound, or (b) deprived nests of midden material. To test the effect of colony-specific HCs on forager return speed, we diluted midden pebbles with expanded Perlite (Scotts Miracle-Grow®, Maryville, OH, USA), a substance similar in structure and size but lacking colony-specific HCs. Perlite is an amorphous volcanic glass that has been softened under extreme heat for commercial use. Perlite consists of 70–75% silicon dioxide, 12–15% aluminum oxide, 3–4% sodium oxide, 3–5% potassium oxide, and trace amounts of iron oxide, magnesium oxide, and calcium oxide. Chemically, there are only minor differences between Perlite and midden pebbles. Midden pebbles found on *P. barbatus* nest mounds and Perlite are both volcanic rocks which contain the same major elements found in all silicate minerals (Elston et al., 1973). Expanded Perlite clumps together to form small balls that are roughly the size of midden pebbles. To deprive nests of midden material, we removed all midden pebbles from the nest mound and left the soil bare.

Nest mounds were divided into 3 zones of approximately similar radii: center (closest to nest entrance), middle, and outer (Fig. 1a). There were 4 treatments: 1) Midden pebbles in the center and middle zones were diluted with Perlite. The center and middle zones were combined because the

results of the experiment described in the previous section showed no significant difference between the zones in HC concentration. 2) Midden pebbles in the outer zone were diluted with Perlite. 3) Midden pebbles for all 3 zones were diluted with Perlite. In treatments 1, 2, and 3, we applied Perlite to the indicated zones twice, on days 1 and 4 of the 6-d observation period. Perlite was reapplied after 2 day because in all colonies, the Perlite treatment was almost completely removed by d 3, probably due to wind, rain, and the activity of nest maintenance workers in the afternoon, when no observations of foraging activity were made. 4) All midden pebbles were removed, leaving the bare soil that had been covered by midden pebbles.

An alternative design would have been to remove pebbles, extract HCs and return them to mounds. This was not feasible due to the large quantity of pebbles on the mound and because extracted pebbles would have been difficult to return to the mound in a homogenous manner.

Data Collection after Experimental Manipulation We measured the speed at which foragers returned to the nest entrance from the edge of the mound using the methods described above once a day for 6 day from 17–22 August 2009. In a total of 76 observations from 11 of the 20 colonies, the colony was not foraging actively, and these observations were excluded. A total of 284 observations of forager return speeds from the 20 colonies were used in the analysis.

Statistical Analysis For each treatment, we compared forager return speeds before and after treatment within each treatment group using unpaired *t*-tests. Data were square-root transformed to meet the assumptions of normality.

To compare forager return speeds in the 4 treatment groups, we first normalized for variation among colonies in forager speed. We found for each colony the difference between mean forager return speed before and after treatment, and divided this difference by that colony's mean forager return speed. Using the normalized mean colony difference in forager return speed before and after treatment, we tested for differences among treatments using an ANOVA.

Results

1 Are there Hydrocarbons (HCs) in the Midden?

Midden pebbles contained HCs tentatively identified as identical to those found on the ants' cuticles (Fig. 2). Twenty-one HCs that occur on the *P. barbatus* cuticle were detected in the midden (Table 1). It is possible that other HCs were also present in trace amounts. Hydrocarbon compounds were of four classes, *n*-alkanes, monomethylalkanes, dimethylalkanes, and alkenes. HCs present in the highest abundances were *n*-alkanes. The 5 most abundant HCs in the midden were *n*-hentriacontane (C31), *n*-nonacosane (C29:0), *n*-pentacosane (C25:0), *n*-heptacosane (C27:0), and *15-, 13-, 11-, 9*-methylhentriacontanes (C31:0), respectively. There was an overall difference among midden zones in individual HC concentration for all HCs except *n*-tricosane and *n*-tetracosane. There was a significant difference among midden zones in the average concentration of HCs for each HC class (Kruskal Wallis tests: alkanes: H_3=8.87, N=10, P=0.012; monomethylalkanes: H_3=11.66, N=10, P=0.003; dimethylalkanes: H_3=10.55, N=10, P=0.005; alkenes: H_3=12.21, N=10, P=0.002). There were about 10 times more HCs on one forager's cuticle than on 5 g of midden pebbles; the mean concentration of HCs in ng (SD) on one forager's cuticle, and on 5 g of midden pebbles were 562.30 (252.27) and 57.81 (67.78), respectively.

