Insect. Soc. (2009) 56:251–260
DOI 10.1007/s00040-009-0018-0

Insectes Sociaux

RESEARCH ARTICLE

Intraspecific nestmate recognition in two parabiotic ant species: acquired recognition cues and low inter-colony discrimination

F. Menzel · T. Schmitt · N. Blüthgen

Received: 9 January 2009 / Revised: 20 April 2009 / Accepted: 22 April 2009 / Published online: 9 May 2009

Abstract Parabiotic ants—ants that share their nest with another ant species—need to tolerate not only conspecific nestmates, but also nestmates of a foreign species. The parabiotic ants *Camponotus rufifemur* and *Crematogaster modiglianii* display high interspecific tolerance, which exceeds their respective partner colony and extends to alien colonies of the partner species. The tolerance appears to be related to unusual cuticular substances in both species. Both species possess hydrocarbons of unusually high chain lengths. In addition, *Cr. modiglianii* carries high quantities of hereto unknown compounds on its cuticle. These unusual features of the cuticular profiles may affect nestmate recognition *within* both respective species as well. In the present study, we therefore examined inter-colony discrimination within the two parabiotic species in relation to chemical differentiation. *Cr. modiglianii* was highly aggressive against workers from alien conspecific colonies in experimental confrontations. In spite of high inter-colony variation in the unknown compounds, however, *Cr. modiglianii* failed to differentiate between intracolonial and allocolonial unknown compounds. Instead, the cuticular hydrocarbons functioned as recognition cues despite low variation across colonies. Moreover, inter-colony aggression within *Cr. modiglianii* was significantly influenced by the presence of two methylbranched alkenes acquired from its *Ca. rufifemur* partner. *Ca. rufifemur* occurs in two varieties ('red' and 'black') with almost no overlap in their cuticular hydrocarbons. Workers of this species showed low aggression against conspecifics from foreign colonies of the same variety, but attacked workers from the respective other variety. The low inter-colony discrimination within a variety may be related to low chemical differentiation between the colonies. *Ca. rufifemur* majors elicited significantly more inter-colony aggression than medium-sized workers. This may be explained by the density of recognition cues: majors carried significantly higher quantities of cuticular hydrocarbons per body surface.

Keywords *Camponotus rufifemur* · *Crematogaster modiglianii* · Cuticular hydrocarbons · Interspecific associations · Nestmate recognition cues

Introduction

Nestmate recognition is one of the key features that maintain integrity of insect societies and prevent or reduce the invasion of parasites, enemies or competitors (Hölldobler and Wilson, 1990). Social insects discriminate between nestmates and alien conspecifics using olfactory signals provided by cuticular substances. These odor signals are in part genetically determined, but often heavily influenced by environmental factors such as diet or nest material (Heinze et al., 1996; Lenoir et al., 1999; Liang and Silverman, 2000; Richard et al., 2004; Sorvari et al., 2008). In ants, these signals are mostly hydrocarbons (Lahav et al.,

F. Menzel · N. Blüthgen (✉)
Department of Animal Ecology and Tropical Biology,
Biocenter, University of Würzburg, Am Hubland,
97074 Würzburg, Germany
e-mail: bluethgen@biozentrum.uni-wuerzburg.de

F. Menzel
e-mail: menzel@biozentrum.uni-wuerzburg.de

T. Schmitt
Department of Evolutionary Biology and Animal Ecology,
Institute of Biology I (Zoology), University of Freiburg,
Hauptstr.1, 79104 Freiburg, Germany
e-mail: thomas.schmitt@biologie.uni-freiburg.de

1999; Wagner et al., 2000). Aggression between conspecific colonies is often directly correlated to the differentiation of cuticular hydrocarbons (e.g. Suarez et al., 2002). Most ant species show high aggression against members of foreign conspecific colonies. However, among invasive ant species, intraspecific aggression is low or even absent, which results in a unicolonial population structure and is a major cause for their ecologically devastating impact (Holway et al., 2002). Their high intraspecific tolerance is probably caused by a low genetic inter-colony differentiation, which translates into lower differentiation of the chemical recognition cues (Suarez et al., 2008; Tsutsui et al., 2000; Tsutsui et al., 2003). A possibly similar mechanism has recently been described for non-invasive ants with unusually low intraspecific aggression (Foitzik et al., 2007).

Parabiotic ants, however, who share their nest with another ant species, often tolerate their partner species despite completely different cuticular hydrocarbons (Menzel et al., 2008b; Orivel et al., 1997). In Southeast Asian parabioses of *Crematogaster modiglianii* and *Camponotus rufifemur*, the tolerance between species often goes beyond the parabiotically associated colony and extends to other colonies of the partner species. The high tolerance coincides with highly unusual cuticular profiles (Menzel et al., 2008a). The cuticle of *Cr. modiglianii* possesses a set of hereto unknown compounds, which can reduce aggressiveness of its *Ca. rufifemur* partner (Menzel et al. submitted). Moreover, both *Cr. modiglianii* and *Ca. rufifemur* possess hydrocarbons that are considerably heavier than in non-parabiotic species of the same respective genera (Menzel et al., 2008a; unpubl. data). Hydrocarbons of high chain lengths are likely to hamper recognition between ant species due to their low volatility (Brandstaetter et al., 2008; Lambardi et al., 2007). The species *Ca. rufifemur* occurs in two sympatric but chemically distinct varieties ('red' and 'black' variety, Menzel et al., 2008a). They show almost no hydrocarbon overlap and may thus represent distinct, cryptic species. However, until genetical evidence exists we will more conservatively speak of two varieties.

These unusual features of the cuticular profiles are likely to affect nestmate recognition *within* both respective species as well. We therefore studied the role of cuticular hydrocarbons versus unknown compounds for intraspecific nestmate recognition in *Cr. modiglianii*. For both *Cr. modiglianii* and *Ca. rufifemur*, intraspecific inter-colony aggression was examined and related to their respective chemical variability. In the red *Ca. rufifemur* variety, we additionally investigated inter-individual hydrocarbon variation between colonies and worker castes in relation to inter-colony aggression.

