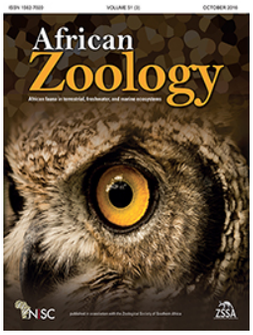

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"You are not always what you eat": diet did not override intrinsic nestmate recognition cues in Argentine ants from two supercolonies in South Africa

Natasha P Mothapo* and Theresa C Wossler

DST-NRF Centre of Excellence for Invasion Biology, Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa
** Corresponding author, email: mothapo@sun.ac.za*

Nestmate recognition in ants is based on cuticular hydrocarbons (CHCs), which are heritable and may also be acquired from the environment (i.e. diet and nest environment). In Argentine ants (*Linepithema humile*), diet and a homogenous environment have been shown to affect nestmate recognition by altering the CHC profile and consequently intraspecific aggression. In our study, Argentine ants were collected from field nests representing two supercolonies in South Africa. Individuals were paired in aggression assays and their CHC profiles analysed. The same nests used in the aggression assays were maintained in the laboratory for five months on a shared diet of crickets and sugar water, in soil-free nests. We predicted that aggression between previously aggressive paired individuals from different nests would decrease over time through the homogenisation of CHCs as a consequence of the shared diet and similar nesting environment. Our data showed that ants maintained in the laboratory readily absorbed prey-derived hydrocarbons and experienced a loss in the number of cuticular compounds compared with their original CHC profiles. However, the changes in CHCs did not impair nestmate recognition with non-aggressive paired interactions maintained while previously aggressive paired interactions persisted. The persistence of aggression between previously aggressive pairs despite environmental homogeneity supports the notion that intrinsic nestmate recognition cues are not overridden by extrinsic cues in the recognition system of Argentine ants.

Keywords: cuticular hydrocarbons, intraspecific aggression, *Linepithema humile*, nestmate recognition, supercolonies

Introduction

Cuticular hydrocarbons (CHCs) are chemical olfactory cues used in social insect colonies to maintain cooperation and colony integrity through a well-developed nestmate recognition system (Hölldobler and Wilson 1990; Astruc et al. 2001; Payne et al. 2004; Ozaki et al. 2005). Individuals within a colony share similar CHC profiles (commonly referred to as the gestalt odour (Crozier and Dix 1979)) derived from the mixing of individual CHCs as well as those acquired from the environment (Boulay et al. 2003; Katsav-Gozanzky et al. 2004; van Zweden et al. 2010), with varying contributions of each (Dahbi and Lenoir 1998; Stuart and Herbers 2000; Lenoir et al. 2001; Lucas et al. 2004; Hefetz 2007). Moreover, newly acquired CHCs are rapidly integrated into the colony odour through physical behaviours, such as trophollaxis and allogrooming (Boulay et al. 2000; Lenoir et al. 2001). Nestmate recognition typically follows a label–template matching model, where a worker expresses a label made up of a set of recognition cues and a template that consists both of an innate odour and shared colony odour (see Newey 2011) and when she encounters an individual, the label is then compared to the template after which she may accept or reject the encountered individual (Vander Meer and Morel 1998; Lenoir et al. 1999). Aggression is typically displayed towards individuals whose CHC profiles do not match that of the recipient's recognition template (Dani et al. 1996; Lenoir et al. 2001; Lucas et al. 2005; Buczkowski and Silverman 2006; Newey et al. 2010).

There is no doubt that CHC profiles in ants are affected by external inputs from the environment as well as the age of the colony and queen number (Bagneres et al. 1991; Liang et al. 2001; Martin et al. 2009). Polygyny has been shown to increase genetic diversity in colonies, with a concomitant increase in the diversity of the intrinsic component associated with nestmate recognition (Vander Meer and Morel 1998). This is thought to broaden the nestmate recognition template resulting in lower levels of aggression and leads to higher acceptance rates in these colonies (Van der Meer et al. 1989; Bagneres et al. 1991; Heinze et al. 1996; Lahav et al. 1999; Liang et al. 2001; Tsutsui et al. 2003; Buczkowski et al. 2005; Sorvari et al. 2008; Martin et al. 2009; Vonshak et al. 2009). In polydomous nests of *Cataglyphis iberica*, nestmates can become slightly aggressive to each other when re-establishing contact after short periods of separation during hibernation (Dahbi and Lenoir 1998), likely due to loss of highly volatile CHCs concomitant with not sharing diet or the same environment, resulting in CHC divergence through changes in compound assimilation from a shared diet or environment (Dahbi and Lenoir 1998; Liang et al. 2001). Therefore, the changes that occur on the profile may cause both slight and/or major shifts in the recognition

template resulting in increased probability of aggression (Dahbi and Lenoir 1998; Liang and Silverman 2000). Van Zweden et al. (2010) suggested that heritable cues potentially have higher transfer rates among nestmates and thus are expected to remain unchanged despite external influences from the environment. Although some studies have shown that if CHCs are acquired from the environment in sufficient quantities, the intrinsic components could be overridden resulting in a loss of intercolony differences (Downs and Ratnieks 1999; Buczkowski et al. 2005; Vonshak et al. 2009), but it is still generally expected that heritable nestmate recognition cues remain stable (Hölldobler and Wilson 1990; Heinze et al. 1996; Holway et al. 1998; Vander Meer and Morel 1998; van Zweden et al. 2009; Vonshak et al. 2009).

The Argentine ant, *Linepithema humile* (Mayr, 1868), is a notorious invasive species that has successfully established in many parts of the world, including oceanic islands, through human-assisted dispersal (Suarez et al. 2001). Argentine ants form geographically vast super-colonies that show low genetic variability and lack of interspecific aggression within supercolonies (Suarez et al. 2002; Sunamura et al. 2009a; van Wilgenburg et al. 2010). The presence of geographically vast and inter-continental colonies suggests that nestmate recognition in this ant species has a strong intrinsic component, rather than being environmentally determined, which is thought to explain the widespread absence of intraspecific aggression (Tsutsui et al. 2000; Giraud et al. 2002; Buczkowski et al. 2004; Corin et al. 2007; Brandt et al. 2009; Sunamura et al. 2009a; van Wilgenburg et al. 2010; Vogel et al. 2010). Of the two intercontinental supercolonies (van Wilgenburg et al. 2010), as well as the pattern expressed by a large and small supercolony studied, where multiple introductions are common (Helanterä et al. 2009; Sunamura et al. 2009a), the smaller supercolonies tend to be characterised by low genetic variability (Sunamura et al. 2009b). This low genetic variability is possibly associated with a more narrow cuticular profile (Helanterä et al. 2009), low tolerance thresholds and therefore stricter nestmate recognition, which results in high levels of aggression towards non-nestmates.

