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Geographical variation in Argentine ant aggression behaviour mediated by environmentally derived nestmate recognition cues

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Social insects use a complex of recognition cues when discriminating nestmates from non-nestmate conspecifics. In the Argentine ant, *Linepithema humile*, recognition cues can be derived from exogenous sources, and *L. humile* acquires prey-derived hydrocarbons that are used in nestmate discrimination. We studied Argentine ant population-level distinctions in response to external recognition cues. Ants belonging to a California population were strongly affected by the imposition of prey-derived hydrocarbons, with spatially isolated colony fragments that had been fed different cockroach prey (*Blattella germanica* or *Supella longipalpa*) showing high and injurious intracolony aggression when reunited. In contrast, colonies of Argentine ants from the southeastern U.S. showed only moderate and noninjurious aggression when subjected to the same treatment. Field-collected colonies of *L. humile* had hydrocarbons in the range of those provided by *S. longipalpa*, and colonies from the southeastern U.S. had significantly higher initial levels of *Supella*-shared hydrocarbons. When fed cockroaches, Argentine ants from both regions acquired additional amounts of *Supella*- and *Blattella*-specific hydrocarbons, with a significant increase in levels of *Blattella*-specific hydrocarbons. Therefore, diet partitioning produced a greater change in the proportion of prey hydrocarbons in the California than in the southeastern U.S. populations, which may be responsible for the altered behaviour observed in the California population. Identifying factors underlying geographical variation in cue expression and/or perception may bring us closer to elucidating the selective forces driving nestmate recognition systems.

In social insects, colony integrity is maintained through a well-developed system for discriminating nestmates from non-nestmate conspecifics, and the recognition signals are chemical and are genetically and/or environmentally based (Crozier & Dix 1979; Gamboa et al. 1986a; Howard & Blomquist 2005). Although recognition cues can be any aspect of an individual's phenotype that reliably signifies colonial membership, chemical cues are widely used because they are potentially information-rich and require little energy to produce, because they are metabolic by-products or passively acquired from the environment. In addition, quantitative and qualitative variations in the levels of chemical signals assure their required complexity. Cuticular hydrocarbons have long been implicated in nestmate recognition in social insects (Vander Meer & Morel 1998) with recent evidence coming from ants (Lahav et al. 1999; Boulay et al. 2000; Liang & Silverman 2000), termites (Clèment & Bagnères 1998) and wasps (Gamboa et al. 1996; Singer 1998). In social insects, cuticular hydrocarbon composition is highly diverse and may vary with age, geographical origin, sex, season, caste, rank and nest (e.g. Vander Meer et al. 1989; Ichinose 1991; Howard 1993; Sledge et al. 2001). Such variation may occur at the individual, colony or population level.

Elucidating factors responsible for variation in cuticular hydrocarbon composition and interpreting the meaning of such variation have been an active area of research for two reasons. First, cuticular hydrocarbons are critical for nestmate recognition, and colony-level variation may provide information about a wide range of parameters, such as territoriality, breeding patterns and foraging ranges (e.g. Kaib et al. 2002; Delphia et al. 2003; Haverty et al. 2003). Second, variation in cuticular hydrocarbon patterns can be used in chemotaxonomic studies, whereby groups of chemical compounds are used as genetic indicators in the establishment of taxonomic relationships (Lockey 1991). Many taxa of social insects show

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intraspecific variation in the types and proportions of cuticular hydrocarbons. The potential of this variation for chemotaxonomic studies has been demonstrated in ants (Oldham et al. 1994; Akino et al. 2002), wasps (Dapporto et al. 2004) and most notably termites (e.g. Kaib et al. 1991; Haverty et al. 2000), where morphological and behavioural approaches often prove unrewarding.

Nestmate recognition systems using cues originating from endogenous and/or exogenous sources provide individuals with accurate information about colony membership (Gamboa et al. 1986b). The relative contribution of genetic and environment-based recognition cues is often addressed in laboratory studies, where the environmental component is kept constant and aggression between individuals is measured. In ants, decreases (Obin 1986; Stuart 1987; Crosland 1989; Boulay et al. 2000; Chen & Nonacs 2000; Buczkowski et al. 2005) and no change or increases (Le Moli et al. 1992; Heinze et al. 1996; Holway et al. 1998; Stuart & Herbers 2000; Suarez et al. 2002) in aggression between colonies reared under uniform laboratory conditions have been reported. A significant decrease in aggression suggests that nestmate discrimination is influenced largely by environmental cues, and maintenance of aggression implies a strong genetic component. Gamboa et al. (1986b), however, cautioned that evidence of dominance of genetic cues in the laboratory is not evidence that genetic cues are more important than environmental factors in field contexts. The relative importance of genetic and environmental factors ultimately depends on the organism's genetic structure, ecology and social context (Buczkowski & Silverman 2005).

