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Colonial recognition of fungus in the fungus-growing ant *Acromyrmex subterraneus subterraneus* (Hymenoptera: Formicidae)

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Summary. Leaf cutting ants live in symbiosis with a basidiomycete fungus that is exploited as a source of nutrients for the ant larvae. Tests of fungus transport demonstrated that *Acromyrmex subterraneus subterraneus* workers discriminate concolonial fungus from alien fungus, and rejected the latter. Larvae and pupae of the ant were used as controls. Chemical analysis of the fungus revealed a great similarity between its hydrocarbon profile and that found on the ant brood. Experiments with lures showed that chemical extracts from the fungus are responsible for this discrimination process. Moreover, the presence of brood inside the fungus seemed to be important for discrimination of the fungus by workers. Resident workers accepted concolonial broodless fungus less than concolonial fungus inoculated with brood odor. Fungus seems to acquire colonial odor passively, simply by contact with the brood. The impact of fungus volume present in the nest on closure of the colony is discussed. We show here for the first time the importance of a symbiotic vegetal organism in colonial recognition in social insects.

Key words. Leaf-cutting ants – symbiosis-colony odor – fungus

Introduction

Leaf-cutting ants are well known for their obligatory mutualism with a basidiomycete fungus. The fungus culture is one of the most remarkable behaviors of the Attini tribe, where all the species are obligate fungus farmers and are considered to have co-evolved with the fungus (Wilson 1971; Weber 1972, 1982). Ants of the genera *Atta* and *Acromyrmex* cultivate fungus on fresh leaves, which are harvested, cut and brought into the underground nest. The fungus is exploited as the sole source of nutrients for the brood in all species, as worker-larva trophallaxis is absent (Weber 1972). In *Atta* and *Acromyrmex* only 5% of the energy needs of adult workers is provided by fungus eating, because workers drink the plant sap (Quinlan & Cherrett 1979). However, the fungus constitutes a significant part of the workers' diet in primitive attines such as *Cyphomyrmex rimosus* and *Myrmicocrypta ednaella* (Murakami & Higashi 1997).

The fungus cannot live independently of the ants, which protect it against contamination by alien fungi and bacteria through their antibiotic production (Bass & Cherrett 1994) and through the use of antibiotic-producing bacteria (Currie *et al.* 1999a,b). The ants have also a role in the recycling and secretion of certain enzymes that are essential for the fungus' development (Hölldobler & Wilson 1990; Bass & Cherrett 1996a). Moreover, the ants perform a mechanical action in the preparation of the substrate: they remove the waxes present on the surface of the leaves, thus facilitating inoculation of the substrate by the fungus and also removing or inhibiting micro-organisms (Quinlan & Cherrett 1977).

A recent phylogenetic study on fungus cultivars showed that fungus-growing ants successfully domesticate multiple cultivars. They are capable of switching to novel cultivars: a single ant species can farm a diversity of cultivars and these cultivars are occasionally shared among distantly related ant species, probably by an unknown mechanism of lateral transfer between colonies (Mueller *et al.* 1998). As is the rule in social insects, ants are able to discriminate between nestmates and alien individuals of the same species by the chemical cues that form the so-called colonial odor (Hölldobler & Wilson 1990). We believe that in order to be integrated into the colony, chemical modifications of the fungi must have appeared during evolution to match the olfactory and gustatory capabilities of ants, and we investigated this hypothesis.

It is generally accepted that cuticular hydrocarbons play an important role in nestmate recognition, at least in ants and wasps (Lorenzi *et al.* 1996; Singer 1998; Vander Meer & Morel 1998; Lenoir *et al.* 1999). In several ant species colony brood recognition has also

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been demonstrated. The concolonial brood is accepted and fed by workers. In contrast, allocolonial brood may be rejected or, if accepted, it may temporarily receive quantitatively less care than the concolonial brood (Lenoir 1981, 1984; Febvay *et al.* 1984; Isingrini *et al.* 1985; Bonavita-Cougourdan *et al.* 1989). In addition to morphological cues and typical behavior, the brood possesses chemical cues that allow its recognition by workers (Morel & Vander Meer 1988; Vander Meer & Morel 1988). In attine ants little is known about brood recognition. In *Atta cephalotes* there is some evidence that nestmate brood is distinguished (Robinson & Cherrett 1974). In *Atta sexdens rubropilosa*, workers rejected allocolonial larvae and pupae; even eggs were rejected when they came from the fungus garden, in contrast to those taken directly from previously isolated queens (Araujo *et al.* 1996). In *Acromyrmex octospinosus*, workers are able to recognize their nestmate brood. In tests with artificial baits, allospecific and conspecific larvae showed that several stimuli are successively utilized by workers: visual recognition (attractivity at close range), tactile examination (essential before an object can be carried) and chemical recognition (for transporting into the nest) (Febvay *et al.* 1984).