Methods

Study site and ants

Experiments and sample collection were carried out at Danum Valley Conservation Area. The area is located at 5°N 117°50′E and approximately 100 m a.s.l. in Sabah (Malaysian Borneo) and represents one of the major remaining patches of Sabah's primary lowland rainforest. Aggression bioassays were carried out at parabiotic nests of *Cr. modiglianii* and *Ca. rufifemur*, which were located in hollow, living tree trunks. The experiments with cuticular extracts were performed with two parabiotic colonies (R0, R1) that were brought into the laboratory and kept in their original nests (small tree trunks) in open plastic boxes with fluon-coated walls.

Bioassays with cuticular extracts

These assays were performed to determine the role of the unknown compounds in nestmate recognition. Two *Cr. modiglianii* laboratory colonies (R0, R1; kept together with their parabiotic partner) were confronted with corpses, extracts or extract fractions (hereafter, 'treatments') of both nestmates and non-nestmates. In each treatment, we measured whether the ants distinguished nestmates from non-nestmates. The four treatments were (1) dead ants from the same and alien conspecific colonies, (2) their respective cuticular extracts, (3) the hydrocarbon (i.e. unpolar) fractions, and (4) the polar fractions (which contained the unknown compounds) of nestmate and non-nestmate extracts. Extracts and fractions were applied onto a dead *Cr. modiglianii* nestmate that had been extracted with hexane and chloroform for 10 min twice each (henceforth termed 'dummy'). For surface extracts, 50 ants were killed by freezing and immersed in hexane for 10 min. Unpolar and polar fractions of these extracts were obtained using conditioned SiOH columns (CHROMABOND, 100 mg, Macherey-Nagel, Düren, Germany) with distilled hexane and chloroform as respective eluents. The chloroform of the polar fraction was subsequently evaporated and the fraction was reconstituted in hexane. GC–MS analyses confirmed that the hexane fractions contained hydrocarbons while the chloroform fractions contained the unknown compounds. The amount of extract per dummy was adjusted such that each dummy carried the extract of five individuals.

In each bioassay, the dummy (or the dead ant) was held with forceps onto the nest trunk of the laboratory colony so that several ants (up to 9) could interact with it simultaneously. During 3 min, all observed interactions with the dummy were counted and classified as peaceful (antennate

or perform trophallaxis), weakly (open mandibles) or strongly aggressive (bite or lock mandibles). To provide more weight to long-lasting interactions, continued interactions were recorded again after every 10 s during the 3 min. This classification is consistent with an earlier study (Menzel et al., 2008b). Different treatments were tested in haphazard order on different places of the nest trunk. We carried out ten replicates for each treatment. Dummies with pure hexane were tested as controls. We conducted experimental series with two different alien colonies (R3, B4) as non-nestmates in the laboratory colony R1 and a series with a third alien colony (R5) for laboratory colony R0.

In situ aggression bioassays

The in situ aggression bioassays estimated aggression against living 'intruder ants' from alien conspecific colonies. They were conducted in arenas directly at the parabiotic nests in the rainforest of the study site. The arenas consisted of plastic rings (∅11.5 cm, height 5 cm) coated with fluon (*Cr. modiglianii* assays) or paraffin oil (*Ca. rufifemur* assays). They were placed on a plastic platform with paper tissue as floor. For tests with *Cr. modiglianii*, ten resident workers were carefully caught with forceps and placed into the arena. After 5 min to calm down, a living intruder ant from another colony of the same site was carefully introduced with forceps. For tests with *Ca. rufifemur*, we used the same method as described in Menzel et al., 2008b. An arena was provided with tuna bait and connected to the nest trunk with a twig such that the ants could walk into the arena. After 1–2 h, the twig was carefully removed without disturbing the foraging workers, and the intruder ant (major or medium-sized worker) was introduced. The *Ca. rufifemur* assays were conducted at night, under red light, since this species is nocturnal. The number or workers in the arena was recorded as a covariate. All interactions of the resident ants towards the intruder were then observed for 3 min as described above.

For *Cr. modiglianii*, we performed intraspecific *Cr. modiglianii* assays using ten parabiotic and three non-parabiotic *Cr. modiglianii* colonies. The aggression assays comprised a total of 44 colony combinations, 31 between parabiotic nests and 13 between a parabiotic and a non-parabiotic *Cr. modiglianii* colony. Five to seven replicates were conducted per colony combination. Within the red *Ca. rufifemur* variety, three allocolonial colony combinations were studied with 12 replicates per combination, i.e. 6 major and 6 medium-sized workers as intruders. In addition, we re-analyzed the data for 11 additional intra- and allocolonial colony combinations of *Ca. rufifemur* from Menzel et al., 2008b, and included only assays with majors as intruders. See Fig. 4a for the number of replicates and colony combinations per *Ca. rufifemur* variety. All studied *Ca. rufifemur* colonies of either chemical variety were separated by rivers and by at least 500 m distance. Due to their much broader heads, *Ca. rufifemur* majors are allometrically distinct from smaller worker castes. Medium-sized workers were defined as non-major workers above 6 mm body length.

Statistical analysis: bioassays

From each bioassay replicate we calculated the sum of all aggressive versus all non-aggressive interactions. For the bioassays with cuticular extracts, we used generalized linear models (GLMs) with quasibinomial error distribution and logit link function. Pairwise comparisons between nestmate and non-nestmate treatments were performed for each test series. For the *Cr. modiglianii* aggression bioassays, we tested whether inter-colony aggression in *Cr. modiglianii* depended on the variety membership of their *Ca. rufifemur* partner. Proportions of strong and total aggression were analyzed using GLMs as described above. In addition, the constant number of workers allowed analyzing the absolute numbers of interactions using a linear mixed-effect model.