Nestmate recognition in the Argentine ant, *Linepithema humile*, integrates both genetic (Tsutsui et al. 2000; Suarez et al. 2002) and environmental (Chen & Nonacs 2000; Liang & Silverman 2000) inputs. Liang & Silverman (2000) examined Argentine ant nestmate recognition and its modification by diet in a California population and showed that hydrocarbons acquired from cockroach prey, *Supella longipalpa* and *Blattella germanica*, were incorporated into the ant's intrinsic cuticular hydrocarbon profile, and that workers from colony fragments of a single colony reared in isolation on different diets displayed high aggression when reunited. Furthermore, by isolating Argentine ant colony fragments for as few as 28 days and feeding them different prey (either *B. germanica* or *S. longipalpa*), re-establishment of internest communication was prevented (Silverman & Liang 2001).

In our ongoing efforts to understand the influence of prey-acquired hydrocarbons on nestmate recognition and colony dynamics in the Argentine ant, we attempted to induce intracolony aggression within a colony of *L. humile* collected from Winston-Salem, North Carolina, U.S.A., by following the methodology of Liang & Silverman (2000). Despite prolonged (4-month) rearing on distinct prey diets, we were unable to induce high aggression in this colony. Former nestmates reared on the different prey displayed only moderate, noninjurious aggression (level 1 or 2 on a scale of 0–4; Suarez et al. 1999), and colony fragments merged readily when reunited.

Based on this experience, we suspected that the phenomenon described earlier by Liang & Silverman (2000) and Silverman & Liang (2001) for a single colony from a single location in California cannot be universally applied. Thus, we hypothesized that between-colony variation occurs in Argentine ant response to prey-derived nestmate recognition cues. More specifically, from recent evidence of differences in *L. humile* population structure between California and the southeastern U.S. (Buczkowski et al. 2004), we predicted regional differences in prey-induced aggression as well. Furthermore, we hypothesized that any colony-level variation in prey-induced aggression behaviour has an underlying chemical basis, and consequently, we examined qualitative and quantitative differences in colony hydrocarbon profiles before and after rearing on *Blattella germanica* or *Supella longipalpa*. We discuss our results in the context of geographical differences in genetic diversity within introduced *L. humile* populations (Buczkowski et al. 2004). A comparison of colonies across populations could provide insights on how regional events affect the interplay of intrinsic and extrinsic nestmate recognition processes.

METHODS

Collection and Rearing of Laboratory Colonies

We collected ants along two 700-km transects. One transect was through an area of a supercolony in California (38.3–33.0°N and 122.2–117.0°W) described by Tsutsui et al. (2000), and the other spanned three states in the southeastern U.S.A.: North Carolina, South Carolina and Georgia (33.0–36.1°N and 72.0–84.4°W). We sampled seven sites in California: Berkeley, Corona, Davis, Escondido, Ojai, Pleasanton and Riverside. In the southeastern U.S. we sampled 11 sites: North Carolina (6: Chapel Hill, Emerald Isle, Greenville, Jacksonville, Shallotte and Winston-Salem); South Carolina (2: Greenville and Greer) and Georgia (3: Barnesville, Fayetteville and Griffin).

For each location, we established two colony fragments each consisting of 5000 workers, several dozen queens and numerous brood. Colonies were maintained in soil-free, Fluon-coated trays containing nests composed of plastic dishes filled with moist grooved plaster. Colonies were provisioned with 25% sucrose solution and artificial diet (Bhatkar & Whitcomb 1970) ad libitum, and hard-boiled egg once a week. For each location, colony fragments were also reared on one of two prey diets: *Supella longipalpa* male and female adults, or *Blattella germanica* female adults. The cockroaches were presented live but injured to facilitate prey handling by the ants. All colonies were maintained at 24 ± 1°C and 50 ± 10% RH, on a 12:12 h light:dark cycle.

Aggression Tests (Nestmate Discrimination Bioassay)

