

# Queen acceptance and the complexity of nestmate discrimination in the Argentine ant

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**Abstract** In most social insect species, individuals recognize and behave aggressively towards non-nestmate conspecifics to maintain colony integrity. However, introduced populations of the invasive Argentine ant, *Linepithema humile*, exhibit pronounced variation in intraspecific aggression denoting diversity in nestmate recognition behavior, which possibly shapes their social structure and the varying levels of unicoloniality observed among these populations. One approach to better understand differential aggression behaviors towards conspecifics and recognition cue perception and response in *L. humile* is to examine variation in nestmate discrimination capability among genetically distinct colonies under different social contexts. Consequently, we investigated the dynamics of queen and worker recognition in southeastern US *L. humile* queenless and queenright colonies by measuring rates of non-nestmate worker and queen adoption and intercolony genetic similarity. Aggression levels between colony pairs differed and were associated with non-nestmate worker, but not queen adoption. Adoption of queens and workers was a function of host colony origin, while colony queen number affected adoption of queens, but not workers, with queens more readily accepted by queenless hosts. Fecundity of adopted non-nestmate queens was comparable to that of rejected non-nestmate and host colony queens, suggesting that queen fecundity did not affect adoption decisions. Genetic similarity between colonies ranged from 30 to 77% alleles shared, with more genetically similar colonies showing lower levels

of intraspecific aggression. Non-nestmate queens and workers that were more genetically similar to host colony workers were more likely to be adopted. We provide the first evidence for the role of *L. humile* colony queen number on queen discrimination and suggest an effect of resident queens on worker conspecific acceptance thresholds. Our findings indicate a role for genetically based cues in *L. humile* nestmate recognition. However, subtle discrimination capability seems to be influenced by the social context, as demonstrated by more frequent recognition errors in queenless colonies.

**Keywords** Argentine ant · *Linepithema humile* · Nestmate recognition · Aggression · Genetic similarity · Non-nestmate adoption

## Introduction

Group and individual recognition play a major role in the social organization and behavior of numerous animal species. Most social interactions such as territorial behavior, care of young, maintenance of social hierarchies, colony defense, and pair bonding rely on the ability to recognize individuals or to discriminate between familiar and unfamiliar groups of individuals or among close kin, distant kin, and unrelated conspecifics (Fletcher and Michener 1987). In social insects, such as termites and several Hymenoptera, discrimination behavior is essential, as it allows an individual's integration within the colony and maintains the integrity of these closed and complex societies. Moreover, if social groups consist of kin, discrimination between members and non-members can increase the indirect fitness of individuals that display altruistic behavior toward group members (Hamilton 1964; Crozier and Pamilo 1996).

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While kin recognition is synonymous with nestmate recognition when the social group consists exclusively of family members and is relatively homogenous genetically, recognition of kin is not necessarily implied in heterogeneous social groups that consist of both relatives and non-relatives (Breed and Bennett 1987). The presence of multiple reproductive queens, or polygyny, common in social insects, particularly ants (Keller and Vargo 1993; Bourke and Franks 1995), has been linked to variable levels of within-colony genetic relatedness (Herbers 1993); hence, recognition systems that rely on genetically based cues are expected to be less efficient in polygynous colonies that exhibit high levels of genetic diversity (Hölldobler and Michener 1980; Bourke and Franks 1995). Nevertheless, if nestmate recognition failures lead to colony fitness costs, selection should favor recognition mechanisms that optimize discrimination and minimize recognition errors—rejecting a desirable conspecific or accepting an undesirable one.

Recognition systems generally consist of a set of cues produced by the encountered individual or cue-bearer, perception of these cues by the recognizing participant or evaluator, and potential discriminatory response by the evaluator (Liebert and Starks 2004). A possible recognition mechanism underlying nestmate discrimination consists of comparing the phenotype of previously unencountered cue-bearers with the evaluator's inner learned template (Lacy and Sherman 1983). In social insects, recognition cues used in this proposed phenotype matching process are presumably chemical in nature and originate from either environmental (diet, nesting substrate), endogenous sources (genetically determined, acquired from queens and workers), or both. The template represents a memory pattern of the colony's recognition cues imprinted upon worker eclosion, derived from the environment, the individual's own phenotype, or collectively from all colony members (Breed and Bennett 1987; Reeve 1989; Vander Meer and Morel 1998). The cue-template matching process guides a behavioral response, acceptance or rejection of the encountered individual, with decisions based upon the degree of internal template-phenotypic cue similarity (Gamboa et al. 1986; Reeve 1989). According to Reeve (1989), conspecific rejection occurs if template-cue dissimilarity is above an acceptance threshold, while conspecifics are accepted if the template-cue dissimilarity is below this threshold. An optimal or evolutionarily stable acceptance threshold is determined by the interaction frequency with foreign conspecifics and the fitness consequences of accepting or rejecting conspecifics, including benefits of accepting and rejecting desirable and undesirable cue-bearers and the costs of making acceptance and rejection errors. Discrimination may thereby vary according to the social and ecological context in balancing the fitness costs of accepting non-nestmates and rejecting nestmates. This plasticity is expected

to minimize the chance of recognition errors in a dynamic ecological and social environment.

The ontogeny of recognition cues varies considerably among ants, with ant queens producing endogenous recognition cues distributed among, and learned by all adult colony members (Carlin and Hölldobler 1986; Brian 1986), or all colony members sharing recognition cues so that each individual bears a mixture of cues or colony gestalt odor (Stuart 1988; Errard and Jallon 1987). Queen-derived recognition cues play a role in nestmate discrimination in *Leptothorax lichtensteini* (Provost 1989), *Messor barbarus* (Provost et al. 1992, 1994), and *Pseudomyrmex pallidus* (Starks et al. 1998), but not in *Rhytidoponera confusa* (Crosland 1990), *Leptothorax ambiguus* (Stuart 1988), and *Solenopsis invicta* (Obin and Vander Meer 1989; Morel et al. 1990). The gestalt label is expected to prevail in polygynous ant species; however, extreme polygyny may limit unique label creation by minimizing intercolony variation and diminishing discrimination among neighboring colonies (e.g., unicolonial ant populations, Hölldobler and Wilson 1990).

