The Journal of Experimental Biology 211, 1249-1256
Published by The Company of Biologists 2008
doi:10.1242/jeb.017301

Cuticular hydrocarbons as queen adoption cues in the invasive Argentine ant

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Accepted 9 February 2008

SUMMARY

In social insects, individuals typically recognize and behave aggressively towards alien conspecifics, thereby maintaining colony integrity. This is presumably achieved *via* a nestmate recognition system in which cuticular compounds, usually cuticular hydrocarbons (CHC), of genetic and/or environmental origin serve as recognition cues. Most invasive populations of the Argentine ant, *Linepithema humile* (Mayr), display minimal nestmate–non-nestmate discrimination, resulting in low levels of intraspecific aggression allowing free movement of workers and queens among nests. However, invasive *L. humile* in the southeastern United States show relatively high levels of intraspecific aggression, and selectively adopt non-nestmate queens. Using behavioral assays and gas chromatography, we found an association between non-nestmate queen adoption and similarity of the CHC profiles of adopted and host colony queens. Also, nestmate and non-nestmate queen CHC profiles became more similar after adoption by queenless colonies. Furthermore, queens treated with non-nestmate queen CHC had distinct CHC profiles and were generally attacked by nestmate workers. We suggest that in *L. humile*, CHC are used as queen recognition cues, and that queen recognition errors are more likely to occur when the CHC profiles of non-nestmate and host colony queens are similar. Our findings provide further evidence for the complex and dynamic nature of *L. humile* nestmate discrimination, which may in part underlie the success of introduced populations of this invasive ant.

Key words: Argentine ant, *Linepithema humile*, nestmate recognition, cuticular hydrocarbons, intraspecific aggression, non-nestmate queen adoption.

INTRODUCTION

In social insects, nestmate recognition allows an individual's acceptance and integration within a colony and prevents non-colony members – both conspecifics and heterospecifics – from invading and exploiting the colony's resources; non-nestmate intruders are recognized and elicit active defensive behaviors (Hölldobler and Wilson, 1990; Vander Meer and Morel, 1998). Nestmate recognition in social insects is adaptive because workers obtain inclusive fitness benefits from aiding nestmates and discriminating against non-nestmates, provided that nestmates are usually more closely related to one another than to members of other nearby colonies (Hölldobler, 1995). Natural selection should favor the use of cues that optimize discrimination because recognition errors – rejecting a desirable conspecific or accepting an undesirable individual – lower the benefits expected from kin interactions (Lehman and Perrin, 2002). Recognition cues in social insects and other animals are primarily chemical in nature and perceived by olfaction or contact chemoreception (Hölldobler and Michener, 1980; Breed, 1998).

One model for nestmate recognition proposes that individuals discriminate colony members from non-members by means of a phenotype matching mechanism in which the phenotype of a newly encountered individual (actor) is compared with the individual's (reactor) inner learned template (Lacy and Sherman, 1983). Phenotypic recognition cues must be reliable, and they originate from either the environment (diet, nesting substrate), endogenous sources (genetically determined), or both (Breed and Bennett, 1987; Vander Meer and Morel, 1998). The template represents a constantly changing memory pattern of the colony's recognition cues, and the process of cue–template matching guides a behavioral response, usually acceptance or rejection of the encountered individual (Reeve, 1989; Gamboa et al., 1986). In addition, recognition of phenotypic cues by allele matching (recognition-allele mechanism), may also occur (Keller and Ross, 1998). For example, in fire ants, *Gp-9* genotypic compatibility seems to regulate queen identity and number *via* production of a distinct chemical label and formation of a specific exclusionary template based on the allelic variant possessed by workers and queens (Gotzek and Ross, 2007).

Appropriate behavioral responses are guided by recognition decision rules concerning the level of dissimilarity between the template and the phenotypic cues of the encountered individual (Breed and Bennett, 1987). Models for decision rules in recognition include (i) an individualistic model in which individuals retain their own cue integrity and score other individuals by comparison with themselves, accepting them based on genotypic similarity; and (ii) a Gestalt model in which cue transfer occurs among colony members resulting in a unique mixture of chemical cues (colony 'odor'), and individuals are classified as colony members based upon the degree to which they possess the odour (Crozier and Dix, 1979; Getz, 1982; Crozier, 1987). Also, decisions may be made according to a recognition threshold so that if the template–odor match is greater than a minimum similarity threshold (or below a dissimilarity threshold) the individual is accepted and treated as nestmate (Gamboa et al., 1986; Reeve, 1989). Interaction frequency with foreign conspecifics and the fitness consequences of accepting or rejecting conspecifics may determine the optimal acceptance threshold (Reeve, 1989); hence, discrimination should vary according to the social and ecological context to balance the fitness costs of accepting non-nestmates and rejecting nestmates. Alternatively, a graded behavioral response depending on the degree of cue and template similarity would suggest a non-threshold model (Vander Meer and Morel, 1998).

In social insect species with large colonies, queens and workers seem to be labeled by a more or less homogenous recognition odor, or colony gestalt label, where each colony member bears a mixture of cues representative of the variation among members of the colony (Stuart, 1988; Errard and Jallon, 1987). This gestalt label is expected to prevail in polygynous ant species, although extreme polygyny may limit the creation of unique labels, thereby reducing intercolony variation. In addition, the presence of multiple nests within a colony (polydomy) can potentially increase within-colony cue diversity leading to a broader template, which may explain the reduced aggression toward alien conspecific workers observed in polydomous ant colonies (van Wilgenburg et al., 2006). The lack of distinct intrinsic colony odors and a broader recognition template may facilitate the formation of unicolonial populations in which colony boundaries are weak or absent, although some odor differences arising from extrinsic factors (e.g. the microenvironment) may still exist (Hölldobler and Wilson, 1990).

