When supercolonies collide: territorial aggression in an invasive and unicolonial social insect

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Abstract

Some species of ants possess an unusual form of social organization in which aggression among nests is absent. This type of social organization, called unicoloniality, has been studied in only a handful of species and its evolutionary origins remain unclear. To date, no study has examined behavioural and genetic patterns at points of contact between the massive supercolonies that characterize unicoloniality. Since interactions at territory boundaries influence the costs of aggression and the likelihood of gene flow, such data may illuminate how supercolonies are formed and maintained. Here we provide field data on intraspecific territoriality for a widespread and invasive unicolonial social insect, the Argentine ant (*Linepithema humile*). We observed abrupt and well-defined behavioural boundaries at 16 contact zones between three different pairs of supercolonies. We visited nine of these zones weekly during a six-month period and observed consistent and intense intercolony aggression that resulted in variable, but often large, levels of worker mortality. Microsatellite variation along six transects across territory borders showed that F_{ST} values were lower within supercolonies (0.08 ± 0.01 (mean ± SE)) than between supercolonies (0.29 ± 0.01) and that this disparity was especially strong right at territory borders, despite direct and prolonged contact between the supercolonies. Matrix correspondence tests confirmed that levels of aggression and genetic differentiation were significantly correlated, but no relationship existed between geographic distance and either intraspecific aggression or genetic differentiation. Patterns of F_{ST} variation indicated high levels of gene flow within supercolonies, but little to no gene flow between them. Overall, these findings are inconsistent with a model of relaxed ecological constraints leading to colony fusion and suggest that environmentally derived cues are not the prime determined of nestmate recognition in field populations of Argentine ants.

Keywords: intraspecific aggression, invasive, *Linepithema humile*, microsatellites, territorial borders, unicoloniality

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Introduction

Explaining patterns of variation in the size, persistence and cohesion of social insect societies remains a continuing

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challenge in evolutionary research (Kaspari 2005; Wilson & Hölldobler 2005). One axis of social variation that remains inadequately understood concerns disparities in aggression between conspecific workers. Multicolonial species maintain populations divided into discrete colonies that recognize behavioural colony boundaries, whereas unicolonial species form large populations that lack behavioural boundaries (Hölldobler & Wilson 1990). While recent studies generally acknowledge that these behavioural categories represent

the endpoints of a continuum, research on unicolonial taxa has received considerable attention because of perceived challenges that these insects pose to kin-selection theory (Sturtevant 1938; Crozier 1979; Bourke & Franks 1995) and because of associations between unicoloniality and ecological dominance (Hölldobler & Wilson 1977, 1990) that are especially strong for many invasive ants (Passera 1994; Holway *et al.* 2002).

A recent focus of attention in this area of research concerns colony-structure variation in the Argentine ant (*Linepithema humile*). This widespread, abundant and damaging invasive ant exhibits extreme unicoloniality in its introduced range where it forms expansive supercolonies (Tsutsui *et al.* 2000; Giraud *et al.* 2002). In fact, despite the prominence of *L. humile* as a pest and an invader, the mere existence of intraspecific aggression was not reported in the literature until fairly recently (Holway *et al.* 1998; Suarez *et al.* 1999; Chen & Nonacs 2000; Liang & Silverman 2000). In contrast, native populations of *L. humile* in Argentina typically display high levels of intraspecific aggression over short (< 100 m) spatial scales (Tsutsui *et al.* 2000).

Tsutsui *et al.* (2000, 2003) and Giraud *et al.* (2002) recently proposed a series of hypotheses to account for the origin of extreme unicoloniality in introduced populations of *L. humile* (see Starks (2003) for a review of this literature). Tsutsui *et al.* (2000, 2003) propose that one or more genetic bottlenecks resulting from the Argentine ant's introduction and rapid range expansion in California reduced the population-level genetic diversity of recognition loci to such an extent that large supercolonies became established. While demonstrating that *L. humile* introduced into Europe also lost genetic diversity, Giraud *et al.* (2002) de-emphasize the role of genetic bottlenecks and instead propose a two-part scenario to account for extreme unicoloniality. After the introduction of *L. humile* into Europe, a release from ecological constraints first resulted in increased colony densities. Elevated colony densities increased costly encounters between non-nestmates and in turn created selection against individuals with diverse recognition loci. The end result of this process was the formation of supercolonies. Despite the differences in these proposed hypotheses, both Giraud *et al.* (2002) and Tsutsui *et al.* (2003) agree that selection against diversity is important for the maintenance of extreme unicoloniality at the population level.

In addition to investigations that address the evolution of unicoloniality, several recent studies demonstrate that the context in which interactions take place contributes to the intensity and polarity of intraspecific aggression in introduced populations of Argentine ants (Buczkowski & Silverman 2005; Thomas *et al.* 2005a). For example, workers show especially high levels of aggression in the lab when they encounter foreign workers inside their nests (Buczkowski & Silverman 2005) and in the field when they have prior experience with conspecific workers (Thomas

et al. 2005a). The context-dependent nature of nestmate discrimination heightens the need for investigations that examine territorial aggression *in situ*. However, no study on Argentine ants has yet examined in detail how established supercolonies interact at points of direct contact in the field. Although recent studies have examined nests of different supercolonies that are relatively close to each other (Suarez *et al.* 1999; Tsutsui *et al.* 2000; Tsutsui & Case 2001; Jaquiery *et al.* 2005), none of these studies focused on actual zones of contact between colonies, where members of different supercolonies can potentially interact. Understanding the nature of such interactions seems key to interpreting the evolution of unicoloniality because direct interactions at territory boundaries will influence the costs of territoriality and the likelihood that colonies exchange genes.

In this study, we examine multiple contact zones between mutually antagonistic supercolonies of *L. humile* from California. We address four questions: (i) are territory boundaries spatially discrete? (ii) If so, do boundaries shift position over time? (iii) Do abutting colonies interact aggressively with one another? If so, how often do colonies fight with one another, and what potential costs are incurred? (iv) If well-delineated behavioural discontinuities exist between adjacent supercolonies, do these behavioural boundaries correlate with levels of genetic differentiation between supercolonies? Since little is known about how established colonies of unicolonial social insects interact when they contact one another under natural circumstances, answers to these questions will add to the growing body of knowledge about colony structure variation in social insects, shed light on the generality of previous experimental studies, and provide insight into the different competing hypotheses that have been proposed to explain the existence of colony structure variation in this and other social insect species.

Materials and methods

Study system

Five supercolonies of Argentine ants are currently known to occur in southern California: the large supercolony (LC), which extends as a nonaggressive behavioural unit for roughly 1000 km throughout much of coastal California (Tsutsui *et al.* 2000; Tsutsui *et al.* 2003), and four more spatially restricted supercolonies: Lake Hodges (LH), Sweetwater (SW), Lake Skinner (LS) (Suarez *et al.* 2002; Tsutsui *et al.* 2003) and Cottonwood (CW) (Thomas *et al.* 2005a). These smaller supercolonies range in size from approximately 11 km² (LH) to < 1 km² (LS). All supercolonies are mutually antagonistic to one another, and patterns of aggression at the colony level exhibit complete transitivity (Holway & Suarez 2004; Thomas *et al.* 2005a). The distribution of the southern California supercolonies is such that

the four geographically restricted supercolonies are not known to contact one another at present. However, because of LC's expansive distribution, it abuts SW, CW and LH and comes close to contacting LS. Because LH occupies a considerably larger area compared to SW, LS and CW, we were able to study the LC–LH border in the most detail. For the purposes of this paper, we use the terms 'contact zone', 'territory border', and 'territory boundary' interchangeably to refer to situations where two Argentine ant supercolonies come into direct contact with one another in the field.