2 Can *P. barbatus* Workers Discriminate between their own Midden and that of Another Nest?

Midden pebbles contain colony-specific HCs that are recognized by workers. Ants were more likely to respond aggressively toward midden pebbles from another colony's nest than toward midden pebbles from their own colony's nest (Fig. 3a; Paired *t*-test: t_{26}=4.1, P<0.001). The mean proportion of ants biting/stinging (SD) midden pebbles from their own and conspecific nests were 0.06 (±0.08) and 0.42 (±0.19), respectively.

Pogonomyrmex barbatus workers recognized the HC blend extracted from midden pebbles of their own colony. Workers distinguished HCs extracted from midden pebbles of their own nest from those extracted from the midden pebbles, or workers of another colony. A higher proportion of contacts were aggressive in response to extracts of midden pebbles and whole ants from other colonies than to extracts of midden pebbles or extracts from ants of their own colony (Fig. 3b; ANOVA: $F_{4,\ 35}$=8.8, P<0.001). The mean proportions of workers biting/stinging (SD) glass blocks treated with nestmate cuticular HCs from same-colony workers, non-nestmate cuticular HCs from foreign-colony workers, HCs from same-colony midden pebbles, HCs from foreign-colony midden pebbles, and solvent were 0.40 (±0.33), 0.84 (±0.18), 0.40 (±0.23), 0.84 (±0.27), and 0.24 (±0.20), respectively [Fisher's PLSD (Protected Least Signficant Difference): non-nestmate (foreign) cuticular HCs (FCHCs), foreign midden HCs (FMHCs): mean difference = 11.606, P=0.262; cuticular HCs from nestmates (CHCs), colony midden HCs (MHCs): mean difference = 17.249, P=0.099; CHCs, solvent: mean difference = 11.953, P=0.249; MHCs, solvent (pentane): mean difference = 5.296, P=0.606; FCHCs vs CHCs, MHCs, solvent: mean difference = 32.511, 49.760, and 44.464, P=0.003, <0.001, and <0.001 respectively;