Although various volatile and nonvolatile compounds have been demonstrated as nestmate recognition cues, most frequently, long-chain cuticular hydrocarbons (CHC) have been shown to serve this role in ants (e.g. Lahav et al., 1999; Thomas et al., 1999; Boulay et al., 2000; Liang and Silverman, 2000; Ozaki et al., 2005; Greene and Gordon, 2007), wasps (e.g. Gamboa et al., 1996; Dani et al., 2001; Ruther et al., 2002), and termites (e.g. Clèment and Bagnères, 1998). The sensory mechanism for detection of CHC in social insects is unclear, however peripheral recognition of specific CHC blends (i.e. nestmate *versus* non-nestmate) by specialized antenna sensillae may be achieved by desensitization of gustatory receptor neurons to nestmate CHC blends (Ozaki et al., 2005).

Introduced populations of the Argentine ant *Linepithema humile* (Mayr), are highly polygynous, polydomous and unicolonial (Newell and Barber, 1913; Hölldobler and Wilson, 1990; Suarez et al., 1999), and exhibit pronounced variation in intraspecific aggression (Tsutsui et al., 2000; Suarez et al., 2002; Giraud et al., 2002; Buczkowski et al., 2004). These populations are, therefore, ideal models to examine nestmate discrimination, and the effects of genetic similarity and social and ecological context on behavioral thresholds (Buczkowski and Silverman, 2005). Nestmate recognition in this widespread invasive species is mediated by endogenous and exogenous CHC (Suarez et al., 2002; Liang and Silverman, 2000), and because the contribution of environmentally derived cues to nestmate recognition varies among introduced populations, it appears that phenology and genotypic diversity affect the expression and perception of components of the *L. humile* recognition system (Buczkowski and Silverman, 2006). Therefore, examining variation in Argentine ant recognition cue diversity and recognition threshold modulation may further our understanding of recognition cue ontogeny, perception and action thresholds.

We have recently demonstrated that unrelated *L. humile* colonies from the southeastern United States selectively adopt foreign queens and fuse (Vásquez and Silverman, 2008a; Vásquez and Silverman, 2008b), thereby potentially altering colony genetic composition and 'eroding' non-nestmate discrimination. In unicolonial *L. humile* populations that exhibit low variation in genetic-based recognition cues, non-nestmates may be accepted if the template–cue dissimilarity is below a rejection threshold (Starks, 2003). Likewise, in more genetically diverse populations, colonies with higher levels of genetic-based recognition cue similarity may accept non-nestmates if the template–cue match is below a dissimilarity threshold.

In this study, we investigated a possible mechanism underlying *L. humile* non-nestmate queen acceptance by comparing queen CHC profiles among colonies and examining the relationship between queen CHC profile similarities and queen adoption rates in queenless and queenright host colonies. We hypothesized that queen CHC similarities are correlated with, and probably guide, behavioral interactions between queens and recipient workers. We thus expected that the CHC profiles of adopted non-nestmate queens would be more similar to host colony queens than the CHC profiles of non-adopted queens. In addition, we examined the chemical profiles of adopted queens to determine whether queens acquired non-nestmate CHC as a means of colony integration. In *L. humile*, workers treated with prey-derived hydrocarbons elicit nestmate worker aggression (Liang and Silverman, 2000) as do cotton balls dosed with extracted nestmate CHC that are supplemented with *n*-alkanes (Greene and Gordon, 2007). Nevertheless, the effect of supplementing the CHC profiles of live ants with non-nestmate CHC on worker aggression has yet to be tested. To determine if queen CHC also modulate worker aggression and serve as nestmate queen recognition cues, we applied purified non-nestmate CHC of queens that were consistently attacked to live queens, recorded nestmate worker behavior, and analyzed treated queen CHC. We expected that application of naturally occurring non-nestmate queen CHC onto queens would also elicit worker aggression.

MATERIALS AND METHODS

Experimental colonies

We used colonies of Argentine ants *Linepithema humile* (Mayr), collected from four sites in the southeastern USA: Chapel Hill (CHH), Research Triangle Park (RTP) and Winston-Salem (FOR) in North Carolina and Greenville (COC) in South Carolina. Distances between collection sites ranged from 27 km (CHH–RTP) to 392 km (RTP–COC). Ants collected from these sites were genetically differentiated, based on seven microsatellite markers, with colony pairs sharing different levels of genetic similarity ranging from 30.3% (CHH–COC) to 72.4% (CHH–FOR) alleles shared (Vásquez and Silverman, 2008a). All colonies were maintained in soil-free, Fluon™-coated trays (40×55×8 cm; AGC Chemicals American Inc., Bayonne, NJ, USA). Nests were plastic Petri dishes (9 cm diameter) filled with moist grooved Castone® (Dentsply International Inc., York, PA, USA) dental plaster. Colonies were provided 25% sucrose solution, artificial diet (Bhatkar and Whitcomb, 1970) *ad libitum*, hard-boiled egg once a week and a water source. All colonies were maintained at 25±1°C and 50±15% relative humidity, on a 12 h:12 h light:dark cycle. Source colonies from each of the four locations containing ants not used to set-up the experimental colonies were also maintained as described above.