Transects across territory boundaries

In October 2003 and 2004, we sampled Argentine ant workers from nine transects, each of which ran roughly perpendicular to a contact zone. We used aggression assays to identify territory borders (Breed 2003). By testing pairs of workers collected from different locations at varying distances from one another, we were able to isolate colony boundaries. Of the nine transects, six intersected the LC–LH border, two intersected the LC–SW border, and one intersected the LC–CW border. Transects were at least 200 m apart from one another and varied in length depending on the spatial dimensions of the smaller supercolonies. For all transects we collected workers from locations that were 1, 25 and 100 m away from territory boundaries. Because of the large size of LH, we were also able to collect workers that were 500 m away from the contact zone for the six transects that intersected the LC–LH border. We also collected workers from the following distances ('Far' category): > 1 km from the LC–LH boundaries, approximately 700 m from the LC–CW boundaries and approximately 150 m from the LC–SW boundaries. Differences in the 'Far' category for each supercolony pairing reflect the unequal size of the small supercolonies. We chose transects so they did not intersect more than one contact zone and did not cut across territory boundaries at points where physical barriers (e.g. paved roads) might limit opportunities for supercolonies to interact. Six transects were in suburban residential areas (five LC–LH and one LC–CW) and three were in riparian habitats (one LC–LH and two LC–SW).

We collected workers for behavioural analyses at sites along each transect and, for the six LC–LH transects, we also collected workers for genetic analyses. For the behavioural survey, we sampled workers along each transect on a single day and conducted aggression assays within two days of collection. All assays were conducted blind; the observer was unaware of the identity (i.e. transect number or distance category) of any of the ants in any of the trials. For each transect, we performed three replicate assays for every pairwise combination of distances and used the mean of these three assays as data points in all analyses. To

test how aggression levels vary as a function of spatial distance and genetic differentiation, we use all pairwise distance combinations in matrix correspondence tests (MCTs; see *Analysis of molecular data*). In the presentation of the behaviour data, we focus primarily on differences in aggression levels between and within supercolonies, in addition to changes in aggression intensity between opposing supercolonies as a function of distance away from territory borders. For a LH–LC transect, for example, we considered the following distance pairings for aggression levels, (i) between supercolonies, 1 m × 1 m, 25 m × 25 m, 100 m × 100 m, 500 m × 500 m, and Far × Far; and (ii) within supercolonies, we used nearest neighbour comparisons, 1 m × 25 m, 25 m × 100 m, 100 m × 500 m and 500 m × Far.

To estimate levels of intraspecific aggression between workers from paired sites, we used five-on-five behavioural assays (see Thomas *et al.* (2005b); technique modified from Roulston *et al.* (2003)). For each trial, we placed five workers from each location in a 10-cm^2 Petri dish with Fluon-lined inner walls to prevent ants from escaping. We then scanned the dishes once every minute for a total of 10 min and classified each worker's behaviour into one of four categories: (i) no interest, ignore; (ii) mandible gaping, avoidance, or intense antennation; (iii) aggression (a physical attack by one or both workers, including lunging, biting, holding or pulling of legs or antennae); and (v) fighting (aggression resulting in death or severe injury; includes the use of chemical defensive compounds).

A single aggression index ranging from 1 (no aggression) to 4 (intense aggression) was calculated from these 10-min trials. We determined the proportion of ants involved in each behavioural category at every minute increment and then calculated the average for each category. We next multiplied the average of each behaviour category by the aggression level for that behaviour (i.e. 1–4), and the sum of these numbers gave the final aggression index. For example, an index of 1 indicates no aggression during a trial, whereas an index of 4 means that all ants were fighting at the highest level within the first minute.

Spatial movement, intraspecific aggression and mortality at territory boundaries over time

We monitored 16 territory borders between April and September 2004 in order to determine (i) the spatial position of territory boundaries over time; (ii) to what extent abutting colonies engaged in territorial skirmishes; and (iii) whether or not mortality resulted from territorial battles. Of the 16 boundaries, 12 were between LC and LH (the six transect sites described above, as well as six additional sites), two were between LC and SW, and two were between LC and CW. All but three sites were in suburban residential areas; the three remaining territory boundaries (one LC–LH site and two LC–SW sites) were in

riparian habitats. We visited seven of the 16 sites monthly; primarily to determine changes in the position of boundaries. At the remaining nine territory boundaries (two LC–SW sites and seven LC–LH sites), we made weekly visits to obtain more detailed information. These nine sites were selected because they lacked physical barriers (e.g. paved roads) that might hinder contact between supercolonies. Weekly visits took place between the hours of 0600 and 1100 at ground temperatures suitable for Argentine-ant foraging.

On our weekly visits to the nine contact zones, we examined an area of several square metres that contained a segment of territory border where ants from opposing supercolonies directly contacted one another. In other words, each site was a tiny section of a much larger territory boundary. During every visit, we recorded the following: (i) the distance and direction of shifts in territory borders relative to their location the previous visit; (ii) the number of dead workers; and (iii) the intensity of fighting. To measure shifts in territory boundaries, we first used fighting assays to identify the location of each border, and then marked its location such that changes could be measured during subsequent visits. All boundary shifts were measured along an axis running perpendicular to the contact zone. Dead workers were collected only in the immediate vicinity of where ants were observed fighting. We used an aspirator to retrieve dead workers, which were later counted in the lab. For the purpose of comparison, we also searched for dead workers approximately 10 m away from each contact zone (but on substrates resembling those at the territory border). Despite comparable sampling effort in areas located away from territory boundaries, we located no dead workers at these sites. We measured fighting intensity at each territory border by observing ant activity in an area of 50×30 cm over a 5-min period. We positioned this observation area as closely as possible to where recruitment trails of opposing supercolonies intersected. During each 5-min period, we counted the number of workers engaged in each of three different levels of escalating aggression: 1 = avoidance (contacts between pairs of workers that resulted in one or both ants recoiling and running away), or use of chemical defenses (a worker lifting its gaster and exuding (or threatening to exude) chemical defensive compounds when confronting an opponent); 2 = holding (a physical attack by one or both workers, including lunging, biting or pulling of legs or antennae); and 3 = fighting (aggression that often resulted in impairment or death). For every 5-min period, we multiplied the number of workers observed in each aggression category by the level of aggression (i.e. 1, 2 or 3) and summed these products to generate a measure of fighting intensity.

Based on the empirical distribution of fighting-intensity measurements obtained during weekly visits to territory borders, we scored these measures on a five-point scale.