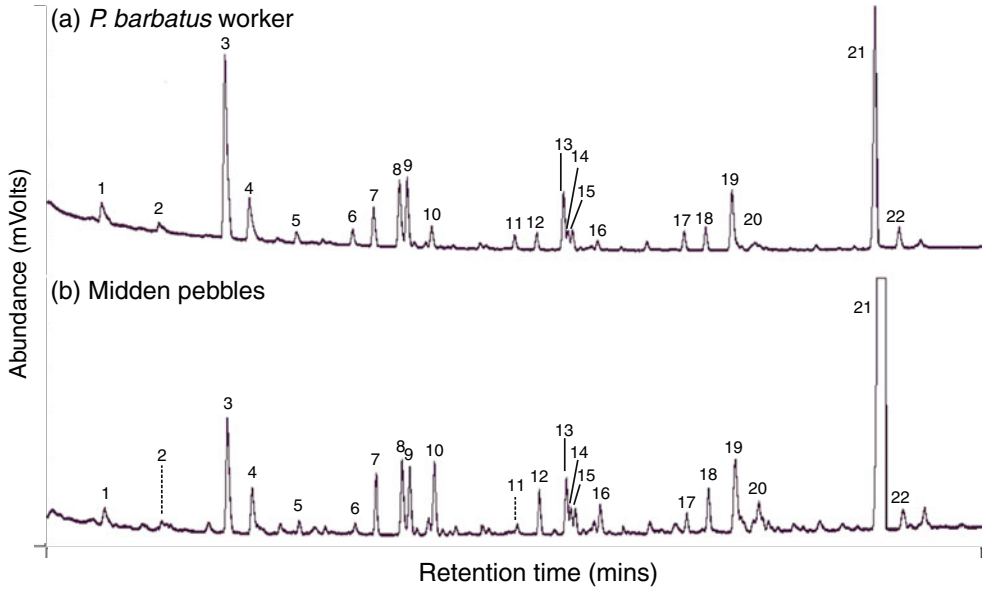

Fig. 2 Gas chromatograms of *Pogonomyrmex barbatus* forager (**a**), and of midden pebbles from a *P. barbatus* nest mound (**b**). Each number identifies a hydrocarbon peak: **1**– *n*-tricosane, **2**– *n*-tetracosane, **3**– *n*-pentacosane, **4**– 13-methylpentacosane*, **5**– *n*-hexacosane, **6**– heptacosene*, **7**– *n*-heptacosane, **8**– 13-methylheptacosane*, **9**– 7-methylheptacosane*, **10**– 7,23-dimethylheptacosane*, **11**– nonacosene*, **12**– *n*-nonacosane, **13**– 15-methylnonacosane*, **14**– 9-methylnonacosane*, **15**– 7-methylnonacosane*, **16**– 7,13-dimethylnonacosane*, **17**– hentriacontene*, **18**– *n*-hentriacontane*, **19**– 15-,13-,11-,9-methylhentriacontanes*, **20**– 13,17-, 11,15-, 9,13-dimethylhentriacontanes*, **21**– *n*-triatriacontane (internal standard), **22**– 17-,15-,13-,11-,9-,7-methyltritriacontanes*. *Compounds tentatively identified based on mass spectral interpretation and prior literature

FMHCs vs CHCs, MHCs, solvent: mean difference = 20.905, 38.154, and 32.858, P=0.047, <0.001, and 0.003, respectively].

3 Is there a Spatial Pattern of Hydrocarbon Abundance in Midden?

There is a HC concentration gradient on the midden pebbles and on the soil below the midden pebbles on the surface of nest mounds. Hydrocarbon concentration in both the midden pebbles and soil is higher near the nest entrance (Fig. 4). The mean total HCs in ng (SD) for the midden pebbles were 82.75 (56.00), 73.73 (92.97), and 16.94 (14.03) for the center, middle, and outer zones, respectively (Kruskal Wallis test, H_3=10.41, N=10, P=0.006); the center zone differed significantly from the outer zone (Dunn Multiple Comparison test, difference in rank sum = 12.2, P<0.01). The mean total HCs in ng (SD) for the soil were 40.08 (13.84), 11.91 (6.15), and 8.20 (2.14) for the center, middle, and outer zones, respectively (Kruskal Wallis test: H_3=9.78, N=5, P=0.008); the center and outer zone differed significantly (Dunn Multiple Comparison test, difference in rank sum = 8.6, P<0.01).

4 Does the Hydrocarbon Gradient in the Harvester Ant Midden Influence Foraging Behavior?

The HCs on the nest mound facilitate the return of foragers to the nest. Diluting midden pebbles with Perlite reduced the speed with which returning foragers reached the nest entrance (Fig. 5; Unpaired t-test: t_{522}= 3.689, P<0.001). The mean speed at which returning foragers reached the nest entrance (SD) before and after treatment was 5.9 (3.0) cm/s (N=240) and 5.0 (3.0) cm/s (N=284), respectively. The average forager speed to nest entrance (SD) in the center and middle zones (treatment 1) was 6.4 (3.3) cm/s before treatment and 5.1 (3.1) cm/s after treatment (*Unpaired t-test:* t_{132}=2.21, P=0.029). In the outer zone (treatment 2), the average speed to the nest entrance (SD) before treatment was 5.4 (2.3) cm/s and 4.0 (2.0) cm/s after treatment (Unpaired t-test: t_{136}=3.99, P=0.001). In all 3 zones (treatment 3), the average speed to the nest entrance (SD) before treatment was 6.6 (3.5) cm/s and 5.1 (3.0) cm/s after treatment (Unpaired t-test: t_{124}=2.589, P=0.011). The speed of returning foragers to the nest entrance increased slightly but not significantly after the removal of pebbles, with bare soil remaining (treatment 4). When all pebbles were removed, the average speed of returning foragers to the nest entrance (SD) before treatment was 5.1 (2.7) cm/s and 5.8 (3.2) cm/s after treatment (Unpaired t-test: t_{124}=1.17, P=0.243).