Non-nestmate queen adoption assay and sampling of queen cuticular hydrocarbons

A non-nestmate Argentine ant queen was introduced into either queenless or queenright CHH, COC, FOR and RTP experimental colonies, and worker behavior towards the introduced queen was recorded for 24 h (Vásquez and Silverman, 2008a). We established a total of 12 experimental colonies with ants collected at each of the four locations in 2003; for each location there were three colonies each with a different queen number (zero, one or six queens), 100 pieces of brood, and ~3000 workers (1 g). Six queens from each of the four source colonies were marked with a water-based paint for identification, introduced individually into each queenless and queenright (single and six-queen) experimental colony, and left in place for 24 h. The response of the host workers toward the intruder queen was scored as 0 (no aggressive response), 1 (physical attack)

or 2 (intruder killed). Adoption was regarded as having occurred if after 24 h, intruder queens were found in the nest being tended by workers (response scored as 0). Data were analyzed as the average recipient colony response score and as the percentage of queens adopted. The adoption assay was replicated twice across time, and a total of 144 queens per source colony were tested in all six colony–pair combinations.

We collected 10 queens from each of the source colonies used in this assay for CHC analysis. Queens were placed individually in glass vials and stored at –20°C until extraction, purification and analysis of cuticular lipids as described below. Queen CHC profiles were compared (see below) to determine queen CHC similarities between colonies, and to relate similarities of queen CHC to worker behavioral response and percentage queen adoption at 24 h.

To examine CHC profiles of queens before and after adoption, we developed, validated and employed a non-destructive CHC sampling method. A hexane-extracted air-dried cotton ball (2 mm diameter) held by a pair of hexane-rinsed forceps was gently stroked against a queen's abdomen for 3 min then stored in a glass vial at –20°C. We individually sampled nine queens per colony from the CHH, COC, FOR and RTP colony fragments collected in 2004. CHC profiles of queens sampled using the non-destructive method were compared with those of the 40 solvent-extracted source queens from the 24 h adoption assay.

We then introduced individual COC and CHH queens into queenless and multiple queen FOR and RTP colonies. These queen/recipient colony combinations (COC/FOR and CHH/RTP) were selected based on the high adoption rates observed in the previous adoption experiment. Three queenless and three multiple-queen (six queens) experimental colonies were established from FOR and RTP ants collected in 2004, each with 100 pieces of brood, and ~3000 workers. Recipient colony response was recorded as previously described, and queens surviving after 24 h were left in place for 2 weeks. Nestmate queen introductions (FOR and RTP) were also performed. The assay was replicated four times. We used the non-destructive CHC sampling method to examine CHC profiles of nestmate and non-nestmate queens before and after adoption. We sampled all queens tested (96) 24 h prior to introduction, and all queens adopted by queenless colonies (42) 2 weeks after introduction. Because non-nestmate queen adoption rates in queenright colonies were low, the few samples collected were not included in the analysis. CHC profiles of adopted COC and CHH queens were compared before and after introduction with those of FOR and RTP queens adopted by their nestmate colonies, respectively, to determine if changes in CHC occurred after adoption.

Application of non-nestmate queen cuticular hydrocarbons to queens: effects on nestmate worker aggression

To test if CHC are used as cues in *L. humile* nestmate queen recognition we compared worker aggressive behavior towards nestmate queens treated with purified nestmate and non-nestmate queen CHC. We selected the FOR queen/RTP recipient colony combination based on the consistent rejection of FOR queens by RTP recipient colonies. Three multiple-queen experimental colonies (same brood and worker size as in queen adoption assay colonies) were established from RTP source colonies collected in 2005. RTP queens were treated with purified CHC extracts of FOR or RTP queens, or with hexane as control. Purified CHC from six queens (cuticular lipid extraction and CHC isolation procedures detailed below) were resuspended in 100 μl hexane, applied to the inside surface of a 12×32 mm glass vial, and the solvent allowed to evaporate. Three vials were coated per treatment and each vial was used to treat three individual queens. Each queen was anesthetized by brief exposure to CO_2, placed individually in a treated vial, rotated gently for 3 min, allowed 15–30 s to recover and then introduced to one of three RTP multiple queen experimental colonies. Each colony received a total of three queens per treatment. Worker behavior was scored as non-aggressive (antennation, queen moving into nest without being attacked) or aggressive (biting, pulling, lunging, gaster flexion) during a 3 min period by an observer blinded to the type of treatment applied to queens and unfamiliar with the hypothesis being tested. All tested queens were killed by freezing (–20°C). CHC profiles of all queens were compared to determine if they differed between treatments, and between attacked and non-attacked queens.

