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Trail discrimination signal of *Lasius japonicus* (Hymenoptera: Formicidae)

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Summary. Trail-following behavior of *Lasius japonicus* was colony-specific in the field, while trail pheromone activity was not. We found that the footprint substance caused colony-specific trail-following behavior only when working in conjunction with the trail pheromone. The footprint substance alone did not lead the workers to follow trails. The substance consisted mainly of hydrocarbons with composition almost identical to that of cuticular hydrocarbons, except for the absence of n-alkanes. Nestmate workers shared footprint hydrocarbon profiles as well as cuticular hydrocarbons, but the profiles differed among colonies. We therefore consider that the footprint hydrocarbon profiles serve as the trail discrimination signal in *L. japonicus.*

Key words. *Lasius japonicus* – trail pheromone – trail following behaviour – footprint hydrocarbons – cuticular hydrocarbons

Introduction

Trunk trail network systems are well developed in some ant species, including *Atta, Formica*, and *Lasius* (Hölldobler & Wilson 1990). The network system often delineates territory, and entails ritualized confrontation that prevents mutually damaging and lethal confrontations (Hölldobler 1976; Davidson 1977; Harrison & Gentry 1981). In such species the workers are aggressive against intruder ants, especially conspecific foreigners, on their own territory. However, they tend to avoid confrontations on foreign territories as much as possible, and therefore usually travel only their own trails. Such colony specificity in trail-following behavior is caused by the trail pheromone in *Lasius neoniger* (Traniello 1980), but the roots of this mechanism in other ant species are still unknown.

Lasius japonicus Santschi has long been represented as *Lasius niger*, a species widely distributed in Eurasia. Recent study by Seifert (1992) formally identified it as *L. japonicus*. This species has a well-developed trunk trail network system. The workers continuously travel on trails, mainly to collect honeydew excreted by aphids. This does not include travel on other trails established by conspecific foreign colonies in the field. This is presumably because the workers have an ability to distinguish their own trails from foreign ones, or because they may be always expelled by the foreign workers that are the owners of the trails.

Similar trail network system is also confirmed in *Lasius nipponensis* (Akino & Yamaoka 1999). This species has territoriality mainly based on their trails, and it is based on a pheromonal signpost (Akino *et al*. 2005). Its chemical basis is considered to be footprint hydrocarbons (FHC), presumably secreted from tarsi, because covering the tarsi with nail varnish results in drastic decrease of the FHC amount. Despite lack of n-alkanes, the hydrocarbon composition and profile were well similar between FHC and cuticular hydrocarbons (CHC), which causes the colony specific territorial recognition in *L. nipponensis*. While *L. nipponensis* mainly distributes in the forest, *L. japonicus* is a much common species in Japan distributing both in the grassland and forest (Japanese Ant Database Group 2003). These two species have similar habitat in the forest, so that *L. japonicus* could be a host species for *L. nipponensis* when the inseminated queens established a colony by temporal social parasitism (Akino 2002).

This study aims to investigate what signals cause the colony-specific trail-following behavior in *L. japonicus*. This species might use FHC for colony specific discrimination of trails as it was reported in *L. nipponensis* (Akino *et al.* 2005). The trail pheromone compound was not yet identified in *L. japonicus*, but it would be contained in the Dufour gland and eluted with 15 % ether-in-hexane when chromatographed on silica (Ueda, personal communication). We developed a Y-maze bioassay to separate the trail pheromone compounds, and checked whether the pheromone was specific to colonies. We also tested the effect of FHC of the workers on their trail-following behavior, and also on confrontations between workers.

Materials and methods

Ants

Three *L. japonicus* colonies were collected in Kyoto, Japan in 1992. Colony A consisted of at least four queens and approximately 10,000 workers. The two other colonies both consisted of one queen and approximately 3000 workers (colonies B and C, respectively). Additionally, four colonies nested in rotten tree trunks were collected in Tsukuba, Japan, two colonies each from

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2001 and 2004. All consisted of thousands of workers and brood, and were designated as colonies D, E, F and G, respectively.

Each colony was reared in a plastic container (200 × 160 × 40 mm) with a 10 mm plaster layer on the bottom. The container was always covered with a cardboard box to maintain darkness, and was connected to another container (350 × 260 × 40 mm) via a plastic tube (7 mm diam. × 500 mm length). Aqueous honey solution and dead insects were placed into the latter container every two days. All containers were kept at room temperature.

Extraction and separation

For preparation of the trail pheromone (TP) of *L. japonicus*, 100 workers from each of three colonies were killed by being placed into a refrigerator. Gasters of the workers were immersed in 1 ml of hexane for 10 min, and the extract was concentrated and chromatographed on approximately 1 g of silica (230-400 mesh, Merck), using a disposable Pasteur pipette. Compounds were successively eluted with 3 ml of hexane, and 5 %, 15 %, 30 %, and 50 % ether in hexane, ether, and methanol. The solvent was evaporated and the residues were dissolved with 1 ml of hexane. All the samples were kept at −20 °C in a deep freezer until usage.

Approximately fifty workers from each colony were directly released on a clean glass Petri dish (60 mm diam., 15 mm depth) for 4 h, and workers were also released on a clean filter paper placed in a glass dish. After removal of the workers, the conditioned dish was rinsed with 3 ml of hexane, and the rinse was concentrated and chromatographed on approximately 0.50 g of silica gel and successively eluted with 1 ml of hexane and ether. This material was stored at −20 °C until usage. The conditioned filter paper was cut into pieces (8 × 50 mm) for use in the bioassay.