Nestmate recognition in *L. humile* is potentially influenced by both intrinsic and extrinsic cues (Holway et al. 1998; Liang and Silverman 2000; Tsutsui et al. 2000; Silverman and Liang 2001; Giraud et al. 2002; Suarez et al. 2002; Buczkowski et al. 2005; Brandt et al. 2009). However, the contribution of genes and the environment on cue phenotype of Argentine ants within laboratory studies are ambiguous because there is no consensus on whether intrinsic or extrinsic cues are more important in Argentine ant nestmate recognition. A strong genetic component for nestmate recognition in this ant is supported by work of Holway et al. (1998) and Suarez et al. (2002), who showed that Argentine ants maintained in the laboratory on the same diet under uniform environments for as long as 18 months maintained their original behaviour in that aggressive colony pairs remained so and vice versa, whereas Chen and Nonacs (2000) showed a complete loss of aggression after only two months in the laboratory. It comes as no surprise that where aggression persisted, intrinsic cue similarity was considered the most important contributor to nestmate recognition, and where aggression was lost the extrinsic component was considered more important. Therefore, the aim of this study was to investigate the influence of homogenous environmental conditions on the CHC profiles of the South African populations of Argentine ant and subsequent impacts on the patterns of intraspecific aggression and nestmate recognition. We are aware that a number of studies investigating Argentine ant nestmate recognition have been published, though without consensus (Holway et al. 1998; Chen and Nonacs 2000; Liang and Silverman 2000; Liang et al. 2001; Suarez et al. 2002; Buczkowski et al. 2005; van Wilgenburg et al. 2010; Berville et al. 2013). Furthermore, the two supercolonies identified in South Africa (Mothapo and Wossler 2011) do not correspond chemically (CHCs) or genetically to any of the other introduced populations (Brandt et al. 2009; van Wilgenburg et al. 2010; Vogel et al. 2010), and are thought to be primary introductions from South America (van Wilgenburg et al. 2010). The ants are not well studied in South Africa and it is likely that they behave differently as has been observed for small supercolonies in Japan (Sunamura et al. 2011), giving credence to this study. We tested the hypothesis that the environment is important in nestmate recognition for these introduced Argentine ant populations within South Africa, therefore aggression between ants maintained in the laboratory under uniform environmental conditions would be lost through the homogenisation of CHCs. We conducted aggression assays and analysed the CHC profiles of ants from freshly collected field colonies, and repeated this five months later with ants from the same colonies maintained in a homogenous laboratory environment. We assessed whether CHC profiles and intraspecific aggression changed over time. In this study we use the term nest to refer to ants sampled within the same supercolony but from geographically distinct areas.

Methods

Argentine ants were first introduced into South Africa in the early 1900s and have since spread throughout most of the country in both urban and natural areas (de Kock and Giliomee 1989; Prins et al. 1990; Luruli 2007); and form two distinct supercolonies with a boundary at Elim (Lado 2008; Mothapo and Wossler 2011). In our previous study (Mothapo and Wossler 2011) we sampled Argentine ants within the Western Cape, South Africa from eight sites (Figure 1): Elim (small supercolony), Stellenbosch, Bellville, Caledon, Somerset West, Jonkershoek, Bredasdorp and Porterville (all from the large supercolony) (see Figure 1). Pairwise aggression tests between individuals from nests within and between sites were conducted in the field and one experimental nest from each site was excavated and maintained under uniform laboratory conditions for five months, after which pairwise aggression bioassays were repeated to compare changes in levels of aggression. Cuticular hydrocarbons were extracted from ants from the freshly excavated nests and again five months later from the laboratory-maintained nests (Mothapo and Wossler 2011).

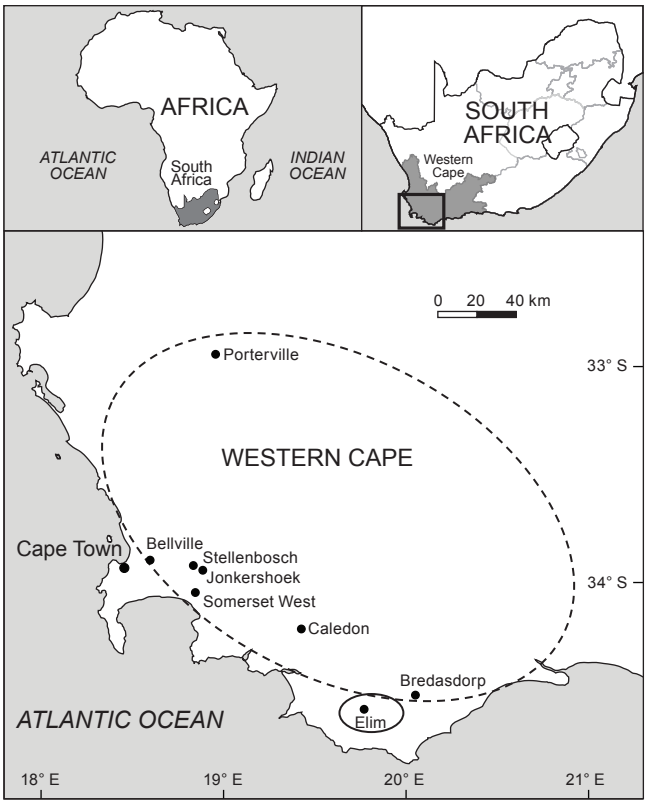

Figure 1: Map of the sampling localities for Argentine ants used in this study. The two supercolonies that are found in the Western Cape, South Africa, are demarcated by the dashed circle (large supercolony) and closed circle (small supercolony)

Maintenance of laboratory colonies

Ants were kept in soil-free nests and fed an uniform diet in order to standardise nest environments. Ants were removed from soil using a flooding technique, slow drip method (Suarez et al. 1999). Artificial nests were housed in a plastic container coated with Fluon® Fluoropolymer Dispersion (Whitford Plastics, Runcorn, UK) on the sides and consisted of two glass tubes half-filled with water, plugged with cotton wool and covered with foil. The colony set-up was 1 000 workers, two or three queens and brood pieces. The ants were fed three times a week on a diet consisting of pin-head crickets; water and 0.25 M sugar water were provided *ad libitum*. The colonies were kept in the laboratory for five months from November 2007 to March 2008, and exposed to 12 h/12 h (light/dark) cycles at temperatures ranging from 24 °C to 28 °C.