Scores of 0 were assigned to border visits in which no fighting was observed. Scores of 1 were given to visits in which workers were observed at the territory boundary in a state of apparent agitation, but no fighting was observed during the 5-min observation period. Scores of 1 were also given to situations in which intercolony aggression was observed but not in the 5-min observation period. Scores from 2 to 4 corresponded to escalating levels of intercolony aggression: 2 = fighting intensity measurements from 1 to 10; 3 = measurements from 11 to 49; and 4 = measurements ≥ 50. For data analysis, we summed each site's score across six months of weekly visits. Summed aggression scores index the potential for mortality to result from intercolony aggression. We then used a Spearman rank correlation to test the relationship between the cumulative number of dead workers collected and these summed aggression scores. We used weekly sites as independent replicates ($n = 8$) in this analysis. We excluded one riparian site because deep leaf litter prevented the collection of behavioural data.

Microsatellite genotyping

At the six LC–LH transects described under *Transects across territory boundaries*, we collected approximately 100 workers at each distance category on the same day that we collected workers for behavioural analysis. The 100-worker samples were placed in 95% ethanol immediately after collection. DNA was later extracted from these workers following the protocol of Sunnucks & Hales (1996). Polymerase chain reactions (PCR) were carried out using four microsatellite loci (Krieger & Keller 1999): Lhum-11 (GenBank Accession no. AF093517), Lhum-18 (AF093521), Lhum-19 (AF093522) and Lhum-52 (AF093531). Forward primers were fluorescently labelled at the 5′ end either with HEX (Lhum-11, Lhum-52) or 6-FAM (Lhum-18, Lhum-19).

Each locus was amplified independently in a 10 μL PCR. Reaction conditions were as follows for Lhum-18, Lhum-19 and Lhum-52: 1.0 μL of PCR buffer (containing 1.5 mM $MgCl_2$), 0.08 μL of dNTP (25 mM each base), 0.2 μL of fluorescently labelled forward primer (10 mM), 0.2 μL reverse primer (10 mM), 0.1 μL Taq (5 u/mL), 0.5 μL of template DNA and 7.92 μL PCR water. After an initial denaturation at 94 °C for 2 min, we ran 35 cycles at the following temperatures: denaturation at 92 °C for 50 s, annealing for 50 s, elongation at 72 °C for 1 min. These cycles were followed by a final extension step at 72 °C for 5 min. Annealing temperatures were 58 °C for Lhum-19 and Lhum-52, and 53 °C for Lhum-18. The size of each amplified region was determined using an ABI Prism 3700 sequencer at the Illinois Genetic Marker Center. Fragments on each gel were compared to the ABI ROX 500 size standard. Analyses were conducted using the program GENESCAN 3.1.2 (Applied Biosystems).

For Lhum-11, the PCR conditions were changed slightly so that the denaturing, annealing and extension steps were

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all 30 s instead of 50 s, the annealing temperature was 48 °C, and 0.1 microlitres each of the forward and reverse primers were used per reaction (instead of 0.2 microlitres each). PCR reactions were diluted to 1/20 (0.5 microlitres product + 9.5 microlitres of water) and cleaned using Promega binding plates. Fragment sizes for Lhum-11 were determined at the W.M. Keck Center for Comparative and Functional Genomics using an ABI Prism 3730xl Analyser, the size standard LIZ500, and analysed using GENEMAPPER 3.7 (Applied Biosystems).

Analysis of molecular data

We used ARLEQUIN 2.0 (Schneider *et al.* 2000) to calculate estimates of genetic differentiation (F_{ST}) between ants from all pairs of nests along each of the six LC–LH transects. To test for associations between intraspecific aggression, spatial distance and genetic differentiation, we used matrix correspondence tests (MCTs) as implemented in the program PERMUTE 3.4 (Legendre *et al.* 1994). We first determined whether or not F_{ST} increased with spatial distance. We then used pairwise MCTs to examine variation in aggression as a function of spatial distance and genetic differentiation, and used a partial MCT to assess the relative contributions of these two factors. In all cases we determined statistical significance by performing 10 000 random permutations (Legendre *et al.* 1994; Manly 1997).

We used a Bayesian model-based clustering analysis, as implemented in the program STRUCTURE, to identify population genetic patterns along each transect (Pritchard *et al.* 2000; Falush *et al.* 2003). We conducted 10 000 iterations of burn-in, followed by an additional 10 000 iterations of data collection, and we applied a model of correlated allele frequencies (Falush *et al.* 2003). In each case, we set the number of hypothetical populations (K) to 2, since our goal was to test the assignment of each individual to each of the two putative supercolonies. We visualized the results using the program DISTRUCT (Rosenberg 2004).

Results

Transects across territory boundaries: intraspecific aggression

When all pairwise distance comparisons were considered (945 assays for all 9 transects), we observed that aggression scores were low for within-supercolony comparisons (1.1 ± 0.02 (Mean ± SE)) and high for between-supercolony comparisons (2.89 ± 0.05). At all nine transects, we also observed well-defined and abrupt territorial boundaries between opposing supercolonies (Fig. 1). In fact, levels of aggression exhibited by workers from territory boundaries were consistently higher than those of workers farther away from the boundaries (Fig. 1). For example, If each

of the nine transects is considered as an independent replicate, levels of aggression between nests immediately on either side of the colony boundaries (aggression score = 3.17 ± 0.37) were higher than between nests located 100 m on either side of the colony boundary (2.50 ± 0.22) (paired t-test: $t = 2.88$, d.f. = 8, $P < 0.05$). Comparisons between workers collected from the immediate vicinity of the territory border, on one hand, and 500 m (or the 'Far' categories) on the other, also show a similar discrepancy in aggression levels.

Spatial movement, intraspecific aggression and mortality at territory boundaries over time

Although all 16 of the territory boundaries that were monitored for six months were consistently abrupt and well defined, their spatial position changed during the course of the monitoring period (Table 1). However, these movements typically resulted in little net movement over the entire period of observation, and there was no evidence of one supercolony steadily encroaching upon the area occupied by an adjacent supercolony (Table 1).

Abutting supercolonies of Argentine ants commonly engaged in intraspecific conflicts, and these battles resulted in considerable worker mortality. On average, we observed fights between supercolonies on 40% of visits (range across the 16 sites = 6–100% of visits). During the six-month survey period, mortality occurred in every month with no pronounced seasonal peak (Fig. 2a, c, e), but mortality may have been higher from July to September than from April to June (paired t-test: $t = 2.039$, d.f. = 8, $P = 0.076$; mortality counts summed across three-month intervals). The cumulative number of dead workers collected throughout the six-month observation period ranged from 26 to 208 534; the median count was approximately 15 000 workers (a LC–SW site) (Fig. 2). These cumulative mortality estimates were positively correlated with the sum of weekly aggression scores (Spearman rank correlation, $r_s = 0.857$, $n = 8$, $P < 0.01$). At the three contact zones where fights were infrequent and relatively little mortality was detected (Fig. 2; all three were LC–LH sites), Argentine-ant densities were low, presumably because the local physical environment was too dry to support higher numbers.