For the comparison of FHC among individual workers, twelve workers from each colony were individually placed in a clean glass tube (7 mm i.d., 35 mm length, Maruemu Japan) for 10 min. After removal of the workers, the inner glass surface of each tube was rinsed with approximately 500 µl of hexane. Individual workers were then immersed in 100 µl of hexane to obtain CHC. Both the rinse and the extract were separately chromatographed on 0.5 g of silica gel and eluted with 1 ml of hexane. They were concentrated to ca. 100 µl (or 10 µl if necessary) by evaporating the solvent. One µl of each sample was subjected to gas chromatography (GC).

To confirm whether the FHC were actually secreted along trails in the field, glass rods (4 mm diam., 200 mm long) were placed along the trails of field colonies. A week later the rods were collected and rinsed with 5 ml of hexane. The rinse was chromatographed on 0.5 g of silica gel to separate hydrocarbons.

Y-maze bioassay

A Y-maze bioassay was used to check whether *L. japonicus* workers have an ability to distinguish their own trails. The letter "Y" was drawn with a pencil on a sheet of high quality paper (Kokuyo, Japan. 100 × 150 mm). The stem and arms of the "Y" were 50 mm long, and the angle between arms was 90°. An intact paper sheet was placed on the bottom of a glass Petri dish (60 mm diam., 15 mm depth), and then approximately 50 workers were released on the paper for 4 h. After removal of the workers, both the conditioned sheet and another untreated sheet were cut into pieces (50 × 10 mm). The two types of pieces were then pasted on opposite arms of the Y character drawn on another intact paper by pencil. Each experiment used the identical base procedure.

Test sample solutions were applied along the line from the arms to the stem with a 10 µl volume micro-syringe. At the stem the sample was applied at 1-2 mm intervals. After the solvent had evaporated, approximately 20 to 30 workers were successively led across a wooden bridge between the artificial nest and the end of the stem. At the end of each arm, the workers were trapped in a piece of cotton that contained aqueous honey solution to prevent escape back to the nest, and their numbers were counted. The treatment of the samples on the arms was reversed, and the experiments repeated at least five times. The results were not so different among the five replicates in each experiment, the data was pooled for further analyses.

Experiment 1: Quantities of TP were prepared from the gaster extract each of colonies A, B, and C workers, and named TP-A, -B, and -C, respectively. Two of them were applied on a Y-maze, one on the right arm and the other on the left arm, and both on the stem. Approximately 0.1 ant equivalent (AE) of TP and gaster extracts was applied.

Experiment 2: We conditioned several pieces of filter paper with colony A and B workers. A piece of the filter paper was then cut and pasted on one arm of the Y, and a piece of untreated filter paper was pasted on another arm. TP-C was then applied on the Y. Colony A and B workers were then separately tested.

Experiment 3: We conditioned glass Petri dishes with *L. japonicus* workers of colonies A and B, separately, and rinsed the dishes with hexane after removal of the workers (hereafter A and B rinses). The A rinse was applied on one arm of the Y, and the B rinse on the other. TP-C was then applied on the Y.

Experiment 4: We applied 0.1 AE of CHC on one arm of the Y, almost the same quantity of FHC on the other arm, and then applied TP-C to the Y.

Experiment 5: Based on the amount of n-nonacosane and n-hentriacontane in CHC-A and B, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 AE of n-alkanes were added to both FHC-A and -B. We applied the mixed FHC on one arm of the Y, the original FHC on the other arm, and then TP-C on the Y.

Experiment 6: Based on the amount of 15-methyl hentriacontane and 15-methyl tritriacontane in FHC-D and -E, 0.1, 0.5, 1, 5, and 10 AE of 15-methyl alkanes were mixed with both FHC-D and -E. Mixed FHC was applied on one arm of the Y, the original FHC-D on the other arm, and then TP was applied on the Y.

Experiment 7: FHC-A and -B were mixed at a ratio of 100:1, 20:1, 10:1, 2:1, and 1:1. One of the FHC mixtures was then applied on one arm of the Y, and one of the two original FHC mixtures on the other. TP-C was then applied on the Y.

Behavioral interaction (Experiment 8)

To confirm whether the FHC potentially serve as territory pheromone in this species, behavioral interaction between two workers was checked on the previously conditioned dish in the same manner with Akino *et al.* (Chemoecology, in press).

Two workers were gently placed in a previously conditioned glass dish, and their behavioral interaction was observed for 5 min. Behavior was categorized into four classes based on our preliminary observation: (1) Obliviousness: workers paid no attention to each other, (2) Alert: a worker opened its mandibles to menace the other and often attempted to bite, (3) Dodge: a worker turned away from the other, (4) Amity: workers touched each other with their antennae and often regurgitated nutrient fluid. Frequencies of their encounters and of each behavior were recorded. The observation was repeated 12 times with different pairs of workers.

This experiment was repeated by placing the workers on the dish treated with the chromatographic fraction that was obtained from the hexane rinse of the conditioned dish. The fraction was eluted with hexane from the silica gel column.

