Journal of Chemical Ecology, Vol. 26, No. 10, 2000

HARVESTER ANTS UTILIZE CUTICULAR HYDROCARBONS IN NESTMATE RECOGNITION

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(Received December 30, 1999; accepted May 18, 2000)

Abstract—Cuticular hydrocarbons appear to play a role in ant nestmate recognition, but few studies have tested this hypothesis experimentally with purified hydrocarbon extracts. We exposed captive colonies of the harvester ant *Pogonomyrmex barbatus* to small glass blocks coated with whole cuticular lipid extracts and the purified hydrocarbon portion of extracts from nestmate and nonnestmate workers. As an estimate of agonistic behavior, we measured the proportion of ants in contact with blocks that flared their mandibles. Blocks coated with cuticular extracts from nonnestmates were contacted by more workers in one of two experiments and elicited higher levels of aggression in both experiments than blocks bearing extracts from nestmates. The cuticular hydrocarbon fraction of extracts alone was sufficient to elicit agonistic behavior toward nonnestmates. The results demonstrate that harvester ants can perceive differences in cuticular hydrocarbon composition, and can use those differences in nestmate recognition.

Key Words—Cuticular hydrocarbons, Formicidae, Nestmate recognition, *Pogonomyrmex barbatus*.

INTRODUCTION

Workers of many social insect species can distinguish nestmate from nonnestmate individuals. For some taxa, including ants, discrimination appears to be based on differences in cuticular lipid composition (e.g., Jutsum et al., 1979;

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0098-0331/00/1000-2245$18.00/0

Dahbi et al., 1996; Howard, 1993; Singer, 1998). Ant colonies of a given ant species often differ in the proportion, but not the identity, of lipid compounds present on the cuticle (Obin, 1986; Bonavita-Cougourdan et al., 1987; Vander Meer et al., 1989; Nowbahari et al., 1990; Gamboa et al., 1996; Wagner et al., 1998). This variation among colonies in cuticular composition might be used by ants to distinguish nestmates from nonnestmates. Experimental studies have demonstrated that ant workers of several species react aggressively when presented with whole lipid extracts from the cuticle of nonnestmate workers (Bonavita-Cougourdan et al., 1987; Nowbahari and Lenoir, 1984; Nowbahari et al., 1990). In these studies, hydrocarbons were the most abundant compounds present and were therefore implicated in nestmate recognition. However, insect cuticular lipids typically contain more than hydrocarbons: fatty acids, alcohols, esters, glycerides, sterols, aldehydes, and ketones may be present in low abundance (Lockey, 1988). Unless hydrocarbons were purified prior to the bioassay, it is not possible to dismiss the possibility that small amounts of nonhydrocarbon lipids were responsible for nestmate recognition (Vander Meer and Morel, 1998).

There is a small but growing body of direct experimental evidence that cuticular hydrocarbons play a central role in ant nestmate recognition. Recent studies on the ant species *Iridomyrmex purpureus* (Thomas et al., 1999) and *Cataglyphis niger* (Lahav et al., 1999) demonstrated that the purified hydrocarbon component of cuticular extracts elicited nestmate recognition. Ants are a diverse group, and it is not yet possible to generalize about the mechanism of nestmate recognition from one species to another. However, cuticular hydrocarbons are clearly a promising focus for studies of the mechanism of nestmate recognition in other ant taxa.

Harvester ants (*Pogonomyrmex barbatus*) sometimes behave aggressively toward workers from different colonies. Under natural conditions, workers from different colonies encounter one another when searching common areas for food, and such encounters sometimes lead to fights (Gordon and Kulig, 1996). Aggression toward nonnestmates occurs when colonies are kept in the laboratory as well (Brown and Gordon, 1997).

Harvester ant colonies differ significantly in their cuticular hydrocarbon composition (Wagner et al., 1998). Do harvester ants use differences in cuticular lipids in general, and the hydrocarbon component in particular, in nestmate recognition? We first examined worker behavioral response to the total cuticular extracts of nestmates and nonnestmates placed on small glass blocks. We predicted that ants would investigate blocks coated with harvester ant lipids more than control blocks lacking cuticular lipids. Furthermore, we predicted that ants would direct agonistic behavior more frequently toward blocks bearing nonnestmate odors.