Introduced populations of the Argentine ant, *Linepithema humile* (Mayr), are highly polygynous and display considerable diversity in nestmate recognition behavior, evidenced, in part, by pronounced variation in intraspecific aggression (Tsutsui et al. 2000; Suarez et al. 2002; Giraud et al. 2002; Buczkowski et al. 2004). Diminished intraspecific aggression in introduced populations from California and southern Europe may have resulted from a reduction in allelic diversity at recognition loci, either via a genetic bottleneck (Tsutsui et al. 2000) or selection against less common alleles (Giraud et al. 2002), thereby leading to reduced phenotypic variability in the cues underlying nestmate recognition. In contrast, high intraspecific aggression in southeastern US *L. humile* populations may be due to a relatively high diversity of genetic-based recognition cues (Buczkowski et al. 2004). Moreover, the contribution of environmentally derived cues, an important component of the *L. humile* recognition system (Liang and Silverman 2000) to nestmate discrimination seems to vary among introduced populations in relation to their genotypic diversity (Buczkowski and Silverman 2006). Following Reeve's (1989) acceptance threshold model, non-nestmates may not be rejected in unicolonial *L. humile* populations with low variation in genetically based recognition cues, as levels of template-cue dissimilarity are generally below the acceptance threshold (Starks 2003). Colonies may not reject non-nestmates sharing more genetically based recognition cues in populations that have not experienced drastic reductions in genetic diversity (e.g., Buczkowski et al. 2004); therefore, the frequency of recognition errors should correlate with levels of between colony genetic similarity. These populations are useful to examine how the levels of

genetic similarity between colonies affect the expression and perception components of the recognition system and to underscore the effect of social and ecological context on action thresholds (Buczowski and Silverman 2005).

A first step to better understand the complexity of the nestmate recognition process in *L. humile*, and probably other unicolonial ants, would be to carefully examine the variation in aggression behavior towards non-reproductive and reproductive individuals from different colonies in different social contexts. In this study, we investigated whether mutually aggressive Argentine ant colonies integrate non-nestmate queens and workers into colonies via adoption and if queen number influences non-nestmate adoption. We hypothesize that the social context (queen number) has an effect on nestmate discrimination, with non-nestmate acceptance thresholds increasing when queens are absent. Given that the degree of worker aggression among nests decreases with increasing genetic similarity in *L. humile* populations from Argentina and California (Tsutsui et al. 2000), we expect southeastern US Argentine ants to accept non-nestmate queens and workers from colonies that are genetically more similar if nestmate recognition is based on heritable cues. To test our hypothesis on the effect of queen number on nestmate discrimination and confirm the genetically based recognition mechanism of Tsutsui et al. (2000), we conducted behavioral assays and performed genetic analyses on queens and workers. Although in nature dispersal of single individuals is unlikely, this approach allows us to examine in more detail the mechanisms underlying *L. humile* nestmate recognition. We show, using *L. humile* colonies from the southeastern USA displaying various levels of intraspecific aggression, that non-nestmate queen and worker adoption can occur under different social contexts and that genetic factors influence the recognition process.

## Materials and methods

### Collection and rearing of laboratory colonies

We collected colony fragments of Argentine ants (*L. humile*) from the following sites in the southeastern USA: Cary (CAR), Chapel Hill (CHH), Research Triangle Park (RTP), and Winston-Salem (FOR) in North Carolina, and Greenville (COC) in South Carolina. Distances between collection sites ranged from 10 km (CAR-RTP) to 402 km (CAR-COC). We established three experimental colonies from each location, each consisting of different queen number (zero, one, or six queens), 100 pieces of brood, and about 3,000 workers (1 g). Colonies were maintained in soil-free, Fluon<sup>TM</sup>-coated trays (40×55×8 cm). Nests were plastic Petri dishes (9-cm diameter) filled with moist grooved Castone<sup>®</sup> dental plaster.

Colonies were provided 25% sucrose solution, artificial diet (Bhatkar and Whitcomb 1970) ad libitum, hard-boiled egg once a week, and a water source. All colonies were maintained at 25±1°C and 50±15%RH on a 12:12 h light/dark cycle. Source colonies from each of the five locations containing ants not used in the experimental colonies were also maintained as described above.

### Non-nestmate adoption assay

We initially assessed the level of worker-worker aggression among six colony pairs (CHH-COC, CHH-FOR, CHH-RTP, FOR-COC, RTP-COC, RTP-FOR) following Roulston et al. (2003). Briefly, individual intruder workers were collected on a toothpick and introduced into trays containing a resident colony. We allowed the intruder up to 25 encounters with resident ants, and aggression was scored using the 1–4 scale of Tsutsui et al. (2000). Colony pairs included workers from queenless colonies matched against workers from other queenless and queenright (one- and six-queen) colonies and workers from queenright colonies matched against workers from other queenless and queenright colonies. Twelve replicates per colony pair were performed; six replicates with colony 1 as the resident and six replicates with colony 1 as the intruder. Levels of aggression were measured 1 week after we established experimental colonies and 48 days after the start of the non-nestmate adoption assay in those pairs where adoption occurred. Data were analyzed as the maximum score per trial. Results of these trials established two aggression categories, moderate (less than 3.0) and high (greater or equal to 3.0). A score lower than 3 (avoidance, prolonged antennation) was not injurious, while a score of 3.0 or higher (pulling, biting, and abdomen curling in an attempt to spray defensive compounds) was usually injurious.

We then assessed the ability of workers from queenless, single queen, and six-queen colonies to discriminate non-nestmate from nestmate workers and queens. Workers and queens from each of four source colonies (CHH, COC, FOR, RTP) were marked on the thorax and abdomen, respectively, with a water-based paint for identification, and all queens were examined for egg production before introduction to conspecific colonies. Each introduction consisted of a single marked intruder transferred to the recipient colony with soft forceps and left in place for 24 h. Six workers and six queens per source colony were introduced sequentially into each queenless and queenright (single- and six-queen) experimental colony (12 colonies total), thereby testing all six colony pair combinations. The response of recipient workers toward the intruder was recorded at 15 and 30 min, every hour thereafter for 6 h, and at 24 h, and scored as 0 (no aggressive response), 1 (physical attack), or 2 (intruder killed). Adoption occurred if after 24 h, intruder queens were found in the nest being tended by workers, and intruder workers were tending

host brood or queens, foraging for food or piling debris. Surviving queens were then transferred to trays (12×12×5 cm) with 10–15 workers from the recipient colony to check for offspring production over 35 days, which indicated successful queen adoption. Data were analyzed as the average score within the first 6 h, the final score at 24 h, and as the percentage of queens and workers adopted by each recipient colony. The adoption assay was replicated twice across time.