Chemical analysis of cuticular hydrocarbons in laboratory-maintained ants

Worker ants were chilled in the fridge for 10 min or until they stopped moving, which reduces the chances of contamination with stomach contents during extraction with the solvent hexane. Ten workers were placed in a 2 ml glass vial and soaked in 100 µl hexane for 10 min, a total of six extractions were done per nest. The workers were then rinsed in an additional 100 µl of hexane for 2 min and the two hexane extracts were combined to produce a 200 µl cuticular lipid extract. Four crickets were individually washed in hexane in a similar manner as the ants to ascertain whether any cricket compounds were assimilated by the ants. The extract was purified using silica gel minicolumns constructed from glass Pasteur pipette tubes filled with ±500 mg silica gel (grade 22, 60–200 mesh; SIGMA-Aldrich, St Louis, MO, USA), plugged with glass wool and pre-wetted with 2 ml hexane prior to loading the cuticular lipid extract (Buczkowski et al. 2005). The 200 µl cuticular lipid extract was reduced to 100 µl under a stream of nitrogen and then loaded onto the pre-wetted silica gel minicolumn. The hydrocarbon fraction was eluted with 6 ml hexane, evaporated to dryness under a stream of nitrogen and re-dissolved in 25 µl hexane from which 1 µl was injected into a gas chromatograph (Agilent 6850) fitted with a splitless inlet, flame-ionisation detection and a DB–5 capillary column (30 m × 0.32 mm × 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA). The injection port and the detector were set at 290 °C and 320 °C, respectively. The initial oven temperature was 80 °C and held for 2 min, then ramped to 270 °C at the rate of 10 °C min^{-1} and finally increased to 310 °C at 3 °C min^{-1} and held for 20 min. Helium was used as the carrier gas and nitrogen as the make-up gas. Compounds were identified using electron impact mass spectra (Agilent 5975B mass spectrometer). The compounds were identified by comparing their mass spectra with those of pure compounds accessed via the NIST and WILEY databases. Only compounds with a match of 90% or more to those accessed in the library were positively identified. All compounds in trace quantities were not included because their abundances could not be calculated.

Behavioural assays

Standard behavioural bioassays (Suarez et al. 1999) were conducted to determine the levels of intraspecific aggression between ants from two different nests. Aggression assays between paired individuals within a nest (control) were conducted first, followed by aggression assays between paired individuals from different nests (all combinations tested). Aggression assays were conducted in the field and repeated five months later, after nests had experienced a homogenous environment, to assess whether aggression levels had changed in relation to changes in the CHC profile. Aggression assays were conducted as follows: two randomly picked workers were paired in a neutral arena for 10 min. Roulston et al. (2003) showed in their study that aggressive responses did not differ significantly between pairwise and group interactions, even though they suggest group interactions are likely to show increased likelihood of aggression, which can show context-dependency of aggression (Buczkowski et al. 2005). When in pairs, ants may avoid getting into a fight, but when they do show aggression it does not differ in severity than when they are in a group (Roulston et al. 2003). Behavioural interactions between the two ants were observed over the 10 min, 10 trials were conducted per colony pair which included nestmate vs nestmate (control) and nestmate vs non-nestmate. During each trial the observer recorded all interactions between the pair of workers. Aggressive interactions included mandible gaping

response, lunging, biting, pulling and fighting. At the end of the trial, the number of aggressive interactions were counted and then used to calculate the aggressivity index as shown below.

Following the method of Martin et al. (2009), the frequency distribution of aggression level was used to calculate the aggressivity index per trial:

$$\text{Aggressivity index} = \frac{\text{Total number of aggressive interactions}}{\text{Total number of interactions}}$$

For graphical illustration, the aggressivity index was converted to percentage. For each nest pair, assays were repeated 10 times and each worker-pair was used only once. The nest origin was unknown to the observer.

Statistical analysis

Chemical analyses

The cuticular hydrocarbon composition of ants maintained in the laboratory were identified and compared with CHC profiles found initially in their field counterparts to determine the effects of a homogenous environment on the hydrocarbon profiles. Peak areas were standardised by calculating the percentage contribution of each compound in relation to the total number of compounds producing the cuticular hydrocarbon blend. Peak areas represent compositional data, therefore the standardised peak areas were transformed to logcontrasts using Aitchison's (1986) formula: $Z_{ij} = \ln[Y_{ij}/g(Y_j)]$, where Z_{ij} is the standardised 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. To determine whether CHC profiles of Argentine ants from different nests and supercolonies converge having been maintained in a homogenous environment for five months and whether they differ from their field counterparts, a principal components analysis (PCA) with varimax rotation to reduce the number of describing variables followed by a discriminant analysis (DA) was applied. Any reduced aggression levels between individuals from different nests compared with the original field aggression levels could potentially indicate CHC convergence and loss of nestmate recognition cues.

Behavioural assays

The Wilcoxon matched-pairs test was used to ascertain whether aggression levels changed between individuals from laboratory-maintained and field-based nests by comparing the aggressivity indices. All analyses were conducted in IBM SPSS Statistics for Windows 22 (IBM Corporation, Armonk, NY, USA), and statistical significance was accepted at $P < 0.05$.

Results

The CHC profiles of laboratory-maintained ants were affected by exposure to the homogenous environment and the shared diet. There was a reduction in the total number of compounds present in the CHC profiles of laboratory-maintained ants compared with their field counterparts (15 compounds from field-collected ants were absent in the laboratory-maintained ants); however, three novel compounds were assimilated in laboratory-maintained ants (Table 1 and see Mothapo and Wossler 2011). Similar to field CHC profiles, the profiles of laboratory-maintained ants consisted largely of straight-chained alkanes followed by clusters of methyl-branched alkanes ranging in chain length from C17 to C43, which have also been found in previous studies of Argentine ants (see Liang et al. 2001 and Brandt et al. 2009 (C14–C43) for similar compound identifications). Nine compounds were found in the CHC profile of the crickets, of which three were shared with the ants, suggesting that ants assimilated prey hydrocarbons (see compounds highlighted in bold in Table 1).

