ABSTRACT

VASQUEZ VILCHEZ, GISSELLA MARIA. Non-nestmate adoption and colony fusion in the Argentine ant, *Linepithema humile*. (Under the direction of Jules Silverman.)

The Argentine ant, *Linepithema humile,* is a widespread invasive species that forms vast and dominant supercolonies responsible for its ecological success in the introduced range. Lack of intraspecific aggression typical of introduced populations has been linked to supercolony formation. Inability to recognize nestmates from non-nestmates through reduced nestmate recognition cue variation has been linked to intraspecific aggression loss. To gain an understanding of mechanisms underlying nestmate recognition in the Argentine ant and their implications in supercolony formation, I studied non-nestmate queen and worker adoption and colony fusion between aggressive colonies and the genetic and chemical factors regulating these processes.

In Chapter I, I examined non-nestmate queen and worker adoption in conspecific queenless and queenright *L. humile* colonies displaying varying degrees of intraspecific aggression. I determined that non-nestmate adoption was a function of host colony origin and queen status, and that non-nestmate queens were more readily adopted by queenless host colonies than non-nestmate workers. I suggest that host colonies utilize both colony-specific and queen-derived cues in their adoption decisions. I also determined that levels of aggression between colonies were positively associated with non-nestmate worker but not queen adoption. I then compared queen fecundity between adopted and rejected non-nestmate queens, and between adopted non-nestmate and host colony queens. My results show that queen fecundity does not differ between adopted and rejected non-nestmate

queens, and between adopted non-nestmate and host colony queens. I also examined levels of genetic similarity between non-nestmate workers and queens and host colony workers and found a correlation between genetic similarity and non-nestmate adoption. I suggest that non-nestmate queen adoption may broaden host worker's recognition template thereby reducing non-nestmate discrimination and contributing to unicoloniality in introduced Argentine ant populations.

In Chapter II, I examined interactions between mutually aggressive Argentine ant colonies displaying varying levels of intraspecific aggression and genetic similarity. I determined that numbers of workers fighting and killed were higher in highly aggressive than moderately aggressive colony pairs, and that all moderately aggressive pairs fused whereas highly aggressive pairs fused selectively. My results also show that levels of intercolony aggression decrease after fusion and that both intraspecific aggression and colony fusion are correlated with genetic similarity between colonies. I also determined that interactions between moderately aggressive colony pairs in the field also led to fusion under controlled conditions. My results suggest that fusion of initially aggressive colonies sharing moderately to relatively high levels of genetic similarity may be a proximate mechanism leading to extreme unicoloniality in introduced Argentine ant populations in the absence of constraints preventing intercolony interactions.

In Chapter III, I examined the effects of fusion between initially aggressive and genetically distinct Argentine ant colonies on colony size and productivity. I discovered that colony pairs that fused produced more brood and workers than colony pairs that did not fuse, in contrast to the comparably low per capita productivity observed in both fused and non-fused colony pairs. I also tested whether queens in fused colony pairs

contributed equally to new worker production by genotyping queens and worker pupae. My results show that queens in fused unrelated colonies contribute equally to worker production. I suggest that fusion of unrelated colonies may lead to changes in colony's genotypic composition. These results support the idea that fusion of unrelated colonies may result in increased fitness benefits through higher brood and worker production. I suggest that fusion between introduced aggressive colonies may be a mechanism leading to the formation of less aggressive colonies that through increased worker numbers may become ecologically successful supercolonies.

In Chapter IV, I examined queen and worker cuticular hydrocarbon similarities between aggressive Argentine ant colonies and whether these levels of chemical cue similarities modulate worker intraspecific aggression, non-nestmate queen adoption and colony fusion. I determined that queen cuticular hydrocarbon profile similarity was associated with non-nestmate queen adoption and colony fusion, and that worker cuticular hydrocarbon profile similarity was associated with intraspecific aggression and colony fusion. I also examined the relationship between queen and worker cuticular hydrocarbon profile similarity and levels of intercolony genetic similarity. I concluded that hydrocarbon profile similarity was associated with both queen-queen and worker-worker genetic similarity. I also examined whether non-nestmate queens adopted by queenless host colonies and queens and workers in fused colony pairs changed their cuticular hydrocarbon profile composition. Results show that non-nestmate queens adopted by queenless colonies do not change their hydrocarbon profile composition, unlike queens in fused colonies that alter their composition to have a profile intermediate between the two source colonies, while hydrocarbon profiles of workers in fused colony

pairs can be distinguished from their original colonies. In contrast, chemical profiles of workers and queens in non-fused pairs do not diverge from those of their source colonies. I also tested whether cuticular hydrocarbons are queen recognition cues by applying non-nestmate queen hydrocarbons on queens and recording nestmate worker aggressive response to treated nestmate queens. Results of behavioral assays show that non-nestmate queen hydrocarbons alter nestmate recognition and lead to nestmate rejection, while results from chemical analysis corroborate that application of non-nestmate hydrocarbons altered queen hydrocarbon composition. My results demonstrate that non-nestmate queen adoption and fusion between unrelated colonies are governed by cuticular hydrocarbon similarities and that queen cuticular hydrocarbons are recognition cues and suggest that recognition cue variation is genetically determined. I suggest that Argentine ant nestmate recognition flexibility may play a major role in shaping the social organization of introduced Argentine ant populations by means of acceptance of non-nestmates that could expand recipient's recognition template and formation of new colonies sharing common chemical recognition cues that may become more open to unrelated colonies possessing similar cues.

NON-NESTMATE ADOPTION AND COLONY FUSION IN THE ARGENTINE ANT, *LINEPITHEMA HUMILE*

by

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BIOGRAPHY

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TABLE OF CONTENTS

Page

LIST OF TABLES ix

LIST OF FIGURES xi

CHAPTER I: ADOPTION OF NON-NESTMATE ARGENTINE ANT CONSPECIFICS AND THE COMPLEXITY OF NESTMATE DISCRIMINATION 1

Abstract 2

Introduction 4

Materials and Methods 10

Collection and Rearing of Laboratory Colonies 10

Aggression Tests 10

Non-nestmate Adoption Assay 11

Adopted Queen Fecundity 12

Genetic Similarity between Colonies 13

Statistical Analyses 14

Results 17

Intercolony Worker-worker Aggression 17

Non-nestmate Queen and Worker Adoption 18

Fecundity of Non-nestmate Adopted Queens 20

Levels of Genetic Similarity 21

Discussion 23

Acknowledgements 30

References Cited....31
Tables....40
Figures....42
CHAPTER II: INTRASPECIFIC AGGRESSION AND COLONY FUSION IN THE INVASIVE ARGENTINE ANT....47
Abstract....48
Introduction....50
Materials and Methods....56
Ant Colonies and Rearing Conditions....56
Levels of Worker Aggression....57
Laboratory Colony Fusion Assay....59
Fusion under Controlled Field Conditions....59
Genetic Similarity between Colonies....61
Statistical Analyses....61
Results....64
Levels of Worker Aggression....64
Laboratory Colony Fusion Assay....64
Field Experiments....66
Genetic Similarity between Colonies....67
Discussion....68
Acknowledgments....77
References Cited....78
Tables....85

Figures 86
CHAPTER III: COLONY FUSION AND ITS FITNESS IMPLICATIONS IN THE INVASIVE ARGENTINE ANT....91
Abstract....92
Introduction....94
Materials and Methods 101
Collection and Rearing of Laboratory Colonies 101
Colony Fusion, Total Colony Size, and Per Capita Brood and Worker Values 102
Microsatellite Analysis of Queens and Offspring in Fused Colonies....103
Statistical Analyses 104
Results....107
Colony Fusion, Total Colony Size, and Per Capita Brood and Worker Values 107
Microsatellite Analysis of Queens and Offspring in Fused Colonies....113
Discussion....115
Acknowledgements....124
References Cited....125
Tables....133
Figures 136
CHAPTER IV: CUTICULAR HYDROCARBONS AS RECOGNITION CUES IN NON-NESTMATE QUEEN ADOPTION AND COLONY FUSION IN THE ARGENTINE ANT 145

Abstract....146
Introduction....148
Materials and Methods....154
Experimental Colonies....154
Behavioral Assays and Sampling of CHC....154
Application of Non-nestmate Queen CHC on Queens and Nestmate Worker Aggression....154
Non-nestmate Queen Adoption....155
Colony Fusion....156
Extraction, Isolation, and Chemical Analysis of CHC....157
Genetic Similarities between Colony Pairs....158
Statistical Analyses....159
Results....161
Queens Treated with Non-nestmate Queen CHC: Chemical Profiles and Worker Aggression....159
CHC Profiles of Queens in the Queen Adoption Assay....162
CHC Profile Similarities versus Queen Adoption and Queen Genetic Similarity....163
Queen Adoption and Changes in Queen CHC....163
CHC Profiles of Workers in the Colony Fusion Assay....165
Worker CHC Profile Similarities versus Worker-worker Aggression, Colony Fusion, and Genetic Similarity....166
Colony Fusion and Changes in Queen and Worker CHC....166

Discussion.....170

Nestmate Recognition, Queen Adoption, and Queen CHC.....171

Intraspecific Aggression, Colony Fusion, and CHC Profile Flexibility.....174

CHC and Argentine Ant Colony Signature.....177

Acknowledgements.....181

References Cited.....182

Tables.....191

Figures.....193

LIST OF TABLES

Page

CHAPTER I

Table 1. Nestmate and non-nestmate queen and worker adoption (Mean ± SE) in queenless and queenright colonies 40

Table 2. Total number and proportion of alleles of introduced non-nestmate queens identical and different from alleles of workers from queenless host colonies 41

CHAPTER II

Table 1. Category of worker-worker aggression, number of replicates that fused, and time of fusion for ten colony pairs 85

CHAPTER III

Table 1. Colony fusion during a 6 month period and queen survival at 24 hours, 120 days and 150 days for ten Argentine ant colony pairs.....133

Table 2. Allele (alle) frequencies (freq) by locus in colonies used in the fusion assay (first trial) genotyped at eight microsatellite loci.....134

Table 3. Alleles of queens (Q) and worker pupae (P) in fused colony pairs genotyped at eight microsatellite loci 135

CHAPTER IV

Table 1. Generalized squared distances between colony means (centroids)

calculated by discriminant analysis of CHC of queens from colonies used in the queen adoption assay extracted by solvent or sampled using a non-disruptive method.....191

Table 2. Generalized squared distances between colony means (centroids) calculated by discriminant analysis of CHC of workers and queens from colonies used in the fusion assay 192

LIST OF FIGURES

Page

CHAPTER I

Figure 1. Mean ± SE levels of recipient colony response (0 = no aggressive response, 1 = physical attacked, 2 = intruder killed) to intruder queen and worker 24 h after introduction for four *L. humile* recipient colonies with different queen numbers (queenless, single queen, and multiple queens): CHH (A), COC (B), FOR (C), and RTP (D). See Table 2 for colony abbreviations. N = 12. NS = nonsignificant ; $*P < 0.01$; $**P < 0.0001$ 42

Figure 2. Relationship between recipient colony response to intruders and worker aggression levels averaged across 6 hours (●) and at 24 h (○) for workers (A) and queens (B) 44

Figure 3. Relationship of non-nestmate worker (●) and queen (○) adoption vs. genetic similarity (% alleles shared) 45

Figure 4. Relationship of non-nestmate worker (●) and queen (○) adoption vs. genetic diversity (number of alleles) 46

CHAPTER II

Figure 1. Mean (±1 SE) worker-worker level of intercolony aggression at the beginning of (Initial) and 6 mo after (Final) the laboratory colony fusion assay. Set of bars with different letters indicate significant differences in aggression changes across colony

pairs ($p < 0.05$, LSD). Aggression levels decreased significantly (-) in five colony pairs throughout the experiment. See Table 1 for colony abbreviations 86

Figure 2. Mean (±1 SE) number of workers fighting and killed (out of 1000) in highly aggressive and moderately aggressive colony pairs in first 24 h of contact. *$p < 0.05$ 87

Figure 3. Relationship between number of ants fighting (A) and killed (B) in the first 24 h of contact and colony fusion within 24 h 88

Figure 4. Relationship between initial aggression levels between colony pairs and whether or not colonies fused within 6 mo 89

Figure 5. Relationship of pairwise F_{ST} (A) and % alleles shared (B) between colonies vs. colony fusion at 6 mo 90

CHAPTER III

Figure 1. Mean (±1 SE) total worker (A), brood (B), and queen (C) number recorded during the 6-month colony fusion assay. Set of bars with different letters indicates significant differences in numbers of workers, brood, or queens among colony pairs (LSMeans, $P < 0.05$). See Table 1 for colony abbreviations....136

Figure 2. Mean (±1 SE) per capita brood production for queens (A) and workers (B), and per capita worker production for queens (C) and workers D) for colony pairs vs. their unpaired controls on days 30, 90, and 180. See Table 1 for colony abbreviations. *P

< 0.05; **P < 0.005; ***P < 0.0001; ns = nonsignificant.....138

Figure 3. Mean (±1 SE) total brood and worker production (A) and total queen number (B) for all colony pair replicates that fused vs. those that did not on days 30 and 180. *P < 0.05; **P < 0.005; ns = nonsignificant.....143

Figure 4. Mean (±1 SE) per capita worker production for workers for all colony pair replicates that fused vs. those that did not on days 1, 90 and 180. *P < 0.05; ns = nonsignificant.....144

CHAPTER IV

Figure 1. Discriminant analyses of seven (A) and five (B) variables (relative proportions of cuticular hydrocarbons) selected from MANOVA and by stepwise discriminant analysis, respectively, for three groups of *L. humile* queens each treated with nestmate queen hydrocarbons (RTP), non-nestmate queen hydrocarbons (FOR), and hexane (HEX). The centroid of each group is marked by a + ...193

Figure 2. Discriminant analyses of six (A) and four (B) variables selected from MANOVA and by stepwise discriminant analysis, respectively, for two groups of treated *L. humile* queens that were either attacked or not attacked by nestmate workers. The centroid of each group is marked by a +.....194

Figure 3. Discriminant analysis of 12 variables selected by stepwise

discriminant analysis for queens from four *L. humile* colonies (CHH, COC, FOR, RTP). The centroid of each group is marked by a + ...195

Figure 4. Relationship between queen cuticular hydrocarbon profile similarities (generalized square distance between colony centroids) based on 12 variables and recipient colony response to non-nestmate queens introduced in queenless (◇), single queen (□), and multiple queen (△) colonies.....196

Figure 5. Relationship of queen cuticular hydrocarbon profile similarities based on 12 variables vs. % alleles shared (A) and pairwise F_{st} (B).....197