#### Adopted queen fecundity

We measured the number of eggs laid by adopted and rejected non-nestmate queens and compared it with those of adopted nestmate queens to determine if queen fecundity has an effect on queen adoption decisions. Six CHH and six COC queens were anesthetized, weighed (mg), and placed individually in glass tubes (10×75 mm) containing 10–15 nestmate workers to count the total number of eggs laid per queen in 24 h (queen fecundity). CHH and COC queens were then marked and introduced individually along with 10–15 nestmate workers into six (three queenless, three five-queen) RTP and six (three queenless, three five-queen) FOR experimental colonies, respectively. These queen/recipient colony combinations were selected based on the higher adoption rates observed in the previous adoption experiment. One week and 2 weeks after introduction, we measured queen fecundity as follows. Introduced and host colony queens (in the case of queenright host colonies) plus ten recipient workers were transferred to a glass tube (10×75 mm) provisioned with food (25% sucrose in capillary tubes) and capped with a removable screen (0.14×0.14 mm). Individually caged queens were immediately reintroduced into the recipient colony, left in place for 24 h, and then released. Thus, we could estimate introduced queen fecundity without removing them from their respective recipient colony. Introduced queens were removed from experimental colonies 15 days after introduction, placed into small trays with 10–15 workers from the recipient colony, and further monitored for offspring (worker or males) production for 2 months as an indicator of colony integration. Nestmate queen fecundity from FOR and RTP (control) colonies was also measured, and the assay was replicated four times. Host colony response (queen adopted or killed at 24 h) in this study was highly correlated with colony response recorded in the previous non-nestmate adoption assay ( $r=0.872$ ,  $N=12$ ,  $P=0.0002$ ) regardless of the differences in how queen introductions were performed (with or without nestmate workers).

#### Genetic similarity between colonies

We assessed genetic similarity between introduced queens and recipient workers from colonies used in the non-

nestmate adoption assay (CHH, RTP, FOR, and COC) plus another colony (CAR) using microsatellite markers. Genomic DNA was extracted from 40–46 introduced queens per source colony and ten recipient workers from each of the experimental colonies (30 workers per location) using the DNeasy tissue kit (Qiagen, Valencia, CA, USA) and analyzed at seven microsatellite loci: Lhum-11, Lhum-13, Lhum-19, Lhum-28, Lhum-35, Lhum-39 (Krieger and Keller 1999) and Lihu-T1 (Tsutsui et al. 2000). Polymerase chain reactions were performed as described by Buczkowski et al. (2004). Products were separated on 6.5% KB<sup>Plus</sup> polyacrylamide sequencing gels using a 4000L Li-Cor DNA sequencer. Microsatellite alleles were scored using GeneMagIR software (Scanalytics, Billerica, MA, USA). Levels of genetic similarity between workers from these five different colonies and between introduced non-nestmate queens and resident workers were estimated based on the percentage of alleles shared between these groups (Tsutsui et al. 2000). The number of identical alleles between non-nestmate queens and workers from queenless colonies was additionally reported. Genetic differentiation ( $F_{ST}$ ) between queenless and queenright experimental colonies from the same location and different locations was estimated with the program FSTAT v.2.9.3.2 (Goudet 1995). We also examined the relationship between non-nestmate adoption rates vs genetic similarity and total number of alleles of the recipient colony. Aggression and adoption assays for colonies paired with CAR were conducted as previously described to complement the behavioral data used in these analyses.

#### Statistical analyses

All behavioral data analyses were performed using SAS 8.2 statistical software (SAS 2000). Differences in initial levels of aggression were determined with a split-split plot analysis of variance (ANOVA) with colony pair (whole plot factor), intruder source by recipient source nested within colony pair (subplot factor), and worker status and recipient status (sub-subplot factors), where status refers to the worker source or recipient colony queen number, as fixed effects replicated in two trials. Maximum initial aggression score of each colony pairing averaged across replicates was the dependent variable. Mean separation was carried out by the least significant difference test (LSD) for colony pair and least square means (LSMeans) for colony pair by recipient status interactions. To determine if levels of aggression varied as a function of exposure to non-nestmates, a similar split-split plot ANOVA was carried out with increase in maximum levels of aggression as the dependent variable.

A split block design with recipient colony and colony status treated as whole-plot factors stripped across each other was used in the non-nestmate adoption assay. Caste

and colony source of introduced individuals was treated as repeated measures subplot factors randomized within a combination of recipient and recipient status. Two trials were conducted, the first one from April through June 2003, and the second one from August through November 2003. Data for the two castes for the 6-h average and final (24 h) scores of each recipient colony averaged across replications were subjected to ANOVA using Proc GLM with appropriate TEST statements and means were separated with LSMeans. ANOVA on averages was justified by first inspecting for time effects (trends for colony response scores to increase or decrease with time) by comparing scores grouped into three periods of two introductions each. A similar split block ANOVA was conducted for each caste (queen or worker) with colony source of introduced individuals as the repeated measures subplot factor. We also carried out an ANOVA on the percentage adoption (arcsine transformed) of nestmate and non-nestmate introduced individuals averaged across recipient colonies followed by mean separation by LSD.

Pearson correlation coefficients ( $r$ ) were used to determine the relationship between aggression levels and average recipient response to non-nestmate workers and queens. Spearman rank correlation coefficients ( $r_s$ ) were used to determine relationships between genetic similarity vs intraspecific aggression, non-nestmate worker adoption, and non-nestmate queen adoption with correlation coefficients tested by Mantel's (1967) test in GENEPOP using 10,000 permutations.