To test whether colony partitioning alone had an effect on aggression, we divided a colony (Winston-Salem, 20 000 workers) into two equal-size fragments. We then tested for aggression between the fragments 6 months after rearing in isolation under identical environmental and dietary conditions. We also examined aggression towards workers that had been removed from and immediately reintroduced to their original colony. We tested for aggression at two time points: day 0 (within minutes of splitting the colony) and 6 months later. Sixty worker introductions were performed for each of two time points, 30 introductions with workers from one colony acting as intruders and 30 with workers from the other colony acting as intruders. We used an assay that measured the level of aggression in single worker introductions into a resident colony (Roulston et al. 2003). Aggression was scored on a 0–4 scale (Suarez et al. 1999: 0: ignore, 1: touch, 2: avoid, 3: aggression (lunging, brief bouts of biting and/or pulling), 4: fighting (prolonged aggression, also abdomen curling to deposit defensive compounds)). The same assay was also used to test for changes in intracolony aggression in colony fragments raised on distinct prey. Ten aggression tests per colony per interval were conducted, five with *Blattella*-fed workers acting as intruders, and five with *Supella*-fed workers acting as intruders. For each test, we allowed the intruder up to 25 encounters with resident ants. Each instance of direct physical contact between the intruder and any resident was regarded as an encounter. Individual ants were not tested in more than one trial. Assays were conducted blind: the observer who recorded worker aggression levels did not know the source of the interacting colony fragments and was unfamiliar with the hypothesis being tested. Data were analysed as the maximum score of 25 encounters (Roulston et al. 2003). We also report the average incidence of injurious aggression for each region. We defined injurious aggression as aggression involving direct physical contact (level ≥ 3) characterized by behaviours such as biting, pulling legs and/or antennae and spraying defensive chemicals. If the intruder and the resident ants engaged in a highly aggressive behaviour (level 4) for more than 10 s, a fluoned ring was placed around the fighting individuals. The trial was permitted to continue for 2 h and the physical condition of the intruder was examined every 1–4 min. Mortality of the intruders was recorded. Aggression assays were performed 12, 22, 32, 42, 52, 82, 112, 132 and 162 days after the colonies were first provisioned with prey.

Extraction, Isolation and Chemical Analysis of Cuticular Hydrocarbons

Ants were killed by freezing (−20 °C) before hydrocarbon (HC) extraction. External lipids were extracted from the cuticle by immersing 10 whole thawed ants in 1 ml of hexane for 10 min, followed by a brief second rinse. The samples were gently shaken for the first and last 20 s of the soak period. Hexane extracts were concentrated under nitrogen to about 100 μl and applied to prewetted (hexane) Pasteur pipette minicolumns filled with 500 mg of silica gel (63–200 mesh size, Selecto Scientific, Georgia, U.S.A.). The HC fraction was eluted with 6 ml of hexane. The extract was resuspended in 5 μl of hexane and 1 μl was injected (two ant equivalents). Capillary gas chromatography (GC) was carried out using an HP 5890 gas chromatograph equipped with a DB-1 column (30 m × 0.25 mm × 0.25 μm film thickness) and interfaced with a G1045A Chemstation (version A05.01). Oven temperature was held at 40 °C for 2 min, then increased to 200 °C at a rate of 20 °C/min and finally to 310 °C at 40 °C/min. Injector and flame-ionization detector were at 270 °C and 320 °C, respectively. Helium was the carrier gas, and the make-up gas was nitrogen. Quantitative data were obtained by integrating the peaks and calculating the percentage area under each peak. Specific peak identity was determined with hydrocarbon standards and by matching diagnostic peaks with those from earlier studies (Jurenka et al. 1989; Liang et al. 2001).

Data Analysis

All behavioural and hydrocarbon data analyses were performed using SAS 8.1 statistical software (SAS 2002). Aggression scores were averaged in the following order: (1) over replicates within a colony ($N = 10$), (2) over colonies within a time period (California: $N = 7$; southeastern U.S.: $N = 11$) and (3) over time periods ($N = 9$). Regional differences in levels of aggression induction were compared on days 12 and 162 and for an average of all time periods using PROC TTEST, which examines the equality of variances. We report results of one of two types of *t* tests, depending on the equality of variances. Results of a Student's *t* test are reported when the variances were homogenous. In cases where the variances were unequal, we used the Welch *t* test with a Satterthwaite correction (Zar 1999). Next, we determined the slope for aggression increase over time for each colony (PROC REG) and tested the hypothesis that the slope for each region (averaged over colonies) is not different from zero (PROC UNIVARIATE). Subsequently, we compared regression slopes between regions using PROC TTEST. We used values averaged over all periods to compare the incidence of injurious aggression and the number of dead workers between regions.

To determine whether Argentine ants acquired key prey-specific hydrocarbons, we first identified key diagnostic hydrocarbons provided by each prey. For *B. germanica*, we selected peaks corresponding to 11- and 13- and 15-methylnonacosane and 3-methylnonacosane. Both hydrocarbons are relatively abundant in adult *B. germanica*, constituting approximately 14.5 and 10.3% of the total hydrocarbons, respectively (Jurenka et al. 1989). Furthermore, our preliminary analysis indicated that both hydrocarbons were acquired by Argentine ants. For *S. longipalpa*, we selected four hydrocarbons: 15,19-dimethylpentatriacontane, 5,9- and 5,11-dimethylpentatriacontane, 13- and 15- and 17- and 19-methylheptatriacontane, and 15,19- and 17,21-dimethylheptatriacontane. These hydrocarbons are present in *S. longipalpa* at 19.0, 9.1, 8.5 and 25.7%, respectively, and each is acquired by Argentine ants from *S. longipalpa* (Liang & Silverman 2000; Liang et al. 2001).