Extraction, isolation and chemical analysis of cuticular hydrocarbons

Nonpolar cuticular lipids of thawed queens and cotton samples collected in all behavioral assays were extracted by immersion in 1 ml hexane for 10 min, followed by a brief second rinse in 100 μl hexane. Samples were lightly shaken for the first and last 15–20 s of the immersion period. The solvent was removed under a gentle stream of high purity N_2, the vial rinsed twice, each with 100 μl hexane, and the concentrated extract (200 μl) was applied to a hexane-prewetted Pasteur pipette mini-column filled with 500 mg of silica gel (100–200 mesh). The hydrocarbon fraction was eluted with 6 ml hexane and the solvent was evaporated with N_2. Capillary gas chromatography (GC) was carried out using a Hewlett-Packard (Rockeville, MD, USA) HP5890 gas chromatograph equipped with a DB-XLB column (30 m× 0.25 mm×0.25 μm film thickness) for analyses of CHC of source queens from the 24 h queen adoption assay, and a DB-5 (30 m×0.25 mm×0.5 μm) for analyses of CHC of cotton samples taken in the 2-week queen adoption assay and of queens treated with non-nestmate and nestmate CHC. The change in columns was based on column availability and the two columns gave identical results for split samples (data not shown). Extracts were introduced into a split-splitless injector operated at 300°C in splitless mode (2 min purge) and with a helium carrier gas average linear velocity of 30 cm s^{-1}. The oven temperature was held at 80°C for 2 min, increased to 270°C at a rate of 20°C min^{-1}, then to 310°C at 3°C min^{-1} and held at 310°C for 20 min. The flame-ionization detector was operated at 310°C with nitrogen make-up gas at 30 ml min^{-1}. Whole queen extracts were resuspended in 20 μl hexane, and 0.5 μl (0.025 queen equivalents) was injected. Cotton sample extracts were resuspended in 4 μl of octane and 2 μl (0.5 queen equivalents) were injected by an automatic injector. Quantitative data were obtained by integrating the area under each peak and calculating its percentage of the total CHC; only peaks with a mean percentage area across all colonies of 1% or higher were used for data analysis. All selected peak areas were standardized to 100%. The identity of discriminating peaks was determined by matching *L. humile* n-alkanes with external hydrocarbon standards (n-C23 – n-C36) and diagnostic peaks were confirmed by GC-MS with those from previous studies (Liang et al., 2001; de Biseau et al., 2004). GC-MS analyses of queen cuticular hydrocarbons were performed on a HP6890 GC equipped with a HP-5MS column (30 m×0.25 mm×0.25 μm film thickness), and connected to a HP5973A mass selective detector. The injector was operated at 300°C in splitless mode with a helium carrier gas average linear velocity of 45 cm s^{-1} (2 min purge). Data were recorded in electron ionization scan mode (25–550 m/z).

Statistical analyses

Data analyses were performed using SAS 9.1 statistical software (SAS, 2004). Standardized selected peak areas were transformed following Aitchison's formula: $Z_{ij}=\ln[Y_{ij}/g(Y_j)]$, where Z_{ij} is the standardized peak area i, for individual j, Y_{ij} is the peak area i for individual j, and $g(Y_j)$ is the geometric mean of all peaks for individual j. We performed a multivariate analysis of variance (MANOVA) and tested the homogeneity of variance of these transformed variables with Brown and Forsythe's test (Brown and Forsythe, 1974) using PROC GLM. We performed a stepwise discriminant analysis (stepwise DA) on transformed variables that met the assumptions of homogeneity of variance in MANOVA using PROC STEPDISC followed by DA on the selected peaks using PROC CANDISC to determine whether the predefined groups (colonies or treatments) could be discriminated on the basis of their chemical profiles. Pairwise generalized square distances between groups and classification error rates were calculated using PROC DISCRIM. Distances between group means (centroids) were used as an estimate of the degree of CHC differentiation between colonies or treatments.

Correlations between queen CHC similarities and recipient colony response and percentage non-nestmate queen adoption were performed using Pearson correlation coefficients (Pearson's r).

RESULTS

Non-nestmate queen adoption assay and queen cuticular hydrocarbon profiles

Queen cuticular hydrocarbon profile differentiation among colonies

Queens from source colonies used in the 24 h queen adoption assay were distinguished based on 24 transformed peaks that differed among groups according to MANOVA with function 1 and function 2 explaining 81.0% and 11.2% of the total variation in the analysis (Wilks' λ=0.0004, F=7.73, d.f.=69, 42.7, P<0.0001). The stepwise DA selected 12 variables that clustered all queens according to their colony of origin (Wilks' λ=0.0027 0.01, F=13.11, d.f.=36, 74.6, P<0.0001) with function 1 (86.1% of variation) separating CHH and RTP from both COC and FOR, and function 2 (9.5%) further separating CHH from RTP and COC from FOR (Fig. 1A); and all queens were correctly classified. Discriminating compounds selected in the stepwise DA were identified as *n*-heptacosane (*n*-C27), *n*-nonacosane (*n*-C29), 5-methylnonacosane (5-MeC29), 5-methyltriacontane (5-MeC30), 5-methylhentriacontane (5-MeC31), hentriacontene (xC31:1), tritriacontene (xC33:1) and 5-methyltetratriacontane 5-MeC34, while four compounds remained unidentified.

As a quantitative estimate of the degree of CHC differentiation between colonies we calculated pairwise generalized square distances between colony means (centroids) on DA canonical variables obtained in the analysis of the 12 transformed peaks identified by stepwise DA (Table 1). These data show that queens from different colonies possess statistically unique CHC profiles of varying degrees of differentiation, and these profiles could mediate worker–queen nestmate recognition.

Queen adoption in relation to similarities of queen cuticular hydrocarbon profiles

CHC similarities between colonies (distances between colony centroids) were positively correlated with recipient colony response (0=queens adopted, 1=queens attacked, 2=queens killed) in queenless (Pearson's r=0.86, P=0.0276) and single-queen host colonies (Pearson's r=0.90, P=0.0154) with non-nestmate queens more likely to be attacked and killed with increasing distances between queen CHC profiles (Fig. 2). By contrast, we found no association between CHC similarities and recipient response in multiple queen colonies (Pearson's r=0.66, P=0.1504). Also, queen CHC profile similarities between colonies were inversely associated with non-nestmate queen adoption (percent) in queenless (Pearson's r=–0.85, P=0.0329) and single queen colonies (Pearson's r=–0.89, P=0.0154), but not in multiple queen colonies (Pearson's r=–0.66, P=0.1503).