## Results

### Non-nestmate queen and worker adoption

Using the maximum aggression scores (mean±SE) 1 week after experimental colonies were established, we identified three colony pairs with moderate aggression (<3.0), RTP–FOR (2.21±0.11), CHH–FOR (2.25±0.09), and FOR–COC (2.81±0.12), and three colony pairs with high aggression (>3.0), CHH–RTP (3.06±0.11), RTP–COC (3.78±0.05), and CHH–COC (3.89±0.03). Aggression between colony pairs differed ( $F_{5,5}=25.34$ ,  $P=0.0015$ ), and a colony pair by

recipient colony status (zero, one or six queens) interaction ( $F_{10,48}=6.07$ ,  $P<0.0001$ ) was found. Differences in aggression levels were observed between six-queen vs single-queen, and six-queen vs queenless recipient colonies in one colony pair with high aggression, CHH–RTP ( $t_{48}=3.50$ ,  $P=0.001$ , and  $t_{48}=3.85$ ,  $P=0.0004$ , respectively), and in one colony pair with moderate aggression, FOR–COC ( $t_{48}=4.40$ ,  $P<0.0001$ , and  $t_{48}=3.32$ ,  $P=0.002$ , respectively). Aggression between single queen FOR–COC was high, while aggression between single queen and between queenless CHH–RTP was moderate. Lower scores were recorded in queenless vs single queen ( $t_{48}=-3.87$ ,  $P=0.0003$ ) and six-queen ( $t_{48}=-3.97$ ,  $P=0.0002$ ) FOR–RTP; however, all scores were <3.0.

Overall, worker–worker aggression recorded 48 days after the adoption experiment started tended to be higher than that before the start of the experiment. However, these changes were not consistent across trials for colony pairs ( $F_{2,3}=10.89$ ,  $P=0.042$ ). The pair by recipient status interaction ( $F_{4,60}=4.49$ ,  $P=0.003$ ) indicated that not all colony pairs consistently increased aggression in queenless, single, and six-queen colonies. Multiple queen FOR–COC became more aggressive (3.1), while aggression decreased between single queen recipient colonies (2.8;  $t_{60}=3.46$ ,  $P=0.001$ ). When aggression was averaged across all queen number levels, both CHH–RTP and CHH–FOR showed higher aggression (from 3.0 to 3.7 and from 2.3 to 2.8, respectively) across both trials at the end of the adoption experiment. However, these changes in aggression scores still lie within the range used for colony pair classification into high and moderate aggression.

We found an effect of recipient colony status (zero, one, or six queens) on intruder adoption ( $F_{2,8}=12.27$ ,  $P=0.004$ ), varying across specific intruder by recipient colony ( $F_{9,33}=121.37$ ,  $P<0.0001$ ) and specific intruder by recipient colony and colony status ( $F_{18,33}=2.26$ ,  $P=0.020$ ). We found no differences between periods when recipient colony response scores were averaged over 6 h in worker ( $F_{2,288}=2.11$ ,  $P=0.123$ ) and queen ( $F_{2,288}=0.16$ ,  $P=0.849$ ) introductions, and for final scores in worker ( $F_{2,288}=2.47$ ,  $P=0.087$ ) and queen ( $F_{2,288}=0.09$ ,  $P=0.913$ ) introductions, indicating that previous exposure to non-nestmate intruders had no effect on recipient colony response.

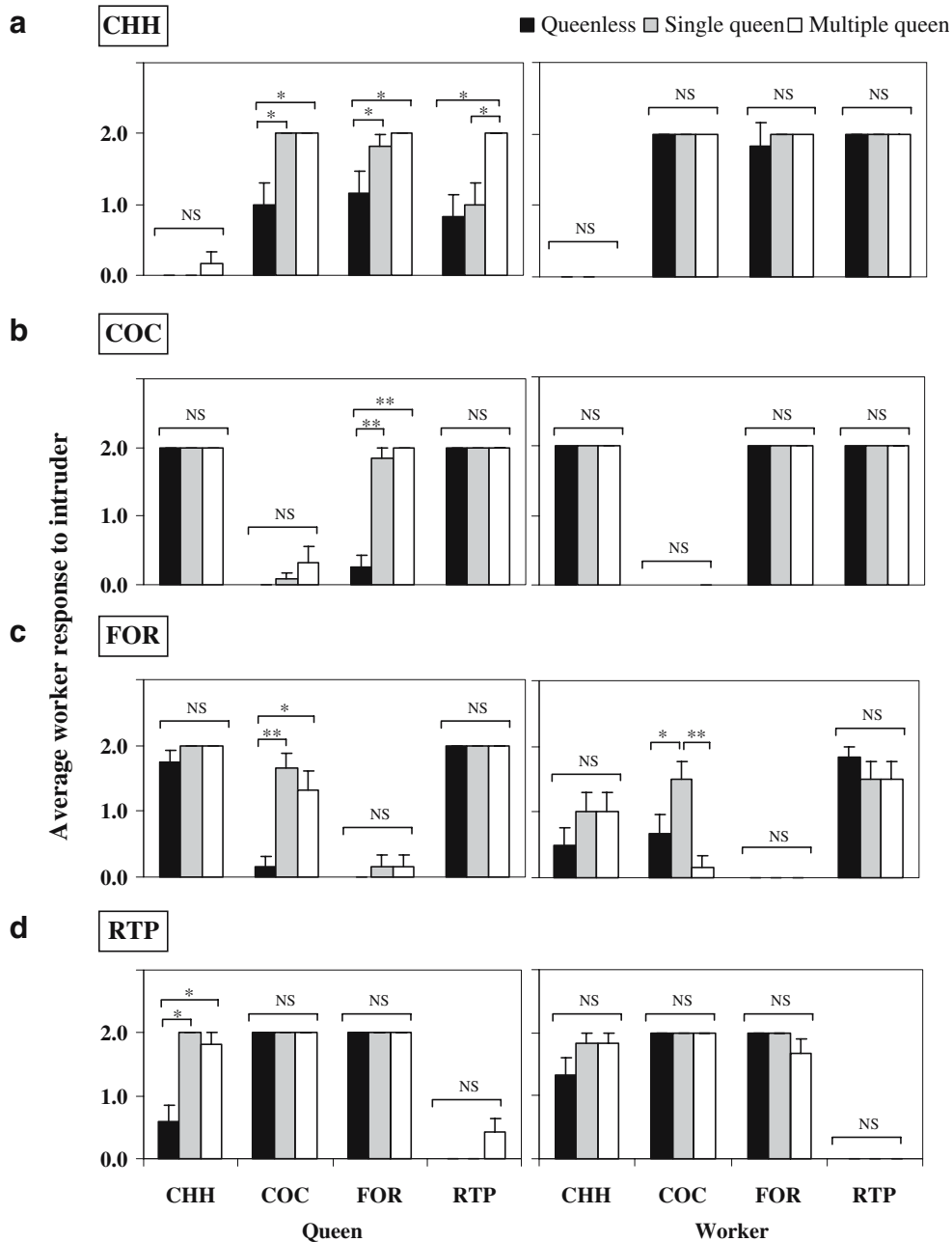
**Table 1** Nestmate and non-nestmate queen and worker adoption (mean±SE) in queenless and queenright *L. humile* colonies

Host colony status	Nestmate adoption (%)		Non-nestmate adoption (%) <sup>a</sup>	
	Queen	Worker	Queen	Worker
Queenless	100.00±0.00 a	100.00±0.00	34.03±8.83 a	14.59±6.05 a
Single queen	97.91±5.89 ab	100.00±0.00	6.95±4.36 b	9.03±4.70 b
Multiple queen	89.58±8.63 b	100.00±0.00	3.47±2.83 b	14.58±6.21 ab

<sup>a</sup> Means within a column followed by a different letter are significantly different (LSD,  $P<0.05$ ).  $N=48$  (nestmate) and 144 (non-nestmates).