Gas chromatography (GC) and gas chromatogrphy-mass spectrometry (GC-MS) analyses

GC analyses were performed on a Shimadzu GC-14A equipped with an apolar capillary column (Shimadzu CBP1, 25 m long, 0.2 mm diam., and 0.25 µm film thickness) and a flame ionization detector. Helium was the carrier gas, at a column head pressure of 65 kPa. Injection was made at split/splitless mode for 0.75 min

Table 1 Colony specificity of the trail pheromone in *Lasius japonicus*

Test workers	Trail pheromone applied on the Y			
	TP-A	TP-B	TP-C	No choice
Colony A workers	49	53		6
		34	38	3
	54		58	9
Colony B workers	46	51		10
		34	39	8
	43		35	9
Colony C workers	50	43		6
		41	38	9
	32		35	7
	Gaster extract from colony F	Gaster extract from colony G		No choice
Colony F workers	38	43		12
Colony G workers	31	36		5

There were no significant differences in the numbers of the workers' choice

splitless duration. Temperature of the injection and detector ports was 300 °C. The temperature program of the column oven was 80 °C for 5 min, 80 °C to 300 °C at 10 °C/min, and then constant at the final temperature for 10 min. GC-MS analyses were performed on a Shimadzu QP-1000EX equipped with a Shimadzu GC-14A. EI-mass spectrum was obtained at 70 eV. GC condition was the same in the GC analyses.

Resemblance of the hydrocarbon profiles was evaluated by multivariate analyses. The amounts of single hydrocarbons in each sample were presented as peak areas by the FID detector. All the data were standardized by the method proposed by Aitchinson (1986): $Zij=\ln(Yij/g(Yj))$ where Zij is the standardized peak area I for individual ant j, Yij is the observed peak area I for individual ant j, and $g(Yj)$ is the geometric mean of all peak areas for ant j included in the analyses. An analysis of quantitative variation in peak volumes was then done with principal component analyses (PCA). All the multivariate analyses and other non-parametric statistics were performed with the "Black-Box package" for data analyses (Aoki 2003).

Resemblance coefficient

Resemblance of the hydrocarbon profiles was estimated by Nei's distance (Ferguson 1980). Profiles were presented as 23-tuple vectors, and each element corresponded to the amount of the hydrocarbon components. The Nei's distance c is defined as the cosine coefficient, by which the similarity of the two profiles can be expressed as the cosine of the angle between them. For larger values of c, similarity is considered to increase and *vice versa*.

Chemicals

Authentic hydrocarbons, *i.e.* n-nonacosane and n-hentriacontane, were purchased from Sigma-Aldrich Chemicals Co. USA. Methyl-branched alkanes, *i.e*. 15-methyl hentriacontane and 15-methyl tritriacontane, were provided by Dr. Sadao Wakamura of the National Institute of Agrobiological Sciences.

Results

Preparation of the trail pheromone

For the preparation of TP, one-tenth equivalent (AE) of each chromatographic fraction of gaster extract was applied to a drawn 100 mm line of a Y-maze. Hexane was applied as a control. Only the fraction eluted with 15 % ether-in-hexane was active, *i.e.* 90 % of the workers (107/119) followed the line. No workers followed the line applied with any other fractions. Thus, the fraction eluted with 15 % ether-in-hexane contains TP of this ant, though the chemical compounds are not yet identified.

Experiment 1: Colony specificity of the trail pheromone (Table 1)

Colony A workers almost equally chose TP-A, -B, and -C when two of them were presented (Table 1). There were no significant differences in the numbers of workers. This was also true when workers of colonies B and C were tested. Thus, the workers followed any TP almost equally, regardless of origin (Chi-square test, $P>0.05$). This suggests that TP is not specific to colonies in this species. When the gaster extracts were tested by colony F and colony G workers, extracts were chosen almost equally, regardless of origin (Chi-square test, $P>0.05$). This suggests that the gaster extract might not contain colony specific compound.

Experiment 2: Effect of footprint marking (Table 2)

When TP was applied on the conditioned and untreated paper, the workers chose the former more often than the latter (Chi-square test, $P<0.01$). Thus, the paper conditioned by the nestmate workers caused colony specific trail following behavior in workers of both colonies A and B. However, without applying TP, no workers followed the line even on the conditioned paper (38 colony A workers and 31 colony B workers were tested). This indicates that TP is necessary to evoke trail following behavior in workers.

Experiment 3: Effect of footprint chemicals (Fig. 1)

When colony A workers were tested, 93 and 8 workers chose the arms applied with the A and B rinses, respectively, and 5 did neither. In contrast, when colony B workers were tested in the same manner, 29 and 72 workers chose the arms treated with the A and B rinses, respectively, and 8 did neither. No workers followed the line applied with the rinses when TP was not treated (35 colony A workers and

Table 2 Effect of footprint marking on worker preference of the trail pheromone

Test workers	Footprint making source on the filter Paper	TP		No choice
		conditioned paper	untreated paper	
Colony A workers	Colony A workers	76*	21	3
Colony B workers	Colony B workers	81*	15	4

*Significantly more workers chose TP applied on the conditioned paper (Chi-square test, P<0.01)

Fig. 1 Experiment 3. Effect of the footprint hydrocarbons (FHC) on choice of trails by the workers. FHC-A and -B are the chromatographic fractions that contain footprint hydrocarbons of colonies A and B, respectively. TP is trail pheromone

47 colony B workers were tested). After separation by silica gel column chromatography, the fractions eluted with hexane produced a similar effect on the workers when TP was present (Fig. 1). The fractions obtained from the A and B rinses were hereafter named FHC-A and-B, respectively. Workers from colonies A and B apparently preferred TP treated with the rinse containing their own FHC (Chi-square test, P<0.01). No workers traveled on the lines if TP-C was not present.

Experiment 4: Comparison of the effect of CHC and FHC

When FHC and CHC from colony A were tested, 47 and 54 colony A workers traveled on TP with FHC and CHC, respectively, and 4 did neither. In contrast, 8 and 13 colony B workers chose the FHC and CHC, respectively, and 79 did neither. When both FHC and CHC were from colony B workers, 21 and 24 colony A workers chose FHC and CHC, respectively, and 46 did neither. When colony B workers were tested, 46 and 51 workers chose FHC and CHC, respectively, and 6 did neither. There were no significant differences in the numbers of the workers choosing FHC and CHC (Chi-square test, P>0.1), but more workers followed TP when CHC and FHC were of nestmates rather than foreigners (Chi-square test, P<0.01).