Forager return speed did not differ according to the midden zone from which Perlite was added. Diluting the midden with Perlite (treatments 1–3) reduced forager return speeds more than the removal treatment (treatment

Table 1 The mean concentration (ng) of all hydrocarbons present in each midden zone (center, middle, and outer) and on the cuticle of *Pogonomyrmex barbatus* foragers

Hydrocarbons	Formula	Hydrocarbon concentration (ng)[a]					
Alkanes	C_nH_{2n+2}	Center	Middle	Outer	p-value[b]	All Zones[c]	*P. barbatus* forager[d]
1. *n*-Tricosane	$C_{23}H_{48}$	**3.46** (3.15)	**2.53** (2.08)	**1.96** (1.42)	*P*=0.31	**2.65** (2.33)	**31.37** (15.15)
2. *n*-Tetracosane	$C_{24}H_{50}$	**1.22** (0.91)	**1.10** (1.20)	**0.51** (0.43)	*P*=0.07	**0.94** (0.93)	**12.42** (6.43)
3. *n*-Pentacosane	$C_{25}H_{52}$	**13.56** (10.32)	**7.56** (8.48)	**2.95** (1.39)	*P*=0.005**	**8.02** (8.69)	**208.26** (105.76)
5. *n*-Hexacosane	$C_{26}H_{54}$	**1.91** (0.99)	**1.04** (0.99)	**0.64** (0.59)	*P*=0.008**	**1.20** (1.00)	**11.28** (5.49)
7. *n*-Heptacosane	$C_{27}H_{56}$	**6.98** (4.35)	**6.34** (5.65)	**1.98** (1.35)	*P*=0.02*	**5.10** (4.63)	**37.29** (20.52)
12. *n*-Nonacosane	$C_{29}H_{60}$	**5.92** (4.45)	**17.37** (32.60)	**2.03** (2.46)	*P*=0.04*	**8.44** (19.54)	**13.63** (7.07)
18. *n*-Hentriacontane*	$C_{31}H_{62}$	**6.01** (5.09)	**20.66** (33.84)	**2.88** (5.14)	*P*=0.02*	**9.85** (20.82)	**14.24** (8.21)
Mean alkanes	C_nH_{2n+2}	**39.06** (23.43)	**56.60** (76.87)	12.96 (10.02)	*P*=0.012*	**155.17** (110.04)	**331.83** (163.53)
Total alkanes	C_nH_{2n+2}	356.42	566.00	129.59		1086.15	16424.45
Methyl alkanes	$C_{n+1}H_{2n+5}$						
4. 13-Methylpentacosane*	$C_{26}H_{55}$	**4.25** (3.72)	**1.54** (1.86)	**0.66** (0.61)	*P*=0.005**	**2.03** (2.66)	**30.37** (16.66)
8. 13-Methylheptacosane*	$C_{28}H_{59}$	**5.83** (5.27)	**2.11** (3.02)	**0.70** (0.79)	*P*=0.01**	**2.72** (3.87)	**33.71** (16.08)
9. 7-Methylheptacosane*	$C_{28}H_{59}$	**5.17** (4.57)	**1.71** (2.55)	**0.63** (0.95)	*P*=0.009**	**2.36** (3.38)	**34.00** (16.26)