The aim of the present work was to test colonial discrimination of the fungus in *Acromyrmex subterraneus subterraneus* in order to shed light on the co-evolutionary pathways in attines and their fungi. We hypothesized that the symbiotic fungus bears the colony odor and is considered as a member of the colony. As the brood has the colony odor and is reared inside the fungus, we hypothesized that the fungus is chemically contaminated by contact with the brood. Accordingly, we conducted chemical studies of the colonial fungus odor and colony behavior.

Material and methods

Ants

We used 13 young colonies of *Acromyrmex subterraneus subterraneus* (Forel 1893) (Attini) and one colony of *Acromyrmex crassispinus* (Forel 1909) for allospecific comparisons. Colonies were collected in Viçosa (MG, Brazil) between 1993 and 1996. The colonies were maintained at 25°C ± 2°C, 75% relative humidity, and a photoperiod of 12:12. Fresh leaves (bramble, privet) and roses when available were supplied every two days. The nests were constructed of dark plastic domes on a platform linked by a tube to an external area. For larger colonies, a second external area placed 60 cm from the entrance of the nest was linked to the first one by a bridge. As *Acromyrmex* are essentially nocturnal, observations were performed during the dark, under red light (Viana 1996).

Behavioral tests

Experiment 1: brood and fungus nestmate recognition
Since nestmate brood discrimination had already been reported in *A. octospinosus* (Febvay *et al.* 1984), we investigated this phenomenon in *A. subterraneus*. In three colonies we observed the transport by workers of brood and of small pieces of fungus of different origins (concolonial, allocolonial and allospecific *A. crassispinus*). Larvae, pupae and pieces of fungus were collected from their colonies a few minutes before the tests. All the brood and workers present in the pieces of fungus were removed with needles. The pieces of fungus weighed 16 mg (the mean weight of the media workers) and were equivalent to the size of a larva in order to make a portable fragment.

Tests consisted of offering either 10 larvae, 10 pupae or 10 pieces of fungus placed on a glass slide in the external area. Concolonial, allocolonial or allospecific items were used. For a maximum of 30 minutes, we observed the behavior of workers, resulting either in the transport of the items into the nest (= acceptance), or in the transport to the middens (= rejection). Ten trials per colony and per element were carried out.

Experiment 2: Role of brood odor in fungus recognition
We hypothesized that fungus containing larvae has the colony odor and therefore is carried into the nest if concolonial. Consequently, we tested the role of ant brood in fungus recognition. The tests were conducted on three colonies. Workers were offered small pieces (16 mg) of their own fungus. According to the terminology of Bass (Bass & Cherrett 1996b), we distinguished between two kinds of fungus: mature and young. Mature fungus was taken from the center of the garden containing the brood, and young fungus from the upper part of the garden where fresh substrate has been added by the ants but does not yet contain brood. Brood present in the mature fungus was removed before the experiments as previously indicated. Twenty pieces of fungus of each kind were offered successively to the colonies in the foraging area, on a glass slide. Acceptances and rejections were observed for a maximum of 30 minutes.

Experiment 3: Role of the fungus presence in the colony
In order to study the effects of fungus deprivation on the fungus acceptance behavior of workers, groups of 200 media workers from three colonies were isolated from their colony (and their fungus) during different periods and placed in a nest similar to their original nest. Con- and allocolonial fungus discrimination by these isolated groups was tested after different separation times (1 hour, 24 hours, 48 hours, 72 hours and one week). Twenty pieces (100 mg) of con- and allocolonial broodless fungus were offered in the foraging area of these fungus-deprived groups. A control test was performed on the three mother colonies containing their own fungus. Acceptances and rejections were recorded for a maximum of 30 minutes.

Experiment 4: Transport of lures
To isolate the chemical cues used in brood and fungus discrimination, we extracted the chemicals and reapplied them onto lures.