Figure 6. Discriminant analysis of 13 variables selected by stepwise discriminant analysis for queens from four *L. humile* colonies (CHH, COC, FOR, RTP). Cuticular hydrocarbons were sampled using a non-destructive method. The centroid of each group is marked by a + ...198

Figure 7. Discriminant analysis of three variables selected by stepwise discriminant analysis for queens from two *L. humile* colonies (COC and FOR). Cuticular hydrocarbons of queens were sampled 24 h before introduction and 2 weeks after adoption by a FOR queenless recipient colony using a non-disruptive method. The centroid of each group is marked by a + ...199

Figure 8. Discriminant analysis of 11 variables selected by stepwise

discriminant analysis for queens from two *L. humile* colonies (CHH and RTP). Cuticular hydrocarbons of queens were sampled 24 h before introduction and 2 weeks after adoption by a RTP queenless recipient colony using a non-destructive method. The centroid of each group is marked by a +.....200

Figure 9. Discriminant analyses of 18 variables selected by stepwise discriminant analysis for workers from five *L. humile* colonies (CAR, CHH, COC, FOR, RTP). The centroid of each group is marked by a + ...201

Figure 10. Relationship between worker cuticular hydrocarbon profile similarities based on 18 variables and levels of worker intraspecific aggression.....202

Figure 11. Relationship of worker cuticular hydrocarbon profile similarities based on 18 variables vs. % alleles shared (A) and pairwise F_{st} (B).....203

Figure 12. Relationship between source (A) and control (B) worker cuticular hydrocarbon profile similarities based on 18 and 10 transformed variables, respectively, and colony fusion at 24 h (△) and six months (□).....204

Figure 13. Discriminant analysis of nine variables selected by stepwise discriminant analysis for queens from five *L. humile* colonies (CAR, CHH, COC, FOR, RTP). The centroid of each group is marked by a +.....205

Figure 14. Discriminant analysis of 12 variables selected by stepwise

discriminant analysis for queens from five *L. humile* colonies (CAR, CHH, COC, FOR, RTP) and five *L. humile* colony pairs (CAR-CHH, CAR-COC, CAR-RTP, CHH-FOR, CHH-RTP). The centroid of each group is marked by a +.....206

Figure 15. Linear discriminant functions plotted for queens of fused colony pairs (CAR-CHH, CAR-RTP, CHH-RTP) and their respective unpaired controls (CAR, CHH, RTP) based on nine variables.....207

Figure 16. Linear discriminant functions plotted for queens of non-fused colony pairs (CAR-COC, CHH-FOR) and their respective unpaired controls (CAR, CHH, COC, FOR) based on nine variables.....208

Figure 17. Discriminant analysis of 11 variables selected by stepwise discriminant analysis for workers from five *L. humile* colonies CAR, CHH, COC, FOR, RTP) and five *L. humile* colony pairs (CAR-CHH, CAR-COC, CAR-RTP, CHH-FOR, CHH-RTP). The centroid of each group is marked by a +.....209

Figure 18. Linear discriminant functions plotted for workers of fused colony pairs (CAR-CHH, CAR-RTP, CHH-RTP) and their respective unpaired controls (CAR, CHH, RTP) based on 10 variables.....210

Figure 19. Linear discriminant functions plotted for workers of non-fused colony pairs (CAR-COC, CHH-FOR) and their respective unpaired controls (CAR, CHH, COC, FOR) based on 10 variables.....211

CHAPTER I

Adoption of non-nestmate Argentine ant conspecifics and the complexity of nestmate discrimination

Abstract. In most social insect species, individuals behave aggressively towards non-nestmate conspecifics to maintain colony integrity. However, in invasive Argentine ant, *Linepithema humile*, populations intraspecific aggression is largely absent, resulting in the formation of expansive colonies. Two hypotheses explaining this phenomenon have been proposed, genetic bottleneck and selection against diverse recognition loci, with both agreeing that a reduction in the diversity of genetically-based recognition cues may have contributed to extreme unicoloniality. In addition, the multi-component nature of nestmate recognition requires corresponding changes in internal recognition template development, perceived cues processing, and cue-template matching. One approach to better understand mechanisms underlying cue perception and response, and their implications in unicoloniality, is to examine variation in nestmate discrimination capability at the individual level and under different social contexts in invasive *L. humile* populations. Consequently, we investigated the dynamics of queen and worker adoption in conspecific queenless and queenright *L. humile* colonies. Adoption of queens and workers was a function of host colony origin and queen status, with queens more readily adopted by queenless hosts, suggesting that host colonies utilize both colony-specific and queen-derived cues in their adoption decisions. Intraspecific aggression levels between colony pairs were positively associated with non-nestmate worker but not queen adoption. Queen fecundity did not differ between adopted and rejected non-nestmate queens, and adopted non-nestmate queen fecundity was comparable to that of host colony queens. Genetic similarity between non-nestmates and host workers ranged from 30.3% to 77.4 % for workers and from 37.3% to 76.2% for queens, and non-nestmate queens and workers that were more genetically similar to host colony workers were more likely

to be adopted. Adoption of unrelated *L. humile* queens could enhance a colony's fitness if their offspring are used in the worker force or serve as mates, thereby gradually changing the host colony's genetic composition. We propose that in addition to a reduction in recognition loci and external cue diversity, broadening of the neural template through the adoption of unrelated queens reduces non-nestmate discrimination capability and may contribute to unicoloniality in *L. humile* populations.

Keywords: Argentine ant, *Linepithema humile*, nestmate recognition, aggression, queen adoption.

Introduction

Group and individual recognition play a major role in the social organization and behaviour of numerous animal species. Most social interactions such as territorial behaviour, 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 & Michener 1987). In social insects, such as termites and several Hymenoptera, discrimination behaviour is essential as it allows 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 behaviour toward group members (Hamilton 1964, Crozier & Pamilo 1996).

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 & Bennett 1987). The presence of multiple reproductive queens, or polygyny, is common in social insects, particularly ants (Keller & Vargo 1993, Bourke & Franks 1995). If queens of a polygynous colony are more related to each other than are queens from other colonies, nestmate recognition may promote fitness through kin selection (Vander Meer & Morel 1998). However, levels of genetic relatedness within a colony are variable among polygynous ant species (Herbers 1993), and 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 & Michener 1980, Bourke & Franks 1995). Failure in the nestmate recognition system in societies of ants is costly since colony resources will not be properly directed, although adoption of unrelated individuals may occur as a strategy of desperation when the colony's survival is at high risk (Sudd & Franks 1987). Furthermore, nestmate recognition systems are dynamic, with plasticity in discrimination varying according to the social and ecological context to balance the fitness costs of accepting non-kin and rejecting kin (Reeve 1989).

Queens of the same ant species can produce endogenous recognition cues, which are distributed among, and learned by, all adult colony members (Carlin & Hölldobler 1986, Brian 1986). Queen number and, presumably queen-derived discriminators, affect intraspecific worker aggression 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), *L. ambiguous* (Stuart 1988), and *Solenopsis invicta* (Obin & Vander Meer 1989, Morel et al. 1990). Multiple queen colonies may possess a less distinctive odour if the queens have different chemical signatures, thereby diminishing discrimination among neighboring colonies (Vander Meer & Morel 1998).

Queens and workers may also be labeled by a colony gestalt odour where nestmates share recognition cues, and each colony member bears a mixture of cues representative of the variation among members of the colony (Stuart 1988, Errard & Jallon 1987). This gestalt label is expected to prevail in polygynous ant species, although extreme polygyny may limit unique label creation thereby minimizing intercolony

variation. Unicolonial populations may result from the lack of distinct intrinsic colony odours, although some odour differences arising from extrinsic factors may still exist (Hölldobler & Wilson 1990).

Introduced populations of the Argentine ant, *Linepithema humile* (Mayr), are highly polygynous and display considerable diversity in nestmate recognition behaviour 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). These populations are useful to examine the differential behaviours toward conspecifics based on the degree of genetic similarity, and the effect of social and ecological context on action thresholds (Buczkowski & Silverman 2005). The study of behavioural interactions, particularly aggression, between colonies from these introduced populations may allow us to better understand possible mechanisms by which *L. humile* social structure is shaped.

While intraspecific aggression between Argentine ant workers has received considerable attention (e.g. Tsutsui et al. 2000, Suarez et al. 2002, Roulston et al. 2003), worker-queen interactions have only been examined within the same colony (e.g. intranest queen attraction, Keller & Passera 1989, and queen execution, Keller et al. 1989, Reuter et al 2001). It has been suggested that Argentine ant queens do not affect nestmate recognition since high levels of aggression toward non-nestmate workers occur in both queenless and queenright colonies (Caldera & Holway 2004). Nevertheless, it is unclear whether *L. humile* workers discriminate between non-nestmate and nestmate queens as they do between nestmate and non-nestmate workers, and whether queen number can affect the aggressive response of workers to non-nestmate queens and their subsequent adoption into a foreign colony. Foreign queen adoption and offspring

production could directly affect the genetic make-up of the colony by decreasing intranidal relatedness. These changes in genetic structure may modify levels of intraspecific aggression and, possibly, social structure, where the entry of each foreign queen produces an increasing breakdown in nestmate discrimination. Here, we investigated whether mutually aggressive Argentine ant colonies accept non-nestmate queens and workers and if queen number influences non-nestmate adoption. We hypothesize that social context (queen number) has a greater effect on nestmate discrimination in colony pairs with lower levels of intraspecific aggression and that non-nestmate acceptance thresholds increase when queens are absent. The queen-replenishment hypothesis (Brown & Keller 2000) suggests that below some threshold of queen number, new queens are recruited to enhance colony survival and productivity. Although this hypothesis was proposed to explain colony sex-ratio specialization in polygynous ants (preferential production of gynes in colonies with low queen numbers), we extend this to our system and suggest that higher queen recruitment into conspecific queenless colonies may prevent egg and brood-limited production as long as a certain level of genetic similarity exists. In Argentine ants, the degree of aggression among nests decreases with increasing genetic similarity (Tsutsui et al. 2000). Therefore, we hypothesize that Argentine ants are more likely to accept non-nestmates from colonies that are genetically more similar if nestmate recognition is based on heritable cues.

Explanations for the origins of *L. humile* unicoloniality, or absence of intraspecific aggression allowing workers and queens to move freely between nests, center on widespread loss of intraspecific aggression resulting from a genetic bottleneck rendering workers unable to distinguish nestmates from non-nestmates (Tsutsui et al.

2000) or by release from ecological constraints leading to increased non-nestmate encounters with subsequent selection against diverse recognition loci (Giraud et al. 2002). Both hypotheses suggest that changes in the social structure of introduced *L. humile* populations arose from a reduction in recognition loci diversity leading to reduced phenotypic variability in the cues underlying nestmate recognition and reduced intercolony aggression. In contrast, recent evidence reveals that both native and introduced populations appear to be unicolonial, although native supercolonies are drastically smaller (Pedersen et al. 2006). Unlike introduced populations from California (Tsutsui et al. 2000) and southern Europe (Giraud et al. 2002), *L. humile* in the southeastern U.S. occupy relatively small territories, are genetically more diverse, show high intracolony aggression and, therefore, may represent a transitional stage between native and the aforementioned introduced populations (Buczkowski et al. 2004). If the diversity of recognition cues is reflected in the overall high genetic diversity in the southeastern U.S., then the high intraspecific aggression exhibited by this population may follow Reeve's acceptance threshold model (1989), where conspecific rejection occurs if template-cue dissimilarity is above an acceptance threshold. Similarly, non-nestmates may not be rejected in unicolonial *L. humile* populations that exhibit low variation in genetic-based recognition cues as this might reduce the template-cue dissimilarity below the acceptance threshold (Starks 2003). In addition to the role of external recognition cues, specifically reduced cue diversity, we predict that expansion of the internal recognition template would decrease rejection of non-nestmates in populations that have not experienced a drastic loss of genetic diversity (Giraud et al. 2002, Buczkowski et al. 2004). According to this model, the recognition template is the neural representation of

the colony's recognition cues, either innate or learned, and in polygynous ants, exposure to a wider range of phenotypic cues results in a broader template (Vander Meer and Morel 1998) leading to acceptance of a broader range of recognition cues. A first step to better understand possible mechanisms that could be involved in recognition template broadening would be to carefully examine the variation in aggression behaviour towards individuals from different colonies and towards non-reproductive and reproductive individuals under different social contexts. Although this approach does not allow us to elucidate a direct mechanism by which *L. humile* could form expansive supercolonies since groups rather than individuals would encounter and interact in nature, we considered that due to the complexity of the nestmate recognition process, responses examined at the individual level better reflect the variability in decisions and actions taken by discriminating individuals. We show using *L. humile* colonies from the southeastern U.S. that display various levels of intraspecific aggression that foreign queen and worker adoption can occur under different social contexts, and that genetic factors influence adoption decisions, implying that changes in nestmate discrimination are due at least in part to the broadening of the recognition template.

Materials and Methods

Collection and Rearing of Laboratory Colonies

We collected colonies of Argentine ants (*Linepithema humile*) from five 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 9.7 km (CAR – RTP) to 402.3 km (CAR – COC). We established three experimental colonies from each location, each consisting of different queen numbers (zero, one, or six queens), 100 pieces of brood, and ca. 3000 workers (1 g.). Colonies were maintained in soil-free, Fluon™-coated trays (40 x 55 x 8 cm). Nests were plastic petri dishes (9 cm diameter) filled with moist grooved Castone® dental plaster. Colonies were provided 25% sucrose solution, artificial diet (Bhatkar & Whitcomb 1970) *ad libidum*, 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.

Aggression Tests

We assessed the level of worker-worker aggression between 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). The intruder was removed and discarded after each trial. Colony pairs included workers from queenless colonies matched against workers from other queenless and queenright (single queen and multiple queens) 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. The observer who recorded the aggression scores did not know the identity of the interacting colonies and was unfamiliar with the hypothesis being tested. Levels of aggression were measured one 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), for the subsequent non-nestmate adoption study. Aggression categories are based on a score lower than 3 (avoidance, prolonged antennation) being not injurious, while a score of 3.0 or higher (pulling, biting, and abdomen curling in an attempt to spray defensive compounds) was injurious.

Non-nestmate Adoption Assay

We assessed the ability of workers from queenless and queenright (single and multiple queen) colonies to discriminate non-nestmate from nestmate workers and queens. Six workers and six queens from each of four source colonies were introduced sequentially into each queenless and queenright experimental colony. Each introduction consisted of a single intruder transferred to the recipient colony with forceps and left in place for 24 h.