Twelve principal components (PCs) with eigenvalues larger than 1 explaining 80.6% of the total variance were produced by the PCA from CHC profiles of freshly collected and laboratory-maintained ants. The CHC profiles of freshly collected ants were distinct from those ants maintained in the laboratory (Wilks' $\lambda = 0.000$, $\chi^2 = 832.92$, df = 180, $p < 0.0001$; Figure 2), and separated strongly along discriminant function 2. The ants collected from field nests in Elim were the most aggressive and chemically distinct (Figure 2; see also Mothapo and Wossler 2011). However, after five months in the laboratory, the CHC profiles of ants from Elim converged with those of ants from the nests belonging to the large supercolony that had been maintained in the laboratory (Figure 2).

The high levels of aggression observed in field-collected ants from Elim were maintained in the laboratory even after the CHC profiles of these ants converged with those of nests from the large supercolony (Figure 3). There was one exception, however, with aggression levels between laboratory-maintained ants from Somerset West and Elim showing a significant reduction (Wilcoxon signed ranks: $Z = -2.49$, $p = 0.01$; Figure 3). The persistence of aggression between ants from Elim and those from all other sites (despite the convergence of the CHC profiles of the Elim laboratory-maintained ants), except Somerset West which we cannot explain, suggested that nestmate recognition abilities were not impaired by exposure to the homogenous environment nor the changes imposed on the CHC profile.

Discussion

We found that the nestmate recognition cues, expressed as a CHC profile, of the Argentine ant populations established in South Africa were affected by external environmental factors, such as diet and nest environment, in accordance with previous studies (Liang et al. 2001; Buczkowski et al. 2005; Vonshak et al. 2009); however, there was very little change in intraspecific aggression patterns after spending five months in a homogenous environment. Laboratory-maintained ants assimilated prey-derived hydrocarbons (HCs), notably lost several HCs that were present on their CHC profile prior to laboratory maintenance (Mothapo and Wossler 2011), yet the high levels of aggression between the ants from Elim and those from other locations were maintained. Previous studies on Argentine ants also showed a reduction in the number of CHC compounds of ants maintained in the laboratory (see Liang and Silverman 2000; Buczkowski et al. 2005), and the assimilation of prey-derived compounds (Liang and Silverman 2000; Liang

Table 1: Hydrocarbon composition of laboratory-maintained Argentine ant colonies from eight geographic sites within the Western Cape. Percent contribution (mean ± SE) of each compound is presented for each of the nests (n = 6 runs per nest) from the field and after maintenance in the laboratory. Compounds highlighted in bold are compounds derived from pin-head cricket (prey). Compounds not detectable within ant CHC profiles are denoted by '–'