The *Ca. rufifemur* aggression bioassays were analyzed using GLMs with quasibinomial error distribution and logit link function. The first model considered only assays with major workers as intruders. It included the parameters 'within/across variety' (intruder and resident from the same or different *Ca. rufifemur* varieties), 'intra-/allocolonial', as well as 'colony combination', and the number of workers present in the arena. A second model, which considered only allocolonial confrontations within the red *Ca. rufifemur* variety, analyzed the effects of 'caste' (major/medium-sized worker) and 'colony combination' on aggression.

The influence of each parameter was determined by likelihood ratio tests (*F* tests). In all bioassays, strong and total (including weak) aggression were analyzed separately. Since the statistical results of both analyses were very similar, we will report the latter and mention the former only if different.

Chemical analysis

Extracts for the analysis were prepared by immersing 10–90 ants killed by freezing in hexane for 10 min. Substance quantities in *Cr. modiglianii* were too low to allow individual extracts. All samples for analysis contained an internal standard of 2 µg octadecane. We studied the cuticular hydrocarbons of nine *Cr. modiglianii* colonies with one to nine sample replicates per colony. All surface hydrocarbons had been identified in an earlier study (Menzel et al., 2008a). Quantification was carried out with

a high-resolution ThermoQuest Trace GC-FID with H_2 as carrier gas in order to achieve a better separation of the substances. We used an unpolar capillary column [DB-1 (J&W Scientific, Folsom, CA, USA), 20 m × 0.18 mm ID, 0.18 µm film thickness]. Temperature was kept at 60°C for 2 min then increased by 60°C/min up to 200°C and subsequently by 4°C/min to 320°C, where it remained constant for 10 min. A split/splitless injector was installed at 260°C in the splitless mode for 30 s. The flame ionization detector (FID) was kept at 340°C. Peak areas were computed with Chrom-Card 1.19 (CE Instruments, Milan, Italy).

Colony and caste differentiation (major or medium worker) within the red *Ca. rufifemur* variety was studied using analogous extracts of single individuals. We analyzed four to six individual extracts for each of two worker castes (major and medium workers) and the three colonies tested in aggression bioassays (total $n = 29$). Quantification of these hydrocarbons was carried out using a Hewlett Packard 5890 GC-FID with an unpolar capillary column [DB-1 (J&W Scientific, Folsom, CA, USA), 30 m × 0.25 mm ID, 0.25 µm film thickness] and helium as carrier gas. A split/splitless injector was installed at 260°C in the splitless mode for 30 s. The flame ionization detector (FID) was kept at 340°C. The temperature programme was specified as above. The acquired data were used for discriminant analysis. Based on the internal standard, absolute hydrocarbon quantities per individual were compared among the two worker castes. The quantities were then related with two body size metrics (head width and hind tibia length, Hölldobler and Wilson, 1990) acquired from 28 workers from 2 of the 3 focal colonies and an additional colony.

Statistical analysis: surface hydrocarbons

In order to estimate inter-colony variation of cuticular profiles, we used data acquired by high-resolution GC-FID from multi-individual extracts. We calculated the mean relative abundances of cuticular substances for nine (*Cr. modiglianii*), nine (red *Ca. rufifemur*) or four (black *Ca. rufifemur*) colonies, respectively. For each substance with mean abundance >3%, the coefficient of variation between colonies was calculated as CV = SD/mean relative abundance.

The correlation of chemical differentiation and inter-colony aggression in *Cr. modiglianii* was estimated using the relative abundances of 28 major hydrocarbon peaks in the *Cr. modiglianii* profile (Menzel et al., 2008a). We calculated the Bray–Curtis indices of dissimilarity for the profiles of nine different *Cr. modiglianii* colonies. The obtained distance matrix was compared to a matrix of inter-colony aggression (obtained from the in situ aggression bioassays) between the same nine colonies. Since not all possible colony combinations had been tested, we used a Mantel test adjusted for missing values. As aggression measures we used relative proportions as well as absolute numbers of strong or total (including weak) aggression. All computations were performed in R Version 2.7.0 (R Development Core Team, 2007).

Statistical analysis: discriminant analysis

We analyzed the relative abundance of seven substance peaks, which were the only ones detectable in single-individual extracts. These were five methylbranched alkene peaks and two non-identifiable substances with retention indices 33.53 and 38.79 (see Table 1; Menzel et al., 2008a for details). All peak areas were standardized according to $A_p' = \ln[(A_p + 0.0001)/g(A_p)]$, where A_p is the peak area and $g(A_p)$ is the geometric mean of all peak areas of the respective sample (Aitchinson, 1986), in order to correct for the high interdependence of this type of data. The constant 0.0001 was added to provide non-detectable substances with a small non-zero value as recommended by Aitchinson (1986). The transformed data were entered into a step-wise forward

Table 1 List of all substances detectable in individual surface extracts of the red *Ca. rufifemur* variety

Reference number	Substance	Relative abundance (%)	Retention index
–	Unknown	0.27 ± 0.04	33.73
37	25-MeC37-14-ene, 25-MeC37-16-ene[a]	0.29 ± 0.04	36.96
43	x(25,26,27)-MeC38-y(13,14,15,16)-ene[a,b]	2.24 ± 0.68	37.93
49	Unknown	0.14 ± 0.1	38.79
52	27-MeC39-14-ene, 27-MeC39-16-ene	90.79 ± 0.76	39.02
56	27-MeC40-14-ene, 27-MeC40-15-ene, 27-MeC40-16-ene[a]	3.19 ± 0.13	39.97
61	x(27,29)-MeC41-y(14,16,18)-ene[a,b]	3.08 ± 0.28	40.94 (extrapolated)

The reference number refers to Table 1 in Menzel et al., 2008a. The first compound had not been regularly detected in earlier studies. Relative abundance is given as relative peak area (mean and standard error)

[a] Position of double bond tentative

[b] Number of substances and their exact structure could not be further determined

discriminant analysis. We report Wilks' λ values and the percentage of correctly assigned samples (classification matrix). The discriminant analysis was performed using Statistica 7.0.