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). Introduced queens and workers were marked on the thorax and abdomen, respectively, with a water-based paint for identification. 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 x 12 x 5 cm) with 10-15 workers from the recipient colony to check for viable offspring production (worker pupae) over 35 d, which indicated successful queen adoption. All tested queens (144 per source colony) produced eggs prior to introduction. 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 non-nestmate queens that integrated within a foreign colony. Twenty-four COC queens and 24 CHH queens were individually introduced into nine queenless and nine multiple queen (5 queens) FOR and RTP experimental colonies, respectively. The total number of eggs laid within 24 h by queens placed individually in glass tubes (10 x 75 mm) and attended by 15 workers, and queen weight (mg) were measured prior to introduction. Individual queens were anesthetized and weighed in a CAHN 27 automatic electrobalance (CAHN Instruments Inc., Cerritos, CA, USA). Individual marked queens were introduced into recipient colonies

accompanied by 10-15 nestmate workers and left in place for 15 d. Host colony response (queen adopted or killed) in this study was highly correlated with colony response recorded in the previous non-nestmate adoption assay ($R^2 = 0.7637$, $P = 0.0002$) regardless of the differences in how queen introductions were performed. One week and two weeks after introduction, we measured queen fecundity as follows. Introduced and host colony queens (in the case of queenright host colonies), each accompanied by 10 recipient workers, were individually transferred to a glass tube (10 x 75 mm) that was provisioned with food (25% sucrose in capillary tubes) and capped with a removable screen (0.14 x 0.14 mm). Caged queens were immediately reintroduced into the recipient colony, left in place for 24 h and then released. Thus, we could count the total number of eggs produced in 24 h by the enclosed queens without removing them from their respective recipient colony. Nestmate queen fecundity from FOR and RTP (control) colonies was also measured. Marked queens were removed 15 d after introduction, placed into small trays with 10-15 workers from the recipient colony and further monitored for viable offspring (workers or males) production as an indicator of colony integration.

Genetic Similarity between Colonies

We assessed genetic similarity between introduced queens and recipient workers from five colonies: CAR, CHH, RTP, FOR and COC using microsatellite markers. Genomic DNA was extracted from 40-46 introduced queens per source colony and 10 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 & Keller

1999) and Lihu-T1 (Tsutsui et al. 2000). PCR reactions were performed as described by Buczkowski et al. (2004). Products were separated on 6.5% KB^{Plus} polyacrylamide sequencing gels using a 4000L Li-Cor DNA sequencer. Microsatellite alleles were scored using GeneImagIR software (Scanalytics Inc., Billerica, MA, USA). Levels of genetic similarity between introduced non-nestmate queens and resident workers and between non-nestmate workers and resident workers were estimated based on the percentage of alleles shared between these groups (Tsutsui et al. 2000). The proportion of identical alleles between queens and recipient workers over the total number of queen alleles was estimated for non-nestmate queens introduced into queenless host colonies. We also compared the total number of alleles present in recipient colonies and non-nestmate adoption rates to examine the relationship between genetic diversity and the likelihood of intruder conspecific adoption. Genetic differentiation (F_{ST}) between queenless and queenright experimental colonies from the same location and colonies from different locations was estimated with the program FSTAT v.2.9.3.2 (Goudet 1995).

Statistical Analyses

All behavioural 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 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) as fixed effects replicated in two trials. Maximum initial aggression score of each colony pair averaged across replicates was the dependent variable. Mean separation was carried out by least significant difference test (LSD) for colony pair and least square

means (LSMeans) for colony pair by recipient status interaction. To determine if levels of aggression varied as a function of exposure to non-nestmate queens, 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 aggressive 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 were used to determine the relationship between aggression levels and average recipient response to non-nestmate workers and queens using SAS statistical software. Spearman rank correlation coefficients were used to determine relationships between genetic similarity vs. non-nestmate worker adoption and

non-nestmate queen adoption with regression coeffiecients tested by Mantel's (1967) test in GENEPOP using 10,000 permutations.

Results

Intercolony Worker-worker Aggression

We identified three colony pairs with moderate (< 3.0), RTP-FOR, CHH-FOR, and FOR-COC, and three colony pairs with high aggression (3.0 or higher), CHH-RTP, RTP-COC, and CHH-COC, when averaging maximum aggression scores recorded one week after experimental colonies were established. Aggression between colony pairs across the aggression categories differed ($F_{5,5} = 25.34$, $P = 0.0015$). We also found a colony pair by recipient colony status (queenless or queenright) interaction ($F_{10,48} = 6.07$, $P < 0.0001$). Although levels of aggression were not significantly different between queenless and queenright colonies in most colony pairs, differences in aggression levels were observed between multiple queen versus single queen, and multiple queen versus queenless recipient colonies in one colony pair with high aggression, CHH-RTP ($P = 0.001$ and $P = 0.0004$, respectively), and in one colony pair with moderate aggression, FOR-COC ($P < 0.0001$ and $P = 0.0017$, respectively). Aggression between single queen FOR-COC was high, while aggression between single queen and queenless CHH-RTP was moderate.

Intraspecific aggression in experimental colonies did not decrease throughout the non-nestmate adoption assay according to the levels of aggression recorded 48 d after the experiment started. Although levels of aggression tended to increase over the course of the adoption experiment, these changes were not consistent across trials for colony pairs where adoption occurred ($F_{2,3} = 10.89$, $P < 0.0421$). There was a pair by recipient status interaction ($F_{4,60} = 4.49$, $P = 0.0031$) indicating that not all colony pairs consistently increased aggression in queenless and queenright colonies. Multiple queen FOR-COC

became more aggressive (3.1) while aggression decreased between single queen recipient colonies (2.8) ($P = 0.0010$). When aggression was averaged across all queen number status 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.

Non-nestmate Queen and Worker Adoption

We found an effect of recipient colony status (queen number) on intruder adoption ($F_{2,8} = 12.27$, $P = 0.0037$), varying across specific intruder by recipient colony ($F_{9,33} = 121.37$, $P < 0.0001$) and specific intruder by recipient colony and colony status ($F_{18,33} = 2.26$, $P = 0.0204$). We found no differences between periods when recipient colony response scores were averaged over 6 h in worker ($F_{2,288} = 2.11$, $P = 0.1232$) and queen ($F_{2,288} = 0.16$, $P = 0.8493$) introductions; and for final scores in worker ($F_{2,288} = 2.47$, $P = 0.0867$) and queen ($F_{2,288} = 0.09$, $P = 0.9131$) introductions, indicating that previous exposure to non-nestmate intruders had no effect on recipient colony response.

In general, reintroduced nestmate queens and workers were not attacked and 10.5% of nestmate queens were rejected by multiple queen colonies (Table 1). Most non-nestmate workers were attacked by recipient workers of queenless and queenright colonies, with less than 15% surviving an introduction. In contrast, non-nestmate queen adoption was greater in queenless versus queenright colonies ($F_{2,8} = 9.28$, $P = 0.0082$) (Table 1). Specific colony pair (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 pair 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, $P = 0.0026$) (Fig 1). When CHH was the recipient colony, foreign queen adoption was observed with FOR and RTP queens introduced to queenless and single queen colonies, and also with COC queens introduced to queenless colonies, however, non-nestmate workers were consistently killed (Fig. 1A). Similarly, COC workers killed CHH, FOR and RTP worker intruders, while only FOR queens were adopted by queenless and single queen colonies (Fig. 1B). Unlike CHH and COC colonies, FOR workers did not kill all introduced non-nestmate workers and adopted only CHH and COC queens (Fig. 1C), while RTP colonies adopted only foreign queens from CHH and a few workers from CHH and FOR (Fig. 1D). Rates of non-nestmate worker adoption in FOR and RTP colonies ranged from 8.3% (RTP) to 83.3% (COC), and from 0% (COC and FOR) to 16.7% (CHH), respectively. Rates of non-nestmate queen adoption varied across all queenless colonies, ranging from 41.7% (FOR) to 58.3% (RTP) in CHH colonies, from 0% (CHH and RTP) to 91.7% (FOR and COC) in both COC and FOR colonies, and from 0% (COC and FOR) to 66.7% (CHH) in RTP colonies. Therefore, it appears that the selective non-nestmate queen adoption observed in queenless colonies is regulated by interactions between specific colonies. Also, queen adoption at 24 h showed close correspondence with viable offspring production (worker pupae) over 35 d further supporting non-nestmate queen integration.

We found an association between initial aggression level and mean and final colony response to non-nestmate workers averaged across recipient colony status ($P < 0.0001$ and $P = 0.0043$, respectively) (Fig. 2A), but no association between worker intraspecific aggression and mean and final colony response to non-nestmate queens ($P = 0.4080$ and $P = 0.6168$) (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 24 h prior to introduction was not different between adopted (12.4 ± 12.7 eggs/24 h) and rejected non-nestmate queens (9.9 ± 8.2 eggs/24 h) (*t*-test: $t_{42} = 0.81$, $P = 0.2105$); adopted nestmate queens produced fewer eggs (5.9 ± 4.0 eggs/24 h) than adopted (*t*-test: $t_{34} = 2.27$, $P = 0.0149$) and rejected non-nestmate queens (*t*-test: $t_{50} = 2.09$, $P = 0.0207$). Fecundity measured one week after queen introduction did not differ between non-nestmate (17.1 ± 12.7 eggs/24 h) and nestmate queens (12.9 ± 12.0 eggs/24 h) (*t*-test: $t_{39} = 1.57$, $P = 0.0613$). Similarly, no differences were detected between non-nestmate (20.7 ± 5.2 eggs/24 h) and nestmate (14.9 ± 2.8 eggs/24 h) queen fecundity averaged over week one and week two (*t*-test: $t_2 = 1.60$, $P = 0.1249$), indicating that foreign queens contributed equally to colony productivity. Our queen fecundity measurements could have been affected by our experimental set-up, however, the number of eggs laid by caged queens in this study was within the 1 to 60 eggs/d range reported in larger single queen laboratory colonies (Newell & Barber 1913). Tending of non-nestmate offspring by recipient workers, and non-nestmate worker or male eclosion in colonies that were set-up using individual queens from the two-week fecundity trial

further suggest colony integration of adopted foreign queens and their offspring. Queen weight measured prior to adoption was not different between adopted foreign queens (3.19 ± 0.6 mg) and adopted nestmate queens (3.13 ± 0.6 mg) (*t*-test: $t_{39} = 0.29$, $P = 0.3816$). Also, we did not find a significant correlation between queen weight and queen fecundity ($R^2 = 0.0585$).

Levels of Genetic Similarity

While queenless and queenright laboratory colonies from the same location were not genetically different (F_{ST}CARY = 0.032 ± 0.045, F_{ST}CHH = -0.026 ± 0.006, F_{ST}COC = -0.012 ± 0.012, F_{ST}FOR = -0.003 ± 0.012, F_{ST}RTP = 0.001 ± 0.017), colonies from different locations were significantly differentiated ($F_{ST} = 0.219 \pm 0.047$). We found a positive relationship between pairwise F_{ST} (introduced non-nestmates and recipient workers) and non-nestmate adoption ($R^2 = 0.4736$, $P = 0.0344$). We also estimated percentage of shared alleles between non-nestmate intruders, queens and workers separately, and recipient workers since 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 non-nestmate workers and recipient workers, and from 37.3% (CHH-COC) to 76.2% (CAR-RTP) for non-nestmate queens and recipient workers. A positive association ($R^2 = 0.5224$, $P = 0.0161$) was found between levels of genetic similarity between workers and non-nestmate worker adoption averaged across host colony status (Fig. 3). A positive association was also found between queen-worker genetic similarity and non-nestmate queen adoption averaged across host colony status ($R^2 = 0.4217$, $P = 0.0257$) (Fig. 3). 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 ± 0.12) than that of rejected non-nestmate queens (0.64 ± 0.15) (*t*-test: $t_{21} = 2.53$, $P = 0.0097$). 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 ($R^2 = 0.3426$, $P = 0.0754$) was found between number of alleles in recipient colonies and non-nestmate adoption (Fig. 4).

Discussion

We determined that Argentine ant colonies adopt non-nestmate conspecific queens and that adoption of these non-nestmates may constitute a mechanism promoting extreme unicoloniality in introduced populations. Ant workers eliminate queens if their maintenance is costly (i.e. reduction of colony productivity), if they are unrelated queens, and possibly to reinforce queen dominance hierarchies (Reeve & 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 (Balas 2005). The fitness cost of accepting non-nestmate reproductives decreases in the absence of queens (Reeve 1989) or in colonies producing gynes or with low resident queen numbers (Fortelius et al. 1993, Brown et al. 2003). We demonstrate that queenless *L. humile* colonies accept more non-nestmate queens than queenright colonies further supporting the acceptance of a replacement queen due to queen pheromone deficit (Fletcher & Blum 1983) that affects the acceptance threshold (Reeve 1989) so that subtle recognition cue differences are less likely to be detected. However, we found that adoption of non-nestmate workers by both queenless and queenright *L. humile* recipient colonies was low, suggesting that queens do not influence aggression toward non-nestmate workers. Similarly, queen removal did not reduce intraspecific aggression between *L. humile* colonies in California (Caldera & Holway 2004). It would appear that adoption of non-nestmate queens may further shape *L. humile* social structure by creating assemblages of genetically distinct worker offspring.

We observed considerable variation in adoption rates of non-nestmate queens and workers from colony pairs displaying moderate and low 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 non-nestmate discrimination may be modulated by cuticular hydrocarbons, which differ in *L. humile* queens and workers (de Biseau et al. 2004).

Social organization and group cohesion depend not only on recognition mechanisms between group members but also the pheromonal signals queens convey to the colony (Keller & Nonacs 1993). Chemical manipulation of worker behaviour by queens can increase a queen's fitness at the worker's expense or both workers and queens' inclusive fitness (Keller & Nonacs 1993). Queen presence can influence worker aggressive behaviour (Vienne et al. 1998, Boulay et al. 2003). Moreover, in the case of *S. invicta*, acceptance of replacement queens by workers occurs when levels of queen pheromones circulating in a colony are below an optimal range, as for example in queenless colonies, while higher queen numbers raise the level close or above a tolerance threshold causing workers to behave aggressively toward some queens, probably the ones with the least familiar odour or the least productive ones (Fletcher & Blum 1983, Vander

Meer and Alonso 2002). A similar queen pheromonal effect may regulate acceptance thresholds 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 & Passera 1991, Passera et al. 1995). Therefore, *L. humile* queen pheromones may also influence other aspects of nestmate recognition, including differential aggression towards non-nestmate castes and the adoption of new queens. For example, a drop in levels of *L. humile* primer pheromones following queen seasonal execution (Keller et al. 1989, Reuter et al. 2001) may be followed by adoption of virgin, newly mated, and even non-nestmate queens to restore these levels to the optimal limits.