Experiment 5: Effect of n-alkanes (Fig. 2)

Almost equal numbers of colony A workers chose the original and mixed FHC-A, but they preferred the original FHC-A to a mixed FHC-A containing 10AE of n-alkanes (Chi-square test, P<0.01) (Fig. 2a). Colony B workers did not show any preference between the original and mixed FHC-B (Chi-square test, P>0.1) (Fig. 2b).

Experiment 6: Effect of methyl alkanes (Fig. 3)

As shown in Fig. 3, colony D workers preferred the original FHC-D only when the mixed FHC contained 10AE of 15-methylalkanes. In contrast, colony E workers preferred the original FHC-E when more than 5 AE of 15-methylalkanes were contained in the mixed FHC-E.

Experiment 7: Comparison of the effects of mixed FHC's (Fig. 4)

Between FHC-A and mixed FHC (A/B 100/1), 48 colony A workers chose the former and 52 chose the latter (Fig. 4a). More colony A workers chose the original FHC-A, as mixed FHC contained more FHC-B. There were significant differences in the numbers choosing the original FHC-A when it was mixed with FHC-B at 10:1, 2:1, and 1:1 (Chi-square test, P<0.01). This was also true in colony B workers (Fig. 4b). They significantly preferred the original FHC-A to the mixed FHC at FHC-B:A 10:1, 2:1, and 1:1 (Chi-square test, P<0.01).

Experiment 8: Behavioral interaction on the conditioned dish (Fig. 5)

When two nestmate workers were placed on the conditioned dish, they usually paid less attention to each other but occasionally touched each other with their antennae. On the other hand, when they were non-nestmates, the resident worker (a nestmate of the workers that conditioned the dish) often menaced the intruder (a foreigner to the workers that conditioned the dish).

The latter tended to avoid encounter with the resident worker. This was also true when the glass dish was treated

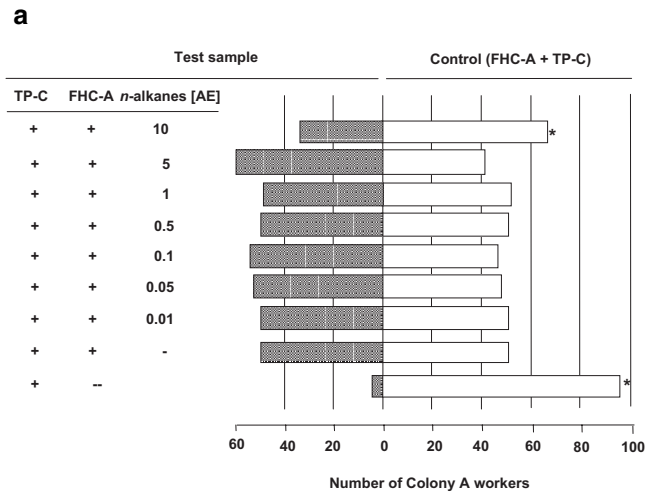

Fig. 2a Experiment 5. Effect of n-alkanes on workers' choice of lines treated with trail pheromone (TP) and footprint hydrocarbons (FHC). Asterisks indicate more frequent choice of control samples than the test samples (Chi-square test, P<0.01)

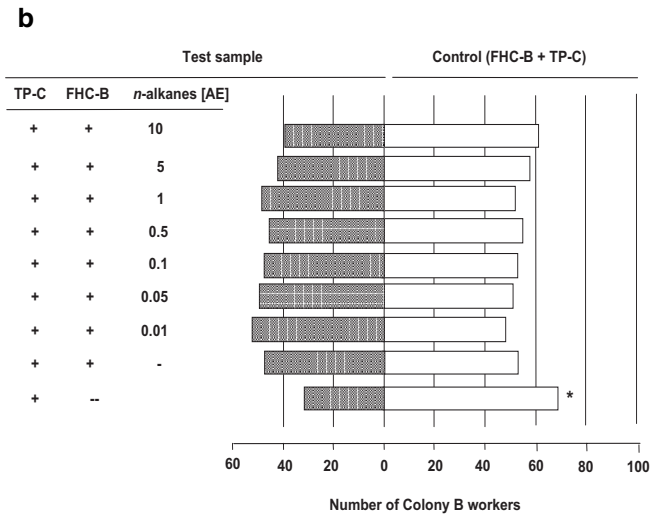

Fig. 2b Experiment 5. Effect of n-alkanes on workers' choice of lines treated with trail pheromone (TP) and footprint hydrocarbons (FHC). Asterisks indicate more frequent choice of control samples than the test samples (Chi-square test, P<0.01)