	Compound	Elim		Bellville		Stellenbosch		Caledon		Porterville		Somerset West		Bredasdorp		Jonkershoek	
		Field	Laboratory	Field	Laboratory	Field	Laboratory	Field	Laboratory	Field	Laboratory	Field	Laboratory	Field	Laboratory	Field	Laboratory
1	Tridecane	2.07±0.60	–	1.73±0.48	0.14±0.14	1.33±0.75	1.70±0.80	2.50±0.96	3.24±181	2.19±0.54	2.97±0.98	2.14±0.63	2.95±1.09	3.06±0.59	1.89±0.65	2.75±0.41	2.00±0.61
2	Tetradecane	0.44±0.11	–	0.34±0.19	–	0	–	0.06±0.07	–	0.56±0.28	–	0.66±0.27	–	0.58±0.21	–	0.46±0.21	–
3	Hexadecane	0.74±0.17	–	0.28±0.11	–	0.35±0.25	–	0.87±0.42	–	0.39±0.27	–	0.73±0.19	–	0.38±0.20	–	0.79±0.22	–
4	Heptadecane	0.47±0.10	0.43±0.37	0.36±0.10	0.14±0.14	0	1.70±0.80	0.18±0.13	3.24±181	0	2.97±0.98	0.61±0.19	2.95±1.09	0.70±0.26	1.89±0.65	0.93±0.33	2.00±0.61
5	Octadecane	0.17±0.08	–	0.10±0.06	–	0	–	0.27±0.26	–	0.26±0.46	–	0.22±0.13	–	0.18±0.11	–	0	–
6	Nonadecane	0.24±0.08	–	0.13±0.07	–	0	–	0.24±0.22	–	0.25±0.35	–	0.18±0.11	–	0.22±0.12	–	0	–
7	Eicosane	0.63±0.04	0.15±0.15	0.52±0.12	0.46±0.31	0.18±0.17	–	0.92±0.38	0.95±0.32	0.66±0.73	0.80±0.68	0.94±0.36	3.13±1.16	0.62±0.26	1.03±0.53	0.50±0.40	0.92±0.24
8	Heneicosane	0.87±0.05	–	0.94±0.07	0.13±0.13	0.67±0.70	0.57±0.37	1.08±0.62	1.06±0.53	0.79±0.27	1.27±0.27	2.05±0.63	1.47±0.43	1.02±0.42	0.55±0.20	0.64±0.23	0.36±0.12
9	Docosane	1.52±0.11	0.10±0.10	1.61±0.12	0.42±0.27	2.04±0.71	–	4.07±1.66	1.19±0.63	2.00±0.29	0.95±0.46	5.88±1.87	6.51±3.87	2.53±1.18	0.51±0.29	1.57±0.35	0.78±0.13
10	Tricosane	2.43±0.19	0.26±0.18	2.71±0.21	0.16±0.16	4.27±1.10	–	5.67±1.95	0.89±0.48	3.15±0.47	1.08±0.46	7.96±2.29	5.77±3.63	3.87±1.32	0.83±0.47	2.89±0.38	0.32±0.15
	2-Methyltricosane	–	1.80±1.80	–	7.12±2.28		8.25±1.87	–	5.05±1.24		5.99±2.75	–	8.37±4.21	–	4.87±2.42	–	7.44±0.85
11	Tetracosane	3.56±0.33	0.64±0.23	3.80±0.30	0.44±0.20	6.25±1.29	–	7.78±2.28	1.22±0.58	4.88±0.63	1.53±0.58	10.15±2.62	6.99±4.55	5.65±1.45	1.21±0.69	4.23±0.52	0.39±0.15
12	Pentacosane	4.66±0.35	3.47±0.87	5.25±0.43	1.58±0.53	6.51±2.55	0.21±0.21	6.12±1.45	2.06±0.99	6.41±0.81	4.92±2.67	5.14±1.70	5.37±2.77	5.83±1.04	5.45±3.20	5.63±0.66	0.99±0.23
13	1,2-Benzenedicarboxylic acid	2.08±0.30	–	1.89±0.44	–	1.53±1.01		1.50±0.69	–	2.01±0.67	–	1.53±0.53	–	3.10±1.03	–	2.01±0.45	–
14	Hexacosane	4.83±0.37	7.18±1.57	5.17±0.34	3.55±1.01	9.53±2.25	0.23±0.23	7.19±1.46	3.65±2.08	6.16±0.73	10.75±5.46	8.33±1.01	9.87±5.55	6.44±0.80	10.73±5.56	5.21±0.62	1.83±0.16
15	Heptacosane	6.94±0.42	24.53±4.92	5.75±0.41	10.34±3.87	10.88±2.73	2.76±0.62	7.21±0.48	7.59±5.24	5.71±0.63	17.48±8.58	6.32±0.32	8.91±5.80	5.96±0.88	9.70±6.80	5.48±0.50	3.21±0.15
	2,6,10,14-Tetramethyl-tetracosane	–	1.11±0.28		2.84±0.61		0.87±0.61	–	5.02±2.33		7.05±2.45	–	4.75±1.16	–	1.63±0.50	–	5.08±1.71
16	Octacosane	4.18±0.25	10.39±5.45	4.80±0.34	4.76±1.66	7.38±1.79	5.02±0.44	5.22±0.54	2.66±0.76	4.92±0.79	4.00±1.85	4.88±0.26	4.27±2.02	4.76±0.52	3.00±1.50	4.45±0.42	4.94±0.27
17	Nonacosane	4.50±0.26	2.12±1.92	4.99±0.38	6.40±2.27	6.82±1.31	8.09±0.79	4.82±0.45	3.84±0.98	4.63±0.44	5.84±2.71	4.27±0.40	6.32±3.06	4.98±0.62	5.02±2.55	4.46±0.40	6.81±0.35
18	Triacontane	3.70±0.15	1.88±1.88	4.41±0.60	9.30±3.64	4.11±1.01	9.30±1.05	3.75±0.45	4.46±1.13	3.53±0.38	6.02±2.75	3.05±0.26	1.49±1.49	3.50±0.45	4.74±2.37	3.48±0.49	9.54±0.45
19	Hentriacontane	3.07±0.22	1.42±1.42	3.73±0.44	5.78±1.85	2.97±0.65	10.10±1.09	3.15±0.42	4.31±1.10	2.81±0.33	4.66±2.12	2.67±0.34	7.62±3.70	2.82±0.41	3.63±1.73	3.13±0.31	8.84±0.81
20	Dotriacontane	3.37±0.48	1.11±1.11	3.60±0.58	6.53±1.52	2.33±0.65	7.12±0.67	2.77±0.42	4.50±1.34	2.45±0.43	1.36±1.36	2.23±0.28	1.62±1.62	2.04±0.47	3.06±1.38	2.90±0.25	7.30±0.49
21	13-Methylhentriacontane	0.26±0.18	2.70±1.88	0.51±0.41	7.72±2.53	3.17±3.45	5.13±0.50	0.44±0.36	2.97±0.81	13.40±6.91	2.65±1.20	1.18±0.94	1.00±1.00	12.97±4.49	4.70±2.23	3.38±2.64	5.48±0.49
	8-Hexylpentacosane	–	1.36±1.36	–	8.08±2.03		7.34±0.80	–	3.85±1.07		4.69±2.12	–	1.38±1.38	–	3.63±1.69	–	8.21±0.48
22	Tritriacontane	2.36±0.16	10.65±1.82	2.45±0.34	4.86±1.95	1.28±0.49	3.94±0.38	2.05±0.34	2.28±0.68	1.68±0.28	1.25±0.80	1.88±0.30	–	1.67±0.38	3.21±1.44	2.11±0.19	4.35±0.36
23	2,6,10,15-Tetramethyl-heptadecane	1.41±0.18	–	2.78±0.28	–	1.56±0.58	–	1.82±0.60	–	2.08±0.34	–	1.89±0.46		2.17±0.46	–	1.88±0.34	–
24	Tetratriacontane	0.33±0.11	10.33±1.82	6.38±0.58	3.05±0.74	7.06±2.44	3.44±0.41	5.26±1.41	2.11±0.59	7.71±1.59	1.14±0.73	4.04±1.29	0.77±0.77	4.47±1.11	2.85±1.26	7.68±0.99	3.29±0.63
25	Pentatriacontane	2.91±0.26	4.92±3.06	2.95±0.46	4.22±2.43	2.32±1.50	5.84±5.84	2.63±0.77	19.62±7.06	2.76±2.09	3.99±1.65	1.35±0.42	–	1.27±0.58	12.72±8.17	2.32±0.82	5.42±3.24
26	Hexatriacontane	1.33±1.33	2.27±1.23	4.09±0.49	3.03±1.26	3.03±1.02	1.61±0.60	2.72±0.76	1.85±0.50	2.77±0.52	0.76±0.48	2.74±0.50	1.10±1.10	2.34±0.45	2.01±0.86	3.28±0.38	2.87±0.30
27	13,17,21-Trimethyl-pentatriacontane	1.62±1.62	1.08±0.49	1.15±0.28	3.99±1.52	0.40±0.35	2.06±0.81	1.13±0.46	2.71±0.85	1.19±0.38	–	0.63±0.35	3.06±1.94	1.36±0.47	5.14±2.70	2.35±0.47	2.84±0.82
28	Heptatriacontane	0.10±0.05	1.94±0.58	1.32±0.21	2.70±0.75	0.29±0.23	8.03±1.64	0.78±0.35	2.24±0.81	0.70±0.28	1.56±1.11	0.83±0.35	–	0.63±0.23	2.58±1.06	1.52±0.32	1.53±0.43
29	Octatriacontane	2.04±0.37	–	1.99±0.29	–	1.33±0.73	–	1.35±0.37	–	0.98±0.31	–	1.35±0.28	–	1.15±0.36	–	1.57±0.21	–
30	17-Methylheptatriacontane	2.19±0.21	–	2.99±0.31	0.75±0.75	1.76±1.08	3.54±2.75	1.89±0.65	1.27±0.10	1.50±0.37	0.53±0.53	2.56±0.64	4.75±2.42	1.94±0.57	–	1.95±0.39	0.40±0.26
31	17, 21-Dimethylheptatriacontane	1.90±0.67	6.88±1.74	6.83±0.78	1.51±0.76	3.45±1.26	4.22±0.83	5.21±1.71	0.66±0.42	4.97±0.87	0.23±0.23	2.81±1.46	–	4.01±0.99	4.10±1.84	7.71±1.13	3.25±0.41
32	13,17,21-Trimethyl-heptatriacontane	2.68±1.21	–	3.40±0.60	–	2.00±0.89	0.64±0.64	2.48±0.86	0.90±0.44	1.62±0.37	0.24±0.16	2.00±0.56	0.85±0.55	1.74±0.53	0.12±0.12	2.32±0.31	0.66±0.16
33	Tritetracontane	5.80±3.38	1.30±0.37	3.23±0.58	0.67±0.40	2.47±0.89	–	2.48±0.48	0.94±0.58	1.82±0.53	6.31±3.10	2.24±1.34	1.70±0.80	2.12±1.57	1.08±1.08	4.84±1.16	0.95±0.36
34	13-Methyltritetracontane	0	–	0.42±0.16	–	0.07±0.11	–	0.28±0.19	–	0.17±0.17	–	0.12±0.09	–	0.46±0.20	–	0.64±0.26	–
35	Unknown	6.45±0.61	–	1.09±0.18	–	0.45±0.33	–	0.46±0.31	–	0.14±0.13	–	0.62±0.28	–	0.68±0.29	–	0.69±0.29	–
36	Tetratetracontane	2.16±0.35	–	0.92±0.20	–	0.24±0.23	–	0.49±0.26	–	0.15±0.15	–	0.97±0.28	–	0.71±0.30	–	0.54±0.21	–
37	1-Bromotetratetracontane	0	–	0.65±0.39	–	0.49±0.36	–	0.85±0.40	–	0.43±0.24	–	0.98±0.29	–	0.45±0.35	–	1.23±0.35	–
38	13-Methyltetratetracontane	0	–	1.40±0.11	–	0.27±0.25	–	0.46±0.29	–	0.34±0.21	–	0.88±0.39	–	0.43±0.20	–	1.33±0.25	–
39	13,17-Dimethyltetratetracontane	0.48±0.48	–	1.17±0.39	–	0.16±0.20	–	0.63±0.56	–	0.26±0.28	–	0.07±0.12	–	0	–	0.88±0.82	–
40	Unknown	7.77±2.58	–	1.61±0.36	–	1.06±0.59	–	1.21±0.41	–	1.57±0.86	–	0.95±0.28	–	1.21±0.50	–	1.18±0.34	–