13. 15-Methylnonacosane*	$C_{30}H_{63}$	**4.05** (3.50)	**1.38** (2.17)	**0.31** (0.52)	*P*=0.006**	**1.82** (2.74)	**26.61** (13.11)
14. 9-Methylnonacosane*	$C_{30}H_{63}$	**1.62** (1.53)	**0.96** (1.52)	**0.10** (0.20)	*P*=0.03*	**0.85** (1.33)	**8.87** (4.21)
15. 7-Methylnonacosane*	$C_{30}H_{63}$	**1.75** (1.44)	**0.57** (0.91)	**0.11** (0.24)	*P*=0.006**	**0.76** (1.13)	**9.11** (4.77)
19. 15-,13-,11-,9-Methylhentriacontanes*	$C_{32}H_{67}$	**6.61** (5.55)	**2.52** (3.09)	**0.57** (0.72)	*P*=0.003**	**3.11** (4.32)	**35.15** (21.59)
21. 17-,15-,13-,11-,9-,7-Methyltritriacontanes*	$C_{34}H_{71}$	**1.62** (1.28)	**0.94** (1.04)	**0.06** (0.19)	*P*=0.004**	**0.85** (1.12)	**10.70** (6.81)
Mean methyl alkanes	$C_{n+1}H_{2n+5}$	**30.89** (26.24)	**11.73** (14.83)	**3.13** (3.96)	*P*=0.003**	**57.19** (28.71)	**191.20** (94.77)
Total methyl alkanes	$C_{n+1}H_{2n+5}$	287.07	117.33	31.32		457.54	9426.45
Dimethyl alkanes	$C_{n+2}H_{2n+8}$						
10. 7,23-Dimethylheptacosane*	$C_{29}H_{62}$	**5.54** (4.54)	**2.12** (2.62)	**0.69** (0.66)	*P*=0.006**	**2.64** (3.42)	**13.32** (16.32)
16. 7,13-Dimethylnonacosane*	$C_{31}H_{66}$	**2.04** (1.86)	**0.77** (1.13)	**0.10** (0.22)	*P*=0.008*	**0.92** (1.41)	**3.71** (2.21)
20. 13,17-, 11,15-, 9,13-Dimethylhentriacontanes*	$C_{32}H_{67}$	**1.84** (1.98)	**0.55** (0.92)	**0.06** (0.20)	*P*=0.012*	**0.79** (1.43)	**4.52** (4.98)
Mean dimethyl alkanes	$C_{n+2}H_{2n+8}$	**9.42** (8.26)	**3.44** (4.58)	**0.85** (0.97)	*P*=0.005**	**45.71** (32.80)	**21.55** (20.54)
Total dimethyl alkanes	$C_{n+2}H_{2n+8}$	87.54	34.38	8.52		137.13	1077.27
Alkenes	C_nH_{2n}						
6. Heptacosene*	$C_{27}H_{54}$	**1.05** (0.71)	**0.33** (0.73)	**0.00** (0.00)	*P*<0.001***	**0.44** (0.69)	**7.24** (4.68)
11. Nonacosene*	$C_{29}H_{58}$	**0.69** (0.59)	**0.81** (1.11)	**0.00** (0.00)	*P*<0.001***	**0.48** (0.78)	**6.48** (3.81)
17. Hentriacontene*	$C_{31}H_{62}$	**1.64** (1.28)	**0.81** (1.12)	**0.00** (0.00)	*P*=0.002**	**0.77** (1.10)	**10.02** (5.74)
Mean alkenes	C_nH_{2n}	**3.38** (2.51)	**1.95** (2.80)	**0.00** (0.00)	*P*=0.002**	**17.78** (5.88)	**23.74** (13.60)
Total alkenes	C_nH_{2n}	31.05	19.53	0.00		53.35	1186.80