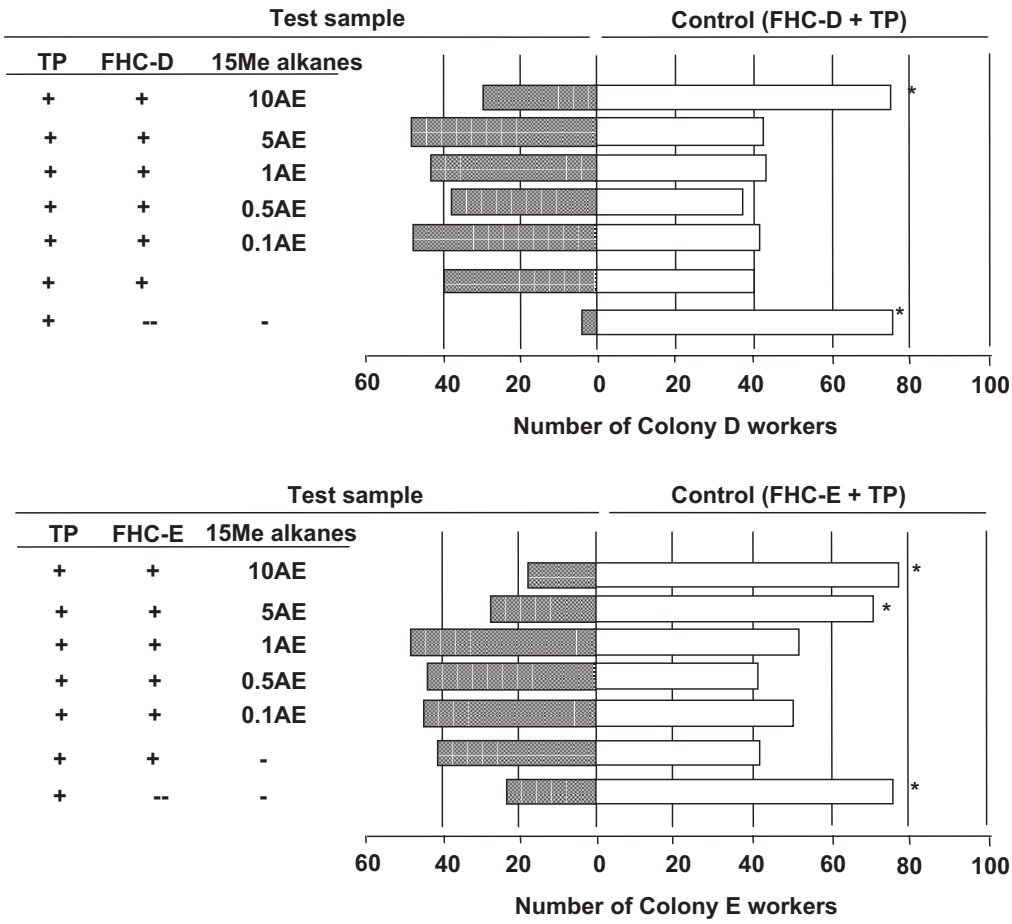

Fig. 3 Experiment 6. Effect of 15-methyl alkanes on workers' choice of lines treated with trail pheromone (TP) and footprint hydrocarbons (FHC). Asterisks indicate more frequent choice of control samples than the test samples (Chi-square test, P<0.01)

with the hydrocarbons that were separated from the hexane rinse of the conditioned dish. Workers from both colonies F and G behaved as residents when the applied hydrocarbons came from their nestmate workers. Thus, footprint hydrocarbons cause an asymmetric aggression between two workers.

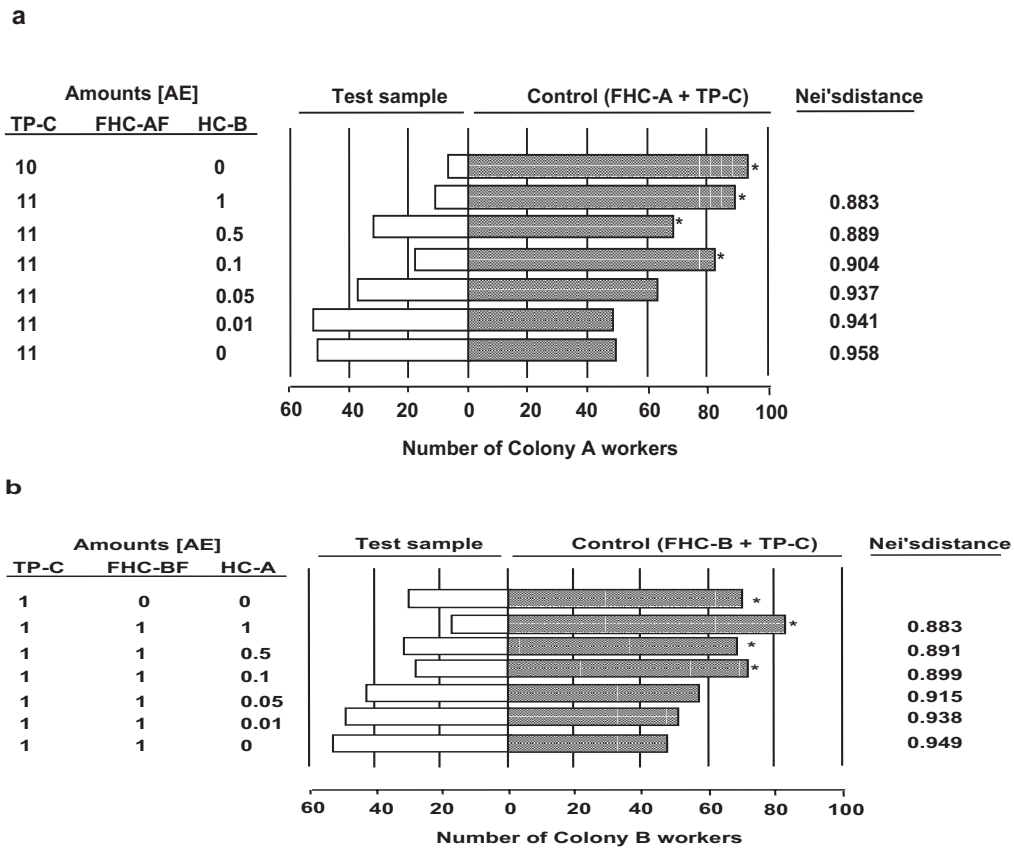

Fig. 4 Experiment 7. Effect of foreign footprint hydrocarbons on workers' choice of lines treated with trail pheromone (TP) and footprint hydrocarbons (FHC), and effect of FHC-B on colony A workers' response (a) and that of FHC-A on colony B workers' response (b). Resemblance between the mixed FHC and original FHC was evaluated as the Nei's distance. Asterisks indicate more frequent choice of control samples than the test samples (Chi-square test, $P<0.01$).