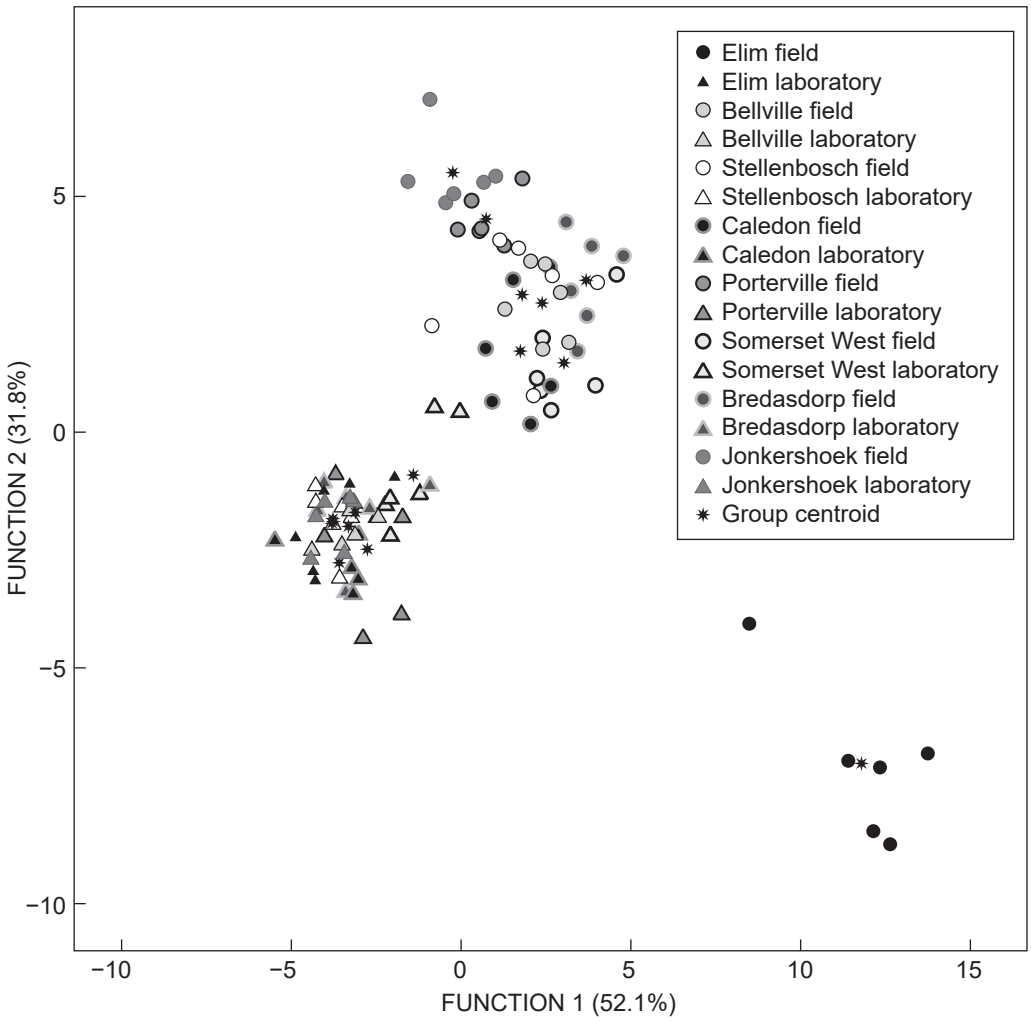

Figure 2: Discriminant analysis of Argentine ant cuticular hydrocarbons extracted from field nests (n = 6 samples per nest) and the same nests maintained in the laboratory for five months (n = 6 samples per nest). The CHC profiles of the field ants from Elim are distinct from the profiles of field-collected ants from all other sites. After five months in the laboratory, the CHCs converge, including those from Elim

et al. 2001). Moreover, the persistence of intraspecific aggression observed in this study supports the idea that the intrinsic component of cues produced (not identified by the study) is particularly important in the nestmate recognition system of this ant as suggested in other studies (Holway et al. 1998; Tsutsui et al. 2000; Suarez et al. 2002; Buczkowski et al. 2005; Brandt et al. 2009; van Wilgenburg et al. 2010; Vogel et al. 2010), emphasising a potential mechanism by which these ants are able to maintain intercontinental distributions (Sunamura et al. 2009a). Our results support a strong genetic component to nestmate recognition in this ant, particularly for the South African population. While Liang and Silverman (2000) and Chen and Nonacs (2000) point to an environmental component driving nestmate recognition, their results cannot explain the ability of ants to recognise each other across different continents (Sunamura et al. 2009a). Therefore, our study is in agreement with those studies that point to a strong genetic component to recognition in this ant species.