Queen adoption is not uncommon among polygynous ant species that mostly recruit queens from within their colony (Hölldobler & Wilson 1990), with a variety of ecological and intrinsic factors modulating this process, including risky dispersal and increased colony survival and productivity (Keller 1995, Brown & Keller 2000). In the Argentine ant, adoption of daughter queens after seasonal queen execution by workers has been documented in introduced populations (Markin 1970, Keller 1988). However, the fitness consequences of non-nestmate queen adoption, and whether it may be a strategy for orphaned colony survival or a behavioral anomaly observed only in introduced populations, remain unclear. Based on the well developed sexual brood discrimination by *L. humile* workers (Vargo & Passera 1991), recipient colonies may rear only worker offspring from adopted non-nestmate queens, and these workers could care for the original colony's sexual brood or perform other tasks that increase colony productivity. This strategy might resemble that of inquilines and slave-making ants,

which rely exclusively on the enslaved workers to raise their sexual offspring (Sudd & Franks 1987, Hölldobler & Wilson 1990), and, in the case of slave-makers, could lead to the genetic death of the enslaved colonies (Foitzik & Herbers 2001). Argentine ant colonies that accept non-nestmate queens could then replenish their work force without permanently changing their genetic composition. Alternatively, ant colonies would benefit by adopting unrelated queens that produce sexuals that can serve as mates for the resident sexuals thereby lessening chances of inbreeding and enhancing offspring fitness. Similarly, birds which accept unrelated hatchlings may increase their fitness not only by having a larger helper force to reduce predation on their own young and enhance their own growth rate, but also by providing their young with ecologically compatible but genetically unrelated mates (Avital et al. 1998).

Investigators using prevailing nestmate recognition assays generally assume that aggressive behaviours (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. Also, the effect of differences in host colony behavioural response towards individual conspecific queens and workers on interactions between colony cohorts warrants investigation.

We were surprised that approximately 10% of queens were killed when reintroduced to their own multiple queen colonies. We recorded no genetic differentiation between queenless and queenright recipient colonies from the same location indicating

that the effect of recipient colony queen status on worker aggression towards both nestmates and non-nestmates was unrelated to differences in colony genetic composition but to queen presence. Therefore, random elimination of excess queens to enhance colony productivity (Reeve & Ratnieks 1993) and to restore queen numbers in compliance with the hierarchical queen pheromone hypothesis (Fletcher & Blum 1983), i.e. elimination of least productive queens, may explain this selectivity. We recorded a positive association between non-nestmate worker and queen adoption and genetic similarity between introduced non-nestmates and recipient workers, which suggests that genetic similarity may also regulate non-nestmate adoption, further supporting a *L. humile* genetically-based recognition system (Tsutsui et al. 2000, Tsutsui & Case 2001). Moreover, the lack of a nestmate queen inhibitory pheromonal effect in queenless colonies suggests that the higher rates of queen adoption observed might be strongly associated with queen-worker genetic similarity. Adopted non-nestmate queens introduced into these colonies had a higher proportion of alleles in common with recipient workers than queens that were not adopted. Also, colonies with lower genetic diversity seem to be less likely to adopt foreign queens. Therefore, it is also possible that the selective adoption of non-nestmate queens in relatively more diverse colonies could broaden the recipient colony template and reduce nestmate discrimination and intraspecific aggression. In contrast, if less diverse colonies are less likely to adopt foreign queens their possibility to expand their already narrow template is minimal. Hence, adoption of non-nestmate queens may change *L. humile* social structure if less aggressive colonies gain selective advantages by reducing the costs of territory defense, and subsequently displace highly aggressive colonies. 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 (Buczkowski et al. 2004) would be useful to clarify whether foreign queen adoption is exclusive to invasive populations that have not yet reached the expansive supercolony level.

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. Instead, colony of origin influenced queen rejection, and colony-derived cues probably play 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 & Blum 1983, Fortelius et al. 1993, Sundström 1997, Keller & Ross 1993). For a specific colony pair, 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 & Keller 2001). Although not recorded, viability of eggs laid by introduced *L. humile* may be a better indicator of queen reproductive status (Vargo & Ross 1989, Chen & Vinson 2000).

Through selective elimination of unrelated queens by workers, levels of within-colony relatedness and social structure (e.g. Krieger & Ross 2002) are maintained in social insect colonies. Adoption of unrelated queens may affect levels of relatedness within the recipient colony thereby changing colony and population genetic structure, and further eroding nestmate discrimination, resulting in acceptance of more foreign individuals (Bourke & Franks 1995). Flexibility in recognition processes allowing the exchange of unrelated queens may represent an early stage in the development of unicoloniality, and the exchange of queens and consequent breakdown in territorial

boundaries may particularly benefit small *L. humile* propagules (Hee et al. 2000), which may otherwise be at a disadvantage against larger native ant colonies. Although our experimental conditions did not fully reflect queen dispersal in nature, i.e. queens usually disperse accompanied by workers during colony budding (Newell & Barber 1913, Keller 1995), habitat disturbance may cause colony fragmentation or trail disruption, thus increasing the likelihood of a solitary queen dispersing and encountering new groups of workers by orienting alone along chemical trails (Aron 1992). In addition, incipient colonies comprising a single queen with few workers or queenless workers from distant locations can potentially encounter and interact via human-mediated jump-dispersal (Suarez et al. 2001), however, whether these mixed individuals can successfully establish remains to be investigated. While gene flow between highly aggressive neighboring field colonies may be limited (Thomas et al. 2006) less aggressive and more genetically similar colonies may exchange queens and workers. Therefore, conspecific queen adoption may contribute to the success of *L. humile,* and possibly other invasive ants, particularly in areas where rates of expansion are not ecologically (Ingram 2002) or geographically (Suarez et al. 2001) constrained. While unclear how adopted queens may impact colony productivity, 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 and colony fusion, two of the pathways that lead to secondary polygyny, act synergistically in shaping *L. humile* social structure.

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Table 1. Nestmate and non-nestmate queen and worker adoption (Mean ± SE) in queenless and queenright colonies.

Host colony status	Nestmate adoption (%)		Non-nestmate adoption (%) [a]	
	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

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

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

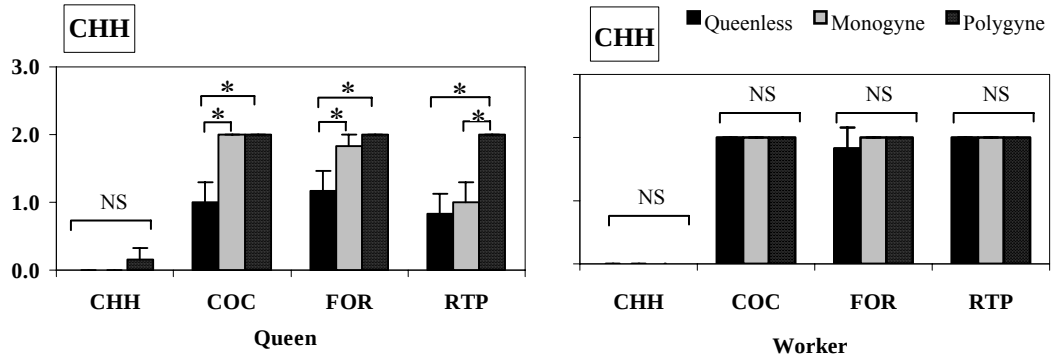
Host colony	Queen	Adoption	Number of alleles[a]		Proportion (I/I+D)
			Identical (I)	Different (D)	
CAR	CHH	Y	15	5	0.75
	COC	Y	11	4	0.73
	FOR	Y	14	4	0.78
	RTP	Y	15	2	0.88
CHH	CAR	Y	11	6	0.65
	COC	Y	7	6	0.54
	FOR	Y	14	4	0.78
	RTP	Y	15	7	0.68
COC	CAR	N	9	11	0.45
	CHH	N	7	10	0.41
	FOR	Y	11	5	0.69
	RTP	N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

CAR: Cary; CHH: Chapel Hill; COC: Greenville; FOR: Winston-Salem; RTP: Research Triangle Park. Y = Yes; N = No.

[a] Total number of alleles across seven microsatellite loci.

Figure 1. Mean ± SE levels of recipient colony response (0 = no aggressive response, 1 = physical attacked, 2 = intruder killed) to intruder queen and worker 24 h after introduction for four *L. humile* recipient colonies with different queen numbers (queenless, single queen, and multiple queens): CHH (A), COC (B), FOR (C), and RTP (D). See Table 2 for colony abbreviations. N = 12. NS = nonsignificant; $*P < 0.01$; $**P < 0.0001$.

A


CHH
Queenless
Monogyne
Polygyne
3.0
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1.0
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NS
*
CHH
COC
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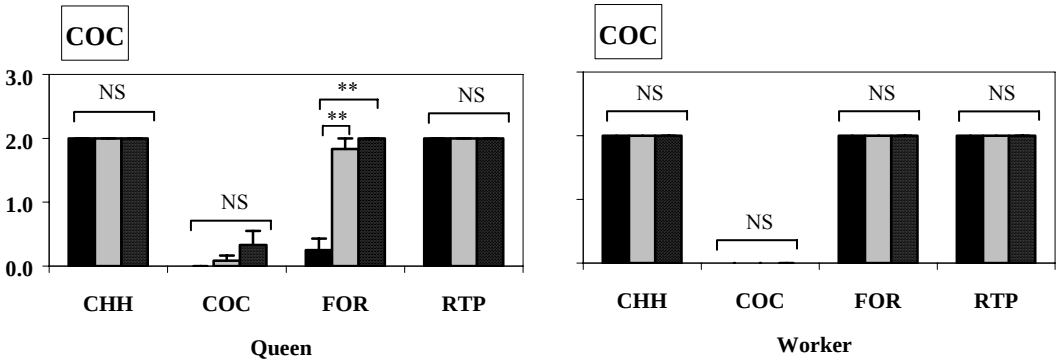
RTP
Queen
Worker

B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CHH
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RTP
Queen
Worker

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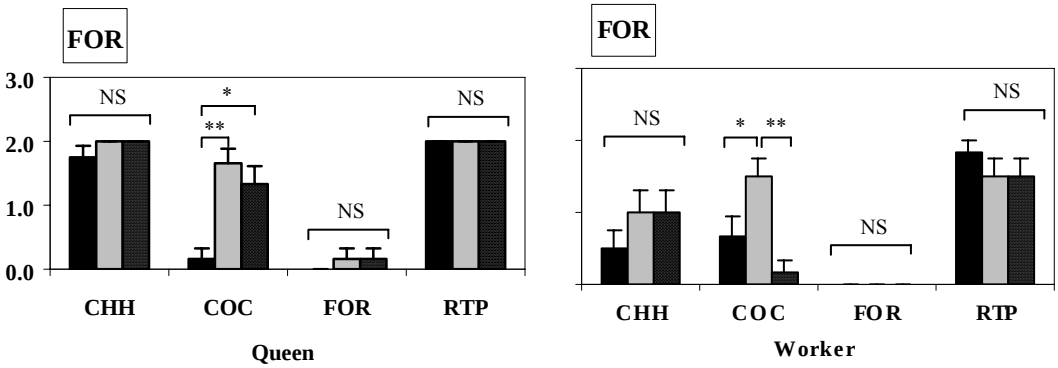
FOR
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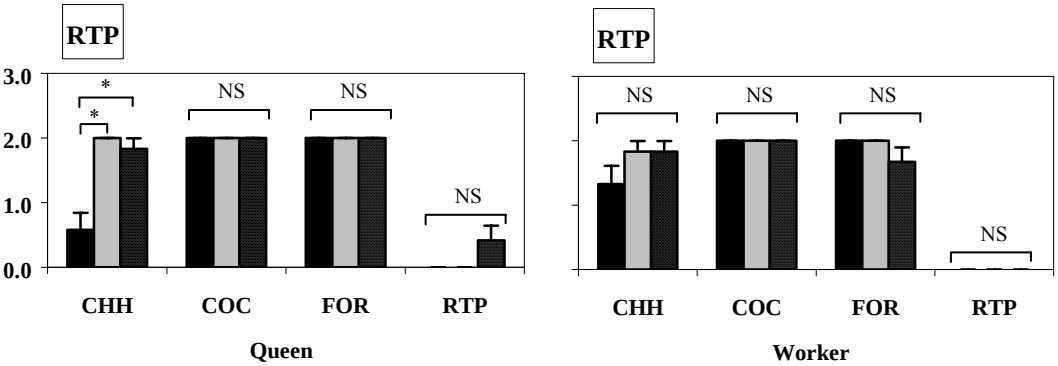
RTP
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Worker

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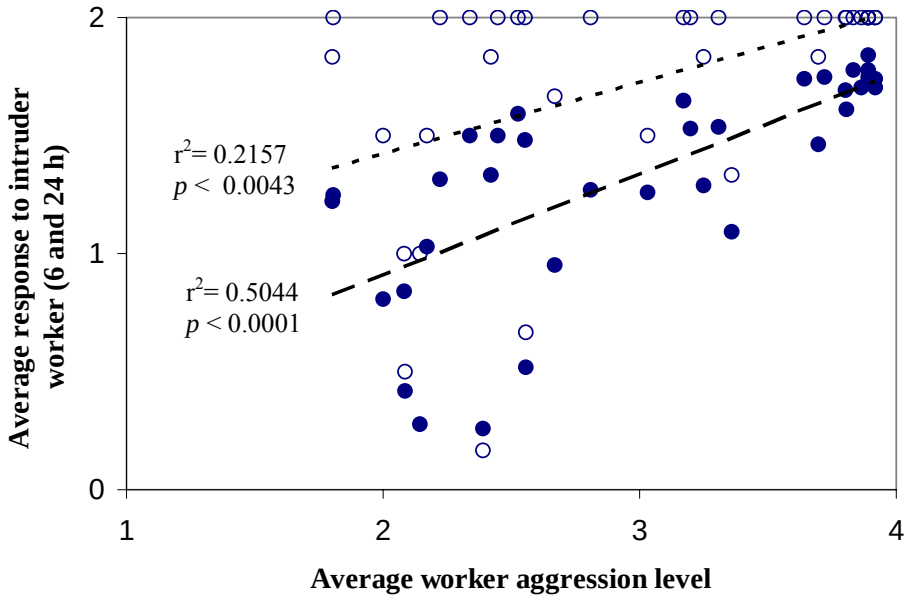

B

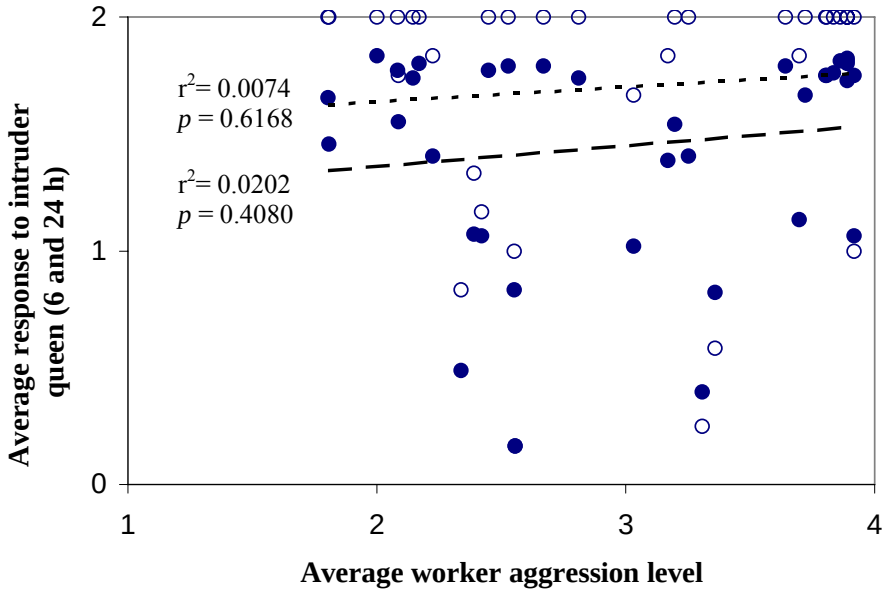

Figure 2. Relationship between recipient colony response to intruders and worker aggression levels averaged across 6 hours (●) and at 24 h (○) for workers (A) and queens (B).