In general, nestmate queens and workers were not attacked and less than 11% of nestmate queens were rejected by multiple queen colonies (Table 1). Most non-nestmate workers were killed by recipient workers of queenless, single-, and six-queen colonies. Similarly, workers of single- and six-queen colonies killed most non-nestmate queens (Table 1). In contrast, queenless colonies adopted a higher proportion of non-nestmate queens than both queenright colonies ( $F_{2,8}=9.28, P=0.008$ ). Specific

colony pairing (specific intruder and recipient colony combination) had a significant effect on recipient colony response to workers ( $F_{9,9}=41.41, P<0.0001$ ) and queens ( $F_{9,9}=32.13, P<0.0001$ ) for 6-h average scores. Likewise, we found a colony pairing effect on colony response to workers ( $F_{9,9}=29.79, P<0.0001$ ) and queens ( $F_{9,9}=39.70, P<0.0001$ ) at 24 h, with recipient colony final response to queens from the same donor colony varying across recipient colony status ( $F_{18,24}=3.45, P=0.003$ ; Fig. 1 a–d). Differ-



**Fig. 1** Mean±SE levels of recipient colony response (0=no aggressive response, 1=physical attack, 2=intruder killed) to intruder queens and workers 24 h after introduction into four *L. humile* recipient colonies with different queen numbers (queenless, single queen, and

multiple queen): CHH (a), COC (b), FOR (c), and RTP (d). See Text for colony abbreviations.  $N=12$ . LSMs: NS Not significant; \* $P<0.01$ ; \*\* $P<0.0001$

ences in colony response towards workers translated into varying rates of non-nestmate worker adoption ranging from 0% (COC and FOR workers) to 16.7% (CHH workers) in COC recipient colonies, and from 8.3% (RTP workers) to 83.3% (COC workers) in FOR recipient colonies. Rates of non-nestmate queen adoption varied across all queenless colonies, ranging from 41.7% (FOR queens) to 58.3% (RTP queens) in CHH colonies, from 0% (CHH and RTP queens) to 91.7% (FOR and COC queens) in both COC and FOR colonies, and from 0% (COC and FOR) to 66.7% (CHH) in RTP colonies. Therefore, the selective non-nestmate queen adoption observed in queenless colonies appears to be regulated by interactions between specific colonies. In addition, adopted queens produced offspring (6–25 worker pupae) when attended by a subset of recipient workers further supporting non-nestmate queen integration.

We found an association between initial aggression level and mean ( $r=0.710$ ,  $N=36$ ,  $P<0.0001$ ) and final ( $r=0.464$ ,  $N=36$ ,  $P=0.004$ ) non-nestmate worker adoption averaged across recipient colony status (Fig. 2a), but no association between worker intraspecific aggression and mean ( $r=0.142$ ,  $N=36$ ,

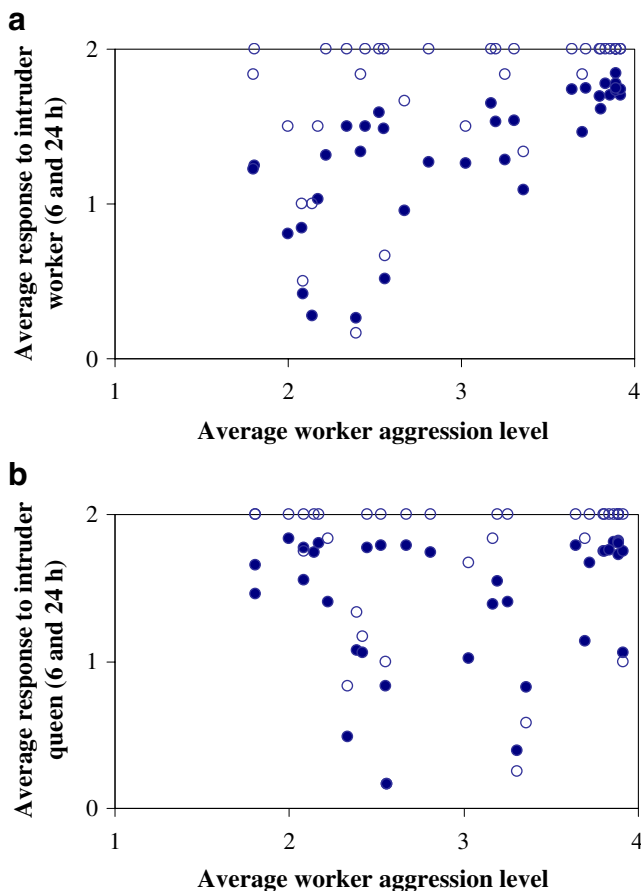
$P=0.408$ ) and final ( $r=0.086$ ,  $N=36$ ,  $P=0.617$ ) non-nestmate queen adoption (Fig. 2b), suggesting that high aggression between workers does not necessarily correspond to complete rejection of non-nestmate queens and vice versa.