We hypothesized that population-specific differences in response to environmental hydrocarbons may result from differences in the initial levels of hydrocarbons (percentage of total) that are within the range of those provided by the prey (i.e. there would be a negative relation between the level of aggression induced and the level of hydrocarbons similar to those provided by the prey). Therefore, we examined colonies in both regions for the presence of *Blattella*-specific and *Supella*-specific hydrocarbons and compared the initial levels of those hydrocarbons between regions (PROC TTEST). We determined whether Argentine ants acquired significant amounts of prey hydrocarbons following different diet provisions by comparing their initial levels of prey-specific hydrocarbons (field-collected colonies; day 0) to levels on day 52 and performing analysis of variance (ANOVA) using PROC GLM. We chose to examine hydrocarbon profiles on day 52 to allow enough time for ants to acquire maximal levels of prey hydrocarbons. Silverman & Liang (2001) reported that colony fragments fed *B. germanica* and *S. longipalpa* acquired prey hydrocarbons within 14 days and aggression between colony fragments was maximal after 28 days of isolation. We compared the relative amounts of prey-specific hydrocarbons acquired by Argentine ants in California and in the southeastern U.S. for possible regional differences in prey hydrocarbon acquisition using PROC GLM. All analyses were performed for each diet in two ways: (1) by considering each hydrocarbon separately and (2) by combining diet-specific hydrocarbons into a single group.

RESULTS

Intracolony Aggression

Colony partitioning did not result in aggression between colony fragments ($\overline{X} \pm \mathrm{SE} = 0.0 \pm 0.0$) and no aggression was observed when workers were reintroduced into their own colony (0.0 ± 0.0). Examination of geographical variation in Argentine ant nestmate recognition behaviour revealed different responses to environmental cues between Californian and southeastern U.S. populations. Colonies from the California population were affected by the imposition of prey-derived hydrocarbons because colony fragments raised on either *B. germanica* or *S. longipalpa* displayed high aggression when reunited (Fig. 1). In contrast, colonies from the southeastern U.S. population displayed only moderate aggression. In California, the mean ± SE level of aggression was 3.22 ± 0.06 (range over time periods 2.91–3.67; range over colonies 3.02–3.42), and in the southeastern population it was 1.68 ± 0.10 (range over time periods 1.26–2.10; range over colonies 1.22–2.37, $P < 0.0001$). A significant difference in the mean ± SE level of induced aggression between regions was already evident by day 12 (California: 2.91 ± 0.09, southeastern U.S.: 1.78 ± 0.20, $P = 0.002$). Over time, the difference between regions grew larger, and by day 162, California colonies became highly aggressive (3.67 ± 0.13), while southeastern U.S. colonies remained relatively nonaggressive (1.60 ± 0.17, $P < 0.0001$). The slopes for change in aggression across days 12–162, however, were not significant for either population (California: $P = 0.108$, southeastern U.S.: $P = 0.260$), and the difference between slopes was also not significant ($P = 0.058$), indicating that colonies from both regions developed their respective aggression levels within the first 12 days of prey exposure. Aggression levels were maintained throughout the study despite slight fluctuations between time periods.

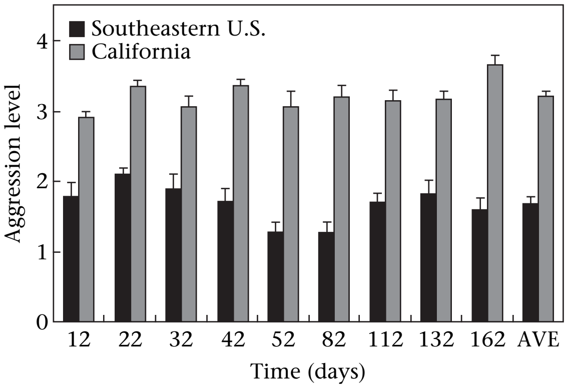

Figure 1. Mean ± SE aggression levels between *L. humile* colony fragments from each population (southeastern U.S.: $N = 11$ colonies; California: $N = 7$ colonies) that were fed different diets. *X* axis shows time in days since colonies were first provisioned with cockroach prey. Initial mean ± SE aggression level (day 0) was 0.0 ± 0.0. AVE: average across all days.