Preparation of extracts
As workers were more selective with pupae than with larvae (even though their responses were similar in these two categories of brood-see results of experiment 1), we chose to test pupae extracts. 250 pupae (mainly of medium size) were removed from the garden with forceps, cleaned with a small brush and immersed in 5 ml of dichloromethane for two minutes. This short time ensured that only cuticular substances were extracted. Extracts were evaporated to dryness under nitrogen flow and preserved in a deep-freeze until the tests. A few minutes before the tests, we added 1 ml of pentane to the dried extract and a concentration of 2.5 pupae equivalent (10 µl) was applied to each lure.

Fungus extracts were obtained using the same methodology. Before collecting pieces of fungus, the colonies were deprived of plant food for 24 hours in order to avoid the presence of fresh pieces of leaves that could contaminate the extracts. Pieces of fungus were cleaned with needles: all the brood and workers were removed and most of the mycelium separated from the substrate to minimize the contamination. For each extraction we used about 500 mg of fungus.

Preliminary tests with concolonial fungus and pupae extracts were performed in order to determine the optimal concentration of extracts. Five extracts per colony and per treatment were prepared.

Preparation of lures
Lures were made from filter paper cut into small pieces and immersed for 24 hours in distilled water to make pulp which was then fashioned into an approximate brood-shape. After drying, lures were immersed in an acetone bath for 1 hour and finally individually rinsed with acetone to remove all impurities. Lures were marked with spots of

water paint, innocuous to the ants. Colors were alternated in order to avoid influencing the ants' choice. Weight of pulp lures was 15 mg. After five minutes of evaporation of the extract or of the solvent on a glass slide, the lures were placed in the external area of the nest and the test began immediately.

Test of transport
For each experiment we used two colonies and conducted 10 trials per colony. For each test 10 lures of each type of extract were offered for a period of 10 minutes. Two kinds of tests were performed: *extract vs. solvent* to measure the attraction potential of the extract and *extract 1 vs. extract 2* to measure the preferences between extracts. We recorded the transports into the nest (= acceptance), transports to the dumping-site or no transport (= rejection). Five replicates were performed per day, for each colony, with one-hour interval between each test.

Statistics
Results were analyzed using the χ^2 test, the Fisher exact test and the Wilcoxon test. Friedman analysis of variance showed that there was no difference between responses of the different tested colonies, which allowed us to pool results for the different colonies.

Chemical analysis

Qualitative GC-MS analysis
Extracts were made from fifteen larvae or fifteen pupae (medium size) or 500 mg fungus washed in 1ml of pentane during 10 minutes. Coupling gas chromatography (Varian 3300) and mass spectrometry (NERMAG R10-10C quadrupole) were used for chemical analyses of the samples. The injector was heated to 280°C and the compounds were separated on a 25 m × 0.32 mm BPX-5 (SGE) non polar capillary column, heated from 100°C to 200°C at 10°C/min and from 200°C to 300°C at 3°C/min.

Quantitative GC analysis
Extracts containing fifteen larvae or fifteen pupae or 500 mg fungus washed in 1 ml of pentane for 10 minutes were done for each analysis. We added an external standard (n-pentadecane, 10^{-5} M/l). The dried extracts were diluted in 30 µl of pentane, and 2 µl were injected to the gas chromatograph (Delsi 300) equipped with a capillary column Chrompack CPSIL 5 WCOT, 25 m × 0.22 mm. The temperature was programmed from 100°C to 280°C at 5°C/min (splitless of 15 seconds), with the injector and the detector at 250°C. The surface of each peak was determined by an integrator Enica 21 and was used to calculate the relative proportion of different compounds. Two colonies of *A. subterraneus* and one colony of *A. crassispinus* were used for quantitative analysis.

Statistics
A Manova was performed on the quantities of different chemical classes after Arcsin (sqrt) transformation for percentages. A discriminant analysis based on the relative proportion of hydrocarbon peaks was made to compare the profiles of the different extracts. Statistica package for Windows was used.