Transects across territory boundaries: genetic differentiation

Patterns of genetic differentiation indicate that the LC and LH supercolonies are genetically distinct from one another and that the abrupt behavioural boundaries illustrated in Fig. 1 are concordant with sharp genetic discontinuities. Estimates of F_{ST} between ants from nests within the same supercolony were consistently lower compared to estimates of F_{ST} between ants from different supercolonies (Fig. 3;
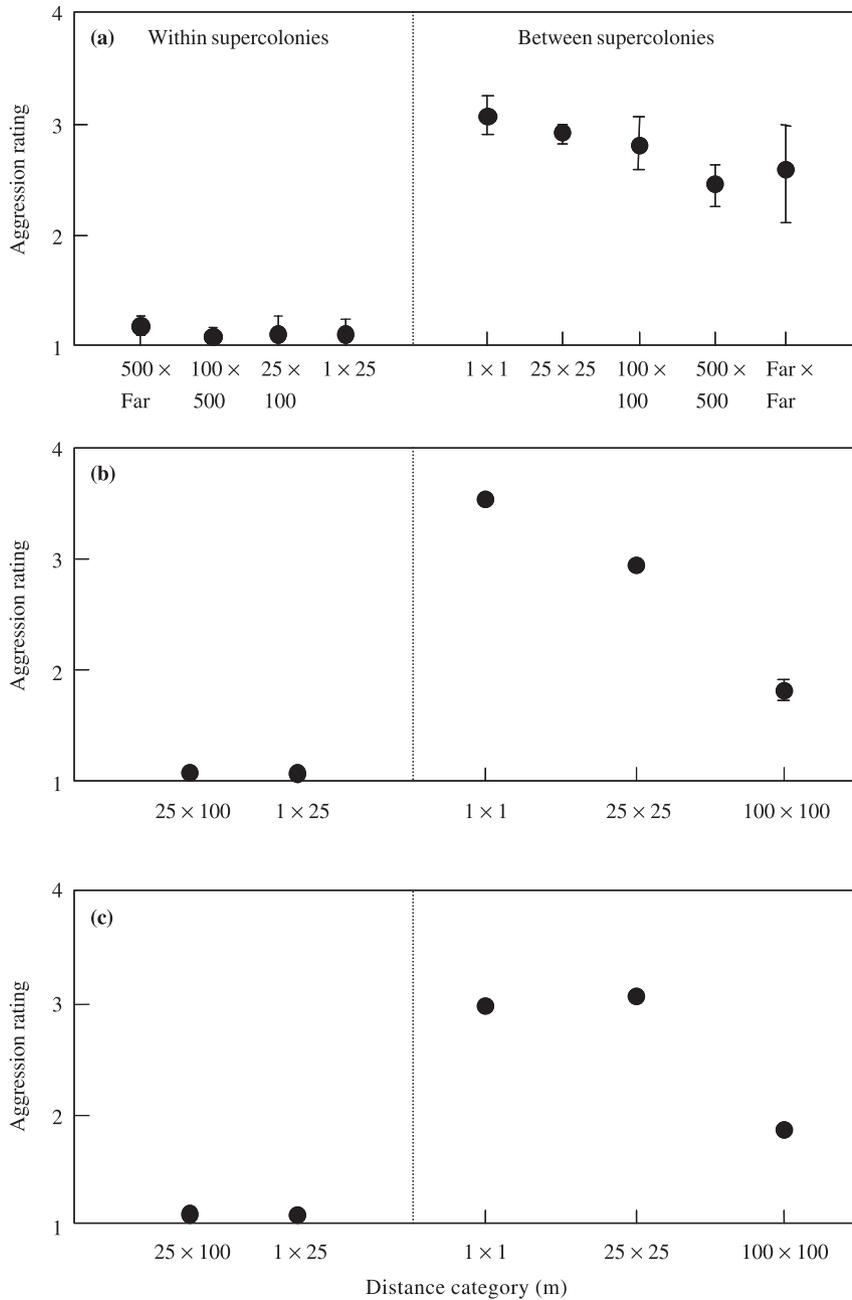
Fig. 1 Mean (± SE) levels of intraspecific aggression between workers from (i) within the same supercolony (left-hand side); and (ii) between opposing supercolonies (right-hand side). For simplicity we only consider the nearest-neighbour distance pairings for aggression levels within supercolonies (1 m × 25 m, 25 m × 100 m (all supercolony pairs), 100 m × 500 m and 500 m × Far (LH and LC only)) and the following distance categories for between supercolony comparisons; 1 m × 1 m, 25 m × 25 m, 100 m × 100 m (all supercolony pairs, 500 m × 500 m, and Far × Far (LH and LC only). Differences in the X-axes are due to size variation between the small supercolonies. a, LH and LC colony pairs; b, SW and LC; and c, CW and LC.

F_{ST} within = 0.08 ± 0.01, F_{ST} between = 0.29 ± 0.01 (mean ± SE)). For all six transects, such disparities in F_{ST} were especially noticeable in the vicinity of territory boundaries, and in four transects estimated F_{ST} values within supercolonies showed little to no overlap with estimated F_{ST} values between supercolonies at any distance (Fig. 3).

Similarly, the Bayesian cluster analysis using STRUCTURE showed that the two colonies along each transect were clearly and unambiguously genetically distinct from each other (Fig. 4). For four transects (1, 2, 3 and 5), there was no indication of historical or ongoing gene flow across the colony boundaries, even among nests just several metres

apart. In transect 4, all ten of the individuals in the Lake Hodges (LH) nests immediately flanking the colony boundary were weakly assigned to LSC, and both of the nests next to the colony boundary in transect 6 (nests LSC1 and LH1) contained some individuals who were assigned with low probability to the opposite colony.

Additional evidence for restricted gene flow between supercolonies comes from the distribution of private alleles in workers collected along each transect (Fig. 5). Although by definition these private alleles will be present in one supercolony and absent from the other, patterns of allele-frequency changes within supercolonies provide

Territory border	Mean (± SE) monthly shift	Maximum monthly shift	Net spread over 6 mos.	In favour of …
LH vs. LC				
1	0.1 ± 2.3	12.4	0.5	LH
2	0.4 ± 4.6	22.3	2.9	LC
3	0.0 ± 0.0	0.0	0.0	—
4	7.0 ± 7.0	48.7	48.7	LH
5	10.1 ± 4.6	35.0	70.8	LH
6	0.4 ± 1.8	19.6	8.5	LC
7	0.4 ± 0.6	14.2	11.5	LH
8	0.2 ± 1.5	24.5	3.8	LC
9	0.6 ± 0.8	13.2	14.7	LH
10	0.5 ± 0.3	7.2	11.2	LC
11	0.4 ± 0.3	3.8	8.7	LH
12	1.0 ± 1.0	16.2	25.7	LC
LC vs. SW				
1	0.1 ± 0.2	3.3	2.3	SW
2	0.2 ± 0.1	2.0	3.9	LC
LC vs. CW				
1	2.5 ± 2.5	15.0	15.0	CW
2	2.1 ± 5.1	30.0	15.0	LC