Harvester ant cuticular lipids contain detectable quantities of nonhydrocar-

bons, such as fatty acids (Wagner et al., 1998), and probably contain trace quantities of other compounds not detected by gas chromatography as well. To distinguish responses to hydrocarbons from responses to other compounds, we tested whether the purified hydrocarbon component of cuticular lipid extracts was sufficient for ants to discriminate between extracts from nestmates and nonnestmates.

METHODS AND MATERIALS

Bioassays. Behavioral assays were conducted at Stanford University with captive colonies of harvester ants. Three ant colonies were used in experiments: Trillian, Kelly, and Sawyer, hereafter abbreviated T, K, and S. All colonies came from a site near Portal, Arizona, USA. Colonies T and K were collected in October 1996, and colony S was collected in August 1998. In the laboratory, each colony occupied a series of interconnected nest boxes filled with plaster. The nest boxes were connected to a 120- × 60-cm foraging arena. At the time of the experiments, each colony contained a queen, brood, and 750–1000 workers.

Agonistic behavior consisted of biting the glass block or standing in contact with the block with mandibles opened and raised. In both cases, the ant flared its mandibles, so to measure the intensity of agonistic behavior we counted the number of ants with mandibles flared that came in contact with blocks. Counts of ants exhibiting aggressive behavior for a given time period were later converted to proportions by dividing the number of ants with mandibles flared by the total number of ants in contact with the block.

Experiment 1. The first experiment, conducted in June and July 1998, tested behavioral responses of workers in colony T to cuticular extracts of workers from T (nestmates) and K (nonnestmates) and to solvent-only controls. Different task groups of harvester ants within a colony differ in hydrocarbon composition (Wagner et al., 1998). To minimize the variation in cuticular extracts presented to ants, we extracted cuticular lipids from midden workers only. Midden workers work primarily outside the nest, moving debris from one place to another (Gordon, 1986). We collected only ants with bits of debris in their mandibles. The removal of large numbers of ants from a single task group at one time disrupts colony operation (Gordon, 1986) and might have affected the ants' reaction to the blocks. We therefore distributed ant collection and bioassays over six trials that took place over nine days. All trials were conducted between 11:00 and 14:00 hr.

Immediately before each trial, we collected six midden workers each from colonies T and K. We killed the ants in the freezer and placed pairs of ants from the same colony into extraction vials. Ants were submerged in 0.5 ml of pentane, and the vials agitated gently for the first minute of the extraction. After 10 min of soaking, the fluid was transferred to a clean vial. The fluid extract from

each vial was then transferred to the upper surface of a 5- × 5- × 10-mm glass block. Fluid was transferred in small volumes by Pasteur pipet and the pentane allowed to volatilize before more extract was added. Controls were prepared by transferring an equal volume of pentane alone to glass pieces.

During each trial, we exposed workers from colony T, the focal colony, to three glass blocks bearing cuticular lipids from nestmate workers, three blocks bearing lipids from nonnestmates, and three solvent-only controls. Nine permanent positions in the arena were designated in advance to maximize the distance between glass pieces, and for each trial, treatments were distributed among positions at random. Data were collected by two observers, each watching blocks in half the arena. Every minute for 10 min, beginning 1 min after the first contact between an ant and a glass block, we recorded the number of ants in contact with each glass block and the number of those ants with mandibles flared.

Experiment 2. The second experiment differed from the first in two ways: (1) we isolated the hydrocarbon component from a subsample of all cuticular lipid extracts and presented both the whole extract and the hydrocarbon component of extracts to ants; and (2) we used a different combination of ant colonies for the bioassay. Colony S served as the focal colony, and colony T as the source of nonnestmate workers. Experiment 2 was conducted from January to April 1999, approximately six months after experiment 1. The average cuticular lipid composition of a colony can change over time (Obin, 1986; Vander Meer et al., 1989; Provost et al., 1993; Liu et al., 1998), so the cuticular composition of colony T during the two experiments may have differed. Hereafter, we refer to colony T at the time of experiment 1 as T1 and at the time of experiment 2 as T2.