#### Fecundity of non-nestmate adopted queens

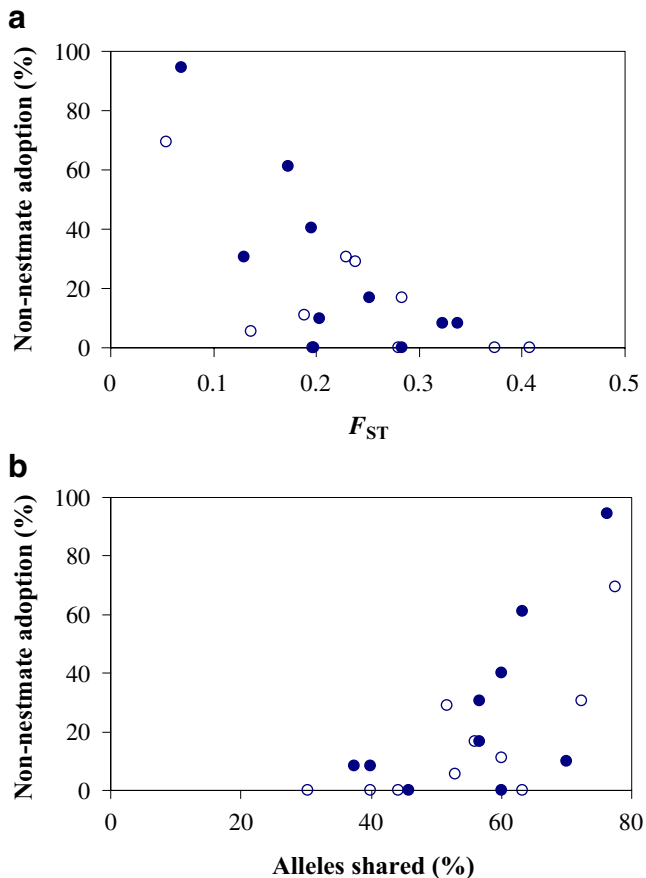
Queen fecundity (mean $\pm$ SE) 24 h before introduction was not different between adopted ( $12.43\pm 3.39$  eggs/24 h) and rejected non-nestmate queens ( $9.87\pm 1.50$  eggs/24 h;  $t_{42}=0.81$ ,  $P=0.211$ ); adopted nestmate queens produced fewer eggs ( $5.91\pm 0.84$  eggs/24 h) than adopted ( $t_{34}=2.27$ ,  $P=0.015$ ) and rejected non-nestmate queens ( $t_{50}=2.09$ ,  $P=0.021$ ). Fecundity measured 1 week after queen introduction did not differ between non-nestmate ( $17.08\pm 3.39$  eggs/24 h) and nestmate queens ( $12.91\pm 2.56$  eggs/24 h;  $t_{39}=1.57$ ,  $P=0.061$ ). Similarly, no differences were detected between non-nestmate ( $20.75\pm 3.67$  eggs/24 h) and nestmate ( $14.86\pm 1.95$  eggs/24 h) queen fecundity averaged over week 1 and week 2 ( $t_2=1.60$ ,  $P=0.125$ ), indicating that foreign queens contributed equally to egg production. Our queen fecundity measurements could have been affected by our experimental setup; however, the number of eggs laid by caged queens in this study was within the 1 to 60 eggs/day range reported in larger single queen laboratory colonies (Newell and Barber 1913) and the 3 to 22 eggs/14 h reported for isolated queens (Keller 1988). Tending of non-nestmate offspring by recipient workers and non-nestmate worker (5–20) or male (1–5) eclosion in colonies that were set up using individual queens from the 2-week fecundity trial further suggest colony integration of adopted foreign queens and their offspring. Queen weight measured before adoption was not different between adopted foreign queens ( $3.18\pm 0.16$  mg) and adopted nestmate queens ( $3.13\pm 0.09$  mg;  $t_{39}=0.29$ ,  $P=0.382$ ), and these values were relatively lower than the 3.3 to 3.8 mg reported for queens in polygynous laboratory colonies (Keller 1988). We found a significant correlation between queen weight and queen fecundity ( $r=0.219$ ,  $N=83$ ,  $P=0.046$ ).

#### Levels of genetic similarity

While queenless, single-queen, and six-queen laboratory colonies from the same location were not genetically different ( $F_{ST}CARY=0.032\pm 0.045$ ,  $F_{ST}CHH=-0.026\pm 0.006$ ,  $F_{ST}COC=-0.012\pm 0.012$ ,  $F_{ST}FOR=-0.003\pm 0.012$ ,  $F_{ST}RTP=0.001\pm 0.017$ ), colonies from different locations were significantly differentiated ( $F_{ST}=0.219\pm 0.047$ ). We found a negative yet non-significant relationship between worker-worker pairwise  $F_{ST}$  and non-nestmate worker adoption (Mantel test:  $r_s=-0.507$ ,  $N=10$ ,  $P=0.080$ ) and an even stronger association between queen-worker pairwise  $F_{ST}$  and non-nestmate queen adoption (Mantel test:  $r_s=-0.778$ ,  $N=10$ ,  $P=0.051$ ; Fig. 3a). We also estimated



**Fig. 2** Relationship between worker aggression levels (1=touch, 2=avoid, 3=biting, pulling, lunging, 4=gaster flexion) and recipient colony response to intruders (0=no aggressive response/adoption, 1=physical attack, 2=intruder killed) averaged across 6 hours (filled circle) and at 24 h (open circle) for workers (a) and queens (b)



**Fig. 3** Relationship of non-nestmate worker (filled circle) and queen (open circle) adoption vs pairwise  $F_{ST}$  (a) and % alleles shared (b) between colonies

percentage of shared alleles between workers from the five colonies and between non-nestmate queens and recipient workers, as it is an absolute measure of genetic similarity between groups (Tsutsui et al. 2000). Levels of genetic similarity varied across colony pairs, ranging from 30.3% (CHH–COC) to 77.4% (CAR–RTP) for workers and from 37.3% (CHH–COC) to 76.2% (CAR–RTP) for non-nestmate queens and recipient workers. Levels of worker–worker aggression were inversely associated with worker genetic similarity (Mantel test:  $r_s = -0.595$ ,  $N = 10$ ,  $P = 0.042$ ). A positive association (Mantel test:  $r_s = 0.682$ ,  $N = 10$ ,  $P = 0.016$ ) was found between levels of genetic similarity between workers and non-nestmate worker adoption averaged across host colony status (Fig. 3b). A positive association was also found between queen–worker genetic similarity and non-nestmate queen adoption averaged across host colony status (Mantel test:  $r_s = 0.553$ ,  $N = 10$ ,  $P = 0.026$ ; Fig. 3b). Moreover, non-nestmate queens adopted by queenless host colonies had an average higher proportion of alleles identical to those of their recipient workers ( $0.78 \pm 0.12$ ) than that of rejected non-nestmate queens ( $0.64 \pm 0.15$ ;  $t_{21} = 2.53$ ,  $P = 0.001$ ). The proportion of identical alleles between

adopted non-nestmate queens and recipient workers varied across recipient colonies, being the lowest for COC queens and queenless CHH (0.54) and the highest for CAR queens and queenless RTP (1.00; Table 2). We found that colonies with lower genetic diversity, COC and CHH, were less likely to accept non-nestmate queens and workers than colonies with higher genetic diversity, CAR, FOR and RTP, and a positive yet non-significant association ( $r = 0.585$ ,  $N = 10$ ,  $P = 0.075$ ) was found between number of alleles in recipient colonies and non-nestmate adoption.