Laboratory-reared colonies of *Cataglyphis niger* are able to progressively change their recognition profile through the addition of a new diet, removing or adding workers and queens, which gradually resulted in shifts in aggression responses between nestmates and non-nestmates (Soroker et al. 1995). The convergence of CHC profiles over time can ultimately lower aggression (Ichinose 1991). In contrast, Ichinose et al. (2009), working on *Aphaenogaster senilis*, found that the initial change in the CHC profile was very rapid (within 5 d) but subsequently remained stable with time, whereas aggression took longer to steadily decline over time. Unlike the common threshold model of acceptance, which suggests that encountered individuals are accepted or rejected at a fixed point, the graded model (proposed by Ichinose et al. 2009) is more dynamic, with flexibility in the levels of acceptance of non-nestmate individuals, thus ants within a colony show variation in aggression towards encountered workers who have similar composition of CHC compounds. The composition of the CHCs may vary slightly with regards to concentrations of the compounds (Greene and Gordon 2007; Ichinose et al. 2009), which may not elicit aggression (Greene and Gordon 2007), but the position of the methyl

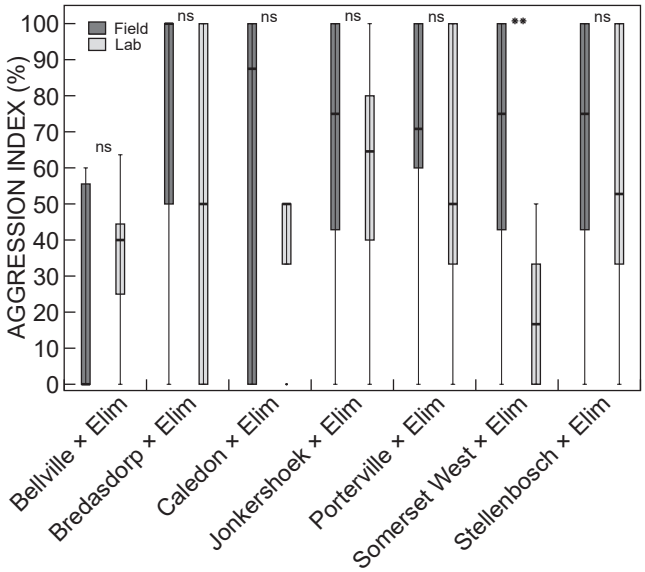

Figure 3: Changes in aggression presented by aggressivity indices as boxplots in percentages (showing the median, interquartile range, minimum and maximum) between ants under field conditions, and again five months later after uniform conditions in the laboratory. Workers from Elim remained aggressive to those from all other sites, with the exception of Somerset West. Significant differences are based on the Wilcoxon signed ranks test for paired samples. ** $p < 0.01$; ns, $p > 0.05$

branch may in fact cause changes on the profile that will lead to aggression (van Zweden et al. 2009). In our study we show a strong convergence of the CHC profiles of laboratory-maintained colonies of Argentine ant, but no change in aggression even after five months, which suggests that despite this change in the CHC profile recognition cues may not be affected. Comparing field and laboratory-maintained ants from the same colony would have added further support to this conclusion. Liang and Silverman (2000) showed that aggression was heightened between nestmates after being reunited with nestmates fed a different diet, but this is only temporary and nestmates were accepted back into their natal colony after some time as was shown by Katzav-Gozansky et al. (2004). In a recent study, Sato and colleagues (unpublished data) showed that aggression between colonies maintained in the laboratory on a diet of German cockroaches, *Blatella germanica*, was very high but those fed on domestic cricket, *Acheta* sp., was initially high but declined within 24 h, after which workers from laboratory-maintained colonies were re-integrated into the freshly collected field colonies from which they were isolated. Their reasoning is that German cockroaches are very oily and cause drastic changes to the profile of the ants, masking the ants CHCs substantially longer than ants fed on a cricket diet. Given that our laboratory-maintained ants were fed a cricket diet, we are of the opinion that these ants would initially have been aggressed by nestmates from their natal field nests but quickly integrated into their colonies.

Previously, it has been suggested that it is the presence and not the absence of certain CHCs on the profile that are used as nestmate recognition cues (Guerrieri et al. 2009). Furthermore, the addition of CHCs should not change cue information (Greene and Gordon 2007). Recent work suggests that recognition follows the undesirable–present model (U–present), which shows that aggression occurs when there is a presence of undesired components on the CHC profile of an encountered worker, but the absence of these components does not affect aggression (van Zweden and d'Ettore 2010). The pattern of loss and gain of CHC compounds in our laboratory-maintained ants, with no resultant change in aggression, suggests that there is not a specific cue involved in nestmate recognition, rather the pattern of the CHC profile confers recognition (see Brandt et al. 2009). Thus, the aggression may have been maintained due to the configuration of the CHC profile regarding the similarity in the combination of branched and unbranched HCs. Greene and Gordon (2007) showed that the combination of branched and unbranched HCs is important in nestmate recognition of the Argentine ant, with methyl alkanes being important in the discrimination between nestmates and non-nestmates. Therefore, the fact that the ants maintained their original levels of aggression suggests that the CHC pattern used for nestmate recognition between the Argentine ant populations in this study were unchanged.