Results

Behavioral tests

Experiment 1: Transport of concolonial, allocolonial and allospecific fungus and brood
Workers presented different behavior towards the offered elements: 90 to 100% of the concolonial brood and fungus were transported directly into the nest and accepted. Allocolonial elements were treated differently: 40% of the larvae were accepted, but 93% of the pupae and 100% of the fungus were rejected directly to the dump-site or not transported (Fig. 1). The allospecific items were always rejected. There was no difference in the behavior of the workers towards pupae and fungus (fungus vs. larvae: $\chi^2 = 8.331$, $P = 0.004$; Fungus vs. pupae: $\chi^2 = 0.553$, $P = 0.457$; larvae vs. pupae: $\chi^2 = 3.814$, $P = 0.050$). Thus, in retrieving tests, the fungus was treated as brood, in particular as pupae.

Experiment 2: Role of brood odor in fungus recognition
The behavior of the workers differed between the concolonial fungus impregnated with brood and that not impregnated (Table 1). The immature fungus, which was not yet in contact with brood, was not accepted by the workers (more 98% of the pieces were rejected). In contrast, mature fungus was accepted, sometimes at 100% (86.7% mean of the pieces for the three tested colonies; $\chi^2 = 84.5$, $P < 0.001$).

Experiment 3: Impact of duration of fungus deprivation on discrimination capacities of the fungus by the workers
In the control experiments (without deprivation), in contrast to experiment 1, all the pieces of fungus (concolonial or allocolonial) were rejected except one (Table 2). After 24 hours of deprivation, the workers of colonies 2 and 3 accepted 100% of con- and allocolonial fungus samples, whereas 48 hours of deprivation were necessary for colony 1 to accept all the different pieces of fungus. It should be mentioned that colonies 2 and 3 were smaller than colony 1.

Experiment 4: Transport of lures

Lures with extracts of pupae (Fig. 2). In the control tests (Con/Solvent) the concolonial extract was attractive for the workers (75% acceptance) while lures soaked with solvent were much less transported (15%), (Wilcoxon test, $Z = 3.92$; $P < 0.001$).

Workers were also able to discriminate between concolonial brood and alien odors. Workers transported into the nest significantly more lures soaked with concolonial extracts than lures soaked with allocolonial extracts (Con/Allocol, $Z = 3.62$; $P < 0.001$) and allospecific extracts (Con/Allosp, $Z = 3.81$; $P < 0.001$).

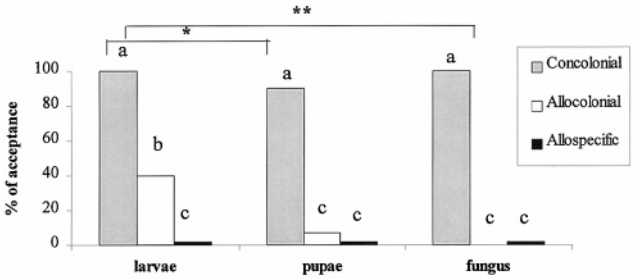

Fig. 1 Percentage of transport into the nest by the workers when we offered concolonial, allocolonial and allospecific larvae, pupae and pieces of fungus. The different letters (a vs. b vs. c) represent inside each group the significant differences (χ^2 test), ($N = 30$). Differences between groups: **: $P < 0.01$; *: $P < 0.05$ (χ^2 test)

Table 1 Number of pieces of fungus accepted by workers of each tested colony ($N = 30$)

Type of fungus	Colony 1	Colony 2	Colony 3	Mean % accepted
Immature fungus which did not contain brood	1	0	0	1.67
Mature fungus which has contain brood	12	20	20	86.7

We did not observe a significant difference between the transport into the nest of allocolonial extracts and allospecific extracts when they were offered in competition (Allocol/Allosp, $Z = 1.59$; $P = 0.11$). In this case, the levels of acceptance were $< 50\%$.

When the items were considered globally, the percentage of acceptance of lures soaked with concolonial extracts was greater (84%) than those with allocolonial pupae extracts (53%), with allospecific extract (31%) or with solvent (15%).

Lures with extracts of fungus (Fig. 3). Workers of the 2 colonies were able to recognize and discriminate odors of their own fungus. In the control test, we verified a significant difference between the acceptance of lures soaked with concolonial extracts and lures soaked with pure solvent (Con/Solvent, $Z = 3.72$; $P < 0.001$).

Workers were able to discriminate between concolonial fungus and alien fungus extracts. There was a significant difference between the acceptance of concolonial and allocolonial lures: lures with concolonial fungus extract were more frequently accepted than lures with the allocolonial extract (Con/Allocol, $Z = 2.95$; $P < 0.01$). Workers also discriminated concolonial lures when they were offered together with allospecific ones (Con/Allosp, $Z = 3.72$; $P < 0.001$). There was no difference in the treatment with alien extracts (allocolonial and allospecific extracts) between the response of workers, which rejected (58% and 76% of lures, Allocol/Allosp respectively, $Z = 1.79$; $P = 0.07$).