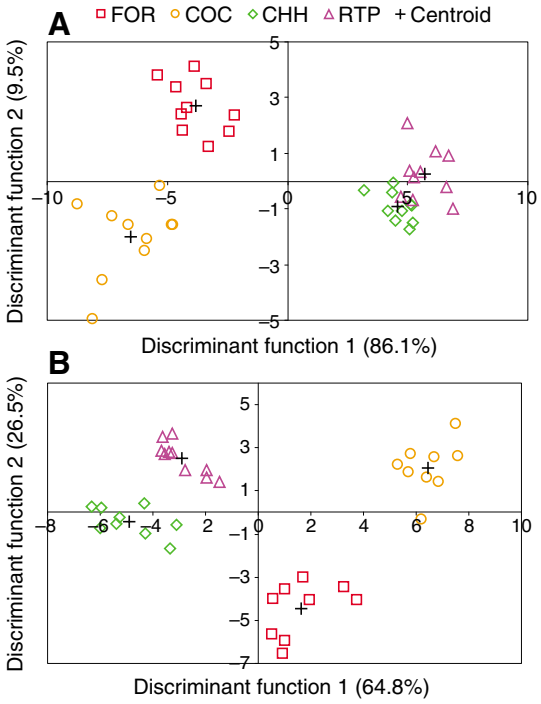

Fig. 1. (A) Discriminant analysis of 12 variables (relative proportions of cuticular hydrocarbons) selected by stepwise discriminant analysis for queens from four *L. humile* colonies (CHH, COC, FOR, RTP) used in the queen adoption assay for which queen cuticular hydrocarbons were extracted with solvent and (B) discriminant analysis of 13 variables selected by stepwise discriminant analysis for queens from the same four *L. humile* colonies for which queen cuticular hydrocarbons were sampled using a non-destructive method. + marks the centroid of each group.

Non-destructive queen cuticular hydrocarbon sampling *versus* solvent extraction

Queens sampled using the non-destructive method were distinguished based on 32 transformed variables that differed among

Table 1. Generalized squared distances between colony means (centroids) calculated by discriminant analysis of cuticular hydrocarbons of *Linepithema humile* queens from source colonies used in the queen adoption assay extracted by solvent or sampled using a non-destructive method

Colony pair	Solvent extraction (12 variables)	Non-destructive sampling (13 variables)
CHH–COC	129.03	136.47
CHH–FOR	85.48	68.86
CHH–RTP	13.23	29.09
COC–FOR	30.16	69.85
COC–RTP	155.67	95.56
FOR–RTP	100.46	70.62

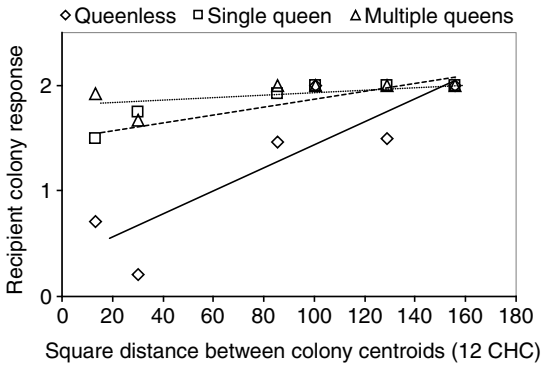

Fig. 2. Relationship between cuticular hydrocarbon profile similarities (generalized square distance between colony centroids) of *L. humile* queens based on 12 variables and recipient colony response (0=adoption, 1=physical attack, 2=intruder killed) to non-nestmate queens introduced in queenless (diamonds), single queen (squares), and multiple queen (triangles) colonies.

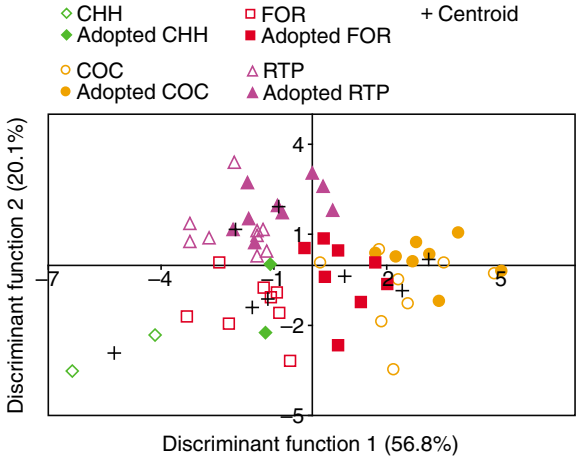

Fig. 3. Discriminant analysis of nine variables selected by stepwise discriminant analysis for *L. humile* queens from four colonies (CHH, COC, FOR, RTP). Cuticular hydrocarbons of queens were sampled 24 h before introduction and 2 weeks after adoption by queenless recipient FOR colonies (COC and FOR queens) and queenless recipient RTP colonies (CHH and RTP queens) using a non-destructive method. + marks the centroid of each group.

colonies according to MANOVA (Wilks' $\lambda<0.0001$, $F=8.80$, d.f.=93, 9.9, $P=0.0004$) with function 1 and function 2 explaining 80.4% and 13.9% of the total variation. DA of CHC sampled by the non-destructive method showed that all queens could be distinguished and correctly classified into their colony of origin based on 13 variables selected by stepwise DA (Wilks' $\lambda=0.0012$, $F=13.95$, d.f.=39, 62.9, $P<0.0001$), with function 1 (64.8% of variation) separating COC and FOR from CHH and RTP, while function 2 (26.5% of variation) distinguished RTP from CHH and COC from FOR (Fig. 1B). Squared distances between colony means obtained by DA of these 13 discriminating peaks followed the same pattern as with solvent extraction (Table 1), although they were not associated with those obtained for queens from sources used in the 24 h adoption assay and extracted by solvent (Pearson's $r=0.78$, $P=0.0689$). Identified discriminating peaks by stepwise DA included seven compounds selected when hexane-extracted queen CHC were analyzed (*n*-C29, 5-MeC29, 5-MeC30, 5-MeC31, xC31:1, xC33:1, 5-MeC34), and three unidentified compounds. These data show that similar results were obtained with the non-destructive approach and with solvent extraction.