The incidence of injurious aggression (≥ level 3) within California colonies was relatively high ($\overline{X} \pm$ SE instances of injurious aggression: 14.6 ± 1.2, $N = 15\,750$ encounters (25 encounters/intruder × 10 intruders × 9 time intervals × 7 colonies)) compared with that of southeastern U.S. colonies (2.7 ± 0.7, $N = 24\,750$ encounters (25 encounters/intruder × 10 intruders × 9 time intervals × 11 colonies); $P < 0.0001$). We recorded 2.2 ± 0.3 dead workers per 10 intruder introductions in fights involving ants from California, whereas no mortality was observed when ants from the southeastern U.S. interacted ($P < 0.0001$).

Comparison of Hydrocarbon Profiles

Field-collected colonies of *L. humile* already possessed hydrocarbons in the range of those provided by both insect prey (Table 1). Colonies in both California and the southeastern U.S. initially had relatively low levels of hydrocarbons shared with *Blattella* (<1% of total hydrocarbons), with no difference between regions ($P = 0.837$). Compared to *Blattella* hydrocarbons, colonies in both regions had higher initial levels of the diagnostic hydrocarbons shared with *Supella* (4–9% of total hydrocarbons). Argentine ants from California had lower initial levels of hydrocarbons shared with *Supella* (mean ± SE = 23.55 ± 1.32%) relative to colonies from the southeastern U.S. (28.60 ± 0.87%; $P = 0.016$).

Argentine ants from both Californian and southeastern U.S. populations that had been fed *B. germanica* acquired significant levels of *Blattella*-derived hydrocarbons (Table 1). When fed *S. longipalpa*, the levels of three of the four *Supella* diagnostic hydrocarbons increased in the California colonies: 13- and 15- and 17- and 19-methylheptatriacontane ($P = 0.024$), 15,19-dimethylpentatriacontane ($P = 0.009$) and 5,9- and 5,11-dimethylpentatriacontane ($P = 0.038$), while the level of 15,19- and 17,21-dimethylheptatriacontane increased ($P = 0.0002$) in the southeastern U.S.

Table 1. Initial cuticular hydrocarbon levels in California and southeastern U.S. colonies and changes in hydrocarbon levels after rearing on *B. germanica* and *S. longipalpa* prey

Geographical region	Cuticular hydrocarbon*	Initial†	Raised on *B. germanica*‡	Net change§	*P***	Raised on *S. longipalpa*‡	Net change §	*P***
California ($N=7$)	BG1	0.00±0.00	4.26±0.37	4.26	<0.0001	0.16±0.16	0.16	0.356
	BG2	0.26±0.26	3.97±0.30	3.71	<0.0001	0.73±0.40	0.47	0.190
	BG1+BG2	0.26±0.26	8.23±0.60	7.97	<0.0001	0.89±0.38	0.63	0.096
	SL1	4.17±0.14	2.80±0.24	−1.37	0.0005	7.30±0.88	3.13	0.009
	SL2	4.41±0.41	4.59±0.29	−0.18	0.699	6.33±0.46	1.92	0.024
	SL3	6.61±0.58	2.74±0.12	−3.86	0.0005	4.41±0.48	−2.19	0.027
	SL4	8.36±0.27	4.64±0.26	−3.71	<0.0001	10.47±0.75	2.11	0.038
	SL1+SL2+ SL3+SL4	23.55±1.32	14.77±0.57	−8.78	0.0002	28.51±1.25	4.96	0.093
Southeastern U.S. ($N=11$)	BG1	0.32±0.18	4.83±0.61	4.51	<0.0001	0.27±0.18	−0.06	0.784
	BG2	0.00±0.00	5.64±0.69	5.64	<0.0001	0.88±0.37	0.88	0.039
	BG1+BG2	0.32±0.18	10.47±1.26	10.15	<0.0001	1.15±0.43	0.83	0.100
	SL1	6.78±0.22	4.60±0.39	−2.18	<0.0001	7.68±0.35	0.9	0.102
	SL2	6.10±0.32	3.58±0.23	−2.52	<0.0001	4.60±0.16	−1.50	0.003
	SL3	7.06±0.26	5.62±0.59	−1.43	0.053	10.74±0.60	3.68	0.0002
	SL4	8.66±0.24	5.98±0.26	−2.68	<0.0001	7.59±0.29	−1.07	0.028
	SL1+SL2+SL3+SL4	28.60±0.87	19.78±1.35	−8.82	<0.0001	30.61±1.17	2.01	0.246

*Hydrocarbon designations: *B. germanica* specific: BG1 = 11- and 13- and 15-methylnonacosane; BG2 = 3-methylnonacosane; and *S. longipalpa* specific: SL1 = 15,19-dimethylpentatriacontane; SL2 = 13- and 15- and 17- and 19-methylheptatriacontane; SL3 = 15,19- and 17,21-dimethylheptatriacontane; SL4 = 5,9- and 5,11-dimethylpentatriacontane. Mean ± SE initial levels of cuticular hydrocarbons in field-collected colonies (day 0) expressed as a percentage of total hydrocarbons.