Table 1 Summary of monthly spatial movements for 16 territory boundaries between abutting supercolonies of Argentine ants. All distances are in metres
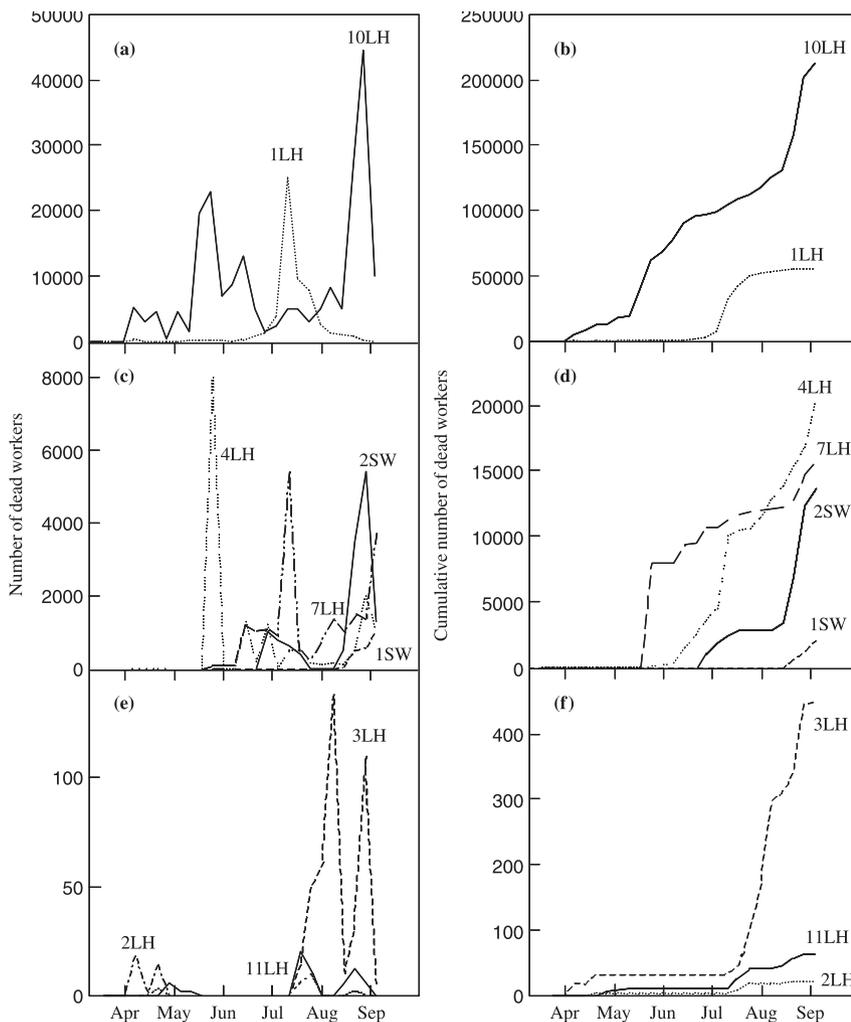
Fig. 2 The number of dead workers collected from nine territory boundaries at weekly intervals over a 6-month observation period. Because the number of dead workers varied considerably from site to site, graphs are divided into high (a–b), medium (c–d) and low (e–f) levels of mortality. Both weekly (a, c and e) and corresponding cumulative (b, d and f) mortality counts are illustrated. Each line represents mortality at a different contact zone and is illustrated by a number and the small supercolony symbol (e.g. LH, SW or CW).
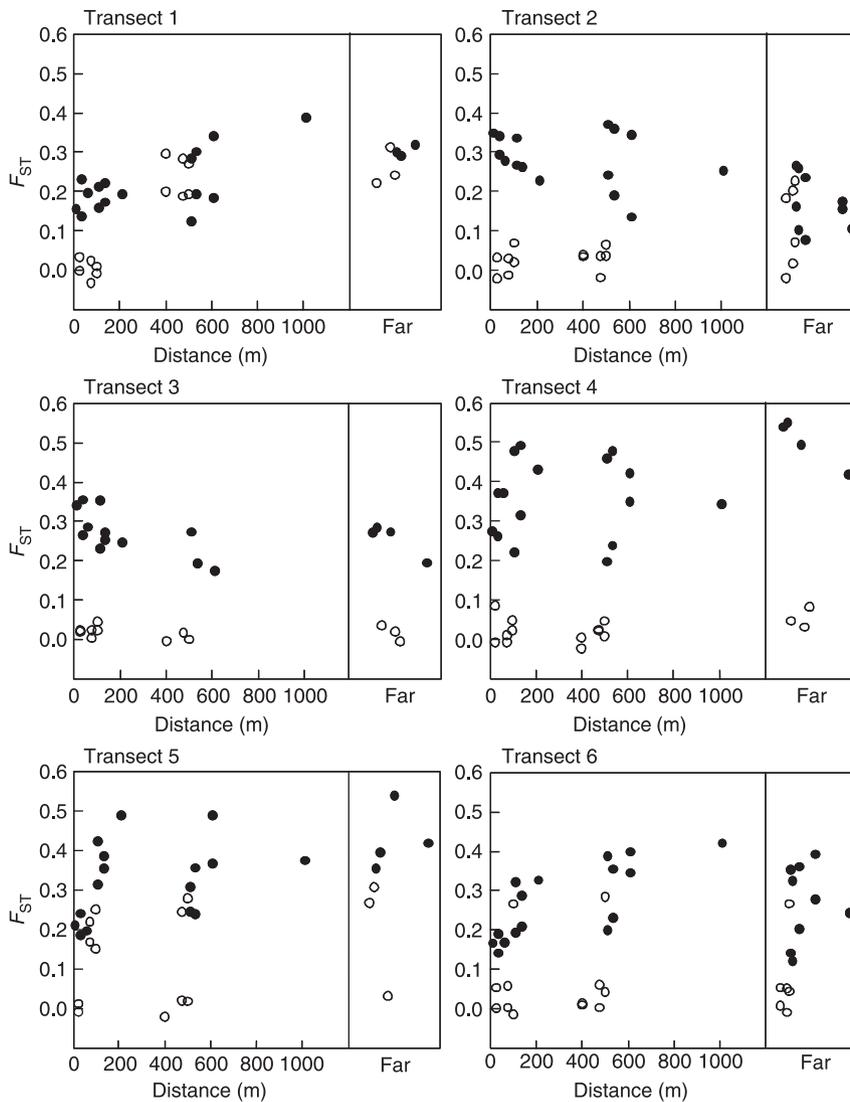
Fig. 3 Pairwise F_{ST} between workers from different nests plotted against distance between nests for six transects that span contact zones between the large supercolony (LC) and the Lake Hodges supercolony (LH). Closed circles show between super-colony comparisons; open circles show within supercolony comparisons.

insight into the degree of genetic connectivity between colonies. If migration (and hence, gene flow) across colony borders is high, then the frequency of private alleles should decrease with increasing proximity to the boundary. Along each LC–LH transect, two to five alleles were found in only one of the two supercolonies. Many of these private alleles were rare, occurring at frequencies lower than 0.10 within a single transect, and were thus uninformative because of potential sampling error. However, 12 private alleles occurred at frequencies greater than 0.10 in the supercolony for which that allele occurred. The frequencies of these alleles do not appear to decrease with increasing proximity to territory borders, further suggesting that gene flow across colony boundaries is low to absent (Fig. 5).

There is no evidence of genetic isolation-by-distance within supercolonies; estimated F_{ST} values between ants from neighbouring nests appear no lower than estimated F_{ST} values between ants from more distant nests in the same supercolony (Fig. 3). These patterns suggest that levels of gene flow are sufficiently high to genetically homogenize nests within the same supercolony.