Argentine ants from Elim are genetically different (Lado 2008) and therefore these data support the hypothesis that nestmate recognition in the Argentine ant is largely genetically determined (Holway et al. 1998; Suarez et al. 2002). Aggression in laboratory-maintained Argentine ants from genetically different populations has been shown to remain unchanged for up to a year (Suarez et al. 2002). Indeed, mixing between supercolonies of Argentine ants has been shown to occur most frequently between colonies that share the most common genetically derived nestmate recognition cues (Tsutsui et al. 2003; Heller et al. 2006; Pedersen et al. 2006; Sunamura et al. 2011). Despite the genetic differences between the Elim and Somerset West colonies, aggression declined significantly after ants were maintained in the laboratory. This was an unpredictable and unexpected outcome that we cannot explain, with similar unpredictable outcomes shown by Langen et al. (2000). This highlights the complexity of nestmate recognition systems, and shows that cues can on occasion be masked and overridden by environmentally derived odours (Tripet et al. 2006; Vonshak et al. 2009). We would like to point out that there are still a number of ongoing studies elucidating nestmate recognition patterns in this ant. Emerging studies show that Argentine ants drive the genetic variation within supercolonies by reducing interbreeding between supercolonies through male selection (Sunamura et al. 2011), which contributes to maintaining the genetic differentiation across the supercolony distribution and possibly the maintenance of aggression among supercolonies. The lack of male acceptance in non-natal supercolonies points to closed breeding units, so that genetic variation is maintained between supercolonies (Sunamura et al. 2011). In ants, *n*-alkanes, *n*-alkenes and methyl-branched alkanes are the structural hydrocarbon classes found on the CHC profiles (Liang and Silverman 2000; Greene and Gordon 2007; Brandt et al.

2009). These hydrocarbon classes may vary both qualitatively and quantitatively between species and between colonies (Lenoir et al. 1999; Dani et al. 2001, 2005; Greene and Gordon 2007; Guerrieri et al. 2009). *N*-alkanes in combination with other structural HCs, particularly methyl-branched alkanes, are important nestmate recognition cues in ants, owing to their complexity and low volatility (Liang and Silverman 2000; Akino et al. 2004; Dani et al. 2005; Greene and Gordon 2007; Bos et al. 2010). Moreover, it has been suggested that these methyl-branched alkanes may be genetically encoded, and compared with *n*-alkanes are easily transferred and homogenised within a colony (see van Zweden et al. 2010). Unlike other studies where only the majority of short-chained HCs were lost, we observed a notable loss in a number of long-chained and methyl-branched HCs in laboratory-maintained ants (Table 1). It is unclear why these compounds were lost, but the *n*-alkanes lost from the CHC profile could possibly be attributed to environmentally derived and highly volatile HCs in the field dissipating during laboratory maintenance (as suggested by Buczkowski et al. 2005). In addition, as shown by this study and that of Liang et al. (2001), methyl-branched HCs can also be acquired from the environment and, in particular, diet. Tissot et al. (2001) found that in the harvester ant, *Pogonomyrmex barbatus*, laboratory-maintained ants experienced a two-fold increase in *n*-alkanes and a three-fold increase in methyl alkanes, which they attributed to overexpression of these compounds as a result of laboratory conditions. They suggest that due to the lower humidity that tends to be experienced under laboratory conditions (Gibbs et al. 1991), the composition of the CHC profile may change when compounds associated with desiccation prevention become overexpressed to compensate for the possible increase in water loss. The study did not relate this to any changes in aggression; however, it does suggest that environmental conditions can cause CHCs to vary both qualitatively and quantitatively.

While diet has been shown to affect nestmate recognition in the laboratory (Liang et al. 2001; Buczkowski et al. 2005), it is unlikely that ants from geographically distant populations feed on similar resources. Argentine ant supercolonies span thousands of kilometres, making it unlikely that these ants all feed on the same diet (Giraud et al. 2002; Richard et al. 2004), yet there is no aggression between ants within these massive supercolonies (Tsutsui et al. 2000; Corin et al. 2007). The diet-breadth hypothesis, as proposed by Vonshak et al. (2009), suggested that in field conditions ants utilise a wide variety of food sources, and although diet-derived cues can be incorporated into their profiles, it is never substantial enough to mask intrinsic cues or to affect nestmate recognition capabilities. In contrast, the diet in laboratory manipulations is often unique, readily available and abundant, and the HCs derived from the diet may accumulate to such an extent as to mask intrinsic cues, even narrowing the template of recognition (Downs and Ratnieks 2000; Buczkowski et al. 2005; Vonshak et al. 2009).

Nestmate recognition in unicolonial populations is not well understood (Chapuisat et al. 2005; Vonshak et al. 2009). Martin et al. (2009) suggested that the lack of intraspecific aggression between spatially separate populations may be reflective of the effects of polygyny and polydomy (Elias et al. 2005; see also Debout et al. 2007). While Helanterä et al. (2009) agree, they further attributed the lack of aggression in polydomous and polygynous invasive populations to sharing a common ancestry, which means the majority of heritable recognition cues are likely to be retained among populations (Beye et al. 1997; Helanterä et al. 2009). The presence of multiple reproductive queens within a colony decreases the level of genetic relatedness amongst workers (Keller and Passera 1989; Starks et al. 1998; Giraud et al. 2002; Debout et al. 2003; Rosset et al. 2007), thus creating a diverse array of CHC profiles (Hölldobler and Wilson 1990; Boulay et al. 2000; Suarez et al. 2002). The workers are more accepting to foreign individuals because they have a wider template of recognition, resulting in reduced intraspecific aggression (Hölldobler and Wilson 1990; Tsutsui et al. 2003) and a more permissive nestmate recognition system (Torres et al. 2007). In South Africa, the two supercolonies in this study, distinct from all other introduced Argentine ant populations studied to date (van Wilgenburg et al. 2010; Vogel et al. 2010), differ in the levels of genetic variability, CHC and levels of aggression (Lado 2008; Mothapo and Wossler 2011). Although both populations are extremely polygynous, the smaller supercolony, to which the Elim nest belongs, showed low heterozygosity and lowered genetic variability compared with the large supercolony (Lado 2008), probably due to fewer foundress individuals for this supercolony (van Wilgenburg et al. 2010; Vogel et al. 2010). Thus, the recognition template for this population is likely narrower with a lower acceptance threshold (Buczkowski et al. 2004) and consequently associated with the high levels of aggression shown by this population (see Tsutsui et al. 2003; Martin et al. 2009; van Wilgenburg et al. 2010), so it is not surprising to see these high levels of aggression even after maintenance in the laboratory. Therefore, this study is in agreement with the concept that genetic variability within supercolonies is the underlying component responsible for the maintenance of unicoloniality typical of Argentine ants globally (Brandt et al. 2009; Sunamura et al. 2009a, 2009b; van Wilgenburg et al. 2010; Vogel et al. 2010). There is a need to decipher the patterns of recognition among the different supercolonies and the mechanisms that drive and maintain this global-scale nestmate recognition system in the Argentine ant.

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