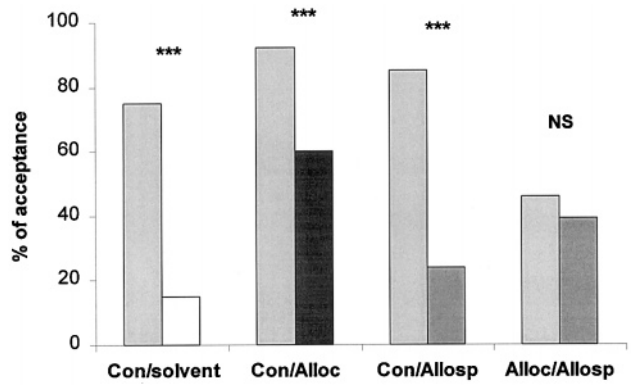

Fig. 2 Percentage of acceptance of lures soaked with concolonial pupae extracts (Con); solvent (pentane); allocolonial extracts (Alloc) and allospecific extracts (Allosp). Each group of 2 histograms represents the tested combination, for example: concolonial vs. solvent ***: $P < 0.001$; NS: non significant difference (Wilcoxon test) ($N = 20$)

Table 2 Number of pieces of fungus transported into the nest (= acceptance) for each tested colony as a function of the time of separation ($N = 20$ for each test)

Time of separation	Kind of fungus	Colony 1	Colony 2	Colony 3	Mean %
control	concolonial	1	0	0	1.67
	allocolonial	0	0	0	0
24 h	concolonial	0	20	20	66.7
	allocolonial	0	20	20	66.7
48 h	concolonial	20	20	20	100
	allocolonial	20	20	20	100
72 h	concolonial	20	20	20	100
	allocolonial	20	20	20	100
One week	concolonial	20	20	20	100
	allocolonial	20	20	20	100

Overall, the percentage of acceptance of lures soaked with concolonial fungus extracts was greater (68%) than those soaked with allocolonial fungus extract (38%), with allospecific fungus (28%) or with solvent (24%).

Chemical analysis

The qualitative analyses revealed no differences between the two species of *Acromyrmex*, although there were differences in relative proportions of compounds (Table 3, all differences between classes significant with Manova, $P < 0.001$). The chemical profile of larvae was simple, with 14 quantifiable peaks. The major constituents of *A. subterraneus* larvae were identified as saturated hydrocarbons from C21 to C35, and four n-alkanes (n-C28, C29, C31, C33) were dominant (total n-alkanes 86.9%). Monomethyl and dimethyl-alkanes were also found (total 8%). *A. crassispinus* larvae had exactly the same profile but in different proportions (n-C29, C31 and C33 dominant, total n-alkanes 76.9%).

The chemical profile of pupae was also very simple, with 15 quantifiable peaks. In *A. subterraneus*, the majority of compounds were n-alkanes, as for larvae (total 36.8%). Other lipids (fatty acids and one ester) were also found in large quantities in pupae (C16 hexadecanoic acid and ethyl ester, C18 octadecadienoic acid, total 43.7%) (Table 3). *A. crassispinus* pupae differed by a lower quantity of these lipids.

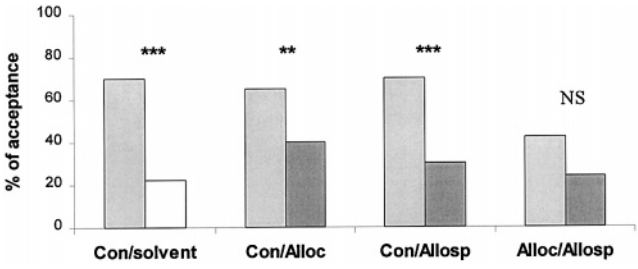

Fig. 3 Percentage of acceptance of lures soaked with homocolonial fungus extracts (Con); solvent (pentane); allocolonial extracts (Alloc) et allospecific extracts (Allosp). Each group of 2 histograms represents the tested combination, for example: concolonial vs. solvent ***: $P < 0.001$; NS: non significant difference (Wilcoxon test) ($N = 20$)