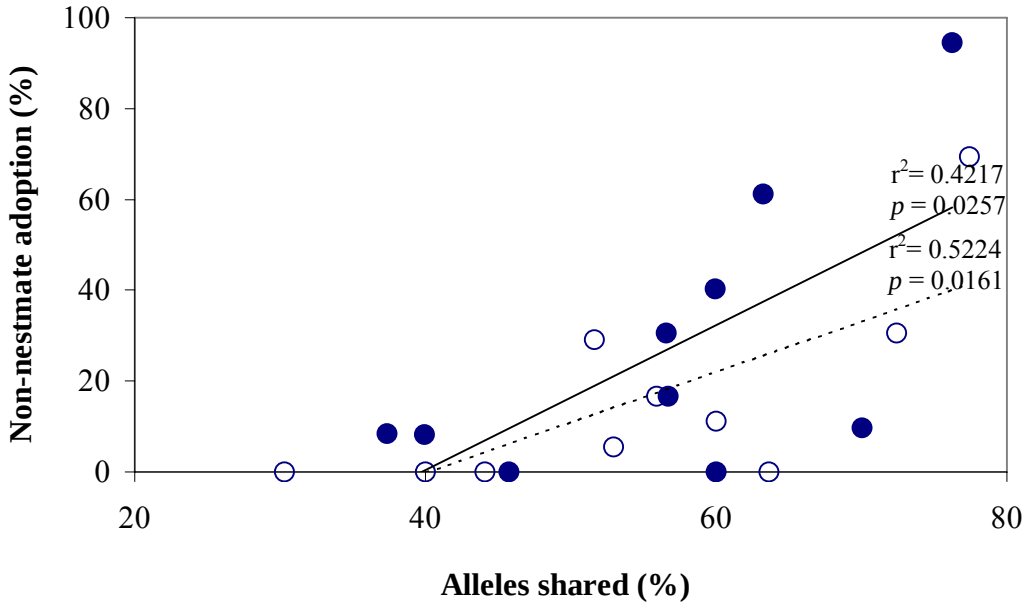

Figure 3. Relationship of non-nestmate worker (●) and queen (○) adoption vs. genetic similarity (% alleles shared).

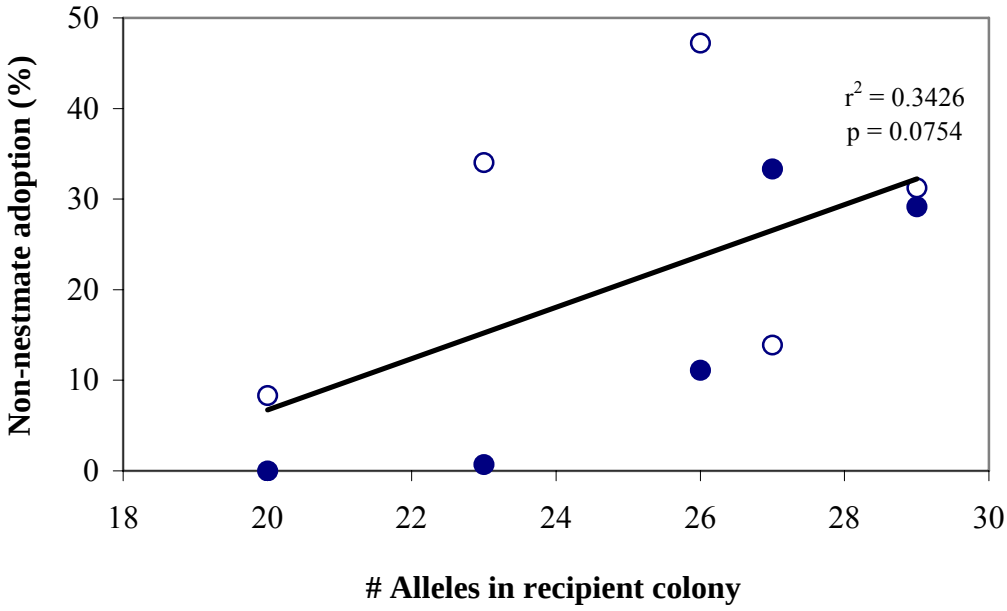

Figure 4. Relationship of non-nestmate worker (●) and queen (○) adoption vs. genetic diversity (number of alleles).

CHAPTER II

Intraspecific aggression and colony fusion in the invasive Argentine ant

Abstract. Unicolonial ants possess an unusual social system characterized by the absence of colony boundaries resulting in expansive networks where individuals move freely among distant nests. The formation of these geographically vast and numerically large unicolonial populations, or supercolonies, has been linked to the ecological success of invasive ants. The Argentine ant, *Linepithema humile*, is one of the few species in which native and introduced populations have been examined to elucidate the origins and maintenance of unicoloniality in invasive ants. Reduced variability in nestmate recognition cues may explain the lack of intraspecific aggression within introduced *L. humile* supercolonies, with loss of genetic diversity via a genetic bottleneck or, alternatively, elimination of colonies possessing rare recognition cues through aggressive intercolony encounters, as possible mechanisms leading to loss of recognition cue diversity. Supercolony formation may thereby result from mixing of either genetically homogenous and non-aggressive colonies, or initially aggressive colonies harboring the most common recognition cues. In this study, we examined interactions between mutually aggressive *L. humile* colonies in the absence of barriers limiting intercolony encounters to determine whether aggressive interactions result in colony elimination or fusion into new non-aggressive colonies. In laboratory experiments we paired moderately and highly aggressive experimental colonies, and recorded number of workers fighting, worker mortality, and fusion events. Higher numbers of workers fighting and killed were observed in highly aggressive colony pairs with fusion occurring in some instances, whereas all moderately aggressive pairs fused. Levels of intercolony aggression decreased after fusion. Using microsatellite markers, we found that genetic similarity between colonies was correlated with both levels of intraspecific aggression and colony

fusion. In the field, the percent of replicates that fused per colony pair was similar to rates recorded in the laboratory. We propose that in the absence of constraints preventing colony interactions, fusion of initially aggressive colonies sharing moderate to relatively high levels of genetic similarity can be a proximate mechanism shaping *L. humile* social structure, thus leading to extreme unicoloniality in introduced populations.

Keywords: Argentine ant**,** *Linepithema humile*, invasive species, unicoloniality, nestmate recognition, aggression, colony fusion, genetic similarity.

Introduction

Most social insect populations contain colonies that recognize nest boundaries and maintain colony integrity by exclusion of both heterospecific and conspecific intruders (Hölldobler and Wilson 1990). In this multicolonial social structure, individuals distinguish nestmates from intruders by means of a nestmate recognition system consisting of genetically determined and/or environmentally derived olfactory cues (recognition label) and a genetically-determined or learned sensory template (Lacy and Sherman 1983; Crozier and Pamilo 1996; Gamboa et al. 1986). A proposed model for nestmate recognition involves matching the label of the encountered conspecific to the individual's inner template so that it can take action (accept or reject) according to the degree of template-cue dissimilarity (Sherman and Holmes 1985; Reeve 1989). High intranest relatedness and low within-colony genetic variation may explain the well-developed recognition system in multicolonial ant species (Breed and Bennett 1987) allowing individuals in these "closed" societies to aggressively defend territories (Sudd and Franks 1987).

In contrast to these closed societies, some ant species form unicolonial populations, whereby colony boundaries are largely absent such that individuals move freely between distant nests (Hölldobler and Wilson 1977, 1990; Bourke and Franks 1995). Limited genetic differentiation between nests in unicolonial populations produces colony odor homogeneity and, therefore, no within-colony aggression (Hölldobler and Wilson 1990), although individuals from other unicolonial populations can be recognized and attacked (Tsutsui et al. 2000; Giraud et al. 2002). Unicoloniality is considered

evolutionarily unstable because altruism among nestmates with levels of relatedness close to zero is inconsistent with kin selection theory (Bourke and Franks 1995). Nevertheless, this unusual social system has been observed in some species of *Anoplolepis*, *Formica*, *Lasius*, *Linepithema*, *Monomorium*, *Pheidole*, and *Wasmannia* (Hölldobler and Wilson 1990; Holway et al. 2002). Interestingly, most of these species thrive in human-altered habitats, and share characteristics including polygyny, colony reproduction by budding, general nesting and dietary requirements, high worker numbers, high nest densities, and superior competitive abilities (Passera 1994; Moller 1996). Moreover, unicoloniality and its associated traits seem to play a key role in the ecological success of invasive ants (Chapman and Bourke 2001; Holway et al. 2002).

Studies comparing native and introduced populations of two highly successful invaders, *Solenopsis invicta* and *Linepithema humile*, have shed light on the origins of unicoloniality and led to the emergence of ecological and genetic hypotheses to explain its evolution in invasive ant species (Ross and Keller 1995; Chapman and Bourke 2001; Holway et al. 2002). It was first proposed that release from natural enemies with subsequent habitat saturation, and a genetic bottleneck that reduced sex-determining locus variation may have both limited independent colony founding in introduced *S. invicta* populations in the U.S., resulting in highly polygynous and dominant colonies (Ross et al. 1996). However, it was later shown that *S. invicta* social organization is genetically regulated with colony queen number being determined by differences at the *Gp-9* locus, with workers bearing the "green beard" allele (*b*) accepting only multiple queens sharing the same allele (Keller and Ross 1998). In *L. humile*, the formation of unicolonial populations may be explained by release from ecological constraints leading

to increased non-nestmate encounters and selection against diverse recognition loci, thereby favoring non-aggressive colonies possessing the most common recognition alleles (Giraud et al. 2002), and lack of nestmate discrimination due to a genetic bottleneck followed by selection against genetic diversity and subsequent loss of recognition cue variation (Tsutsui et al. 2000; Tsutsui et al. 2003).

In addition to the mechanisms proposed in these post-introduction hypotheses, ecological factors shaping the social organization of ant colonies, such as habitat structure, climate, interspecific competition and more efficient exploitation of plant and hemipteran exudates (Herbers 1993; Hölldobler and Wilson 1977, 1990; Davidson 1997), may not only regulate colony size and abundance in invasive ants but also the variation in the expression of unicoloniality among introduced populations. Considerable attention has been paid to the role of intrinsic factors such as loss of intraspecific aggression and colony and nest structure flexibility in the expression of unicoloniality and invasion success of *L. humile* (Holway et al. 1998; Tsutsui et al. 2000; Tsutsui and Case 2001; Ingram 2002a, 2002b; but see Heller 2004). In the absence of intraspecific aggression, high worker numbers are more likely to monopolize resources (Human and Gordon 1996; Holway and Case 2001) while flexibility in nest size, queen number, frequency of colony budding and movement between nests facilitates adaptation to new environments (Ingram 2002a, 2002b). Additionally, colony size and other unicolonial traits are influenced by the ecological context in which they occur, with extreme unicoloniality expressed in the absence of ecological constraints while highly constrained environments restrict its expression.

Therefore, the differential expression of unicoloniality observed in introduced *L. humile* populations (Suarez et al. 2002; Tsutsui et al. 2000; Buczkowski et al. 2004) can be regulated by both abiotic (temperature, humidity, resource availability) and biotic factors (intra and interspecific competition) that affect colony survival, rates of colony expansion, and competitive ability (Holway et al. 2002; Walters and Mackay 2003; Holway and Suarez 2004). Hence, regional differences in *L. humile* social structure in the U.S., i.e. small, patchily distributed, highly aggressive colonies with high genotypic variability in the southeast (Buczkowski et al. 2004) vs. expansive supercolonies with low levels of genetic diversity in California (Suarez et al. 1999; Tsutsui et al. 2000), may be to some extent explained by the dissimilar abiotic and biotic pressures acting on these populations. Unlike populations from California and southern Europe that experience relatively mild winter conditions and also reduced biotic resistance (Human and Gordon 1996; Heller 2004), *L. humile* in the southeastern US are exposed to winter subfreezing temperatures and they possibly compete with *Solenopsis invicta* (Buczkowski et al. 2004). Thus, unfavorable ecological conditions in the temperate southeastern U.S. may restrict colony expansion and subsequent intermixing of individuals resulting in mutually aggressive "diminutive supercolonies", while extreme unicoloniality in regions with Mediterranean or subtropical climate may result from boundary expansion followed by mixing of colony members, thereby creating a blend of recognition cues across the newly fused larger colony.

Colony traits in multicolonial and unicolonial populations may constitute a continuum of variation in social structure in polygynous ant societies (Bourke and Franks 1995), differentially expressed according to specific ecological conditions. Although

reduction in recognition loci diversity offers an evolutionary explanation for extreme unicoloniality in introduced *L. humile* populations (Tsutsui et al. 2000, 2003; Giraud et al. 2002), little attention has been paid to the mechanistic underpinnings of the transition from diminutive to extreme unicoloniality. The mechanisms leading to the development of secondary polygyny in ants, i.e. new gyne acceptance and/or colony fusion (Herbers 1993; Hölldobler and Wilson 1990), could also be involved in the variable expression of unicoloniality among introduced *L. humile* populations. Therefore, studies examining interactions between mutually aggressive *L. humile* colonies in the absence of ecological constraints would shed light on whether the proposed mechanisms, queen adoption and colony fusion, play a role in shaping invasive population social organization. Since intercolony aggression seems to be regulated by levels of genetic and phenotypic similarity (Tsutsui et al. 2000; Suarez et al. 2002), fusion events may depend on the intensity of aggressive interactions between colonies and should vary according to differences in genetic similarity.