## Discussion

We demonstrated that in an introduced Argentine ant population, discrimination of nestmate workers and queens from unrelated conspecifics is affected by the levels of genetic similarity between colonies, and that the social context, specifically colony queen status, further affects queen acceptance presumably via modulation of the action threshold. Nestmate discrimination in this species seems to

**Table 2** Total number and proportion of alleles of introduced non-nestmate queens identical and different from alleles of workers from queenless host colonies

Host colony	Queen	Adoption	Number of alleles <sup>a</sup>		
			Identical (I)	Different (D)	Proportion (I/(I+D))
CAR	CHH	Y	15	5	0.75
	COC	Y	11	4	0.73
	FOR	Y	14	4	0.78
	RTP	Y	15	2	0.88
CHH	CAR	Y	11	6	0.65
	COC	Y	7	6	0.54
	FOR	Y	14	4	0.78
COC	RTP	Y	15	7	0.68
	CAR	N	9	11	0.45
	CHH	N	7	10	0.41
	FOR	Y	11	5	0.69
FOR	RTP	N	8	9	0.47
	CAR	N	11	5	0.69
	CHH	Y	9	1	0.90
	COC	Y	14	3	0.82
RTP	RTP	N	15	5	0.75
	CAR	Y	15	0	1.00
	CHH	Y	10	2	0.83
	COC	N	10	5	0.67
FOR	N	13	5	0.72	

See text for colony abbreviations.

<sup>a</sup> Total number of alleles across seven microsatellite loci

be guided by levels of cue-template similarity between the introduced individual and recipient colony workers, with non-nestmate conspecifics treated as nestmates if the match between the learned template and perceived cue meets a certain level of template-cue similarity (Gamboa et al. 1986; Reeve 1989). Instances of unrelated conspecific adoption may result from an increased frequency in recognition errors due to lower levels of template-cue dissimilarity between individuals belonging to genetically similar colonies. In addition, the effect of social context (queen number) on non-nestmate queen, but not worker acceptance, suggests considerable flexibility in nestmate discrimination with queen recognition modulated by the social environment via more permissive queen acceptance thresholds when the costs of accepting non-nestmates decrease as predicted by the optimal acceptance threshold model (Reeve 1989). The higher incidence of acceptance errors in queenless colonies indicates that acceptance thresholds may shift according to the recognition context.

The positive association between non-nestmate worker and queen adoption and genetic similarity between colonies and the inverse relationship between worker–worker aggression and genetic similarity further support the view that the *L. humile* recognition system has an important genetic component (Tsutsui et al. 2000; Tsutsui and Case 2001). Similarly, genetically determined recognition cues are involved in nestmate recognition in *Leptothorax lichtensteini* (Provost 1991), *Formica polyteca* (Beye et al. 1997), and *F. pratensis* (Pirk et al. 2001). Non-nestmate *L. humile* queen acceptance was affected by queen–worker genetic similarity rather than queen fecundity, with higher rates of queen adoption in queenless colonies where adopted non-nestmate queens had a higher proportion of alleles in common with recipient workers than queens that were not adopted. In addition, it appears that colonies with higher genetic diversity are more likely to adopt foreign conspecifics as previously suggested by Tsutsui et al. (2003); thus, we speculate that the selective adoption of non-nestmates in relatively more diverse colonies could result in a broader template, reduced nestmate discrimination, and, in the long term, decreased recognition cue variation by favoring the most common recognition cues. In contrast, less diverse colonies are less likely to adopt non-nestmates, with the possibility to expand their already narrow template being minimal. Similarly, cuticular hydrocarbon profile similarity in two ant species produced a narrow template in mixed groups, while dissimilarity and a corresponding broader template was followed by lower aggression levels (Errard et al. 2006). Our genetic analyses included a relatively small number of colonies; therefore, examining and comparing other *L. humile* populations with expansive (Tsutsui et al. 2000; Giraud et al. 2002) and restricted colonies (Buczowski et al. 2004) would be useful to clarify whether foreign

conspecific adoption is exclusive to invasive populations that have not yet reached the expansive supercolony level.

We observed considerable variation in adoption rates of non-nestmate queens and workers from colony pairs displaying moderate intraspecific aggression, suggesting a caste-dependent hierarchy in *L. humile* nestmate recognition, whereby colony-derived cues (colony gestalt) are used to discriminate nestmates from non-nestmates, while caste-specific cues signal reproductive status. Where dissimilarity between an individual's recognition template and the encountered conspecific's recognition cues are considerable (e.g., highly aggressive colony pairs), colony-derived cues may solely elicit rejection. However, where the template-cue is only partially mismatched, as may occur in moderately aggressive colony pairs, individual variability in recognition cues (e.g., caste-specific cues) may affect intruder rejection. Caste-specific nestmate discrimination in *L. humile* may be modulated by cuticular hydrocarbons that differ quantitatively and qualitatively between queens and workers (de Biseau et al. 2004). In a Gestalt model, individuals make acceptance decisions according to a certain level of deviation from the gestalt or unique mixture of the colony recognition cues (Breed and Bennett 1987). The distinct cuticular hydrocarbon profiles of queens and workers may suggest that *L. humile* does not form a unique colony odor that is distributed among all colony members but rather subsets for each caste, and different levels of aggression directed towards workers and queens from the same colony may indicate not only variable acceptance thresholds but also two kinds of recognition templates (reproductive and sterile worker). The possibility of separate recognition templates and variable thresholds between castes has been suggested for *Polistes* spp. that develop templates based on colony-specific odors (Gamboa 2004). Dominant females have more restrictive thresholds and respond more aggressively than subordinates, and foraging workers have less restrictive thresholds than workers performing on-nest duties (Gamboa 2004).