Pairwise MCTs showed that genetic relationships between ants from different nests significantly explained the level of aggression that they displayed toward one another (Table 2). In contrast, geographic distance between nests failed to significantly predict either the level of aggression or genetic differentiation (Table 2). Similarly, when the effects of spatial distance were accounted for in the partial MCT, F_{ST} remained a significant predictor of aggression. In contrast, when the effects of genetic differentiation (F_{ST}) were accounted for in the partial MCT, geographic distance between nests did not significantly predict the level of aggression between workers from those nests (Table 2).
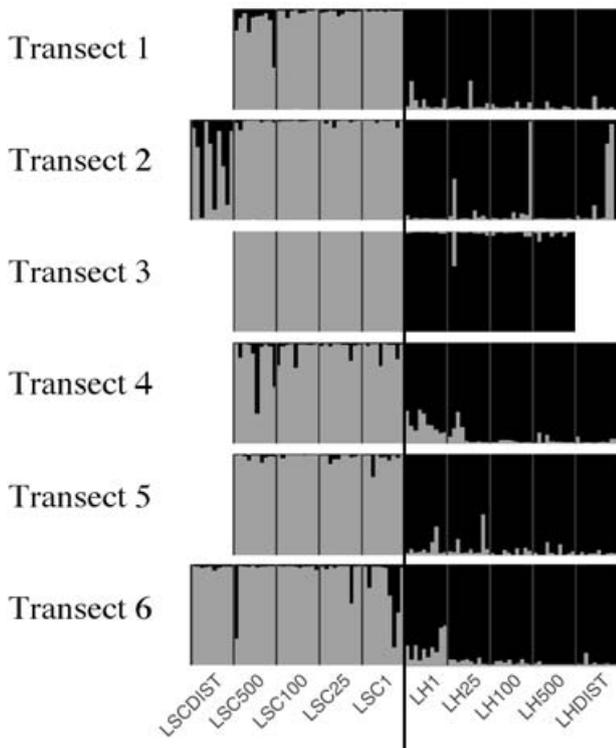
Fig. 4 Results of the genetic analysis using the program STRUCTURE. Each individual is represented by a thin vertical line; the shading of each line indicates the estimated assignment for that individual to one or the other potential populations of origin. The sampling sites are shown in their correct geographic orientation, from one end of each transect, through the colony boundary, to the other end of the transect. The left-to-right ordering of individuals within each sampling site (nest) is arbitrary.

Discussion

Our study provides empirical field data on intra-specific territorial interactions in a unicolonial social insect. In comparisons involving three different supercolony pairings, we observed abrupt behavioural boundaries between opposing supercolonies along all nine transects surveyed; each of which spanned a different border location (Fig. 1). At the nine territory borders visited weekly, we observed colony-level battles between supercolonies during an average of 40% of visits. Fights occurred throughout the six-month observation period, involved intense and conspicuous intercolony aggression, and resulted in variable — but on average large — amounts of worker mortality (Fig. 2). Estimates of mortality were positively correlated to the observed intensity of fighting, indicating that dead workers at the territory boundaries were the products of intercolony conflicts and not of some other cause. Moreover, dead workers were not found at sites located away from the colony boundaries (see Methods), providing additional evidence that the mortality recorded was a direct consequence of the observed aggression.

The well-delineated nature of Argentine ant territory boundaries was also evident from patterns of genetic variation. Along six transects that spanned different parts of the LC–LH contact zone, between-nest F_{ST} estimates, genetic cluster analysis and the frequencies of private alleles indicate little to no gene flow across territory borders, despite direct and prolonged contact between supercolonies on either side of the boundary. The genetic cluster analysis using STRUCTURE revealed a similar pattern. Along all transects, virtually all individuals were assigned with high probability to their putative colony of origin, and there was no apparent gene flow across the colony boundaries in four of the six transects. Along the two transects where there appeared to be a small amount of gene flow between the neighbouring nests that flank the colony boundaries (transects 4 and 6), this gene flow did not appear to penetrate beyond one metre into the recipient colony, and these individuals were all assigned to the opposite colony with low probability. Moreover, for all six transects, F_{ST} estimates between nests on either side of territory borders (separated by only a few metres) were substantially higher compared to F_{ST} estimates between nests within the same supercolony (Fig. 3). A close correlation exists between behavioural and genetic variation: ants within the same supercolony did not fight with one another and had low levels of genetic differentiation, whereas ants from different supercolonies nearly always fought and had relatively high levels of genetic differentiation (Table 2). Geographic

Table 2 Relationships among geographic distance, genetic differentiation (F_{ST}), and aggression for workers from all pairs of Argentine ant nests collected along a transect. For the pairwise matrix correspondence tests (MCTs), we regressed the dependent variable (Y) against a single predictor matrix (X_1). For the partial MCTs, we included a second predictor matrix, X_2. In these cases, b is the partial regression coefficient of the effect of X_1 on Y after accounting for the effects of X_2

Y	X_1	X_2	b	P	R^2
F_{ST}	Geographic distance	—	0.083	0.243	0.007
Aggression	Geographic distance	—	−0.033	0.618	0.001
Aggression	F_{ST}	—	0.584	< 0.001	0.342
Aggression	Geographic distance	F_{ST}	−0.082	0.066	0.349
Aggression	F_{ST}	Geographic distance	0.591	0.001	
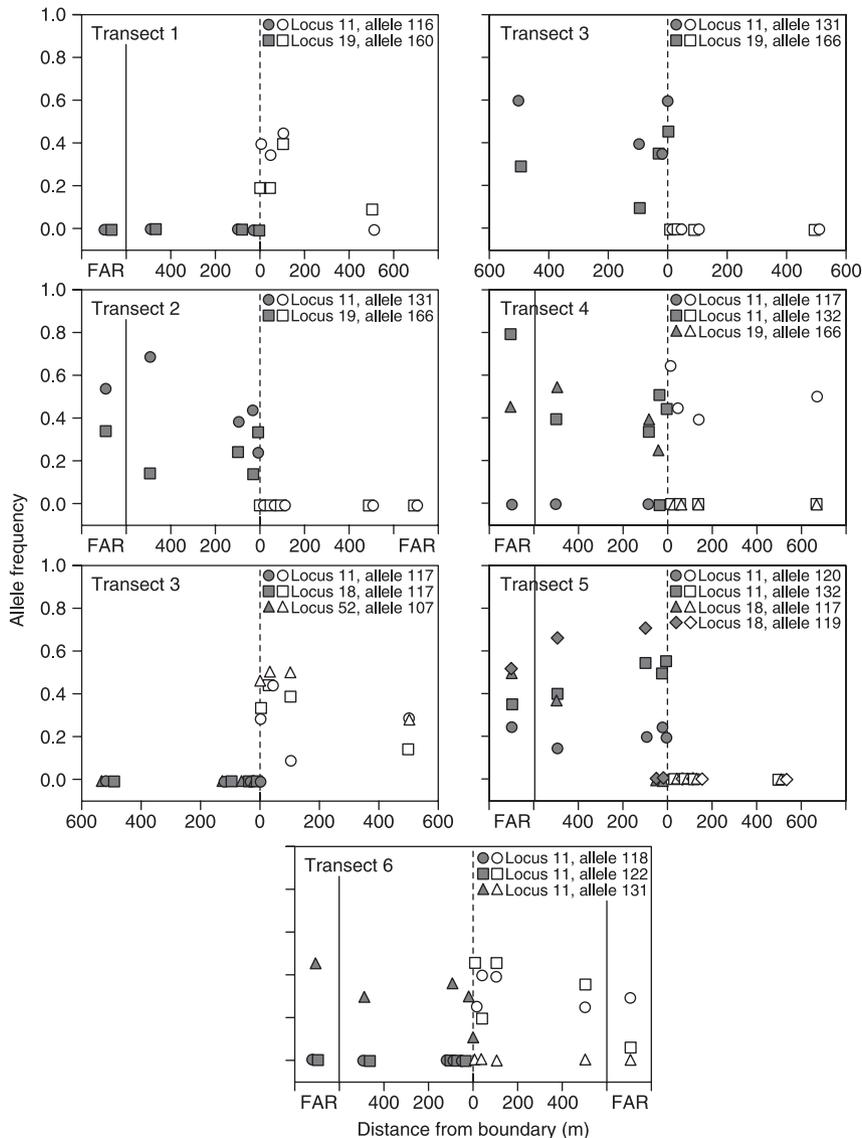
Fig. 5 Frequencies of the 12 most common private alleles plotted against distance from colony boundaries for six transects that span contact zones between the Lake Hodges supercolony (LH, shaded symbols) and the large supercolony (LC, open symbols). Vertical dashed lines indicate the position of territory borders. Allele frequencies at spatially distant nests ('Far' ≥ 1 km from colony boundaries) are shown at the far left and far right of graphs. Overlapping points are slightly offset for clarity.