The objectives of this study were, therefore, to determine if aggressive *L. humile* colonies from the southeastern U.S. can fuse, and if levels of aggression and genetic similarity between colonies influence this process. We predict that low intercolony aggression and high levels of genetic similarity between colonies promote colony fusion. To test this we conducted laboratory assays where we paired experimental *L. humile* colonies showing various levels of intraspecific aggression. We then selected two colony pairs that fused under laboratory conditions to examine the interactions between colony fragments in the field. Although colony fusion might be only one of a variety of mechanisms shaping *L. humile* social structure, we provide evidence for a proximate

mechanism by which unrelated colonies that may have descended from multiple, independent introductions from the native range, fuse to attain extreme unicoloniality in their introduced range.

Materials and Methods

Ant Colonies and Rearing Conditions

We collected colonies of Argentine ants (*Linepithema humile*) from five locations 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. In a colony fusion laboratory assay we tested all 10 pairwise combinations with 5 replicates per pair. Experimental colonies from each location consisted of 5 queens, ca. 100 pieces of brood, and 500 workers. For each colony pair, all queens and 50 workers from each colony were marked on the thorax and abdomen, respectively, with either pink (colony 1) or yellow (colony 2) water-based paint (Apple Barrel Colors®, Plaid Enterprises Inc., Norcross Georgia, USA) using a 10/0 brush to observe individuals mixing and determine fusion events. Colonies were maintained in individual Fluon™-coated trays (17 x 25 x 11 cm) and provided either plastic petri dishes (9-cm diameter) filled with moist grooved Castone® dental plaster (colony 1) or foil-covered glass tubes half-filled with water and stopped with cotton as artificial nests (colony 2), alternating type of nest assigned across replicates in each colony pair. Containers were connected through a 12-cm long vinyl tube with soft earplugs initially inserted at each end to prevent contact between colonies. Each colony was provided with 25% sucrose solution and artificial diet (Bhatkar & Whitcomb 1970) *ad libidum* during a 24-hour acclimation period. Throughout the colony fusion laboratory assay colonies in plaster nests were provided with 25% sucrose solution, artificial diet *ad libidum*, three freshly-killed *Blattella germanica* adults once a week, and a water source. By providing different types of artificial nests and placing food

items in only one of the paired colonies we expected to promote continuous encounters and the likelihood of fusion. Controls consisted of unpaired experimental colonies from each location (5 replicates per location) that were not exposed to any foreign colony. 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.

For a field introduction and colony fusion assay we used Argentine ants from CAR, CHH, and RTP. Eighteen colonies consisting of 2 g of workers, 0.2 g of brood, and 30 queens were placed in individual Fluon™-coated plastic containers (23 x 23 x 9 cm) with their original nesting substrate (500 cc). All queens and a fraction of the workers (ca. 1 out of 10) were marked as previously described, while the brood was marked by feeding the colonies sucrose solution (25%) containing 8mM erioglaucine. Within one week after collection, colonies provided with a water source and 25% sucrose solution were transferred to the field site (RTP) in their plastic containers covered with lids to proceed with the field introduction assay (see below). RTP ants were used as controls.

Levels of Worker Aggression

We assessed the level of worker-worker aggression between all pairwise source colony combinations (CAR-CHH, CAR-COC, CAR-FOR, CAR-RTP, CHH-COC, CHH-FOR, CHH-RTP, COC-FOR, COC-RTP, FOR-RTP) 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). The intruder

was discarded after each trial. Twelve replicates per colony pair were performed; six replicates with colony 1 as the resident and six replicates with colony 1 as the intruder. The observer who recorded the aggression scores did not know the identity of the interacting colonies and was unfamiliar with the hypothesis being tested. Results of these trials established the aggression categories for the subsequent colony fusion study: high aggression if the maximum score was 3.0 or higher (pulling, biting, and abdomen curling in an attempt to spray defensive compounds), and moderate aggression if the maximum score was lower than 3.0 (avoidance, prolonged antennation). Eight colony pairs were highly aggressive: CAR-CHH, CAR-COC, CAR-FOR, CHH-COC, CHH-FOR, COC-FOR, COC-RTP and FOR-RTP, and two colony pairs were moderately aggressive: CAR-RTP and CHH-RTP. To determine if colony fusion could lead to a reduction in intraspecific aggression, we measured aggression levels between fused colonies and their respective controls (unexposed colonies) 6 mo after the start of the colony fusion assay. Levels of aggression between replicates that did not fuse and their respective controls were also measured. Data were analyzed as the maximum score per trial recorded between source colony pairs, and as the difference between maximum aggression scores recorded after 6 mo and initial maximum aggression scores. Analyzing the highest aggression score allows comparison of levels of aggression across colony pairs without the effect of individual variation in the response of resident workers that may arise due to physiological differences that could mask colony recognition abilities (Obin and Vander Meer 1988).

Laboratory Colony Fusion Assay

We conducted a colony fusion experiment to determine if levels of aggression would predict the outcome of intraspecific interactions. After a 24-h acclimation period, the vinyl tube connecting the previously described colony pairs was unblocked and the ants were allowed to interact. We recorded the total number of workers fighting and the total number of dead queens and workers (marked and unmarked) in each container and length of tubing each hour for 6 h and then at 24 h. Colony pairs were inspected for fusion daily, from day 2 - day 30, and monthly, from month 2 - month 6. Fusion was defined as the presence of all queens and brood in the same nest and the mixing of marked workers without fighting. Data were analyzed as the total number of workers fighting and dead workers within the first 24 h and percentage of replicates from the same colony pair that fused 24 h and 6 mo after the experiment started. Three trials were conducted, the first one from August 2003 through February 2004, the second one from May through November 2004, and the third one from March through September 2005. The first two trials included the following colony pairs: CAR-CHH, CAR-COC, CAR-RTP, CHH-FOR, and CHH-RTP (50 pairs and 50 controls); while the third trial included: CAR-FOR, CHH-COC, COC-FOR, COC-RTP, and FOR-RTP (25 pairs and 25 controls).

Fusion under Controlled Field Conditions

Two colony pairs that readily fused in the laboratory assay, CAR-RTP and CHH-RTP, were selected to test whether fusion of field colony fragments could occur after aggressive encounters in the field. CAR and CHH colonies set-up as previously described were placed individually at the base of six red maples, *Acer rubrum*, along a 200 m

transect at the RTP collection site. RTP ants collected at the base of three individual trees along the same transect served as controls. At the start of the experiment, the abundance of RTP ants indicated by the total number of workers trailing/minute across a fixed point on each tree did not differ among assigned treatments (foreign colonies) and controls (F = 2.35, df = 2,2, p = 0.2983). We fastened containers with the introduced colonies to the tree trunk with 130-cm length x 5-mm diameter Nalgene® premium tubing to allow interaction with RTP ants. We banded a section of the trunk (80-cm length) with Tree Tanglefoot® to facilitate the observation of behavioral interactions (i.e. fighting) and prevent foreign ants from escaping. We counted the total number of ants fighting on the connecting tube and on a 5 x 5 cm section around the point of attachment of the tube at 30 minutes and every hour for 6 h to quantify aggressive interactions and compared them with those recorded in the first 6 h of the laboratory assay. After 6 h, we removed the tubing, collected RTP ants nesting at the base of each tree (approx. 500 cc ants and nesting substrate), then placed them in plastic containers (23 x 23 x 9 cm) and transferred colony fragments and introduced colonies to the laboratory to monitor fusion events under controlled conditions. The total number of queens in each of the freshly collected RTP colony fragments were counted and standardized to 30 queens by removing supernumerary queens or adding queens from neighboring colony fragments. All colonies were provided with a water source and 25% sucrose solution dyed with either 8mM erioglaucine (introduced ants and RTP controls) or 8mM amaranth (RTP colony fragments). We replaced the colored sucrose with un-dyed sucrose solution after 14 hours, and connected introduced colonies with their respective RTP colony fragments using 2.5 x 45 cm paper strips as bridges between containers. We checked for mixed

brood, workers and queens (our indicator of colony fusion), and counted the total number of queens alive on each nest at 24 h, 6d and 12 d after colonies were connected. Colonies that did not fuse were determined as those where all queens from one of the colonies were killed. Two trials were conducted, the first one on August 11, 2005, and the second one on September 2, 2005.

Genetic Similarity between Colonies

We assessed genetic similarity between colonies used in the fusion assay (CAR, CHH, COC, FOR, RTP) using microsatellite markers. Genomic DNA was extracted from 15 workers from each of the source colonies using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) and analyzed at eight microsatellite loci: Lhum-11, Lhum-13, Lhum-19, Lhum-28, Lhum-35, Lhum-39 (Krieger & Keller 1999), Lihu-M1 and Lihu-T1 (Tsutsui et al. 2000). PCR reactions were performed as described by Buczkowski et al. (2004). Products were separated on 6.5% KB^{Plus} polyacrylamide sequencing gels using a 4300 LI-COR DNA analyzer. Microsatellite alleles were scored using GeneImagIR software (Scanalytics Inc., Billerica, MA, USA). Genetic differentiation (F_{ST}) between Argentine ants from different locations was estimated with the program FSTAT v.2.9.3.2 (Goudet 1995). Levels of genetic similarity between colonies were estimated based on the percentage of alleles shared between these groups (Tsutsui et al. 2000).

Statistical Analyses

All analyses were carried out using SAS 8.2 statistical software (SAS 2000). Changes in

levels of aggression were determined using PROC MIXED with colony pair as a fixed factor in the model, trials nested within pair as a random variable, and the difference between maximum aggression scores recorded 6 mo after and at the start of the laboratory colony fusion assay as the dependent variable. Differences in changes of aggression between colony pairs with high and moderate aggression was tested using PROC MIXED with aggression category and pair nested within aggression category as fixed factors in the model, trial nested within pair by aggression category as a random variable, and changes in levels of aggression as the dependent variable. Similarly, differences in changes in aggression between colonies that fused and those that did not was tested using PROC MIXED, with fusion after 6 mo and pair nested within fusion as fixed factors in the model and trials nested within pair by fusion as a random variable. Mean separation was carried out by least squares means (LSMeans).

Differences in numbers of workers fighting, worker mortality at 24 h, and the proportion of marked surviving workers (higher over lower survival) in the laboratory fusion assay were determined using PROC MIXED with colony pair as a fixed factor in the model, run nested within pair as a random variable, total workers fighting, workers killed and proportion of surviving workers (after square root transformation) as dependent variables. CONTRAST statements were used to determine differences in workers fighting, worker mortality and proportion of marked surviving workers between highly and moderately aggressive colony pairs, followed by mean separation by LSMeans. Differences in number of workers fighting in the field colony fusion assay were determined with an ANOVA using PROC GLM and means were separated with a least significance difference test (LSD).

To determine if aggressive interactions could explain the results of the colony fusion assay, we performed binary logistic regression with the number of workers fighting and the number of dead workers as independent variables and colony fusion within 24 h as the dependent variable. A similar analysis was performed with the maximum intercolony aggression as the independent variable and colony fusion, within 24 h and after 6 mo, as the dependent variable.

Spearman rank correlation coefficients were used to determine relationships between aggression levels and percent colony fusion (24 h and 6 mo) vs. genetic similarity between colonies (pairwise F_{ST} and % alleles shared). The significance of the regression coefficient was tested by Mantel's (1967) test in GENEPOP using 10,000 permutations. All means reported are followed by standard errors.

Results

Levels of Worker Aggression

Levels of intercolony aggression changed throughout the course of the colony fusion experiment as indicated by aggression levels measured between pairs and their respective control colonies 6 mo after the start of the experiment, and these changes differed among colony pairs ($F = 6.46$, df = 9, 5.5, $p = 0.0213$) (Figure 1). Although these changes did not differ between aggression categories ($t = 2.46$, df = 5.2, $p = 0.0550$), they did for colony pair nested within aggression categories ($F = 6.14$, df = 8, 5.5, $p = 0.0240$). Aggression levels decreased considerably in colonies that fused vs. those that did not fuse ($t = 6.16$, df = 6.5, $p = 0.0006$) when paired with their respective unexposed controls. After 6 mo, aggression remained high between colony pairs that did not fuse while it decreased in colony pairs in which all replicates (100%) fused (CAR-RTP, CHH-RTP, COC-FOR) (Figure 1). Aggression averaged across all replicates also decreased in colony pairs with lower (20-40%) fusion rates (CAR-CHH, CAR-FOR, FOR-RTP) (Figure 1), and even greater changes were recorded only in the fused replicates: CAR-CHH, from 4 to 2.6 ($t = -4.69$, df = 10.8, $p = 0.0007$); CAR-FOR, from 4 to 2.7 ($t = -3.41$, df = 6.8, $p = 0.0118$); and FOR-RTP, from 3.5 to 2.7 ($t = -2.04$, df = 6.8, $p = 0.0827$).

Laboratory Colony Fusion Assay

Fusion of Argentine ant colonies varied across colony pairs, with rates of fusion increasing over time in some highly aggressive pairs (Table 1). All replicates (100% fusion) for one colony pair with moderate aggression, CAR-RTP, and one with high

aggression, COC-FOR, fused within 24 h. Fusion rates increased from 50% at 24 h to 100% after a month in the moderately aggressive CHH-RTP. Fewer FOR-RTP and CAR-FOR replicates fused (40%), while one member of the colony pair was eliminated in the other replicates. Similarly, one of the colonies was eliminated in CAR-CHH replicates that did not fuse, with only 20% of the replicates fusing. All the other highly aggressive colonies, CAR-COC, COC-RTP, CHH-COC, and CHH-FOR, did not fuse with elimination of one of the colonies in all pairs except CHH-FOR where workers were mixed in all replicates while all queens from either one of the colonies were killed. Rates of fusion recorded at 24 h and at 6 mo were highly correlated ($r^2 = 0.7967$, $n = 10$, $p = 0.0005$).

Although the total number of workers fighting and workers killed at 24 h were not different across all colony pairs ($F = 3.99$, df = 9,5, $p = 0.0711$, and $F = 2.38$, df = 9,5, $p = 0.1763$, respectively), we found considerable differences in these two parameters between highly aggressive and moderately aggressive colony pairs ($t = 3.68$, df = 5, $p = 0.0143$ and $t = 10.94$, df = 5, $p = 0.0213$, respectively) (Figure 2). The number of marked surviving workers (survival ratio) in paired colonies with moderate aggression was lower (1.21:1) than the worker survival ratio (3.19:1) in highly aggressive pairs ($t = 2.05$, df = 8, $p = 0.0373$).