Table 3 Composition of chemical compounds extracted from fungus, larvae and pupae of 2 *Acromyrmex species*. $N = 10$ for *A. subterraneus* (2 colonies pooled) and 5 for *A. crassispinus*. Manova on chemical classes after Arsn(sqrt) transformation, all differences with $P < 0.001$. Total inferior to 100% when some substances were unknown

	Fungus *A. subt*		Fungus *A. crass*		Larvae *A. subt*		Larvae *A. crass*		Pupae *A. subt*		Pupae *A. crass*	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
hexadecanoic acid	17.54	12.96	2.19	1.68					23.24	12.11	2.55	1.24
hexadecanoic ethyl ester									5.32	2.76	2.26	0.83
octadecanoic acid	11.18	12.68	4.66	2.72								
octadecadienoic acid									15.12	11.40	3.26	1.16
Total non HC lipids	*28.72*	*18.80*	*6.85*	*3.42*	*0.00*		*0.00*		*43.68*	*1.65*	*8.10*	*0.90*
n-C21	0.62	0.26	0.39	0.34	t		t					
n-C23	1.06	0.46	0.94	0.46	t		t					
n-C24	0.33	0.10	0.36	0.14	t		t					
n-C25	2.24	1.00	3.05	0.21	t		t					
n-C26	0.17	0.09	0.41	0.09	t		t					
n-C27	4.98	2.01	10.81	1.36	3.17	1.03	7.04	1.42	17.63	9.89	27.96	2.77
n-C28	0.13	0.07	0.31	0.09	14.02	10.25	4.70	2.29				
n-C29	2.64	1.86	4.20	1.83	14.72	3.57	19.49	4.99	12.54	6.44	8.68	1.37
n-C30	1.15	0.64	0.50	0.03	4.37	0.77	2.59	0.41	0.80	0.39	3.31	0.88
n-C31	20.49	5.97	20.07	1.09	30.53	3.12	10.25	1.30	4.23	2.07	18.37	2.37
n-C32	0.19	0.14	0.26	0.11	3.79	0.87	4.25	0.82				
n-C33	7.76	6.62	8.86	3.98	14.26	2.42	20.50	2.03	1.57	0.87	8.88	1.43
n-C34					0.88	0.27	3.54	0.26				
n-C35	1.01	1.27	1.09	0.17	1.19	0.38	4.50	2.06				
Total n-alkanes	*42.76*	*14.34*	*51.24*	*1.31*	*86.90*	*2.73*	*76.85*	*1.38*	*36.80*	*15.77*	*67.25*	*2.31*
4Me C26	2.26	2.13	3.89	2.87					1.626	0.67	4.31	0.31
12+14Me C28	0.21	0.11	0.35	0.26								
4Me C28	0.19	0.10	0.97	0.07								
11+13+15Me C29	12.48	6.30	13.48	2.00								
11,15+13,17DiMe C29	1.08	0.85	0.26	0.03								
12+14Me C30	0.52	0.49	2.14	0.49								
4Me C30	2.10	0.70	2.58	0.72								
11+13+15Me C31	0.88	0.45	0.66	0.07					4.12	2.75	0.75	0.10
11,15+13,17DiMe C31	1.28	0.79	0.73	0.23	6.00	1.63	2.82	1.15	1.18	1.33	3.33	0.65
4Me C32	0.50	0.22	1.55	0.38	1.36	0.82	4.64	1.62				
4Me C34					0.63	0.19	6.75	2.64				
Total branched-alkanes	*21.50*	*7.06*	*26.61*	*1.97*	*8.00*	*1.60*	*14.21*	*0.95*	*6.93*	*0.95*	*8.41*	*0.82*
docosanal	2.97	1.59	5.49	7.44								
tetracosanal	0.47	0.18	0.62	0.24								
hexacosanal	0.68	0.27	0.91	0.53								
octacosanal	0.59	0.26	1.00	0.32								
triacontanal	1.41	0.56	0.84	0.26								
? aldehyde	0.90	0.29	6.43	5.13								
Total aldehydes	*7.02*	*2.51*	*15.29*	*4.69*	*0.00*		*0.00*		*0.00*		*0.00*	
Total	100.00		100.00		94.90		91.06		87.42		83.75	

The chemical profile of fungus was more complex, with 31 quantifiable peaks. The majority of products were identified as saturated hydrocarbons ranging from n-C21 to C35 as in the larvae extracts (total 42.8 and 51.2% respectively for the 2 species). Monomethyl-C29 and C31 were dominant. Among the other identified compounds we found two fatty acids (hexadecanoic and octadecanoic acids) in variable quantities and long chains saturated aldehydes (C22, C24, C26, C28 and C30, total 7% and 15% respectively for the 2 species) (Table 3).