We conducted four trials, each separated by approximately 22 days. In each trial, workers from colony S were presented with pentane-only controls, whole lipid extracts from colonies S (nestmates) and T2 (nonnestmates), and the hydrocarbon component of those extracts. For each trial, we collected and freeze-killed eight midden workers each from colonies S and T2 (both midden workers and foragers were used in trial 1). In this experiment, all ants from a single colony were extracted together in 2 ml of pentane. As before, vials were agitated for the first minute of extraction and the fluid extract was transferred to an empty vial after a total of 10 min.

We isolated the hydrocarbon component of half of each volume of extract by passing it through a silica gel column (5 cm long, 70–230 mesh, average diameter 60 Å, Sigma, St. Louis, Missouri) (e.g. Jackson et al., 1974; Page et al., 1990; Lahav et al., 1999), followed by elution with approximately 3 ml of pentane. Both the total lipid extract and the hydrocarbon portion of the extract were reduced to approximately 0.2 ml under N_2 gas. Each vial of reduced fluid was then distributed equally among two glass blocks. As in experiment 1, each glass block received a quantity of cuticular lipids similar to that present on two ants.

Solvent-only controls were prepared and behavioral data scored as in Experiment 1.

Gas Chromatography. We measured the relative abundance of ant cuticular lipids using gas chromatography. During each experiment, we collected six midden workers from each colony involved and stored them at −80°C. Ants from colony T were collected twice, once during experiment 1 and again during experiment 2. Cuticular lipids were extracted from each ant separately by soaking the ant in 1 ml pentane for 10 min, with agitation for the first minute. The extract was then transferred to a clean vial and dried under a stream of N_2. Analytical methods were similar to those used in Wagner et al. (1998). Samples were analyzed using an HP 5890 gas chromatograph. The residue was dissolved in 50 μl of chloroform, and aliquots of 1 μl were introduced by splitless injection onto a SPB-1 fused silica capillary column (30 m ×0.25 mm ID, 0.25-μm film thickness; Supelco, Bellefonte, Pennsylvania); samples were purged after 1 min. Helium was the carrier gas, flowing at 1 ml/min. The injector was maintained at 300°C. The oven was set at 170°C during injection, raised quickly to 220°C at 25°C/min, then more slowly to 300°C at 3°C/min. Samples of a standardized mixture of hydrocarbons were interspersed among ant samples to confirm the consistency of elution times.

To identify unknown compounds and confirm the identity of peaks with retention times and relative positions familiar from previous work, we analyzed the mass spectra of those peaks from a subset of the collected ants. Lipids were extracted from four ants; one from each colony/experiment. Extraction methods were the same as those described above, except that ants were extracted in pairs. Samples were dried, and the residue dissolved in 50 μl chloroform. About 2–4 μl were injected onto a capillary column (DB-1 fused silica, 30 m × 0.025 mm ID, 0.25-μm film thickness; J & W Scientific, Folsom, California). Samples were analyzed on an HP 5890/5970 GC-MS. Instrument temperatures and run times were the same as above, except that the oven was raised to a final temperature at 310°C. We used the mass spectra to identify compounds, using Nelson et al. (1980) as a reference.

In order to ascertain whether hydrocarbon abundance was reduced by the elution through the silica gel column, we compared the abundance of hydrocarbon compounds in stock solutions that had and had not been run through silica gel columns. Stock solutions contained pentacosane, octacosane, and tritriacontane (3 μg/ml each). The relative abundance of each compound was estimated as the sum of the proportional peak area from the total ion chromatograph.