The total number of workers fighting during the first 24 h was a strong predictor of colony fusion within 24 h (Wald $X^2 = 16.45$, $n = 75$, $p < 0.0001$) with fusion decreasing as the number of workers fighting increased (Figure 3A). Fusion generally occurred when worker mortality was low (Wald $X^2 = 11.69$, $n = 75$, $p = 0.0006$) (Figure 3B). An even stronger relationship (Wald $X^2 = 13.32$, $n = 75$, $p = 0.0003$) between worker

mortality and fusion occurred when CHH-FOR, the colony pair in which only workers but not queens mixed and in which worker percent mortality was relatively low (22.27 ± 5.43%), was excluded from the analysis. Colony pairs with moderate levels of aggression, CAR-RTP and CHH-RTP, both fused consistently and had low worker percent mortality at 24 h, 5.60 ± 0.39% and 30.32 ± 3.95%, respectively, while the highly aggressive pair in which all replicates fused, COC-FOR, had higher worker mortality, 40.00 ± 4.10%, than either moderately aggressive pairs (t = 12.13, df = 13, $p < 0.0001$, and t = 1.60, df = 13, p = 0.0667, respectively). Worker mortality at 24 h ranged from 34.24 ± 2.50% (CAR-CHH) to 56.62 ± 1.44 % (CAR-FOR) in highly aggressive colony pairs in which some replicates fused, however, mortality of fused and non-fused replicates was similar (t = 0.6124, df = 18, p = 0.2739). Worker mortality ranged from 38.03 ± 4.27% (CAR-COC) to 53.46 ± 4.83% (CHH-COC) in highly aggressive pairs where no replicates fused. Initial aggression between colony pairs was a robust predictor of fusion at 24 h (Wald X^2 = 20.02, n = 75, $p < 0.0001$) and after 6 mo (Wald X^2 = 20.58, n = 75, p = 0.0004) (Figure 4).

Field Experiments

The number of workers fighting in the field assay differed among colony pairs (F = 159.70, df = 2, 2, p = 0.0062), with the highest numbers recorded for CHH-RTP (226.50 ± 7.86), while fewer and no workers fought in the CAR-RTP pair (83.67 ± 4.58) and RTP control, respectively. Similarly, more CHH-RTP workers (293.00 ± 42.82) than CAR-RTP (22.60 ± 7.50) workers fought in the laboratory assay where worker fights and mortality were strongly correlated (r^2 = 0.52, n = 75, $p < 0.0001$). CAR and CHH nests

fused with RTP nests with rates of fusion ranging from 50.00 ± 16.67% (CHH-RTP) to 83.33 ± 16.67% (CAR-RTP) at six days. These fusion rates were similar to those in the laboratory assay at 24 h, 50.00 ± 10.00% for CHH-RTP and 100 ± 0.00% for CAR-RTP (t = 0.001, df = 2, p = 0.4998 and t = 1.00, df = 2, p = 0.2113, respectively). However, for CHH-RTP, queen survival at 12 d in nests from the field fusion assay was lower (62.83 ± 2.72%) than that at 1 month in laboratory colonies (84 ± 0.00%) (t = 7.78, df = 2, p = 0.0080). Fusion rates between field CHH and RTP ants did not increase with time compared with these same colonies in the laboratory assay, probably due to higher queen mortality in some field fusion replicates.

Genetic Similarity between Colonies

Colonies from different locations were genetically differentiated (F_{ST} = 0.27 ± 0.062). Although we did not find a relationship between pairwise F_{ST} and colony fusion at 24 h (r = -0.5326, n = 10, p = 0.1111), we found a negative relationship between pairwise F_{ST} and colony fusion at 6 mo (r = -0.7615, n = 10, p = 0.0085) (Figure 5A). Levels of genetic similarity varied across colony pairs, ranging from 30.3% (CHH-COC) to 63.3% (CAR-RTP). A positive yet not significant association was found between levels of worker genetic similarity (% alleles shared) and rates of colony fusion at 24 h (r = 0.5884, n = 10, p = 0.0877), while worker genetic similarity and fusion at 6 mo were significantly associated (r = 0.6501, n = 10, p = 0.0492) (Figure 5B). Also, a negative relationship was found between maximum aggression score and genetic similarity between colonies (r = -0.6417, n = 10, p = 0.0151).

Discussion

We provide evidence that introduced *L. humile* colonies fuse in the absence of barriers preventing their encounters, and that colony fusion is regulated by levels of intraspecific aggression and genetic similarity between interacting colonies. We suggest that fusion of unrelated colonies may be a route by which introduced *L. humile* populations can achieve extreme unicoloniality in the absence of ecological pressures, thereby influencing distribution patterns and their ability to dominate new habitats. Current explanations for the formation of invasive *L. humile* supercolonies with reduced intraspecific aggression include a lack of overall genetic differentiation among separated nests following a genetic bottleneck resulting in low genetically-based recognition cue variation and inability to recognize non-nestmates in introduced populations (Tsutsui et al 2000), and increasing encounter rates between colonies in new habitats with relaxed ecological constraints leading to the elimination of colonies with rare recognition alleles and the emergence of supercolonies composed of colonies sharing the most common recognition alleles (Giraud et al. 2002). We demonstrate a range of outcomes between genetically distinct colonies, from complete colony elimination to fusion. These outcomes can be explained by the level of intraspecific aggression and genetic similarity between colony pairs despite distances between collection sites as great as 289.67 km. Consequently, we provide evidence for the Giraud et al. (2002) hypothesis by demonstrating that increased rates of encounters between foreign *L. humile* colonies resulted in the elimination of colonies with higher levels of aggression and genetic dissimilarity, and the formation of new colonies composed of non-aggressive unrelated individuals probably sharing similar

recognition cues. However, unlike Giraud et al. (2002), variability at loci coding for recognition cues seems to be associated with genetic variability at microsatellite markers as revealed by the positive association between overall genetic similarity vs. intraspecific aggression and colony fusion, which supports the genetically-based recognition system proposed by Tsutsui et al (2000).

Colony fusion has been suggested as a route towards polygyny and unicoloniality in ants (Bourke and Franks 1995; Herbers 1993; Crozier and Pamilo 1996) and as a mechanism leading to complex family structure in some termites, particularly *Reticulitermes* species (Matsuura and Nishida 2001; Clèment 1986; Deheer and Vargo 2004). Acceptance of foreign individuals would be expected if the fitness cost of accepting a non-nestmate is low or when the fitness cost of erroneously rejecting a nestmate is high (Reeve 1989). Therefore, colony fusion may be an adaptive tactic if the cost of fusion is lower than that of intercolony fighting, and if it benefits the colony by increasing its labor force and/or expanding their foraging range (Matsuura and Nishida 2001; Su and Scheffrahn 1988). In the Argentine ant, fusion of non-aggressive experimental colony pairs collected from different sites increased rates of resource retrieval, and brood and worker production (Holway et al. 1998), and as colony size increased interference competition and exploitative ability increased as well (Holway and Case 2001). Similarly, we expect that fusion of aggressive colonies would result in larger and more productive colonies if the benefits from increased colony size supersede the costs of initial high mortality.

The outcome of our colony interactions, fusion or complete elimination of one of the groups, was largely determined by levels of aggression and worker mortality,

supporting Roulston et al. (2003) where lower worker mortality resulted in merging and high aggression decreased chances for fusion. However, in contrast to Roulston et al. (2003), we observed fusion between some colony pairs where extensive worker mortality occurred. Fusion between some of our most aggressive colonies may have occurred after the elimination of the most aggressive phenotypes and/or less genetically similar individuals, thereby increasing levels of similarity between groups and favoring merging. In an arboreal nesting termite, high aggression leads to elimination of one of a pair of colonies, while either continuous avoidance or merging results after the elimination of the most aggressive individuals in lower aggression pairs (Leponce et al. 1996). In our study, different outcomes across replicates within the same colony pair may be explained by variation in colony composition (phenotypic heterogeneity, perceptive ability) due to physiological or genetic factors that may cause conflicts that supersede group level benefits.

Our laboratory experiments used relatively small colonies and the restricted space provided and limited food accessibility may have forced interactions. However, it is not clear whether this experimental design biased in favor or against fusion compared to field populations. Our laboratory conditions could have either hindered fusion because of the lack of gradual acceptance with time or increased competition due to restricted food availability, or increased fusion by using small colonies that stopped fighting to prevent complete elimination. Colony size, presence of surrounding nestmates, habituation, and proportion of aggressive individuals can affect aggressive interactions between social insect colonies (Hölldobler and Wilson 1990; Binder 1988; Langen et al. 2000). We found that the total number of workers fighting and workers killed were comparably high

in both highly aggressive pairs that fused and those that did not fuse. Our experimental conditions may partially reflect the magnitude of aggressive encounters, in that highly aggressive colonies stopped fighting after reaching a minimum colony size threshold, past which losing any more workers could be fatal. However, even after the apparent elimination of the most aggressive individuals, possibly older workers (Hölldobler and Wilson 1990), which could have facilitated fusion between the least aggressive individuals (e.g. callows, brood and queens), not all colonies fused, indicating that fusion events are not governed by colony size but by intrinsic colony factors. Although the small size of our experimental colonies do not reflect the numbers of workers found in established field colonies, our results may be most relevant to incipient field colonies or small colony fragments that have budded from the main colony.

The persistence of our fused colony pairs throughout the length of the study indicates that fusion was not a transient event resulting from nestmate recognition inaccuracies. In ants, it has been proposed that the nestmate recognition mechanism consists of matching the phenotypic recognition cues of the encountered conspecific with the individual's neural template, which is a learned representation of colony's recognition cues derived from the environment, the individual's own phenotype, or all colony members (Breed and Bennett 1987). This cue-template matching process results in a behavioral response, usually acceptance or rejection of the encountered individual (Reeve 1989). Therefore, reduced intraspecific aggression towards unpaired control (source) colonies suggest that fused colony pairs posses the most common recognition cues of both sources and/or a broader neural template than either source colony. Merged colonies can form a more homogenous colony Gestalt if individuals in the mixed group bear only

common labels, as revealed by the modified hydrocarbon profiles in queenless *Camponotus fellah* that had fused (Boulay et al. 2003). Alternatively, the high diversity of recognition cues resulting from merging of genetically heterogenous groups could produce an expanded neural template, thereby decreasing label-template dissimilarity and reducing aggression due to acceptance of more labels (Vander Meer and Morel 1998). Unexposed control colonies were less aggressive towards individuals from their respective fused colony, suggesting that the recognition cues in individuals from fused colonies were similar to those present in both groups. However, whether this was achieved by cue transfer and chemical profile homogenization or elimination of individuals bearing labels that made them more easily recognized as foreign remains unclear. Overall, individuals in fused groups may have a broader recognition template and/or more similar genetically-based labels allowing them to mix with both groups, suggesting that fusion between small groups can gradually lead to colony odor homogenization on a larger scale. This recognition system plasticity may improve group success through increased colony size and superior competitive ability.

High levels of aggression and genetic differentiation prevent the exchange of individuals between adjacent introduced *L. humile* colonies (Thomas et al. 2006), thereby suggesting that aggressive *L. humile* colonies do not fuse, while non-aggressive colonies fuse into larger and more productive supercolonies as has been previously demonstrated (Holway et al. 1998). In contrast, we found that this situation is not universal since unrelated and aggressive *L. humile* colonies fused into colonies with reduced levels of intraspecific aggression. These discrepancies may be due to the broader range of aggression and genetic similarity levels between colonies tested in our study. For

example, estimates of genetic differentiation (F_{st}) between colonies that did not fuse in our study were comparable to estimates between aggressive supercolonies in which individuals do not intermix (Jaquiéry et al. 2005; Thomas et al. 2006), however, these values were consistently higher than those from our colony pairs that fused that probably have higher levels of genetic similarity in terms of overall allele sharing than aggressive supercolonies in California and Europe. In *L. humile*, prior experience seems to increase aggression between colonies (Thomas et al. 2005), which could in part explain our different results. However, we did not detect an increase in aggression between distant colony pairs after increasing interactions among individuals, and, moreover, colony pairs that fused decreased their aggression levels suggesting that not only prior experience but also colony genetic composition is an important factor modulating intercolony interactions. Overall, our findings are in line with previous studies supporting a genetic basis for aggressive behavior and territoriality in introduced *L. humile* populations (Tsutsui et al. 2000, Tsutsui et al. 2003). A genetically based fusion mechanism has also been reported for some colonial marine invertebrates (Scofield et al. 1982; Grosberg and Hart 2000).

In addition to the genetic component modulating fusion between unrelated colony pairs, the lower rates of fusion between colonies interacting in the field suggest that additional factors including colony composition, phenology, physiological differences, position relative to colony boundaries, environmentally derived recognition cues, and other environmental and seasonal effects might also influence fusion events. Therefore, long-term studies monitoring fusion in the field at a relatively feasible larger-scale, and at

different times of the year to account for seasonal variation in *L. humile* natural history, would be instructive.

Colony fusion in ants may be proximately explained by a reduction in intraspecific aggression due to loss in recognition cue diversity (Tsutsui et al. 2000, Giraud et al. 2002), colony odor similarity (Astruc et al. 2001), and increased tolerance to foreign conspecifics due to queenlessness (Boulay et al. 2003). Following Reeve's acceptance threshold model for nestmate recognition (1989), colony members will accept conspecifics when levels of cue-template dissimilarity are below the acceptance threshold, consequently fusion between colonies should vary accordingly. Also, the variable rates of fusion recorded in some colony pairs suggests a graded-response model (Provost 1991, Vander Meer and Morel 1998) in which an increased scale of acceptance depends on an increased degree of similarity between the perceived cues and the learned template, or interindividual variability in acceptance thresholds. However, this variation may be due to colony intrinsic differences (worker age, proportion of nurses vs. foragers, queen reproductive status), in which case the response does not necessarily reflect levels of cue-template dissimilarity. In addition, phenotypic similarities between groups may also affect the balance between the costs of fighting vs. merging, with the costs of fighting being higher when levels of similarity, and, therefore, reduced ability to distinguish non-nestmates from nestmates, are considerable.