Changes in queen cuticular hydrocarbon profiles following adoption

FOR and RTP queens sampled 24 h before and 2 weeks after adoption by queenless FOR colonies, and COC and CHH queens sampled 24 h before and 2 weeks after adoption by queenless RTP colonies, were distinguished based on their CHC profiles according to MANOVA performed on 32 variables (Wilks' $\lambda<0.0001$, $F=2.22$, d.f.=217, 121.8, $P<0.0001$). DA on nine variables selected by stepwise DA also showed that queens could be differentiated (Wilks' $\lambda=0.0101$, $F=4.41$, d.f.=63, 220.13, $P<0.0001$; Fig. 3). COC and FOR queens could be distinguished based on CHC sampled before adoption, but two COC queens were classified as FOR queens after adoption. FOR queens were classified as a separate group after adoption by their nestmate queenless workers. RTP and CHH queens were differentiated before and after adoption. The distance between centroids for COC queens before and after adoption (3.85) was not greater than for FOR queens before and after adoption (9.87), suggesting that only slight changes in CHC profiles of COC queens were detected. However, after adoption, FOR and COC queens were less dissimilar than before adoption as indicated by a reduction in the distance between centroids of these two colonies from 22.24 to 8.52, suggesting that queen CHC changes after adoption may have produced more similar profiles. Similarly, the distance between centroids decreased in CHH and RTP queens after adoption (from 40.89 to 17.59). Overall, changes in queen CHC profiles after adoption suggest a reduction in phenotypic cue dissimilarities between colonies. However, a larger sample size and examining a greater number of colonies would be needed to further support this trend.

Queens treated with non-nestmate queen cuticular hydrocarbons: chemical profiles and worker aggression

RTP queens treated with (i) nestmate RTP queen CHC, (ii) non-nestmate FOR queen CHC, or (iii) hexane (control) could be distinguished by DA on four variables (Wilks' $\lambda=0.2277$, $F=5.48$, d.f.=8, 40, $P=0.0001$) selected out of 30 variables by stepwise DA, although these groups could not be distinguished according to MANOVA performed on 24 variables (Wilks' $\lambda=0.0007$, $F=1.61$, d.f.=46, 2, $P=0.4582$). The DA on four variables showed that function 1 (explaining 60.1% of variance) differentiated RTP queens treated with FOR-CHC from queens treated with RTP-CHC and solvent control queens, and function 2 (explaining 39.9% of variance) indicated some differences in the CHC profiles of RTP-treated and solvent control queens (Fig. 4A). The DA correctly classified 85.2% of the individuals. The four discriminating variables were 5-MeC29, 5-methyldotriacontane (5-MeC32), xC33:1 and one unidentified compound.

The proportion of treated RTP queens attacked by RTP workers was higher for queens treated with FOR queen CHC (0.56) than for queens treated with RTP queen CHC (0.22; $t=2.47$, $N=4$, $P=0.0343$) or solvent control (0.22; $t=2.47$, $N=4$, $P=0.0343$). CHC profiles of the solvent-treated queens that were attacked were more similar to the CHC profiles of queens treated with FOR CHC than other solvent-treated queens, whereas profiles of RTP queens treated with RTP CHC that were attacked were less similar to solvent-treated queens (controls) than the treated queens that were not attacked (Fig. 4A). The DA on five variables selected from a total of 31

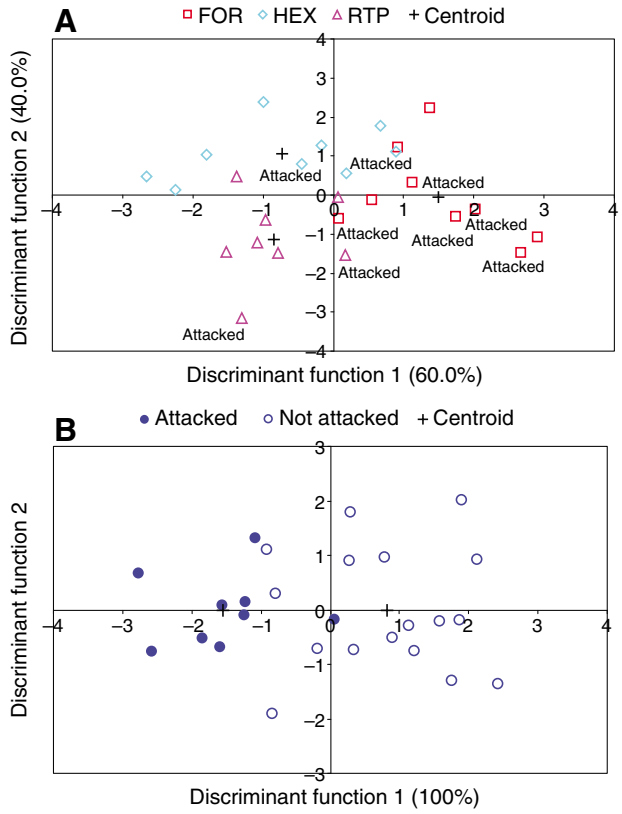

Fig. 4. (A). Discriminant analyses of four variables selected by stepwise discriminant analysis for three groups of *L. humile* queens each treated with nestmate queen hydrocarbons (RTP), non-nestmate queen hydrocarbons (FOR), and hexane (HEX) and (B) discriminant analyses of five variables selected by stepwise discriminant analysis for two groups of treated *L. humile* queens that were either attacked or not attacked by nestmate workers. + marks the centroid of each group.