Data Analysis. The results were similar for all trials within an experiment; there was no effect of trial and no trial × treatment interaction in either experiments 1 or 2 (two-way ANOVA, trial and trial × treatment $P > 0.05$ for all analyses). We therefore combined the data for all trials within each experiment. Proportions were square-root transformed prior to analysis to meet assumptions

of normality. For experiment 1, we compared the effect of cuticular lipid extracts of nestmates, nonnestmates, and controls on the number of ants in contact with blocks and the proportion of ants with mandibles flared using one-way ANOVA. Means were compared after ANOVA using the Tukey-Kramer method (Sokal and Rohlf, 1994). For experiment 2, we first compared the behavioral response of workers to blocks bearing ant cuticular lipids (nestmate or nonnestmate) to controls with a *t* test. We then assessed the effect of hydrocarbon purification on ant contact with blocks and on mandible flaring using two-way ANOVAs, with hydrocarbon purification and colony of origin (nestmate or nonnestmate) as the main effects.

We estimated the relative abundance of each compound as the proportional peak area from the total ion chromatograph and included in the final data set only those compounds that comprised at least 1% of the total ion abundance of at least one ant in any group. We compared the cuticular lipid composition of the two colonies used in experiment 1, and the two colonies used in experiment 2, using linear discriminant analysis. Statistical methodologies were described in detail in Wagner et al. (1998). Data were transformed prior to analysis by taking the arcsine of the square root of the proportional abundance of each compound. Only the five most abundant compounds were used in the analysis, because the use of large numbers of independent variables relative to sample size can lead to falsely significant results (Panel on Discriminant Analysis and Clustering, 1989). Discriminant analyses were performed using the statistical package SPSS (Norusis/SPSS, Inc.).

RESULTS

Experiment 1. Blocks coated with nonnestmate cuticular lipids elicited more ant contact and more agonistic behavior than blocks coated with nestmate cuticular lipids and controls (Figure 1). More workers of colony T contacted blocks bearing ant cuticular lipids (nestmate or nonnestmate) than contacted controls, and more ants contacted blocks bearing nonnestmate lipids than those bearing nestmate lipids ($F_{2,48} = 20.0$, $P < 0.001$; see Figure 1A for comparison among means). Of the ants in contact with glass blocks, a greater proportion flared their mandibles at blocks bearing nonnestmate lipids than at controls or blocks bearing nestmate lipids ($F_{2,38} = 22.0$, $P < 0.001$; Figure 1B).

Experiment 2. As in experiment 1, more workers of colony S contacted blocks bearing ant cuticular lipids, nestmate or nonnestmate, than solvent-only controls. This was true of both whole lipid extracts and the hydrocarbon component of extracts ($t = 3.3$, d.f. = 22, $P = 0.004$ and $t = 2.2$, d.f. = 22, $P = 0.04$, respectively; average number of ants contacting control blocks = 0.16 ± 0.09, see Figure 2 for treatment block averages). No ant flared its mandibles at a control block.

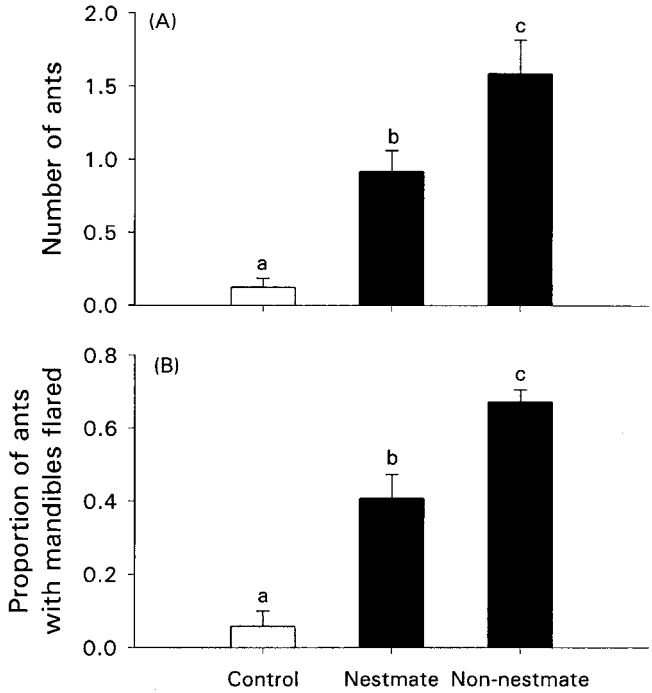

FIG. 1. Behavioral response of ant colony T to glass blocks bearing cuticular lipids extracted from nestmates, nonnestmates (colony K), or solvent-only controls. Error bars are standard errors. Means coded by different lowercase letters are significantly different from one another. (A) The number of ants in contact with blocks in the three treatments. (B) The proportion of ants in contact with blocks that flared their mandibles.