distance between nests, however, was not significantly associated with either intraspecific aggression or genetic differentiation (Table 2).

As previously documented in most other studies of introduced populations of *Linepithema humile* (Tsutsui & Case 2001; Giraud *et al.* 2002; Buczkowski *et al.* 2004; Jaquiery *et al.* 2005; but see Ingram & Gordon 2003), we detected no evidence of genetic isolation by distance within supercolonies. This suggests that gene flow (e.g. from male mating flights or jump-dispersal events) is sufficiently high within supercolonies to prevent ants from different locations from becoming genetically differentiated. In contrast, gene flow between supercolonies appears low or absent, despite the fact that at all six transects for which we genotyped ants, workers from different supercolonies were in direct and prolonged contact with one another at territory borders.

Jaquiery *et al.* (2005) similarly report a lack of gene flow between a pair of mutually antagonistic *L. humile* supercolonies from Spain, but these supercolonies did not have physical contact with one another. In the present study, limited to no gene flow between abutting supercolonies suggests that supercolonies rarely if ever exchange workers or queens across territory borders, and that males probably do not disperse short distances across colony boundaries. However, population-genetic studies of Argentine ants in their native range have shown a pattern of genetic isolation by distance among colonies, indicating that native populations do experience local or regional gene flow (Tsutsui & Case 2001). No such pattern was evident among colonies in the introduced range (Tsutsui & Case 2001).

The intense intraspecific aggression observed at territory boundaries in part corroborates lab studies that examine

how colony structure variation in *L. humile* may influence intraspecific and interspecific competitive performance (Holway *et al*. 1998; Holway & Suarez 2004). Our results, for example, show that intraspecific aggression and the substantial mortality that can ensue are not artefacts of rearing colonies in a structurally simple lab environment. Abutting supercolonies often fought with one another in the field in our study, and colony-level battles resulted in considerable worker mortality (Fig. 2). Week-to-week movements of territory borders (Table 1) further suggest that supercolonies more or less continuously contest space at territory borders, with neither supercolony capturing a considerable portion of the abutting supercolony's territory. It is worth emphasizing that each of our mortality estimates (i) consisted of a one-dimensional slice through a boundary that is several kilometres long; and (ii) represents a minimum measure because we undoubtedly missed many dead workers that were hard to see, scavenged by ants or scattered by the wind. When our estimates are extrapolated over the entire length of the supercolony boundary, the total mortality resulting from territorial aggression would undoubtedly be enormous. For example, the Lake Hodges supercolony has a circumference of about 30.5 km. Even if only one-fourth of its border comes into contact with the main supercolony that surrounds it, over 15 million workers would have died over the six-month period of the study. Site-to-site differences in the level of mortality appeared to be due to variation in the local density of *L. humile*. At territory borders where ant densities were low, presumably as a result of unfavourable physical conditions, minimum mortality estimates were also low.

Translating observed mortality (Fig. 2) into terms of reduced fitness remains difficult owing to the extensive interconnectedness of nests within supercolonies and the likelihood that workers from multiple nests are recruited to contact zones to engage in battles. In all cases, examined supercolonies extended away from territory borders for hundreds of metres, and active conflicts between supercolonies were often fed by more than one recruitment trail. As a result of this territory geometry, one would not expect lowered worker densities in the immediate vicinity of the contact zones; nor would one expect opportunities for native ant establishment to occur as a result. Associations between colony structure, colony density and ecological dominance (Holway *et al*. 1998; Holway & Suarez 2004) clearly require further examination.

Although introduced populations of Argentine ants can form supercolonies that extend for hundreds of kilometres, our data show that boundaries separating different supercolonies are well delineated and aggressively contested, and that colony-level fights take place often and appear to be costly. Supercolony territories seem to correspond to a case of absolute territoriality in the sense of Hölldobler & Lumsden (1980) in that the space surrounding nest sites is continuously and aggressively defended. Since territorial aggression was observed throughout the six-month observation period, it seems unlikely that Argentine ants fight in response to a particular seasonal cue or to seasonally available resource. Our results are also inconsistent with the notion of an inverse relationship between intraspecific and interspecific aggression (Hölldobler & Wilson 1977; Passera 1994). The intense intraspecific aggression observed at territory boundaries in this study seems difficult to distinguish from the hostility that Argentine ants direct towards other ant species (Human & Gordon 1999; Holway *et al*. 2002; Zee & Holway 2006).

The formation and maintenance of supercolonies

The results described in this study contribute important information to an understanding of the evolution of unicoloniality. As other studies have concluded (Tsutsui & Case 2001; Giraud *et al*. 2002; Jaquiery *et al*. 2005), our findings support the notion that mutually antagonistic supercolonies are genetically differentiated from one another. In addition, the well-delineated and aggressively defended colony boundaries observed in this study are broadly consistent with the hypothesis that selection against novel recognition alleles could prevent genetic diversity from increasing (e.g. through mutation, male dispersal or introduction events) in areas where Argentine ants are well established (Giraud *et al*. 2002; Tsutsui *et al*. 2003). Our results do not uphold the idea that territory defence disappears as a result of relaxed ecological constraints and elevated colony densities (Giraud *et al*. 2002; see also Ross *et al*. 1996). Based on the size of the LH supercolony and the length of border it shares with the LC supercolony, the opportunity for these colonies to interact has probably existed for decades. Moreover, the observation that workers from the immediate vicinity of borders interact more aggressively with one another compared to workers away from borders (Fig. 1; Thomas *et al*. 2005a) is the opposite pattern one would expect if territory boundaries were in the process of dissolving.