[a] Values in bold represent the mean hydrocarbon concentration (per 5 g midden pebbles or ant) and values in parentheses represent the standard deviation

[b] *P*-values were obtained by comparing the mean hydrocarbon concentrations among all midden zones using one-way non-parametric Kruskal Wallis ANOVAs

[c] Values represent the mean concentration of each hydrocarbon in all samples (*N*=30)

[d] Values represent the mean concentration of each hydrocarbon for *P. barbatus* foragers (*N*=50)

*Compounds tentatively identified based on mass spectral interpretation and prior literature

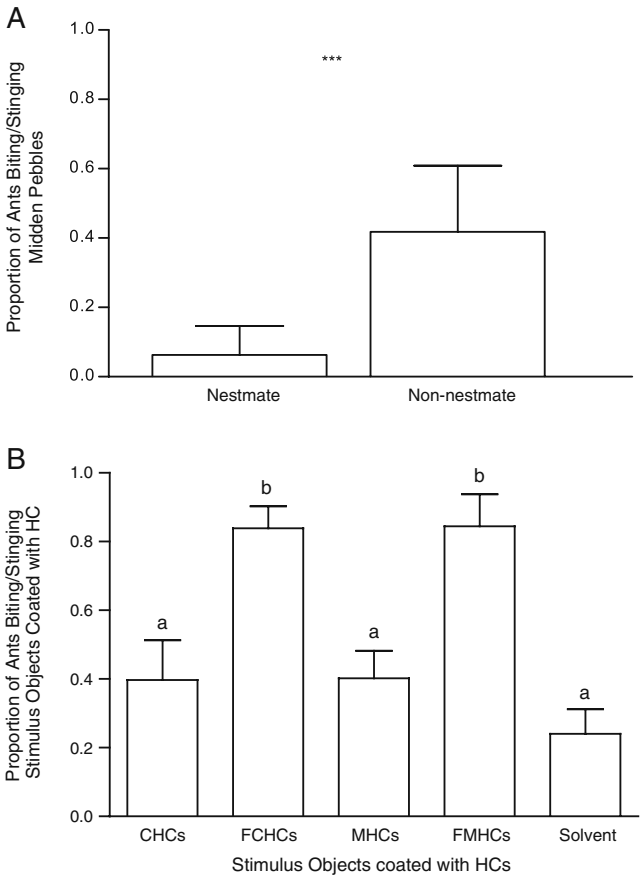

Fig. 3 **a** The proportion of workers that displayed agonistic behavior toward nestmate and non-nestmate midden pebbles. **b** The proportion of workers that displayed agonistic behavior toward glass blocks treated with nestmate or non-nestmate hydrocarbons (HCs) extracted from workers' cuticles or from midden pebbles. Each bar shows the mean proportion of ants that displayed biting or stinging behavior out of all ants that touched the stimulus objects. Lowercase letters indicate significant differences. Error bars represent the standard error of the mean. CHCs– cuticular HCs from nestmates; FCHCs– non-nestmate (foreign) cuticular HCs; MHCs– colony midden HCs; FMHCs– foreign midden HCs; Solvent –pentane

4), but there was no difference among treatments, and thus no effect of the zone from which Perlite was added (*ANOVA*: $F_{3,\ 16}$=1.498, P=0.2531). The average changes in forager speed to nest entrance (SD) for treatments 1–4 were 0.91 (0.12), 0.87 (0.12), 0.84 (0.12), and 1.03 (0.20), respectively.

The Perlite itself did not disrupt the foraging behavior of the ants. To determine that adding inert pebbles to the nest mound, by modifying the nest structure, did not itself affect the speed at which foragers return to the nest, we compared the speeds of returning foragers from colonies with midden pebbles ranging from 50–99% pebble cover to the speeds of returning foragers from colonies whose midden pebbles were removed. The two groups did not differ in the speed of returning foragers (*Unpaired t-test:* t_{262}=0.9241, P=0.356), indicating that the amount of midden pebbles on the nest mound does not affect forager return speed. In response to extraneous material on the nest mound, *P. barbatus* increase the numbers performing nest maintenance and decrease the numbers foraging (Gordon, 1986, 1987). Here, we saw no difference in the number of nest maintenance workers before and after treatment (*Mann–Whitney U test:* U=5841, N_1=100, N_2=120, P=0.727), and the proportion of colonies that did not forage did not differ before and after treatment (before treatment = 19%, after treatment = 19.1%).

Discussion

Our results show that colony-specific chemical cues on the midden pebbles of *P. barbatus* colonies help the foragers return to the nest more quickly. These cues on the midden pebbles are the same HCs that *P. barbatus* workers use in nestmate recognition (Fig. 2; see Wagner et al., 2000). Workers can distinguish their own colony's midden from that of other colonies, and can distinguish their own colony's midden HCs from the midden HCs of other colonies.