The numbers of workers contacting blocks bearing nestmate and nonnestmate extracts did not differ significantly in experiment 2 ($F_{1,28} = 3.2$, $P = 0.08$, Figure 2A). Hydrocarbon purification had no effect on the numbers of ants contacting blocks ($F_{1,28} = 1.2$, $P = 0.3$), and there was no interaction between hydrocarbon purification and colony origin (nestmate or nonnestmate) of the lipids ($F_{1,28} = 1.6$, $P = 0.2$).

As is experiment 1, ants in contact with nonnestmate blocks flared their mandibles more often than those in contact with nestmate blocks ($F_{1,28} = 4.2$, $P = 0.05$; Figure 2B). Hydrocarbon purification had no effect on the proportion of ants with mandibles flared ($F_{1,28} = 0.6$, $P = 0.4$), and there was no interaction between hydrocarbon purification and colony origin ($F_{1,28} = 0.7$, $P = 0.4$).

Isolation of hydrocarbons on the silica gel column caused a 3% (SD = 3%, $N = 9$) reduction in hydrocarbon abundance.

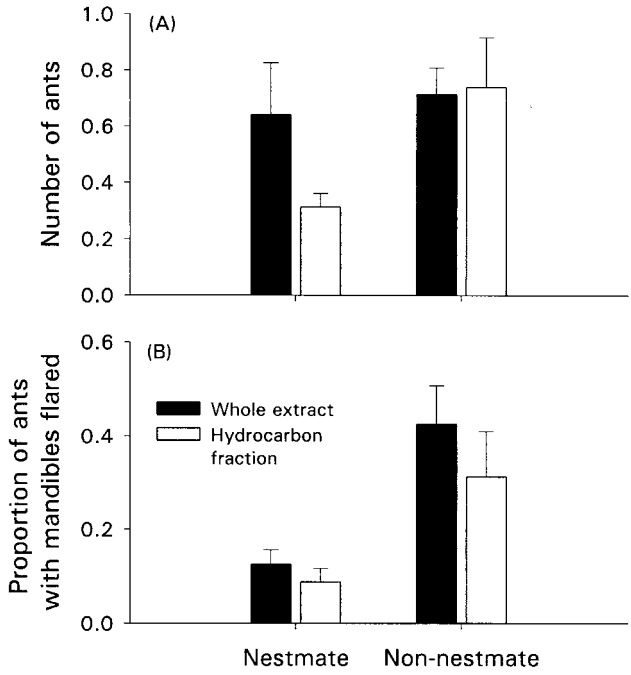

FIG. 2. Response of colony S to whole cuticular lipid extracts and the purified hydrocarbon portion of extracts from nestmates and nonnestmates (colony T). Error bars are standard errors. (A) The number of ants in contact with glass blocks. (B) The proportion of ants contacting blocks with flared mandibles.

Chemical Analyses. Twenty-eight compounds comprised at least 1% of any ant's cuticular lipids in this study (Figure 3, Table 1). In addition to hydrocarbons, we detected several esters (Table 1). The five most abundant compounds, used in the discriminant analysis, were *n*-pentacosane, *n*-tricosane, *n*-heptacosane, 13-methylpentacosane, and 13-methylheptacosane. There was no difference between colonies T1 and K in the relative abundance of these compounds ($\lambda = 0.34$, $\chi^2 = 7.9$, $df = 5$, $P = 0.16$). Colonies S and T2 did differ in relative abundance of the five compounds ($\lambda = 0.33$, $\chi^2 = 14.9$, $df = 5$, $P = 0.01$). There was no difference in the cuticular composition of workers from colony T sampled six months apart ($\lambda = 0.46$, $\chi^2 = 5.9$, $df = 5$, $P = 0.3$).