†Mean ± SE initial levels of cuticular hydrocarbons in field-collected colonies (day 0) expressed as a percentage of total hydrocarbons.

‡Levels of hydrocarbons (day 62) after rearing on *B. germanica* or *S. longipalpa*.

§The change in hydrocarbon levels after rearing on cockroach prey (day 62–day 0).

**Based on results of ANOVA.

population. When all four *Supella*-derived hydrocarbons were analysed as a single group, Argentine ant colonies in California acquired more *Supella*-derived hydrocarbons relative to ants from the southeastern U.S. (4.96 versus 2.01%), however, the net change was not significant in either population. Furthermore, *L. humile* raised on *B. germanica* lost a significant proportion of *Supella*-specific hydrocarbons in both Californian ($P = 0.0002$) and southeastern U.S. ($P < 0.0001$) populations. Little change in levels of *Blattella*-specific hydrocarbons was evident in Argentine ants raised on *S. longipalpa*.

Interregional comparisons cannot be made by simply evaluating changes in *Supella*-specific hydrocarbons in ants fed *S. longipalpa*, because field-collected colonies possess *Supella*-specific hydrocarbons and their proportion declines when fed *B. germanica*. Since we recorded aggression between colony fragments reared on both diets, we must consider changes in *Supella*-specific hydrocarbons in colonies reared on *S. longipalpa* as well as *B. germanica*. The case of 5,9- and 5,11-dimethylpentatriacontane (SL4, Tables 1 and 2) is illustrative. The relative proportion of this hydrocarbon decreased in southeastern U.S. colonies reared on *S. longipalpa* (−1.07%, $P = 0.028$), but increased in California colonies (2.11%, $P = 0.038$; difference between regions $P = 0.001$). Concurrently, the relative proportion of *Supella*-specific hydrocarbons decreased in colonies fed *B. germanica* (decline in southeastern populations: 2.68%, $P < 0.0001$; decline in Californian populations: 3.71%, $P < 0.0001$; difference between regions: $P = 0.018$). Consequently, the difference in proportions of 5,9- and 5,11-dimethylpentatriacontane between the two diet regimes in our California colonies was larger (4.64 versus 10.47%, $P < 0.0001$) than that in our southeastern U.S. colonies (5.98 versus 7.59%, $P = 0.0006$). If 5,9- and 5,11-dimethylpentatriacontane is a key nestmate recognition cue, then population-level differences in the net change of this hydrocarbon may underlie regional distinctions in the induction of aggression behaviour.

When prey-specific hydrocarbons were analysed collectively, ants fed *B. germanica* did not differ in either the amount of *Blattella*-specific cues that they acquired ($P = 0.207$) or the amount of *Supella*-specific cues that they lost ($P = 0.983$), and *Supella*-fed ants acquired similar levels of *Blattella*- or *Supella*-specific cues ($P = 0.764$ and $P = 0.313$, respectively; Table 2). When considered individually, substantial differences between regions were evident especially with respect to the amount of *Supella*-specific hydrocarbons acquired by colonies fed *S. longipalpa* (Table 2). Most notably, changes in levels of three out of four *Supella*-derived hydrocarbons occurred in opposite directions across populations, possibly further contributing to behavioural differences between regions.

Aggression between colony fragments raised on distinct prey diets is probably caused by two factors pertaining to changes in the levels of key nestmate recognition hydrocarbons: net diet-specific changes in the levels of prey-specific hydrocarbons and differences in the level of prey-specific hydrocarbons between diets. Ultimately, aggression may be induced from a combination of these factors. By comparing final prey-derived hydrocarbon levels between diets for each of the two regions, differences became apparent (Table 3). Liang et al. (2001) suggested that certain *Supella*-derived hydrocarbons, especially 15,19-dimethylpentatriacontane, might be critical

Table 2. Comparison of changes in cuticular hydrocarbon levels in *L. humile* colonies fed *B. germanica* and *S. longipalpa*

Diet	Cuticular hydrocarbon*	Region California	 Southeastern U.S.	*P*†
B. germanica	BG1	4.26‡	4.51	0.777
	BG2	3.71	5.64	0.049
	BG1+BG2	7.97	10.15	0.207
	SL1	−1.37	−2.18	0.073
	SL2	−0.18	−2.52	0.0002
	SL3	−3.86	−1.43	0.020
	SL4	−3.71	−2.68	0.018
	SL1+SL2+SL3+SL4	−8.78	−8.82	0.983
S. longipalpa	BG1	0.16	−0.06	0.471
	BG2	0.47	0.88	0.454
	BG1+BG2	0.63	0.83	0.764
	SL1	3.13	0.90	0.025
	SL2	1.92	−1.50	0.0002
	SL3	−2.19	3.69	<0.0001
	SL4	2.11	−1.07	0.001
	SL1+SL2+SL3+SL4	4.96	2.01	0.313