Results

Crematogaster modiglianii

Crematogaster modiglianii workers of both experimental colonies (R0 and R1) significantly differentiated between intra- and allocolonial dead *Cr. modiglianii* workers (Fig. 1). They attacked the latter but mainly antennated the former. The same, significant differentiation was found for dummies that carried either total extracts or hydrocarbon fractions, except for the hydrocarbon fraction of one foreign colony (Fig. 1). However, the workers never discriminated between intracolonial and allocolonial polar fractions, which contained the unknown compounds (Fig. 1), although their composition varied strongly among the used colonies (Bray–Curtis dissimilarities between unknown compounds of colonies R1 and R3: 0.92; R1 and B4: 0.61).

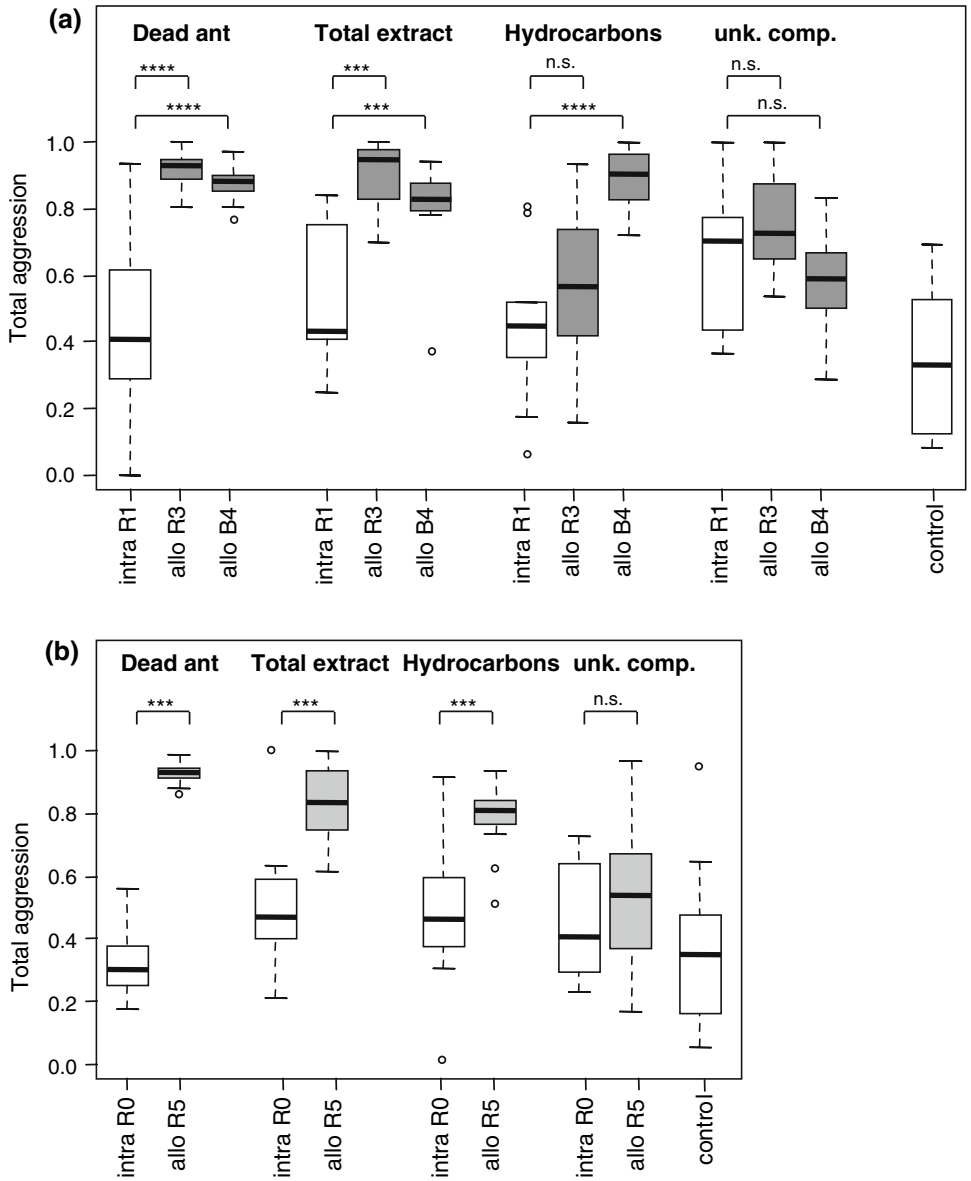

Fig. 1 Total aggression of *Crematogaster modiglianii* (**a**) colony R1 and (**b**) colony R4 against nestmate and non-nestmate extracts and fractions thereof. The *plots* show median, quartiles and range of ten assay replicates each. *Intra* Intracolonial treatments, *allo* allocolonial treatments. '*R*' and '*B*' in the colony code refer to *Cr. modiglianii* colonies associated with red and black *Ca. rufifemur*, respectively. *control* Control bioassays with dummies covered in pure hexane, *unk.comp.* unknown compounds. **** $P < 0.0001$, *** $P < 0.001$, n.s., $P > 0.05$, according to GLM

Aggression between *Cr. modiglianii* workers from different parabiotic or non-parabiotic colonies was generally high. Out of 44 colony combinations, only 3 resulted in peaceful interactions. The Bray–Curtis dissimilarity between nine parabiotically associated colonies did not correlate with allocolonial aggression (adjusted Mantel test with proportions of total aggression: $r = -0.19$, $P = 0.81$, 10,000 permutations, $n = 36$ colony combinations). Similar results were obtained for the absolute number of aggressive interactions. The three mentioned, peaceful colony combinations corresponded to intermediate Bray–Curtis dissimilarity values.