DISCUSSION

The results demonstrate that harvester ants can perceive differences in cuticular hydrocarbon composition and can use those differences in nestmate recog-

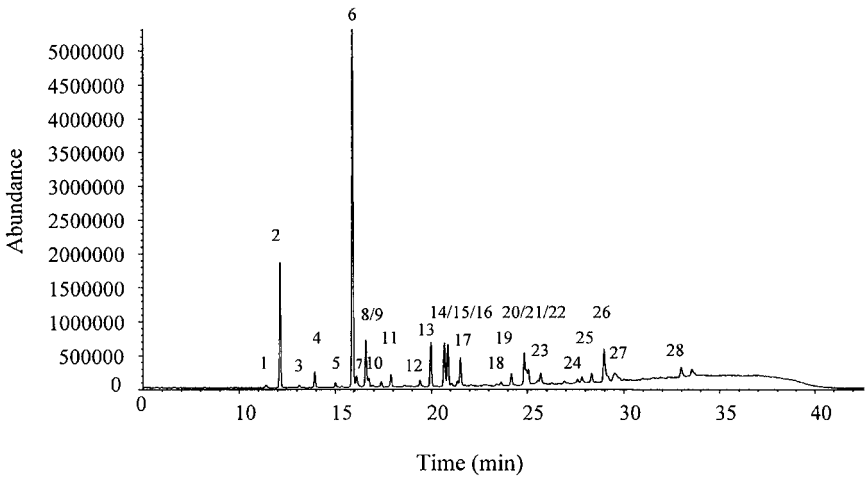

FIG. 3. Total ion chromatogram of the cuticular lipids extracted from *Pogonomyrmex barbatus*. The sample consisted of one ant from each of colonies used in the two experiments: K, T1, T2 and S. Compounds that made up at least 1% of the total ion abundance of the individual ant samples are numbered and identified in Table 1.

nition. Blocks coated with cuticular extracts from nonnestmates were contacted by more workers in one experiment, and elicited higher levels of aggression in both experiments, than blocks bearing extracts from nestmates. The cuticular hydrocarbon fraction of extracts alone was sufficient to elicit agonistic behavior toward nonnestmates. Our results are similar to those of recent studies on two other ant species, which also implicated hydrocarbons as a cue in nestmate recognition (Lahav et al., 1999, Thomas et al., 1999).

Although the two focal colonies used in this study were able to discriminate nestmate and nonnestmate workers using cuticular lipids, only one pair of colonies, colonies S and T2, differed in the relative abundance of the most abundant cuticular hydrocarbons. There are two possible explanations for this. First, ants may not rely on the few common compounds we used in our statistical analyses when discriminating nestmates from nonnestmates. Instead, ants may useless abundant compounds or combinations of compounds. Second, our statistical analysis was based on only six ants per colony and therefore may have lacked the power necessary to detect subtle differences among groups.

The cuticular hydrocarbon composition of the colonies used in this study may have converged while the colonies were in the laboratory, further complicating our ability to discriminate among colonies with gas chromatography. When used in this study, colony T had occupied the laboratory for 20–27 months, colony K for 20 months, and colony S for only six months. The similarity in

TABLE 1. AVERAGE PERCENT COMPOSITION OF CUTICULAR LIPID EXTRACTS FROM HARVESTER ANTS FROM 3 COLONIES[a]