We demonstrated that queenless *L. humile* colonies accept more non-nestmate queens than queenright colonies. Social organization and group cohesion depend not only on recognition mechanisms between group members but also on the pheromonal signals queens convey to the colony (Keller and Nonacs 1993). In *S. invicta*, replacement queen acceptance occurs when levels of queen pheromones circulating in a colony are below an optimal range (e.g., queenless colonies), while higher queen numbers raise the level close to or above a tolerance threshold, causing workers to behave aggressively toward some queens, probably the ones with the least familiar odor or the least productive ones (Fletcher and Blum 1983; Vander Meer and Alonso 2002). In *L. humile*, queen-primer pheromones control gyne production by preventing sexualization of

female larvae and by inducing female sexual brood elimination by workers (Vargo and Passera 1991; Passera et al. 1995). Queen pheromones may also subtly affect nestmate recognition by regulating acceptance thresholds. For example, a drop in levels of *L. humile* primer pheromones after nestmate queen seasonal execution (Keller et al. 1989; Reuter et al. 2001) may allow adoption of new daughter queens (Markin 1970; Keller 1988). Similarly, non-nestmate queen adoption may vary according to circulating pheromone levels within the colony. We were surprised that approximately 10% of queens were killed when reintroduced to their own multiple-queen colonies. Random elimination of excess queens to enhance colony productivity (Reeve and Ratnieks 1993) and to restore queen numbers in compliance with the hierarchical queen pheromone hypothesis (Fletcher and Blum 1983), i.e., elimination of least productive queens, may explain this increased aggression towards nestmate queens.

A flexible acceptance threshold may result from differences in the recognition context (Reeve 1989) and fluctuations in the cost of recognition errors (Liebert and Starks 2004). For example, a shift in *Apis mellifera* acceptance thresholds may result as the cost of accepting non-nestmates decreases with greater nectar availability (Downs and Ratnieks 2000). Ant workers typically eliminate unrelated queens (Reeve and Ratnieks 1993); however, worker uncertainty over maternity and the drive to increase the colony's chance of survival may prevent the elimination of unrelated queens (Sudd and Franks 1987; Balas 2005). A shift to more permissive thresholds to balance the cost of acceptance and rejection errors has been reported for *Pseudomyrmex pallidus* (Starks et al. 1998). Similarly, in Argentine ants, shifted acceptance thresholds, evidenced by an increase in acceptance errors in queenless colonies and an increase in rejection errors in multiple-queen colonies, may occur if the cost of erroneously rejecting nestmate queens is high and the cost of accepting non-nestmate queens is low (Reeve 1989). Increased tolerance of non-nestmate queens could contribute to higher nest productivity and increased competitive ability, particularly in queenless colonies. This is relevant to *L. humile* field populations, given that queenless colony fragments can potentially occur in this species and that field colony expansion may be facilitated by adoption of non-nestmate queens by peripheral colony satellites containing fewer queens than central nests (Ingram 2002). This flexibility may have favored the establishment of incipient colonies in the introduced range and shaped, in part, the social structure of introduced populations exhibiting low genetic diversity and conversely explain, in part, why native colonies are less open and geographically more restricted. Whether non-nestmate queen adoption may be a strategy for orphaned colony survival and colony expansion or a behavioral anomaly

observed only in introduced populations requires further examination.

Like seasonal execution of nestmate queens (Keller et al. 1989), rejection of foreign Argentine ant queens was unrelated to queen weight or rate of egg laying, which is consistent with the absence of a dominance hierarchy among queens in this species (Keller 1988). Instead, we found that colony of origin influenced queen rejection, with colony-derived cues probably playing a major role in a workers' decision to reject a queen. In other polygynous ants, survival of new queens is regulated by colony or nest characteristics (Stuart et al. 1993) and/or queen physiology (Fletcher and Blum 1983; Fortelius et al. 1993, Sundström 1997; Keller and Ross 1993). For a specific colony pairing, variation in *L. humile* queen acceptance could be explained by differences in egg production due to nutritional status (Keller 1988) or reproductive skew for sexual production (Fournier and Keller 2001). Although not recorded, viability of eggs laid by introduced *L. humile* may be a better indicator of queen reproductive status (e.g., Vargo and Ross 1989; Chen and Vinson 2000).

Investigators using prevailing nestmate recognition assays generally assume that aggressive behaviors (e.g., biting, pulling, gaster flexion) inevitably result in intruder mortality because nest entry by non-nestmates is detrimental. Although higher levels of intraspecific aggression were associated with non-nestmate worker rejection (Fig. 2), we found no association between levels of intraspecific aggression and the frequency of non-nestmate queen adoption. Therefore, at least in some instances, initial aggressive encounters are followed by acceptance and subsequent adoption. In addition, the effect of differences in host colony behavioral response towards individual conspecific queens and workers on interactions between colony cohorts warrants investigation.

Through selective elimination of unrelated queens by workers, levels of within-colony relatedness and social structure (e.g., Krieger and Ross 2002) are maintained in social insect colonies. Subsequently, adoption of unrelated queens may affect levels of relatedness within the recipient colony according to their relative contribution to the colony genetic makeup, hence, eroding nestmate discrimination and leading to acceptance of more foreign individuals (Bourke and Franks 1995). Our results reveal that recognition errors in introduced *L. humile* populations are more likely to occur between more genetically similar colonies and in the absence of nestmate queens. The relatively high levels of genetic dissimilarity between colonies in this study fall within those reported for native populations (Tsutsui and Case 2001); hence, the queen and worker discrimination behavior observed may also reflect behavioral interactions between colonies in the native range where this genetically based recognition system presumably

evolved. Moreover, recent findings suggest that both native and introduced populations are unicolonial, differing in supercolony size, a trait that seems to be regulated by intraspecific competition, and with unicoloniality persisting through continuous replacement of supercolonies with more competitive ones (Pedersen et al. 2006). In this context, foreign queen adoption may be a process underlying the success (i.e., increased productivity and competitive ability through increased worker force) or failure (i.e., reduced productivity by unequal sexual and worker production) of a supercolony in both the native and introduced range. While unclear how the infiltration of non-nestmate conspecifics into established colonies may impact colony productivity and colony genetic structure, studies underway exploring interactions at the group level (between colony fragments) may shed light on the evolutionary interests of both workers and queens as a group and whether queen adoption via colony fusion plays a role in shaping *L. humile* social structure by increasing within-colony variability in recognition templates and cues.

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