Identification of the FHC

GC-MS analyses revealed that the FHC mainly consisted of a series of hydrocarbons. On the basis of mass spectrum and retention indices (Fig. 6), they were identified to be alkenes and methyl-branched alkanes with 29-41 carbons. These hydrocarbons were also found in the cuticular hydrocarbons, which contained n-nonacosane and n-hentriacontane in addition to the alkenes and methyl-branched alkanes (Fig. 6b). The footprint hydrocarbons were also found in the hexane rinse of the glass rods taken from sites where *L. japonicus* workers formed trails in the field (Figs. 5c and 5d). Rinse from rods on trails that were not traveled by workers did not have this FHC.

PCA on both footprint and cuticular hydrocarbons reduced the 23 variables (i.e. hydrocarbon components) to 3 principal components that represented 82.3 % of the total variance. On the plots of the first and second, and the first and third principal components, colonies A and B were well separated on the basis of their cuticular hydrocarbon profiles, and also on their footprint hydrocarbon profiles (Fig. 7). However, the profiles were similar within each colony between the cuticular hydrocarbons and footprint hydrocarbons.

Discussion

TP components are chemically identified in the hindgut secretion of European *Lasius* ants, i.e., *L. fuliginosus* (Kern *et al.* 1997) and *L. niger* (Bestmann *et al.* 1992), but not yet in the respective twin species, *L. nipponensis* and *L. japonicus*. In the present study we showed that TP of *L. japonicus* was extractable by hexane and elution with 15 % ether-in-hexane through silica gel column chromatography, as reported in *L. nipponensis* (Akino & Yamaoka 1996). We also confirmed that neither TP nor the gaster extract were colony specific in this ant (Table 1), but that TP caused colony specific trail following behavior of workers when it was deposited on conditioned filter paper (Table 2). Experiment 3 proved this was caused by chemical substances extractable with hexane. GC analyses revealed the substances mainly consisted of hydrocarbons, of which composition and profiles were almost identical with CHC despite lacking of n-alkanes. It suggests that the substances are FHC of *L. japonicus* as reported in *L. nipponensis* (Akino *et al.* 2005). The FHC would be secreted by the workers after their movement, and synergistically work with TP and cause the colony-specific trail following behavior in this species.

The hindgut has long been known as the source of TP in formicine ants (Blum & Wilson 1964), and this is true in both *L. fuliginosus* and *L. niger* (Kern *et al.* 1997; Bestmann *et al.* 1992). In the respective twin species *L. nipponensis* and *L. japonicus,* however, neither TP nor its source were identified. Akino and Yamaoka (1996) propose the Dufour gland as the TP source of *L. nipponensis*, and also of *L. japonicus* (Ueda, personal communication). Further chemical and behavioral studies are necessary for identification of TP and its source in these two species.

Colony specificity in trail following behavior is a well known phenomenon in ants, and often corresponds to territorial behavior (Traniello, 1989; Hölldobler & Wilson 1977; Jaffe *et al.* 1979; Salzemann & Jaffe 1990). It can