Peak	Compound	Colony			
		K	T1	T2	S
1	Dodecanoicacid, nonylester	0.10	0.58	0.37	1.20
2	*n*-Tricosane	12.45	14.82	14.92	15.88
3	Dodecanoicacid, decylester	0.08	0.35	0.26	0.96
4	*n*-Tetracosane	1.82	2.02	2.20	2.00
5	Dodecanoicacid, undecylester	0.16	0.84	0.66	1.54
6	*n*-Pentacosane	40.78	36.16	38.72	34.82
7	13-Methylpentacosane	5.71	5.50	5.42	4.72
8	7-Methylpentacosane	1.03	0.95	0.98	0.59
9	dodecanoicacid, dodecylester	0.09	0.26	0.22	0.27
10	Unknown	0.82	0.68	0.56	0.37
11	*n*-Hexacosane	1.55	1.48	1.70	1.56
12	Heptacosene	1.27	1.15	1.03	0.58
13	*n*-Heptacosane	5.72	4.28	6.46	5.34
14	13-Methylheptacosane	4.85	4.62	4.68	4.61
15	7-Methylheptacosane	4.45	4.24	3.83	3.68
16	Unknown	0.53	0.67	0.24	0.41
17	7,13-Dimethylheptacosane	2.85	2.59	3.00	2.92
18	Nonacosene	0.64	0.51	0.25	0.00
19	*n*-Nonacosane	1.37	1.38	1.92	1.45
20	15-Methylnonacosane	2.87	3.13	2.93	3.07
21	9-Methylnonacosane	1.32	1.21	1.17	1.24
22	7-Methylnonacosane	1.46	1.49	1.59	2.13
23	7,13-Dimethylnonacosane	1.10	1.11	1.26	1.08
24	*n*-Triacontane	1.31	0.59	0.42	0.18
25	13,15-Dimethylnonacosane	0.80	1.02	1.10	0.24
26	Hentriacontene	3.07	3.75	2.95	2.16
27	7-Methylhentriacontane	0.65	0.74	0.00	0.00
28	*x,y*-Dimethyldotriacontane	1.02	1.34	0.37	0.31

[a] Colony T was sampled twice, about six months apart. Peak numbers correspond to those on Figure 3.

hydrocarbon composition of workers of colonies T and K may be due in part to the long period of time spent under similar laboratory conditions. Since being brought to the laboratory, all colonies ate the same foods and occupied nest boxes made from the same materials. Diet can affect insect cuticular lipid composition (Espelie and Bernays, 1989). Similarity of diet reduced aggression in colonies of two species of *Formica*, suggesting that diet may affect cues used in nestmate recognition (Le Moli and Mori, 1990). In the present study, however, there is no indication that the level of aggression between colonies T and K was lower than that between colonies S and T; about 60–70% of ants contacting nonnestmate blocks in both focal colonies flared their mandibles.

In *P. barbatus*, workers in different task groups within a colony differ in cuticular hydrocarbon composition. Foragers and patrollers, which work primarily outside the nest, have a higher proportion of *n*-alkanes and a smaller proportion of branched alkanes and alkenes on the cuticle than nest maintenance workers, which work primarily inside the nest (Wagner et al., 1998). Encounters between nestmates influence an ant's task (Gordon and Mehdiabadi, 1999). Our results suggest that during encounters, a harvester ant could obtain information about another ant's task from its cuticular hydrocarbon composition. Further research is needed to explore the relationship between cuticular hydrocarbon composition and task allocation.

Among social insects, hydrocarbons appear to play a role in nestmate recognition for some taxa and not for others. For example, the wasp *Polistes metricus* uses the hydrocarbon composition of the nest surface as a template for nestmate discrimination (Singer and Espelie, 1992, 1996). Ants, at least the few species studied so far, appear to use cuticular hydrocarbons to distinguish nestmates from nonnestmates. In contrast, there is little evidence that cuticular hydrocarbons play a role in nestmate recognition among termites (Singer, 1998) and honeybees (Smith and Breed, 1995). Work on the recognition systems of additional social insect species will help us to understand the roles of ecology and phylogeny in shaping mechanisms of nestmate recognition.

Acknowledgments—We are grateful to Chris Somerville at the Carnegie Institute of Washington and to Martin Reinhard and Jean-Francois Debroux at the Civil and Environmental Engineering Department at Stanford University for the use of their laboratories. We thank Gert-Jan de Boer, Pierre Broun, Jean-Francois Debroux, Michael Haverty, Christine Johnson, and Lori Nelson for their good advice and assistance with parts of this project. The work was supported by National Science Foundation grant IBN-9603639 to D.M.G.

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