Finally, the genetic and behavioural patterns illustrated in this study appear inconsistent with the use of environmental cues as the primary determinant of colony-specific recognition. Although, in principle, odour cues derived from environmental sources (e.g. nesting material or food) can influence nestmate recognition in Argentine ants (Liang & Silverman 2000), it seems unlikely that such a recognition system could produce abrupt behavioural (Fig. 1) and genetic (Fig. 5) boundaries as well as their week-to-week shifts in position (Table 1). No discernable environmental gradients exist in the vicinity of supercolony borders, and it seems doubtful that the well-delineated territory boundaries documented here correspond to congruent spatial patterns in the availability of insect prey species that also influence nestmate recognition.

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References

Bourke AFG, Franks NR (1995) *Social Evolution in Ants*. Princeton University Press, Princeton, New Jersey.

Breed MD (2003) Nestmate recognition assays as a tool for population and ecological studies in eusocial insects: a review. *Journal of the Kansas Entomological Society*, **76**, 539–550.

Buczkowski G, Silverman J (2005) Context-dependent nestmate discrimination and the effect of action thresholds on exogenous cue recognition in the Argentine ant. *Animal Behaviour*, **69**, 741–749.

Buczkowski G, Vargo EL, Silverman J (2004) The diminutive supercolony: the Argentine ants of the southeastern United States. *Molecular Ecology*, **13**, 2235–2242.

Chen JSC, Nonacs P (2000) Nestmate recognition and intraspecific aggression based on environmental cues in Argentine ants (Hymenoptera: Formicidae). *Annals of the Entomological Society of America*, **93**, 1333–1337.

Crozier RH (1979) Genetics of sociality. In: *Social Insects* (ed. Herman HR), pp. 223–286. Academic Press, New York.

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics*, **164**, 1567–1587.

Giraud T, Pedersen JS, Keller L (2002) Evolution of supercolonies: the Argentine ants of southern Europe. *Proceedings of the National Academy of Sciences, USA*, **99**, 6075–6079.

Hölldobler B, Lumsden CJ (1980) Territorial strategies in ants. *Science*, **210**, 732–739.

Hölldobler B, Wilson EO (1977) The number of queens: an important trait in ant evolution. *Naturwissenschaften*, **64**, 8–15.

Hölldobler B, Wilson EO (1990) *The Ants*. Belknap Press, Cambridge, Massachusetts.

Holway DA, Suarez AV (2004) Colony structure variation and interspecific competitive ability in the invasive Argentine ant. *Oecologia*, **138**, 216–222.

Holway DA, Suarez AV, Case TJ (1998) Loss of intraspecific aggression in the success of a widespread invasive social insect. *Science*, **282**, 949–952.

Holway DA, Lach L, Suarez AV, Tsutsui ND, Case TJ (2002) The causes and consequences of ant invasions. *Annual Review of Ecology and Systematics*, **33**, 181–233.

Human KG, Gordon DM (1999) Behavioral interactions of the invasive Argentine ant with native ant species. *Insectes Sociaux*, **46**, 159–163.

Ingram KK, Gordon DM (2003) Genetic analysis of dispersal dynamics in an invading population of Argentine ants. *Ecology*, **84**, 2832–2842.

Jaquiery J, Vogel V, Keller L (2005) Multilevel genetic analyses of two European supercolonies of the Argentine ant, *Linepithema humile*. *Molecular Ecology*, **14**, 589–598.

Kaspari M (2005) Global energy gradients and size in colonial organisms: worker mass and worker number in ant colonies. *Proceedings of the National Academy of Sciences, USA*, **102**, 5079–5083.

Krieger MJB, Keller L (1999) Low polymorphism at nineteen microsatellite loci in a French population of Argentine ants (Linepithema humile) *Molecular Ecology*, **8**, 1078–1080.

Legendre P, Lapointe F-J, Casgrain P (1994) Modeling brain evolution from behavior: a permutation regression approach. *Evolution*, **48**, 1487–1499.

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

Manly FJB (1997) *Randomization, Bootstrap, and Monte Carlo Methods in Biology*. Chapman & Hall, New York.

Passera L (1994) Characteristics of tramp species. In: *Exotic Ants: Biology, Impact and Control of Introduced Species* (ed. Williams DF), pp. 23–43. Westview Press, Boulder, Colorado.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

Rosenberg NA (2004) DISTRUCT: a program for the graphical display of population structure. *Molecular Ecology Notes*, **4**, 137–138.

Ross KG, Vargo EL, Keller L (1996) Social evolution in a new environment: the case of introduced fire ants. *Proceedings of the National Academy of Sciences, USA*, **93**, 3021–3025.

Roulston TH, Buczkowski G, Silverman J (2003) Nestmate discrimination in ants: effect of bioassay on aggressive behavior. *Insectes Sociaux*, **50**, 151–159.

Schneider S, Roessli D, Excoffier L (2000) ARLEQUIN version 2.000: *A Software for Population Genetic Analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Starks PT (2003) Selection for uniformity: xenophobia and invasion success. *Trends in Ecology and Evolution*, **18**, 159–162.

Sturtevant AH (1938) Essays on evolution. II. On the effects of selection on social insects. *Quarterly Review of Biology*, **13**, 74–76.

Suarez AV, Tsutsui ND, Holway DA, Case TJ (1999) Behavioral and genetic differentiation between native and introduced populations of the Argentine ant. *Biological Invasions*, **1**, 43–53.

Suarez AV, Holway DA, Liang DS, Tsutsui ND, Case TJ (2002) Spatio-temporal patterns of intraspecific aggression in the invasive Argentine ant. *Animal Behaviour*, **64**, 697–708.

Sunnucks P, Hales DF (1996) Numerous transposed sequences of mitochondrial cytochrome oxidase I–II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). *Molecular Biology and Evolution*, **13**, 510–524.

Thomas ML, Tsutsui ND, Holway DA (2005a) Intraspecific competition influences the symmetry and intensity of aggression in the invasive Argentine ant. *Behavioral Ecology*, **16**, 472–481.

Thomas ML, Dixson A, Coggins V, Holway DA (2005b) Food availability and brood number do not influence intraspecific aggression in Argentine ants. *Insectes Sociaux*, **52**, 108–113.

Tsutsui ND, Case TJ (2001) Population genetics and colony structure of the Argentine ant (*Linepithema humile*) in its native and introduced ranges. *Evolution*, **55**, 976–985.

Tsutsui ND, Suarez AV, Holway DA, Case TJ (2000) Reduced genetic variation in the success of an invasive species. *Proceedings of the National Academy of Sciences, USA*, **97**, 5948–5953.

Tsutsui ND, Suarez AV, Grosberg RK (2003) Genetic diversity, asymmetrical aggression, and recognition in a widespread invasive species. *Proceedings of the National Academy of Sciences, USA*, **100**, 1078–1083.

Wilson EO, Hölldobler B (2005) Eusociality: origin and consequences. *Proceedings of the National Academy of Sciences, USA*, **102**, 13367–13371.

Zee J, Holway DA (2006) Nest raiding by the invasive Argentine ant on colonies of the harvester ant, *Pogonomyrmex subnitidus*. *Insectes Sociaux*, **53**, 161–167.

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