Since hydrocarbons are generally considered as nestmate recognition cues, we performed a discriminant analysis on the hydrocarbon profiles after recalculation of the importance of each hydrocarbon component related to the total quantity of hydrocarbons. The discriminant analysis presented in Figure 4 showed significant differences among items, colonies and species (globally $P < 0.001$ and all the differences between groups with $P < 0.001$). The first discriminant variable (67.6% of the variance) separated the different items (fungus, larvae and pupae), that have qualitative different dominant hydrocarbons, confirming the data of Table 3. Variable 2 (19.1%) separated larvae from fungus and pupae. Variable 3 (10.5%, not represented graphically) separated the two colonies of *A. subterraneus*, indicating different hydrocarbon profiles among the colonies.

Discussion

The transport tests showed that the fungus is considered by the ants as an element of the colony and is accepted or rejected like the concolonial brood, particularly the pupae. This discrimination of the fungus indi-

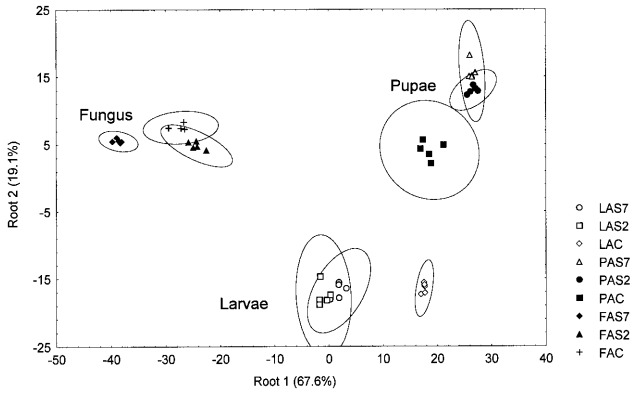

Fig. 4 Discriminant analysis of hydrocarbons for Pupae (P), Larvae (L) and Fungus (F) of *A. subterraneus* colonies 2 and 7 (AS2 and AS7) and *A. crassispinus* (AC). $P < 0.001$. All the distances between groups are significant with $P < 0.001$, including distances between colonies of *A. subterraneus* due to root 3. Ellipses are 95% confidence intervals

cates that it bears a chemical signature that is comparable to the colonial odor. This was confirmed by the fact that lures impregnated with pupae or fungus extracts were treated like live items (respectively 84% and 68%) and were accepted in significantly greater proportions as compared to alien or solvent extracts. Moreover, there was a hierarchy in the acceptance of items. Acceptance increased according to the chemical proximity between the tested item and the colony.

The cuticular chemical profile of *A. subterraneus* larvae was very simple and was dominated by n-alkanes, with fatty acids in the case of pupae. It is known that ant foragers use chemical cues to retrieve organic matter into the nest, such as fatty acids in seed elaiosomes (Lanza *et al.* 1992; Hughes *et al.* 1994), which may enhance retrieving of pupae as food items. A simple complex of alkanes is common to many ant larvae (*Camponotus vagus*, Bonavita-Cougourdan *et al.* 1989; *Myrmica rubra*, Bagnères & Morgan 1991; *Lasius niger* and *L. sakagamii*, Akino & Yamaoka 1998). The myrmecophile larvae of the butterfly *Maculinea rebeli*, which are retrieved into the *Myrmica* nests by foraging workers bear 90% of n-alkanes on their cuticle (Akino *et al.* 1999). We hypothesize that brood n-alkanes may be a stimulus signal to foragers and initiate retrieving behavior.

The fungus possesses all the ant hydrocarbons and particularly large quantities of n-alkanes, the possible signal for retrieving behavior by foragers. Important quantities of branched methylalkanes were also found (more than 20%). Several authors hypothesized that some methyl-branched hydrocarbons could be particularly involved in intercolonial recognition in ants (Bonavita-Cougourdan *et al.* 1987; Provost *et al.* 1992) and in social wasps (Dani *et al.* 1996; Gamboa *et al.* 1996). This may explain colonial discrimination of the fungus. We also found aldehydes in fungus extracts, which may be metabolites of the plants.