The concentration of HCs in midden pebbles and on the soil surface below the midden pebbles increases as a forager moves closer to the nest entrance (Fig. 4). A possible mechanism for the HC gradient is ant density. *P. barbatus* worker densities are greater per cm^2 nearer the nest entrance than away from the entrance (unpublished data). Therefore,

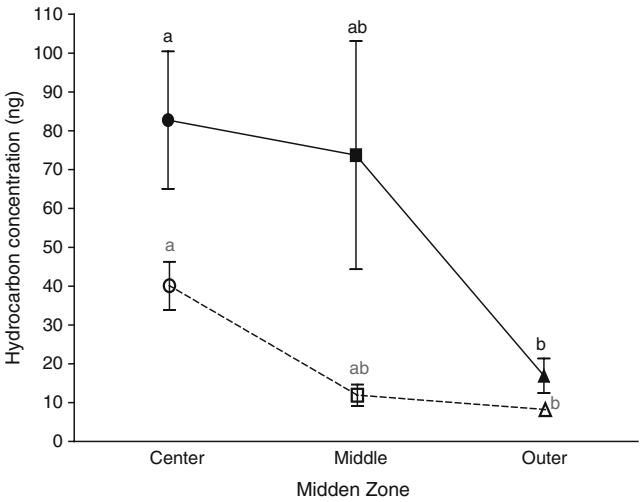

Fig. 4 Mean hydrocarbons in ng per 5 g of midden pebble samples and 2 g of soil samples for the center, middle, and outer midden pebble (closed shapes, solid line), and surface soil (open shapes, dashed line) zones. Center (*circle*), middle (*square*) and outer (*triangle*) zones as in Fig. 1. Lowercase letters above the shapes indicate significant differences. Error bars represent the standard error of the mean

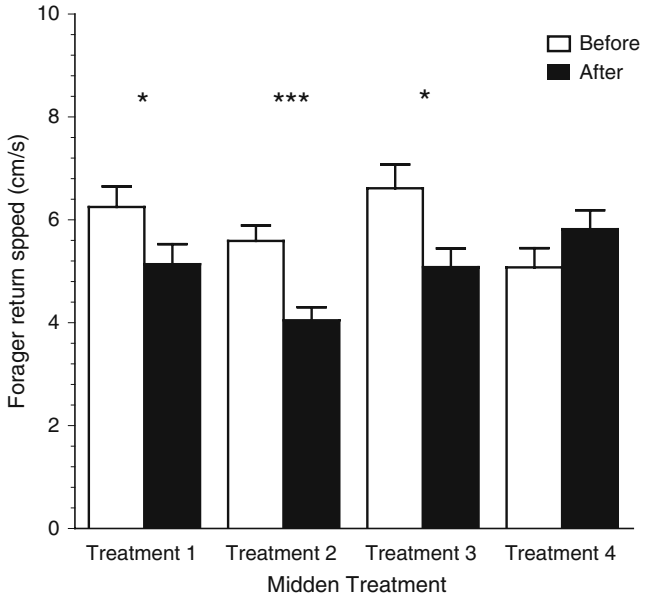

Fig. 5 Comparison of the mean forager return speed (cm/s) before and after treatment within each treatment group. Treatment 1: midden pebbles in the center and middle zones were diluted with Perlite. Treatment 2: midden pebbles in the outer zone were diluted with Perlite. Treatment 3: midden pebbles for all 3 zones were diluted with Perlite. Treatment 4: all midden pebbles were removed, leaving the bare soil that had been covered by midden material. *P*-values were obtained by comparing the square-root transformed forager return speeds before and after manipulation for each treatment group using unpaired t-tests. Error bars represent the standard error of the mean.* $P<0.05$, ** $P<0.01$, *** $P<0.001$

ant footfall or marking behavior occurs more in the center zones than the outer zones. The reduction in the concentration in the outer zones could be due to the difficulty in detecting HCs as their concentrations decrease or due to the greater volatility of certain HCs and the lack of sufficient ant footfall or marking to replace them.

Experimental manipulation of midden pebbles did not disrupt foraging, but it caused a decrease in the walking speed of returning foragers. When midden material was diluted with artificial pebbles lacking the colony-specific HCs, the speed of returning foragers decreased significantly. These effects did not vary according to the region of the nest mound on which the midden was diluted or removed.