variables by stepwise DA distinguished attacked from non-attacked queens (Wilks' λ=0.42, F=5.47, d.f.=5, 20, P=0.0025) with 100% of the variance explained by function 1 and 84.6% of the queens classified to the correct group (Fig. 4B). These five discriminating peaks were identified as *n*-C27, 5-MeC32, xC33:1 and two unidentified compounds.

DISCUSSION

We demonstrated that cuticular hydrocarbons mediate *L. humile* queen discrimination by workers based on the following evidence: (1) queens from different colonies can be statistically distinguished by unique CHC profiles; (2) workers accept non-nestmate queens more readily when the queen CHC profiles are similar to those of nestmate queens; (3) the CHC profiles of introduced and recipient colony queens become more similar after queen adoption; and (4) queens treated with non-nestmate queen CHC elicit worker aggression. Previous studies have shown that CHC mediate nestmate–non-nestmate worker discrimination in *L. humile* (Liang and Silverman, 2000; Greene and Gordon, 2007; Torres et al., 2007), however, behavioral and chemical data supporting the role of CHC in queen discrimination have not been previously reported. We propose that over time, adoption of non-nestmate queens may produce changes in colony genotypic composition and in recognition cues among colony members. Moreover, these changes could increase within-colony cue diversity and lead to recognition template expansion with subsequent reduced aggression towards non-nestmates as suggested for polygynous (Keller and Passera, 1989; Vander Meer and Morel, 1998) and polydomous (van Wilgenburg et al., 2006) ant species. Therefore, non-nestmate queen adoption in *L. humile* could promote lower aggression towards genetically similar colonies, thereby leading to more open colonies.

In queenless and single-queen colonies similarity of the CHC of introduced and resident queens appeared to guide the responses of workers in the resident colony (queens adopted, attacked or killed). Different worker responses to various introduced queens suggests that workers discriminate among queens by matching the cues from newly encountered queens with an internal nestmate queen template, which may persist even in queenless colonies. However, the lack of correlation between worker response towards introduced non-nestmate queens and the degree of CHC profile similarity of introduced and resident queens found in multiple queen colonies, suggests an influence of social context on the acceptance threshold. It is possible that the slight heterogeneity of CHC among multiple queens affects the stringency of the workers' internal template, thus lowering the queen acceptance threshold, or that subsets of workers tend predominantly one or two queens and form a narrow template, thus rejecting new queens. Queen presence does not affect worker aggression toward non-nestmate workers in *L. humile* (Caldera and Holway, 2004); however, queens do affect the aggressive response of workers to non-nestmate queens in this species (Vásquez and Silverman, 2008a). Similarly, queens influence worker aggressive behavior in other ants (Vienne et al., 1998; Provost, 1989; Boulay et al., 2003). Therefore, it is possible that *L. humile* queen pheromones, which influence other aspects of recognition, including aggression towards female sexual larvae (Passera, 1995), may also affect nestmate recognition at different levels. A flexible acceptance threshold may result from differences in recognition context (Reeve, 1989) and fluctuations in the cost of recognition errors (Liebert and Starks, 2004). For example, if a colony's survival is at high risk, a reduction in the cost of accepting foreign conspecifics is expected (Sudd and Franks, 1987). The positive relationship between non-nestmate *L. humile* queen adoption and queen CHC similarity may reflect this cost-benefit trade-off particularly in queenless colonies. Studies on the fitness consequences of non-nestmate queen adoption into queenless colonies should shed light on these colony-level decision processes.

Non-destructive sampling of CHC allowed us not only to detect differences in CHC profiles between colonies, but also revealed slight temporal changes in these patterns. The CHC of adopted queens changed, but contrary to our expectation, the profiles of adopted non-nestmate queens did not change more than those of adopted nestmate queens. The CHC of Argentine ant queens are dynamic, changing quantitatively and qualitatively in relation to queen ovarian activity (de Biseau et al., 2004). Queens have considerable amounts of monomethylalkanes (5-MeC27 to 5-MeC34) and alkenes (C29:1, C31:1, C33:1) (de Biseau et al., 2004), whereas workers do not have these compounds (or have very low amounts) but have dimethylalkanes and trimethylalkanes (diMe- and triMeC33, C35 and C37) as major compounds (Liang et al., 2001). These qualitative differences could result from selective biosynthesis of CHC, with shorter and monomethylalkanes predominantly produced by queens through enzymes that regulate the synthesis of hydrocarbons of different chain length (Blomquist et al., 1998), or by selective transfer of CHC from oenocytes, which produce them, to the cuticle *via* lipophorin (Schal et al., 2003). The distinct CHC profiles of these two castes suggest a limited cue exchange between queens and

workers, and may partially explain the lack of more pronounced CHC changes in adopted non-nestmate queens. Therefore, the subtle profile changes observed in both nestmate and non-nestmate queens adopted by queenless colonies could reflect physiological changes, although we cannot rule out possible acquisition of CHC from host colony workers since queen CHC between colonies were more similar after adoption. Hence, studies examining CHC profiles of non-nestmate queens adopted by queenright colonies may reveal that greater changes in queen recognition cues occur when host colony queens and workers are present.