The quantitative hydrocarbon composition showed a relatively low variation between different *Cr. modiglianii* colonies (Fig. 2). The only exception were 27-MeC39-14-ene and 27-MeC39-16-ene (coefficient of variation 1.39). These two substances, which were not separable by gas chromatography, represent the dominant cuticular hydrocarbons in the red *Ca. rufifemur* variety. They are present in *Cr. modiglianii* colonies associated with red *Ca. rufifemur* variety but absent in those associated with the black one (Menzel et al., 2008a). Composition of unknown compounds was highly variable between colonies (Fig. 2).

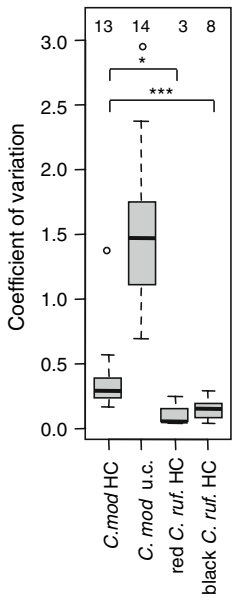

Fig. 2 Coefficients of variation of cuticular substances between colonies. For each substance with mean abundance >3%, the relative proportion of this substance within the profile was compared among colonies by calculating variation coefficients as SD/mean. The *numbers* above each plot indicate number of considered cuticular substances. Data are given for *Cr. modiglianii* unknown compounds and the hydrocarbons of *Cr. modiglianii* ($n = 9$ colonies), red *Ca. rufifemur* ($n = 9$ colonies), and black *Ca. rufifemur* ($n = 4$ colonies). The outlier in *Cr. modiglianii* hydrocarbons represents 27MeC$_{39}$-14-ene and 27MeC$_{39}$-16-ene. *HC* Hydrocarbons, *u.c.* unknown compounds. * $P < 0.05$, *** $P < 0.001$, according to Wilcoxon rank sum test

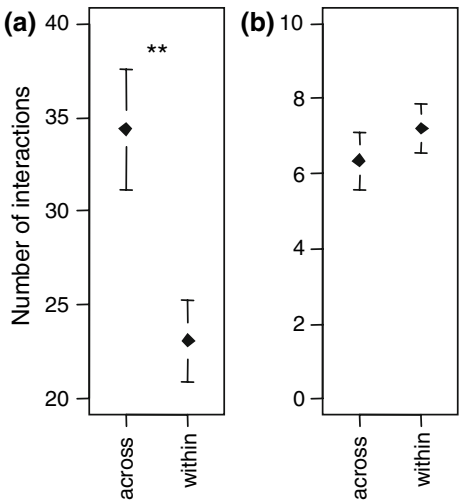

Fig. 3 Aggression bioassays in *Cr. modiglianii*, given as total number of (**a**) aggressive and (strongly or weakly) (**b**) peaceful interactions. The *graphs* show allocolonial confrontations of *Cr. modiglianii* workers associated with the same ('within', $n = 78$ assays) or different ('across', $n = 61$ assays) *Camponotus rufifemur* varieties (mean ± SE). **Significant at $P < 0.01$ according to linear mixed-effect model

While a certain set of these compounds was present in all colonies, others were dominant in some colonies but absent in others, leading to high coefficients of variation.

Crematogaster modiglianii workers were significantly less aggressive if the intruder ant was from a colony associated with the same *Ca. rufifemur* variety. Absolute number of aggressive interactions was significantly higher against intruders associated with the respective other *Ca. rufifemur* variety (linear mixed-effect model: $F_{1,24} = 6.57$, $P = 0.017$), which was not the case for peaceful interactions ($F_{1,24} = 0.38$, $P = 0.55$; Fig. 3). When regarding the relative proportions of strong or total aggression, the effect was marginally significant (both $P < 0.06$).

Camponotus rufifemur

Camponotus rufifemur workers significantly discriminated between intracolonial and allocolonial intruders and only attacked the latter (Table 2; Fig. 4a, regarding majors only). Aggression was especially high against intruders from the respective other variety but significantly lower against those from the same variety. The parameter 'within/across variety' alone explained 40.1% of the total variation in aggression (Table 2; Fig. 4a, majors only). Within the two respective varieties, allocolonial aggression was especially low against medium-sized intruders. Altogether, allocolonial medium workers received no strong aggression at all in 12 out of 22 assays within both respective varieties. Within the red *Ca. rufifemur* variety, they were significantly less attacked than majors (GLM for

Table 2 GLM for the proportion of total aggression in *Camponotus rufifemur*

	Deviance	*df*	*F*	*P*
Within/across variety	985.1	1	65.2	<0.0001
Intra-/allocolonial	426.3	1	37.38	<0.0001
Colony combination	469.1	11	5.296	<0.0001
No. *Camponotus*	44.7	1	6.03	0.017
No. *Crematogaster*	4.89	1	0.659	0.42
Residual error	528.9	68		
Total	2,459.4	83		

Both varieties, but only majors as intruders are considered ($n = 84$ recognition assays)