The reduction in forager return speed is unlikely due to any physical or chemical properties of Perlite, which was of similar size and shape to the midden pebbles. If midden pebbles texture, size, shape, and/or color were significant factors in guiding ants to the nest, we should have seen a decrease, however slight, in the forager return speed in the removal treatment (treatment 4). Instead, there was a slight, although not statistically significant, increase in forager return speed after the removal treatment.

The rate at which inactive foragers leave the nest depends on the rate at which other foragers return with food (Gordon et al., 2008). Midden HCs influence forager return speeds, which influences forager return rates. Foraging activity affects food intake and interactions with neighbors, because encounters between foragers of neighboring colonies help to maintain foraging territory (Gordon, 1992). Thus chemical cues on the midden play a role in food intake, and in relations among conspecific neighbors.

We found a concentration gradient in HCs by zone on the midden, with a higher concentration toward the nest entrance, but our experiments did not elucidate the function of this gradient, at least in returning forager velocity. Our experiments compared the effect of disrupting the gradient by dividing the nest mound into three concentric zones. It may be that the gradient is important, but that the zones we chose arbitrarily do not correspond to areas that are functionally significant for the ants. Small gradients of HCs in the midden may be important, and finer scale manipulations are needed to investigate this. Perhaps, the chemical concentration gradient is used by *P. barbatus* foragers as a primary means of navigating the nest mound.

Chemical extractions from the top soil on the nest mound revealed the presence of a HC gradient on the soil as well as on the midden pebbles (Fig. 4). The presence of HCs, with a concentration gradient in the surface soil of nest mounds suggests that even in the absence of midden pebbles, there were sufficient HCs to be used by returning foragers. A colony usually stays in the same nest for its 20–30 year lifespan (Gordon, 1992). An accumulation of HCs in the midden and on the soil is not unexpected because of the stable nature of these compounds (Martin et al., 2009).

An interesting mechanism explaining the accumulation of HCs on the nest area as a form of colony recognition system was first reported for other harvester ants (see Grasso et al., 2005) whose fecal spots deposited on the nest surroundings contain colony-specific HCs. Interestingly these HCs are the same as those found on the cuticle of the ants. This mechanism also may be valid for *P. barbatus* and could explain the HC accumulation, not only on the pebbles, but also on the soil surface as a consequence of massive area marking with feces by the ants. Hydrocarbons may be deposited by footprints from glands in the legs of ants (Billen, 2009; Lenoir et al., 2009) or by the Dufour's gland located near the tip of the abdomen (Greene and Gordon, 2007). Another possible source of colony specific HCs may be the post-pharyngeal gland which, at least in some ant species, is clearly involved in the formation of the colony specific label (see for example Soroker and Hefetz, 2000). Further research is needed to determine the origin of midden HCs, and whether specific task groups deposit and maintain HCs in the midden.

We do not rule out the role of olfactory cues, apart from HCs, in foraging behavior. When experimentally offered unusually abundant seeds, *P. barbatus* scouts lay phero-

mone trails from the feeding site to the nest with poison gland secretions (Gordon, 1991; Hölldobler et al., 2001). However, although we did not interfere with any marking behavior on the foraging trail or anywhere outside the nest mound, disturbing the midden clearly affected the behavior of returning foragers. This means that short-term cues alone, if they are used at all, cannot be the only means of guiding returning foragers to the nest entrance.

Harvester ants use midden HCs, a chemical cue applied collectively, to aid in the return of foragers. Several ant species use pheromonal or environmental cues when returning to the nest (Cammaerts and Rachidi, 2009; Steck et al., 2009, 2010). Hydrocarbons, on the other hand, are more stable, are colony-specific, and are regularly transferred from ants to their surroundings. Nest mound HCs have been associated with territorial behavior in ants (Grasso et al., 2005; Lenoir et al., 2009). The use of nest mound cuticular HCs contributes to the regulation of foraging activity because it helps foraging harvester ants to return to the nest more quickly, and thus adds to the precision with which forager return rate measures the current availability of food.

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