The transport tests of fungus either impregnated or non-impregnated with brood odor showed that the fungus, after contact with the brood, became attractive to the workers. In the younger parts of the fungus, where there is no contact between fungus and brood, the fungus was not attractive and was rejected as an alien element. In the mature parts of the fungus, when the fungus is in contact with the brood, it may acquire the colonial odor because chemical compounds responsible for the colonial odor present on the brood's cuticle should settle on the fungus. In guest- and parasite-host relationships there are two possibilities of acquiring these cues: chemical mimicry, where the organisms synthesize the cues; and camouflage where the cues are actively obtained from the partner (Howard 1993). Mimicry supposes that co-evolution has selected for fungal capacity to biosynthesize the hydrocarbons of the ant. Recent data on phylogenesis of fungus cultivars showed multiple domestication by the attine (Mueller *et al.* 1998), and in this case mimicry should have evolved numerous times, which is improbable. As camouflage requires active behavior by the guest, the simplest explanation is that the fungus may acquire its colonial odor merely by passive contact with the host. Simple contacts with brood are probably sufficient to obtain the ant odor, as has been observed in many guests of the ant colonies (see reviews in Stowe 1988; Dettner & Liepert 1994). It is also possible that workers transmit the colony odor when they chew the leaves before integrating them into the garden; they may regurgitate the content of their postpharyngeal gland, which is known to be a reservoir of colonial odor (Soroker *et al.* 1994; 1995). As in the attine ants the content of the postpharyngeal gland is not known, and as apparently they do not practice trophallaxis, this phenomenon remains to be demonstrated. Complementary experiments are necessary to verify the hypothesis of passive contamination of the fungus with artificial rearing of these fungi in the absence of the symbiotic ant.

There are a few records noting cross-species of cultivation, such as colonies of *Acromyrmex lobicornis or Trachymyrmex urichi*, which adopted the fungus of *Atta cephalotes* when they were deprived of their own fungus (Weber 1982; Stradling & Powell 1986). In the laboratory, we performed successful fungus adoptions between colonies of *Acromyrmex subterraneus* when the recipient colonies had lost their own fungus. The results of the test of transport of fungus by workers separated from their nest (and so their fungus) showed that deprivation of fungus affects the threshold of acceptance of the fungus by the workers. Workers that are deprived of their fungus may adopt an alien fungus. This is primordial for the colony, which cannot survive in the absence of fungus. The threshold of new cultivar fungus acceptance depends on several factors. The time needed to induce acceptance of a new fungus may be shorter for a young, smaller colony (experiment 3). In experiment 3, surprisingly the concolonial fungus was also rejected. This was probably due to the discrimination capacities, which vary according to the polyethism in the colony. The workers in area 2, which was distant from the nest, may be older foragers and have had a higher threshold, as has been shown previously (Viana

1996) (see also nestmate brood discrimination by nurses and foragers in *Ectatomma tuberculatum* (Fénéron & Jaisson 1992). The age or the size of the colony (and consequently the volume of fungus) seems to influence the duration of deprivation needed to modify the acceptance threshold of the offered fungus. We hypothesize that when the colonies grow, they become closed and selective. In *Atta sexdens rubropilosa*, the larger the fungus volume and the greater the number of workers, the more selective the recognition system becomes. This suggests the existence of a learning process associated with the acquisition and formation of the colonial odor (Bento 1993).

The selective advantage of the ability to discriminate between fungi offers a safety mechanism. As there are several species of fungi cultivated by the attines, it is possible that the discrimination by workers is necessary to prevent contamination by another species, which could induce a competition between the two fungi and thus decrease their fitness. Larvae are particularly vulnerable to coprophilous or saprophytic fungi (Wilson 1971). Recently, garden parasites belonging to the microfungus *Escovopsis* were discovered. These are highly virulent and have the potential for rapid devastation of ant gardens, leading to colony mortality. The parasite is more prevalent in evolved ant lineages like *Acromyrmex* and *Atta*, which cultivate asexual clones (Currie *et al*. 1999a). The reaction towards intruders must be very fast as they will be rapidly impregnated with the colonial odor and become "invisible" to the ant workers. The simplest way to prevent intrusion of fungus competitors is to reject immediately any item that does not bear the exact colonial odor.

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