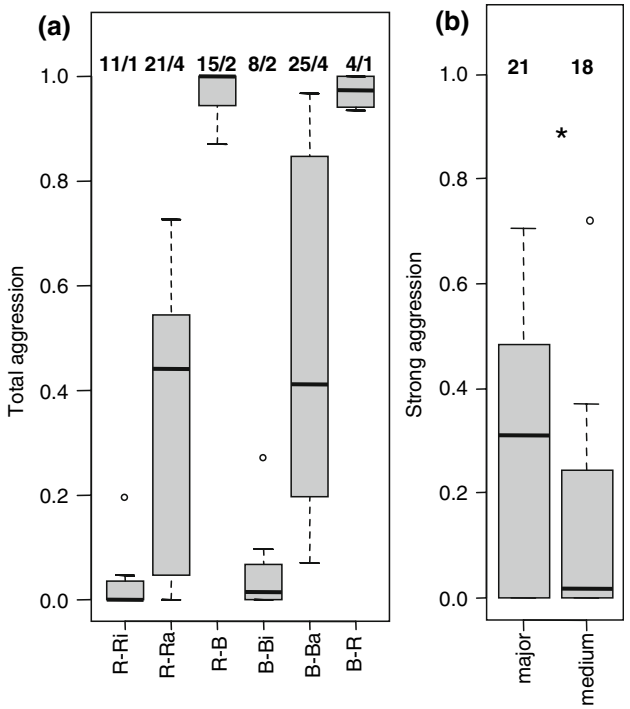

Fig. 4 **a** Aggression of *Camponotus rufifemur* towards majors from different colonies, given as proportions of aggressive interactions. 'R-Ri' red *Ca. rufifemur* towards intracolonial red; 'R-Ra' red towards allocolonial red; 'R-B' red towards black; 'B-Bi' black towards intracolonial black; 'B-Ba' black towards allocolonial black; 'B-R' black towards red. **b** Aggression of red *Camponotus rufifemur* workers against major and medium workers of allocolonial workers of the same variety. The *plots* show the proportion of strong aggression (median, quartiles and range), pooled for three colony combinations. Number of replicates is given above the plots. *$P < 0.05$ according to GLM with binomial error distribution

proportions of total and strong aggression: $P = 0.047$ and 0.019, respectively) (Table 3; Fig. 4b).

The variation in relative hydrocarbon quantities between nine colonies of the red *Ca. rufifemur* and between four colonies of the black variety was significantly lower than in nine *Cr. modiglianii* colonies (Wilcoxon rank sum test: *Ca. ru.* red-*Cr. mod.*: $W = 35$, $P = 0.039$; *Ca. ru.* black-*Cr. mod.*: $W = 95$, $P = 0.0009$; Fig. 2). Major and medium-sized workers in the red *Ca. rufifemur* could be significantly discriminated based on their quantitative cuticular composition. The discriminant model includes five substances (Wilk's $\lambda = 0.379$, $F_{7,21} = 4.91$, $P = 0.0021$, $n = 29$). The model correctly classifies 89.7% of the samples into the two castes. In contrast, the discriminant analysis did not reveal any differentiation between the three colonies (Wilk's $\lambda = 0.91$, $F_{2,26} = 1.21$, $P = 0.31$, $n = 29$).

Table 3 GLM for the proportion of total aggression between colonies of the red *Camponotus rufifemur* variety ($n = 39$ recognition assays)

	Deviance	*df*	*F*	*P*
Caste	40.6	1	4.23	0.047
Colony combination	18.8	3	0.62	0.61
Caste:colony combination	29.4	2	1.49	0.24
Residual	372.6	32		
Total	461.5	38		

Table 4 Individual hydrocarbon quantities and morphometric measures in the red *Camponotus rufifemur* variety

	Hydrocarbon quantity (µg) ($n = 29$)	Head width (mm) ($n = 28$)	Hind tibia length (mm) ($n = 28$)
Major	3.62 ± 2.05	3.30 ± 0.34	3.18 ± 0.26
Medium	0.48 ± 0.22	1.80 ± 0.28	2.31 ± 0.20
Ratio	7.54	1.83	1.39
Ratio^2		3.31	1.93

Data given are mean and standard deviation

Majors of the red *Ca. rufifemur* variety carried more than seven times more hydrocarbons (total quantities) than medium workers (Welch-corrected $t = 5.91$, $df = 14.44$, $P < 0.0001$) (Table 4). In contrast, the two morphometric measures differed by a much smaller factor between the two castes. Their squared major/medium ratios, which roughly reflect surface size ratios, were considerably lower (Table 4). This suggests that majors carry higher hydrocarbon quantities both in absolute terms and per body surface.

Discussion

Crematogaster modiglianii seems to acquire recognition signals from its parabiotic partner

Crematogaster modiglianii workers were significantly less aggressive towards allocolonial conspecifics when they

were associated with the same *Ca. rufifemur* variety. This coincides with the abundance of 27-MeC39-14-ene and 27-MeC39-16-ene in the *Cr. modiglianii* profile. The two methylbranched alkenes are abundant in the red *Ca. rufifemur* variety but absent in the black one. Consequently, they only occur in those *Cr. modiglianii* colonies associated with the red *Ca. rufifemur* variety (see Menzel et al., 2008a; Fig. 2 therein, for a comparison with largely the same colonies as used in this study), and are most probably acquired from its partner (comparable to artificial mixed colonies, Vienne et al., 1995). Regarding the hydrocarbon profile, this represents the only detectable difference between *Cr. modiglianii* associated with red and those associated with black *Ca. rufifemur*. Since nestmate recognition in *Cr. modiglianii* is mediated by hydrocarbons (Fig. 1), and *Cr. modiglianii* shows differential inter-colony aggression depending on the identity of its partner, it appears likely that *Cr. modiglianii* uses these methylbranched alkenes as a recognition signal provided by its parabiotic partner. Ants can adopt nestmate recognition cues from various environmental sources such as food (Sorvari et al., 2008; Richard et al., 2004) or nest material (Heinze et al., 1996). However, to our knowledge it has not been reported previously that ants also adopt intraspecific recognition cues from an associated species.

Except for these two substances, the detectable variation of the remaining cuticular hydrocarbons between nine colonies was low (Fig. 2). However, in contrast to *Ca. rufifemur*, *Cr. modiglianii* was highly aggressive against most alien workers, even against colonies only 2–3 m away. Although the workers thus clearly differentiated between colonies, the chemical differentiation besides the substances acquired from *Ca. rufifemur* is probably too subtle for detection with our methods. This explains why we did not find a correlation between inter-colony aggression and chemical differentiation, although—as revealed by our extract bioassays—*Cr. modiglianii* does use cuticular hydrocarbons as nestmate recognition cues.