*Hydrocarbon designations as in Table 1.
†Based on results of ANOVA.
‡Net change in hydrocarbon levels after rearing on cockroach prey (day 62–day 0, averaged over colonies).

to nestmate recognition in *L. humile*. The difference in the level of 15,19-dimethylpentatriacontane between colonies reared on *B. germanica* and *S. longipalpa* was 62% (2.80 versus 7.30) in the California population, but only 40% (4.60 versus 7.68) in the southeastern U.S. population ($P = 0.01$ between regions).

DISCUSSION

We found profound geographical variation in Argentine ant response to extrinsic nestmate recognition cues; ants from a California population were strongly affected by the imposition of prey-derived hydrocarbons, and colonies from a population in the southeastern U.S. were significantly less affected. In other hymenopteran taxa, various environmental factors affect the expression of social behaviour, including latitude (Packer 1990; Miyanaga et al. 1999; Richards 2000), altitude (Eickwort et al. 1996), food and nest site availability (Banschbach & Herbers 1996; Herbers & Banschbach 1999) and season (Yanega 1993; Richards & Packer 1995). We have also highlighted the potential importance of environmental factors in the evolution and persistence of kin and/or nestmate recognition behaviour and emphasize the importance of laboratory manipulations in revealing phenotypic variation that might otherwise remain undiscovered in studies under natural conditions.

Earlier studies have reported either local variation in the expression of nestmate recognition behaviour (Breed et al. 1999) or variation in the types and proportions of social insect cuticular hydrocarbons for chemotaxonomic purposes (e.g. Brown et al. 1996; Akino et al. 2002; Dapporto et al. 2004). We provide new evidence for a sequence of events that ties intraspecific variation in hydrocarbon levels with colony-level consequences: (1) Argentine ants from the two populations showed intrinsic differences in the levels of hydrocarbons shared with certain prey, (2) an environmental factor (exposure to prey hydrocarbons) differentially affected the two populations, and consequently, (3) different levels of aggression developed in the two populations, producing mortality in one but not the other.

Geographical variation in response to extrinsic environmental cues can result from proximate environmental effects, from underlying genetic factors, as well as from the interaction between the two (Bradshaw 1965; Via &

Table 3. Final levels of prey-specific hydrocarbons in *L. humile* colonies fed *B. germanica* and *S. longipalpa*

Region	Cuticular hydrocarbon*	Diet *B. germanica*	 *S. longipalpa*	*P*†
California	BG1	4.26	0.16	<0.0001
	BG2	3.97	0.73	<0.0001
	BG1+BG2	8.23	0.89	<0.0001
	SL1	2.80	7.30	0.002
	SL2	4.59	6.33	0.009
	SL3	2.74	4.41	0.012
	SL4	4.64	10.47	<0.0001
	SL1+SL2+SL3+SL4	14.77	28.51	0.0002
Southeastern U.S.	BG1	4.83	0.27	<0.0001
	BG2	5.64	0.88	<0.0001
	BG1+BG2	10.47	1.15	<0.0001
	SL1	4.60	7.68	<0.0001
	SL2	3.58	4.60	0.002
	SL3	5.62	10.74	<0.0001
	SL4	5.98	7.59	0.0006
	SL1+SL2+SL3+SL4	19.78	30.61	<0.0001

*Hydrocarbon designations as in Table 1.
†Based on results of ANOVA.