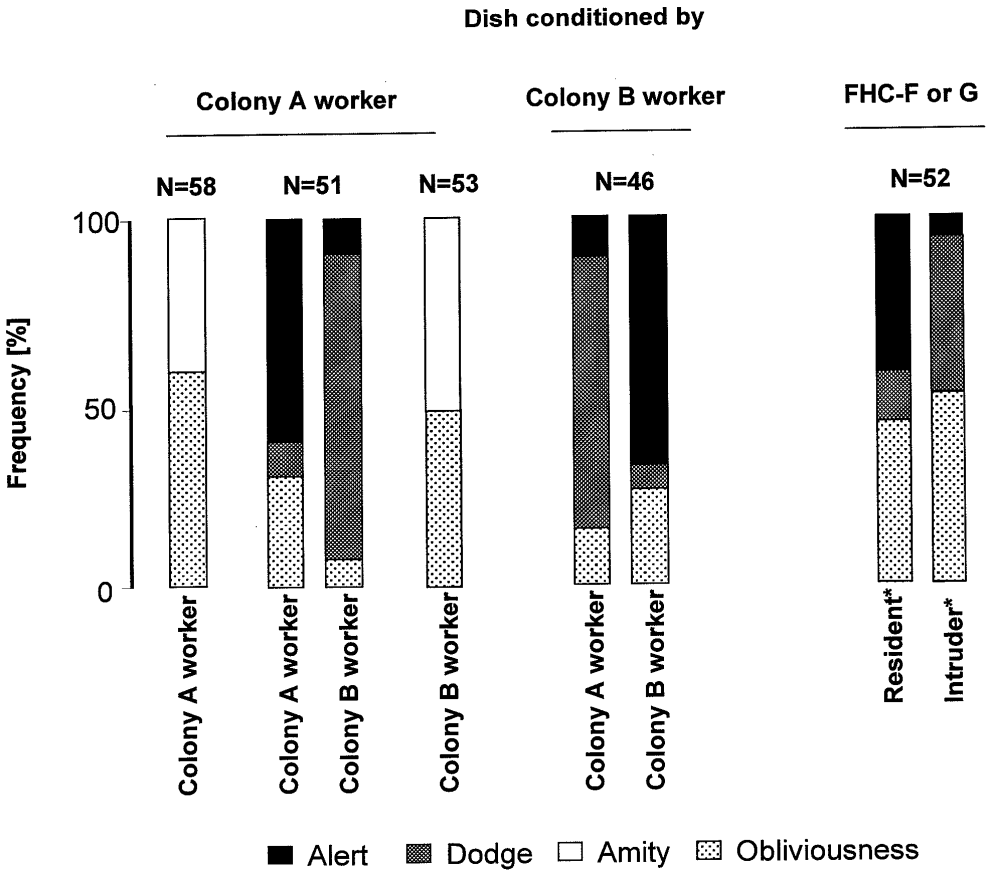

Fig. 5 Aggression between two workers on the glass dish previously conditioned by workers and hydrocarbon components separated from the rinse of the conditioned dish. N indicates the total number of encounters. Intruder and resident refers to colony workers when intruder or resident FHC was applied, respectively

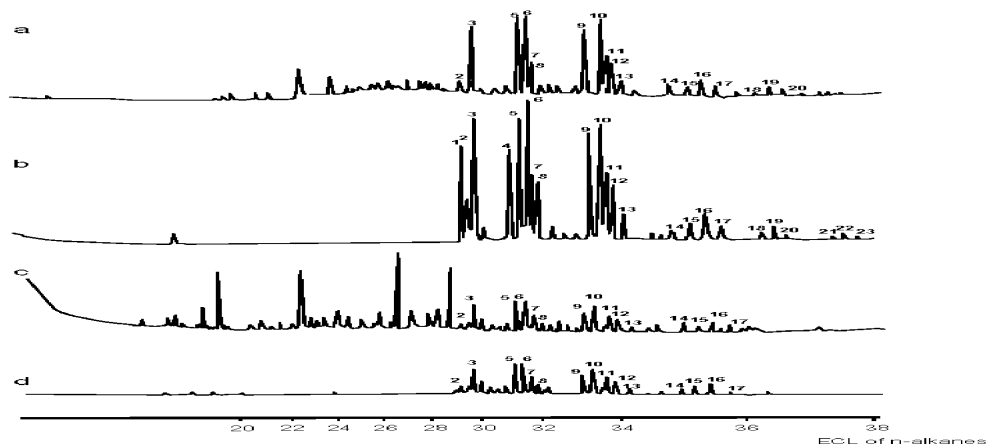

Fig. 6 Comparison of the profiles between the footprint substances and cuticular hydrocarbons: a) footprint substances obtained from a glass dish conditioned by *Lasius japonicus* workers, b) cuticular hydrocarbons, c) footprint substances obtained from glass rods that had been placed on *Lasius japonicus* trails in the field, d) chromatographic fractions of rinse (c) eluded with hexane. Based on the retention index and mass spectra, compounds in each peak were identified as 1) n-nonacosane, 2) 13 and 15-methylnonacosane, 3) 11,15-dimethylnonacosane, 4) n-hentriacontane, 5) 11-, 13-, and 15-methylhentriacontane, 6) 11,15-, and 11,19-dimethylhentriacontane, 7) 11,19-dimethylhentriacontane, 8) 5, 15 and 5,16-dimethylhentriacontane, 9) 13-, 15-, and 17-methyltritriacontane, 10) 13,21-dimethyltritriacontane, 11) dimethyltritriacontane*, 12) 5, 15- and 5,18-dimethyltritriacontane, 13) trimethyltritriacontane*, 14) 11-, 13-, 15-, and 17-methylpenta-triacontane, 15) 11,14-, 11,21-, 11,23-, and 13,21-dimethylpentatriacontane, 16) 5, 13-, 5, 15-, 5, 20- and 5,12-dimethylpentatriacontane,17) 5, x, y-trimethylpentatriacontane*, 18) C37 (methylhexatritriacontane)*, 19) C37 (methylhexatritriacontane)*, 20) C38 (dimethylhexatritriacontane)*, 21) 11-, 13-, 15-, 17-, and 19-methylheptatriacontane, 22) dimethylheptatriacontane*, and 23) 5, x-dimethylheptatriacontane*. Asterisks indicate that the position of the methyl branch is uncertain

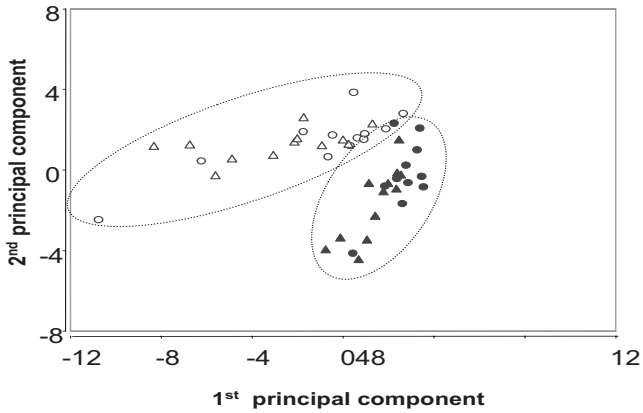

Fig. 7 Principal component maps of cuticular and footprint hydrocarbons from *L. japonicus* workers. White triangles and circles show the footprint and cuticular hydrocarbon profiles, respectively, of individual workers of colony A. Solid triangles and circles show the footprint and cuticular hydrocarbon profiles, respectively, of individual workers of colony B

be explained by the colony-specific trail pheromone as suggested in *L. neoniger* (Traniello 1980; Traniello & Levings 1986). However, it is also explained by two synergistic pheromones: the trail pheromone that actually leads the workers but does not present colony specificity, and the trail discrimination pheromone that causes the colony-specificity. In the case of *L. neoniger*, the colony specificity may be due to the latter pheromone contained in the rectal materials. *L. japonicus* falls into the second category, but the trail discrimination pheromone is not from rectal materials. Although the gaster extract evoked trail following behavior, it was not colony specific one (Experiment 1). Experiments 2, 3, and 4 indicated that the FHC served as the trail discrimination pheromone in this species, as well as in *L. nipponensis* (Akino *et al.* 2005). Although the gaster extract contained CHC in it, its amount was only about 6 % of the total CHC amount. This might be insufficient to cause the colony-specificity in the trail discrimination.