The unknown cuticular compounds, in contrast, do not function as nestmate recognition cues, although they are highly abundant and vary both quantitatively and qualitatively (Fig. 2). Aggression against alien total extracts was similar to aggression against alien hydrocarbons in two out of three cases (Fig. 1). Hence, the unknown compounds most likely do not possess an aggression-reducing effect in intraspecific encounters, as has been shown for interspecific encounters with *Ca. rufifemur* (unpubl. data).

Camponotus rufifemur: low inter-colony aggression within chemical varieties

Albeit higher than against nestmates, inter-colony aggression between *Ca. rufifemur* workers of the same chemical variety was surprisingly low. A notable proportion of allocolonial intruders (especially medium workers) received no aggression at all. This was although the experimental setup—directly at the nest, minimized disturbance, one intruder only—should maximize aggression against alien ants. In contrast, many studies on other *Camponotus* species report high levels of inter-colony aggression, even if these species live in interspecific associations like lestobioses (Carlin and Hölldobler, 1986; Errard et al., 2003; Boulay et al., 2000). However, *Ca. rufifemur* fiercely attacked workers of the respective other chemical variety and often dismembered them within 2 or 3 min.

The low intra-variety aggression cannot be explained by a dear-enemy phenomenon (e.g. Heinze et al., 1996) or a possible polydomous colony structure, since all colonies were distant from each other and separated by rivers. Its causes probably involve the high abundance of long-chain unsaturated cuticular hydrocarbons (C_{37}–C_{49}, Menzel et al., 2008a). Long-chain hydrocarbons are harder to perceive by olfactory receptors due to their low volatility (Gibbs and Pomonis, 1995) and probably blur small differences in the recognition cues (Lambardi et al., 2007). Hence, the workers may be unable to detect small signal differences, e.g. between colonies of the same variety, but still recognize strongly (or qualitatively) different signals (e.g. between the two *Ca. rufifemur* varieties). It has been reported that long-chain hydrocarbons can hamper interspecific discrimination both in social parasites (Lambardi et al., 2007) and between *Cr. modiglianii* and *Ca. rufifemur* (Menzel et al., 2008a). However, to our knowledge it has not been shown previously that high interspecific tolerance also extends to intraspecific tolerance as reported here.

Moreover, there is little inter-colony hydrocarbon variation *within* the two respective *Ca. rufifemur* varieties. Inter-colony coefficients of variation were significantly lower than in *Cr. modiglianii* (Fig. 2). The discriminant analysis did not find colony differences based on individual cuticular extracts. We are aware that lack of detectable differentiation does not necessarily imply lack of differentiation (particularly in single-individual extracts with low substance quantities). For example, *Cr. modiglianii* displayed pronounced nestmate discrimination despite low measureable differentiation. Nevertheless, the fact that hydrocarbon profiles of different colonies are often easily discernible in other ant species (Nielsen et al., 1999; Liu et al., 2001) argues for lower chemical differentiation in *Ca. rufifemur* than in other ant species. The long periods of antennation in within-variety allocolonial encounters (Menzel et al., 2008b) may indicate recognition uncertainty, probably caused by the low chemical differentiation between colonies. This behavior can also be observed towards nestmates that were separated from the colony for hours or days (Menzel et al., 2008b). This corroborates that

the continued antennation may be due to recognition uncertainty, which is different from 'tolerance despite recognition as foreign'. As a matter of principle, however, one cannot infer recognition processes based on behavioral experiments, although this has sometimes implicitly been claimed in earlier studies (e.g. Steiner et al., 2007).

Camponotus rufifemur: Inter-colony aggression depends on worker caste

An unexpected outcome of the aggression bioassays was that medium workers were significantly less attacked than majors. Majors carry on average more than seven times the amount of cuticular hydrocarbons as medium workers. Even when correcting for the larger body surface using two morphometric measures (Table 4), they possess higher hydrocarbon quantities per body surface. The higher availability of recognition cues in majors may make it easier for workers to recognize them as foreign, resulting in the observed higher aggression. The low discrimination between medium castes may thus be a consequence of the low chemical differentiation between *Ca. rufifemur* colonies of the same variety, coupled with low absolute quantities of recognition cues in medium-sized workers.

It is worth noting that both *Ca. rufifemur* and their parabiotic partner *Cr. modiglianii* often fail to discriminate workers from different *Ca. rufifemur* colonies of the same variety (Menzel et al., 2008a). The unusual cuticular compounds found in this species (Menzel et al., 2008a) thus seem to influence both intraspecific and interspecific recognition.

Acknowledgments We are grateful to Heike Feldhaar and Annett Endler for fruitful discussions and support in the GC-FID analysis. The Malaysian Economic Planning Unit (EPU) and the Danum Valley Management Committee (DVMC) kindly gave permission for our research, and Dr. Arthur Chung (Forest Research Center, Sepilok) functioned as our local collaborator. This study complies with current laws of Malaysia. F.M. was supported by a doctoral fellowship of the Bayerische Graduiertenförderung (BayEFG) and the Studienstiftung des deutschen Volkes. This work was also supported by the Sonderforschungsbereich SFB-554 'Mechanisms and Evolution of Arthropod Behaviour' of the Deutsche Forschungsgemeinschaft (DFG).