Lande 1985; West-Eberhard 1989). Although natural selection has been traditionally considered to be very influential in producing phenotypic variants adapted to conditions present in a certain geographical region, evidence for the influence of environmental factors inducing phenotypic plasticity is less common (e.g. Greene 1989; Arnqvist & Johansson 1998). The pattern of geographical variation in nestmate recognition behaviour in *L. humile* may result from diversity in colony-level experiences resulting from ecological differences and/or from differential rates of evolution of genotypic traits affecting agonistic and/or sensory behaviour. Evidence for the contribution of environmental factors comes from our examination of hydrocarbon profiles in *L. humile* from both ranges. Regional environmental differences may especially promote a differential response to prey-derived hydrocarbons. For example, regional distinctions in diet and nesting material diversity might produce qualitative and quantitative differences in cuticular hydrocarbon composition, which might affect worker response to novel nestmate recognition cues, especially if the new cues overlap with similar preexisting cues. Workers may not recognize newly acquired hydrocarbons as foreign if these are already part of their cuticular constituents. These effects might be especially pronounced with threshold levels of hydrocarbons, above which increases in hydrocarbon levels do not cause a change in behaviour. Relative to Argentine ants from California, ants from the southeastern U.S. had higher initial levels of hydrocarbons shared with *Supella*, particularly 15,19-dimethylpentatriacontane and 13- and 15- and 17- and 19-methylheptatriacontane. Levels of these hydrocarbons may be partly responsible for the lack of within-colony aggression induced in ants from this region. Liang et al. (2001) suggested that certain *Supella*-derived hydrocarbons (especially 15,19-dimethylpentatriacontane) might be critical to nestmate recognition in *L. humile*, because their addition alters hydrocarbon ratios within a spectrum that is sensitive to modification. In *Camponotus* spp., the ratios of certain hydrocarbons affect behaviour between worker castes (Bonavita-Cougourdan et al. 1993) as well as between individual workers (Boulay et al. 2000). We found that Argentine ants in the southeastern U.S. not only have higher initial levels of *Supella*-shared hydrocarbons, but they also incorporate lower amounts of some of those hydrocarbons relative to ants from California. *Supella longipalpa* hydrocarbons alter nestmate recognition in the Argentine ant more profoundly than do those from a number of other insects, including *B. germanica* (Liang et al. 2001). Therefore, regional comparisons of *Supella*-specific hydrocarbons might be especially important in explaining population-level behavioural differences. We found that the proportions of three of four *Supella*-shared hydrocarbons increased in *L. humile* from California, but they either decreased or increased only slightly in the southeastern U.S. colonies.

Besides its being derived from environmental sources, geographical variation in the induction of aggressive behaviour may result from regional differences in genetic diversity. The southeastern U.S. *L. humile* population has higher genotypic diversity and intercolony aggression than does the California population that we sampled (Buczkowski et al. 2004; but see Ingram & Gordon 2003), showing that not all introduced populations within the U.S. are genetically and behaviourally uniform.

Regional differences in genetic diversity may also be manifest in differential response to exogenous cues through modification of the structure and operation of the nestmate recognition system. This system consists of three distinct components: (1) expression, (2) perception and (3) action (Gamboa et al. 1986b; Waldman 1987; Reeve 1989). In the Argentine ant, the origins of the expression component (cue ontogeny) are both genetic (Tsutsui et al. 2000; Giraud et al. 2002; Suarez et al. 2002) and environmental (Chen & Nonacs 2000; Liang & Silverman 2000). Although field-collected *L. humile* reveal regional differences in levels of certain hydrocarbons shared with *Supella*, we do not know whether these are due to genetic and/or environmental factors and whether colonies in either region show temporal variation in cuticular hydrocarbon patterns. In some ants, recognition cues are dynamic and may change throughout the life of the colony (e.g. Vander Meer et al. 1989) and show seasonal variation (Ichinose 1991; Nielsen et al. 1999). Differences in cue perception may also reflect regional distinctions in genetic diversity. Perception involves the development of a recognition template and processing of perceived cues. The template is a collection of recognition cues that an individual considers acceptable (Waldman et al. 1988) with the cues based on a referent. The referent can be anything that the individual contacts, including objects in the nesting environment, its diet, and most importantly, other individuals living in the same group (nest or colony). Argentine ant colonies are highly polygynous (Markin 1968, 1970), with each reproductive female possibly contributing unique recognition labels. As a result, workers learn the colony's collective odour cues, thereby creating a uniform template (Crozier & Dix 1979; Breed & Bennett 1987). When the diversity of the referents that form the recognition system is wide, a broader template presumably is formed and more labels are deemed acceptable (Vander Meer & Morel 1998). The breadth of the template ultimately depends on the genetic diversity between individuals that contribute to the collective colony odour. Within the area of California that we sampled, genetic diversity across colonies was lower (23 alleles at 7 microsatellite loci) than that of colonies in the southeastern U.S. (47 alleles at 7 microsatellite loci; Buczkowski et al. 2004). As a result, the template in the California colonies may be relatively narrow, such that addition of *S. longipalpa* and/or *B. germanica* hydrocarbons may trigger changes in perception and provoke aggression between former nestmates. In contrast, in southeastern U.S. colonies, where genetic diversity is higher, *L. humile* may have a wider template and therefore perceive certain exogenous hydrocarbons within this template, such that behaviour is not altered.

Whether variation in expression and/or perception can explain regional responses to exogenous cues, it appears that similar selection pressures, such as perturbation to an ant colony's physical and biotic environment that both fragments the colony and provides potentially novel environmentally derived recognition cues, can have diverse population-level consequences.

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