In our assays, queens acquired queen CHC mechanically from glass surfaces. Similarly, when workers of this species were exposed to large quantities of exogenous CHC they incorporated long-chain CHC (C35-C37) within the range of their intrinsic CHC (Liang and Silverman, 2000). It is not known, however, whether in natural interactions queens or workers would selectively acquire more queen or worker CHC. Transfer of CHC between individuals of the same colony, between mixed species and in dulotic and inquiline species is well documented (Soroker et al., 1994; Howard et al., 1980; Vander Meer and Wojcik, 1982; Kaib et al., 1993). However, it has been suggested that unlike some other ant species in which colony odor is derived from the queen (e.g. Carlin and Hölldobler, 1986) or transferred from worker to queen (Lahav et al., 1998), *L. humile* represents an alternative model for colony odor formation since reproductives and non-reproductives have very different CHC profiles (de Biseau et al., 2004). Therefore, *L. humile* colonies appear to lack a unified colony gestalt odor and instead have two subsets of odors, queen-derived and worker-derived. Thus, minor changes in queen CHC following adoption may reflect these presumed caste-specific Gestalt. Similarly, in some other ants, queens appear not to be important contributors to the colony Gestalt and have queen-specific profiles (Boulay et al., 2003; Dahbi and Lenoir 1998; Dietemann et al., 2003). Furthermore, individuals within a polydomous colony can differ in their cue profiles due to incomplete CHC transfer (van Wilgenburg et al., 2006), suggesting not only the presence of subsets of colony odor but also the formation of distinct templates within a colony. However, we cannot rule out the possibility that a colony gestalt odor based on unknown compounds may exist, and that CHC are used exclusively as caste signals and not as colony recognition cues. Studies examining the role of these caste-specific CHC in *L. humile* nestmate recognition would further support our suggested model.

Worker aggression towards nestmate queens treated with non-nestmate queen CHC supports the view that CHC are important cues in nestmate queen recognition. Queens were distinguished based upon their CHC profiles, with most queens that were treated with non-nestmate CHC grouping together. The few queens treated with nestmate CHC or solvent control that were unexpectedly attacked were either more similar to the non-nestmate CHC-treated queens or less similar to the solvent control than other queens in the group, suggesting that the gentle rotation of ants in glass vials could have affected CHC profiles. For example, while some CHC could be acquired from the glass surface, native CHC could also be lost to the glass surface during this treatment procedure. Alternatively, physiological or behavioral variability among queens within a colony might have affected our results. These concerns could be addressed in future experiments by testing queens of known ages, by optimizing the time of exposure to minimize unintended CHC removal, by working with more inert substrates (e.g. silanized glass) or by direct application of precise CHC quantities to queens. Compounds that appear to be associated – at least statistically – with worker behavior in this assay were monomethyl alkanes and alkenes that are either absent or occur in considerably lower quantities in the CHC of workers.

We found that quantitative variation in queen CHC profiles reflects colony identity, and direct manipulation of queen CHC affected aggression behavior in *L. humile* workers. Additionally, our results indicate that not all CHC but only a statistically derived subset of compounds, could mediate queen discrimination; but whether all or only some of the CHC are indeed important in nestmate recognition remains unknown. Interestingly, the subset of hydrocarbons selected by DA in our experiments belong to at least two structural classes, methyl-branched alkanes and *n*-alkenes, suggesting that a mixture of CHC of different structural classes varying in their relative proportions across colonies rather than a few compounds of a single structural class may be used as nestmate recognition cues. Our results further support the view that colony membership in *L. humile* is conveyed by a mixture of structural classes as suggested by the finding that a mixture of *n*-alkanes supplementing nestmate worker CHC profiles elicited high aggression levels whereas no aggressive response was elicited when the mixture of *n*-alkanes was presented alone (Greene and Gordon, 2007). The role of specific compounds or chemical classes as nestmate recognition cues seems to differ considerably among social insects. For example, methyl-branched alkanes, *n*-alkanes and an alkene and *n*-alkane mixture have been shown to be important colony recognition cues in wasps (Dani et al., 1996; Gamboa et al., 1996), whereas in ants, methyl-branched CHC (mono- and di-methylalkanes and monomethylalkenes) are more colony-specific than *n*-alkanes (Bonavita-Cougourdan et al., 1987; Provost et al., 1992; Astruc et al., 2001; Lucas et al., 2004), although dimethylalkanes seem not to be important in nestmate recognition in *Cataglyphis* species (Dahbi et al., 1996). We cannot rule out that additional recognition-active compounds other than those that seem to be linked to colony chemical profile specificity may also be important. Therefore, chemical supplementation studies testing these presumably important CHC structural classes or the compounds individually or in mixtures, and at different concentrations, could corroborate our findings.

Our combined behavioral and chemical data shed light on the dynamics and complexity of nestmate recognition in *L. humile* and suggest that interspecific variation in CHC and its perception may have colony-level consequences, e.g. the formation of more open colonies. Further investigation on recognition processes in this and other invasive ant species would enhance our understanding of the factors responsible for changes in their social organization and ecological success.

We thank E. Vargo and W. Watson for helpful comments on the manuscript. We also thank C. Brownie for statistical advice; and A. Carper, K. Donohue, P. Labadie, J. Leonard, F. J. Richard and E. Youngsteadt for technical assistance. This study was supported by the Blanton J. Whitmire Endowment at North Carolina State University and the David R. Nimocks fellowship (G.V.).

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