References

Aitchinson J. 1986. *The Statistical Analysis of Compositional Data: Monographs in Statistics and Applied Probability*. Chapman and Hall, London. 416 pp

Boulay R., Hefetz A., Soroker V. and Lenoir A. 2000. Camponotus fellah colony integration: worker individuality necessitates frequent hydrocarbon exchanges. *Anim. Behav.* **59**: 1127–1133

Brandstaetter A.S., Endler A. and Kleineidam C.J. 2008. Nestmate recognition in ants is possible without tactile interaction. *Naturwissenschaften* **95**: 601–608

Carlin N.F. and Hölldobler B. 1986. The kin recognition system of carpenter ants (*Camponotus* spp.) I. Hierarchical cues in small colonies. *Behav. Ecol. Sociobiol.* **19**: 123–134

Errard C., Regla J.I. and Hefetz A. 2003. Interspecific recognition in Chilean parabiotic ant species. *Insect. Soc.* **50**: 268–273

Foitzik S., Sturm H., Pusch K., D'Ettorre P. and Heinze J. 2007. Nestmate recognition and intraspecific chemical and genetic variation in *Temnothorax* ants. *Anim. Behav.* **73**: 999–1007

Gibbs A. and Pomonis J.G. 1995. Physical properties of insect cuticular hydrocarbons: The effects of chain lengths, methyl branching and unsaturation. *Comp. Biochem. Physiol.* **112B**: 243–249

Heinze J., Foitzik S., Hippert A. and Hölldobler B. 1996. Apparent dear-enemy phenomenon and environment-based recognition cues in the ant *Leptothorax nylanderi*. *Ethology* **102**: 510–522

Hölldobler B. and Wilson E.O. 1990. *The Ants*. Springer, Berlin. 732 pp

Holway D.A., Lach L., Suarez A.V., Tsutsui N.D. and Case T.J. 2002. The causes and consequences of ant invasions. *Annu. Rev. Ecol. Syst.* **33**: 181–233

Lahav S., Soroker V. and Hefetz A. 1999. Direct behavioral evidence for hydrocarbons as ant recognition discriminators. *Naturwissenschaften* **86**: 246–249

Lambardi D., Dani F.R., Turillazzi S. and Boomsma J.J. 2007. Chemical mimicry in an incipient leaf-cutting ant social parasite. *Behav. Ecol. Sociobiol.* **61**: 843–851

Lenoir A., Fresneau D., Errard C. and Hefetz A. 1999. Individuality and colonial identity in ants: the emergence of a social representation concept. In: *Information Processing in Social Insects* (Detrain C., Pasteels J.M. and Deneubourg J.L., Eds), Birkhäuser, Basel. pp 219–237

Liang D. and Silverman J. 2000. "You are what you eat": Diet modifies cuticular hydrocarbons and nestmate recognition in the Argentine ant, *Linepithema humile*. *Naturwissenschaften* **87**: 412–416

Liu Z.B., Bagnères A.G. and Yamane S. 2001. Intra-colony, inter-colony and seasonal variations of cuticular hydrocarbon profiles in *Formica japonica* (Hymenoptera, Formicidae). *Insect. Soc.* **48**: 342–346

Menzel F., Blüthgen N. and Schmitt T. 2008a. Tropical parabiotic ants: Highly unusual cuticular substances and low interspecific discrimination. *Front. Zool.* **5**: 16

Menzel F., Linsenmair K.E. and Blüthgen N. 2008b. Selective interspecific tolerance in tropical *Crematogaster–Camponotus* associations. *Anim. Behav.* **75**: 837–846

Menzel F., Blüthgen N., Beuerle T. and Schmitt T. (submitted): Novel cuticular substances in parabiotic ants function as interspecific appeasement signals

Nielsen J., Boomsma J.J., Oldham N.J., Petersen H.C. and Morgan E.D. 1999. Colony-level and season-specific variation in cuticular hydrocarbon profiles of individual workers in the ant *Formica truncorum*. *Insect. Soc.* **46**: 58–65

Orivel J., Errard C. and Dejean A. 1997. Ant gardens: Interspecific recognition in parabiotic ant species. *Behav. Ecol. Sociobiol.* **40**: 87–93

R Development Core Team 2007. R: A language and environment for statistical computing. http://www.R-project.org. Vienna, R Foundation for Statistical Computing 2007

Richard F.J., Hefetz A., Christides J.P. and Errard C. 2004. Food influence on colonial recognition and chemical signature between nestmates in the fungus-growing ant *Acromyrmex subterraneus subterraneus*. *Chemoecology* **14**: 9–16

Sorvari J., Theodora P., Turillazzi S., Hakkarainen H. and Sundström L. 2008. Food resources, chemical signaling, and nest mate recognition in the ant *Formica aquilonia*. *Behav. Ecol.* **19**: 441–447

Steiner F.M., Schlick-Steiner B.C., Moder K., Stauffer C., Arthofer W., Buschinger A., Espadaler X., Christian E., Einfinger K., Lorbeer E., Schafellner C., Ayasse M. and Crozier R.H. 2007. Abandoning aggression but maintaining self-nonself discrimination as a first stage in ant supercolony formation. *Curr. Biol*. **17**: 1903–1907

Suarez A.V., Holway D.A., Liang D., Tsutsui N.D. and Case T.J. 2002. Spatiotemporal patterns of intraspecific aggression in the invasive Argentine ant. *Anim. Behav*. **64**: 697–708

Suarez A.V., Holway D.A. and Tsutsui N.D. 2008. Genetics and behavior of a colonizing species: the invasive argentine ant. *Am. Nat*. **172**: S72–S84

Tsutsui N.D., Suarez A.V., Holway D.A. and Case T.J. 2000. Reduced genetic variation and the success of an invasive species. *Proc. Natl Acad. Sci. USA* **97**: 5948–5953

Tsutsui N.D., Suarez A.V. and Grosberg R.K. 2003. Genetic diversity, asymmetrical aggression, and recognition in a widespread invasive species. *Proc. Natl Acad. Sci. USA* **100**: 1078–1083

Vienne C., Soroker V. and Hefetz A. 1995. Congruency of hydrocarbon patterns in heterospecific groups of ants: transfer and/or biosynthesis? *Insect. Soc*. **42**: 267–277

Wagner D., Tissot M., Cuevas W. and Gordon D.M. 2000. Harvester ants utilize cuticular hydrocarbons in nestmate recognition. *J. Chem. Ecol*. **26**: 2245–2257