The trail discrimination pheromone has potential to serve as the territorial pheromone because of its colony specificity. It may allow the individual workers to practice the principle of bourgeois "fighting on their own territory" (Maynard Smith 1976). Such an asymmetric agonistic effect of the territorial pheromone results in decreasing fatal confrontation on the territory (Hölldobler & Wilson 1977; Salzemann & Jaffe 1990). We confirmed this asymmetric agonistic effect in *L. japonicus*, caused by FHC deposited on the dish (Fig. 5). Therefore we consider that FHC might serve as not only the trail discrimination pheromone but also the territorial pheromone in this species. In the twin species *L. niger*, however, symmetric agonistic interaction is confirmed between the resident and intruder experiment regardless of the type of area marked in the laboratory. In this case, area marking is thought to be for the home range rather than the territory (Devigne & Detrain 2002). Thus, the worker interactions on the marked laboratory terrain differ between these twin species. This difference may come from the amount of the deposited pheromone, because the amount of FHC closely relates to the asymmetry of agonistic interactions, at least in *L. nipponensis* (Akino, unpublished data). Asymmetric interaction was observed only near to trails, nest entrances and feeding sites in the field. The amount of FHC was higher near the trails than other places in the foraging area (Akino *et al.* 2005). To confirm types of territoriality of *L. japonicus*, we need to test the influence of the FHC amount to worker aggression both in field and laboratory. It is also interesting to compare behavioral responses of *L. japonicus* to FHC of *L. nipponensis* and *vice versa*, which will give us a clue to answer why these two species appear to segregate their habitat in the forest (Akino, unpublished data) and how the alate females of *L. nipponensis* approach to workers of its host species including *L. japonicus* (Akino 2002).

The footprint chemicals of *L. japonicus* mainly contained a series of long chained hydrocarbons that consisted of alkanes, methyl alkanes and trace amount of n-alkanes (Fig. 6). These hydrocarbons were also found in CHC. In spite of differences in the amount of n-alkanes, CHC and FHC profiles resembled each other (Fig. 7), and CHC also caused colony-specific trail-following when treated with TP (Experiment 4). This suggests that n-alkanes are not so important for discrimination of the colony specificity. Our experiments confirmed this hypothesis. Authentic n-alkanes influenced the trail discrimination by the workers only when more than 10 AE were added (Fig. 2). Similar results were obtained when 15-methylalkanes were added (Fig. 3). However, the workers distinguished the differences in the hydrocarbon profiles more sensitively when FHCs from two colonies were mixed and presented (Fig. 4). This suggests that it is an entire balance of profiles that is important as a key FHP recognition signal for *L. japonicus*.

It is often argued which hydrocarbon components actually serve as a key signal when the hydrocarbon complex is identified as the pheromone. In *Drosophila* and *Psacithea*, a few components in the hydrocarbon complex serve as the sex pheromone (Scott 1986; Scott *et al*. 1988; Fukaya *et al*. 1996). However, recent studies suggest that the complex itself serves as the key signal (Fukaya *et al*. 2000; Akino *et al.* 2004). The cuticular hydrocarbons have been considered as the nestmate recognition pheromone in ants because of the species specificity of the components and colony specificity of the profiles (Hölldobler & Michener 1980; Yamaoka 1990; Vander Meer & Morel 1998; Lenoir *et al.* 1999). But the profiles shared by workers are not completely identical among nestmates (Vander Meer *et al.* 1989; Nielesen *et al.* 1999; Liu *et al.* 2001; Akino & Yamaoka 2000). This suggests that the hydrocarbon complex, rather than particular hydrocarbons, would be a key recognition signal. This is supported by chemical and behavioral studies on *F. japonica* (Akino et al. 2004). Recently a characteristic sensillum was found in the antennae of *Camponotus japonicus* that is responsible for slight differences, not of particular components but the totality of hydrocarbon compositions (Ozaki *et al.* 2002). It is yet uncertain whether this receptive system is general in ants, but quite likely that it enables discrimination of not only CHC but also FHC complexes.

Several studies indicate that the colony specific marking chemicals are secreted from the Dufour gland

(Salzemann *et al.* 1992; Cammaerts *et al.* 1977). In *L. japonicus*, however, the colony specificity is due to FHC. We confirmed that the source of FHC was not in the gaster, but presumably in the tarsi. In *L. nipponensis*, covering the tarsi with nail varnish results in drastic decrease of the FHC amount, which suggests the presence of an exocrine gland (Akino *et al.* 2005). Some ants have several exocrine glands in their legs (*e.g.* pretarsal gland, basitargal gland) (Hölldobler *et al*. 1992; Billen & Morgan 1998), but further histological study is necessary to confirm the presence of exocrine glands in the legs of *L. japonicus*. The next problem will be to determine how the workers keep the profile of FHC identical to that of CHC.

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