Territoriality and well established foraging ranges in termites and ants may limit opportunities for mixing of workers from different colonies (Vargo 2003; Adams 2003). However, highly saturated habitats may promote colony fusion between initially aggressive ants under natural conditions (e.g. Foitzik and Heinze 1998). Additionally,

traits in highly polygynous and unicolonial ants, including the Argentine ant, like reproduction by budding, constant exchange of individuals between nests, extreme vagility and ability to cope with frequent colony fragmentation (Hölldobler and Wilson 1977), may promote opportunities for colony fusion at natural contact zones. Although our colonies were collected from sites up to 402 km (CAR–COC) apart, thereby limiting encounters under natural conditions, human-mediated dispersal and the ability of incipient *L. humile* colonies to successfully establish in new habitats (Suarez et al. 2001; Aron 2001) may increase the chances for previously geographically distant colonies to encounter and fuse.

In unicolonial ant species, changes in colony structure may be due to powerful colony-level selection, in the context of unusual ecological conditions that seem to have overridden the selfish interests of nest members (Sudd and Franks 1987). Therefore, studies exploring the ecological conditions and the mechanisms giving rise to this form of social organization would greatly contribute to a better understanding of the behavioral plasticity responsible for the variation in expression of unicoloniality in *L. humile* and other invasive ants. This study provides evidence for the role of colony fusion as a proximate mechanism involved in the transition to extreme unicoloniality in *L. humile* populations, and is consistent with the view that colonies harboring the most common recognition cues may selectively mix and form vast, dominant colonies in the absence of ecological constraints limiting their expansion (Giraud et al. 2002). Also, our results suggest that fusion can occur between populations originating from separate introductions, with rates of fusion modulated by levels of genetic similarity. Recent evidence indicates that gene flow between relatively close or contiguous aggressive

supercolonies is absent (Jaquiéry et al. 2005; Thomas et al. 2006). However, this does not preclude that these and other *L. humile* supercolonies were once distinct colonies that subsequently fused. Therefore, behavioral flexibility may reflect the varying degree of interactions occurring between colonies from different origins in their early stages of establishment (recently invaded areas), or between foreign colony fragments encountering newly budded nests from established colonies where mixing of individuals is more likely than between nests of well established colonies (Ingram and Gordon 2003).

Our findings on colony fusion in the Argentine ant raise two major questions: 1) does fusion produce a homogenization of recognition cues i.e. cuticular hydrocarbons, and 2) does fusion of unrelated colonies increase colony productivity and provide clear colony fitness benefits, i.e numerical advantage and greater chances to monopolize resources. A better knowledge of the conditions and factors affecting the expression of unicoloniality in ants would shed light on the selection pressures shaping this social organization, the levels at which selection may be acting upon, and its benefits and costs; and would greatly contribute our understanding of invasive ant species and the complexity of their ecological success.

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Table 1. Category of worker-worker aggression, number of replicates that fused, and time of fusion for ten colony pairs.

Colony pair	*N*	Aggression category[a]	Number of fused replicates				
			24 h	144 h	1 mo	2 mo	6 mo
CAR-CHH	10	H	0	0	0	2	2
CAR-COC	10	H	0	0	0	0	0
CAR-FOR	5	H	1	2	2	2	2
CHH-COC	5	H	0	0	0	0	0
CHH-FOR	10	H	0	0	0	0	0
COC-FOR	5	H	5	5	5	5	5
COC-RTP	5	H	0	0	0	0	0
FOR-RTP	5	H	0	2	2	2	2
CAR-RTP	10	M	10	10	10	10	10
CHH-RTP	10	M	5	9	10	10	10

CAR: Cary; CHH: Chapel Hill; COC: Greenville; FOR: Winston-Salem; RTP: Research Triangle Park. H = high; M = moderate.

[a]Figure 1 provides aggression scores for colony pairs.

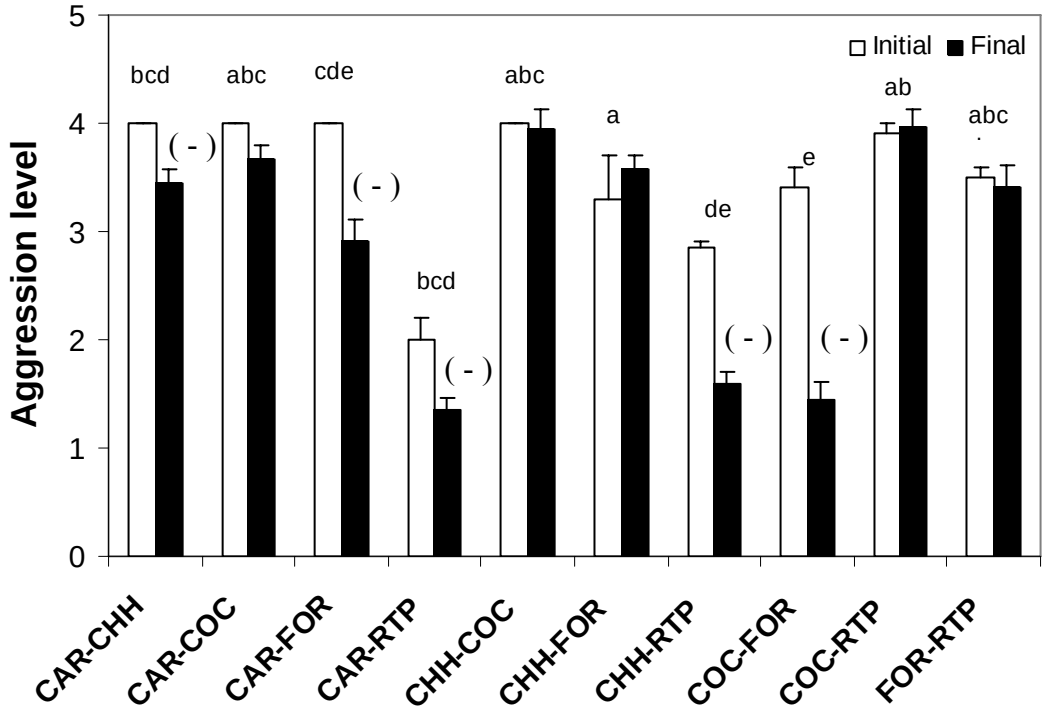


Figure 1. Mean (±1 SE) worker-worker level of intercolony aggression at the beginning of (Initial) and 6 mo after (Final) the laboratory colony fusion assay. Set of bars with different letters indicates significant differences in aggression changes across colony pairs ($p < 0.05$, LSD). Aggression levels decreased significantly (-) in five colony pairs throughout the experiment. See Table 1 for colony abbreviations.

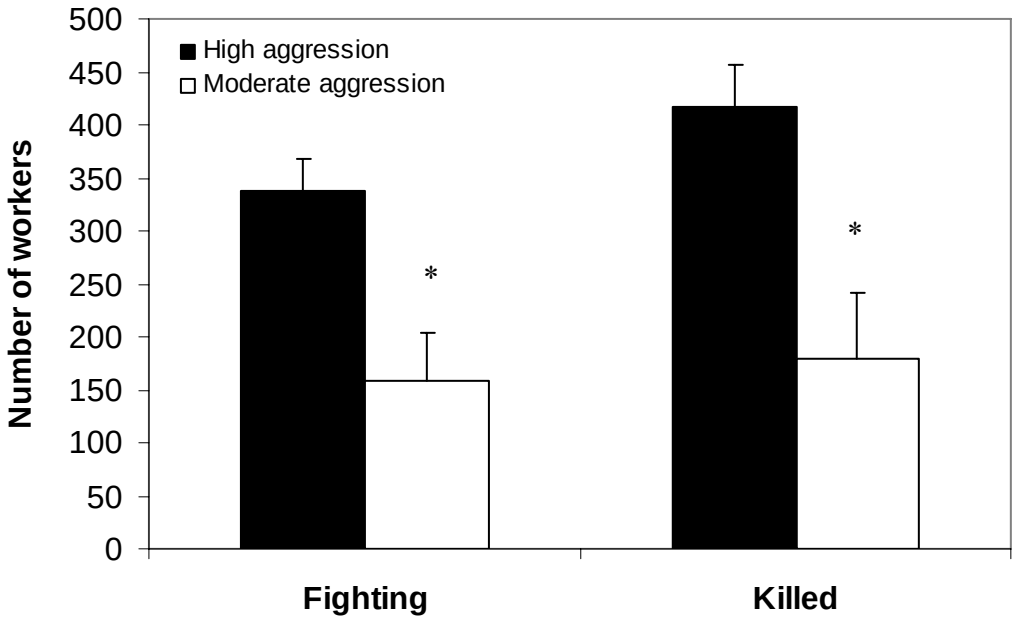
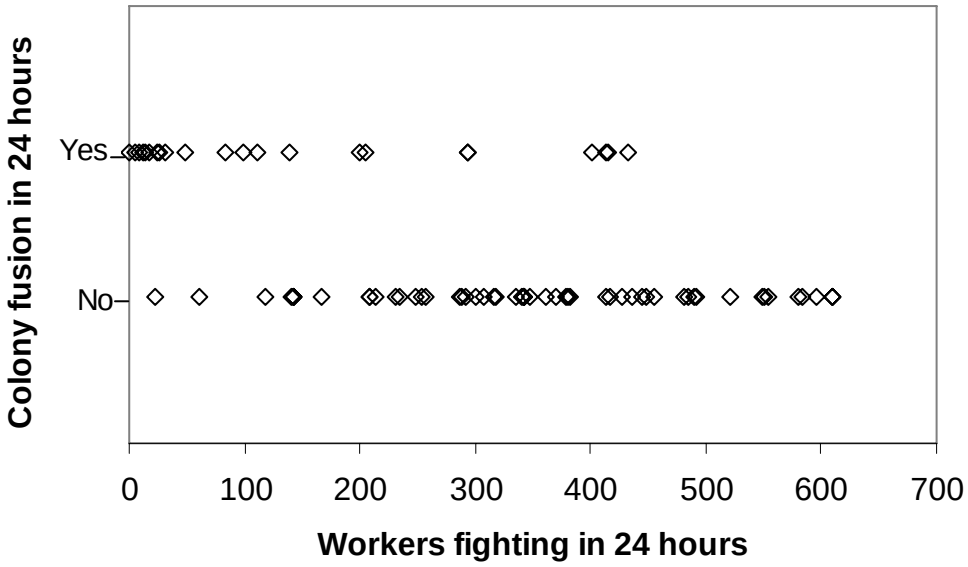


Figure 2. Mean (±1 SE) number of workers fighting and killed (out of 1000) in highly aggressive and moderately aggressive colony pairs within the first 24 h of contact. *$p < 0.05$.

A



B

Figure 3. Relationship between number of ants fighting (A) and killed (B) in the first 24 h of contact and colony fusion within 24 h.

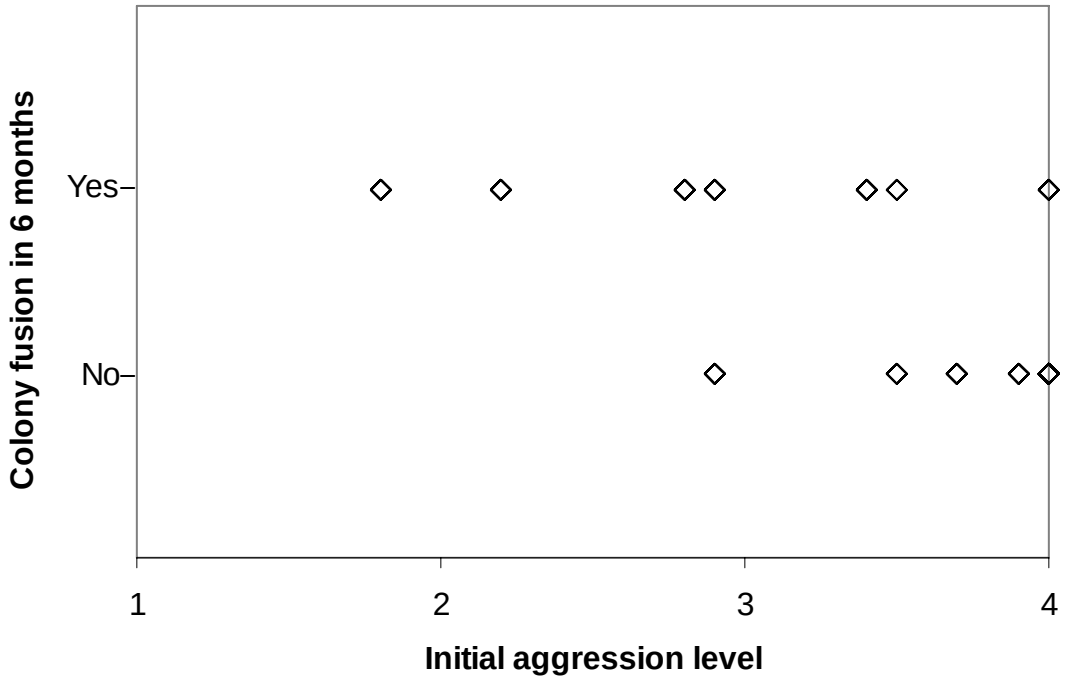
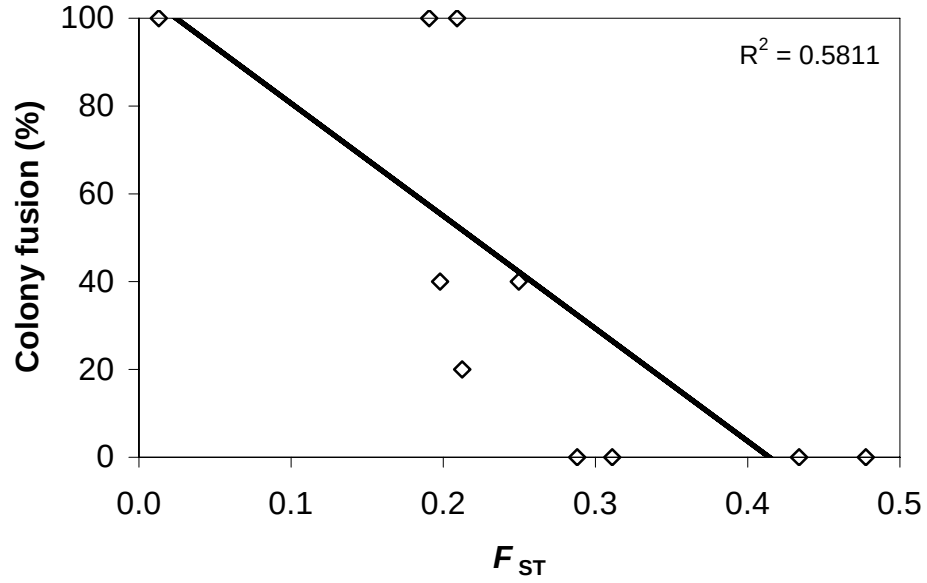


Figure 4. Relationship between initial aggression levels between colony pairs and whether or not colonies fused within 6 mo.

A



B

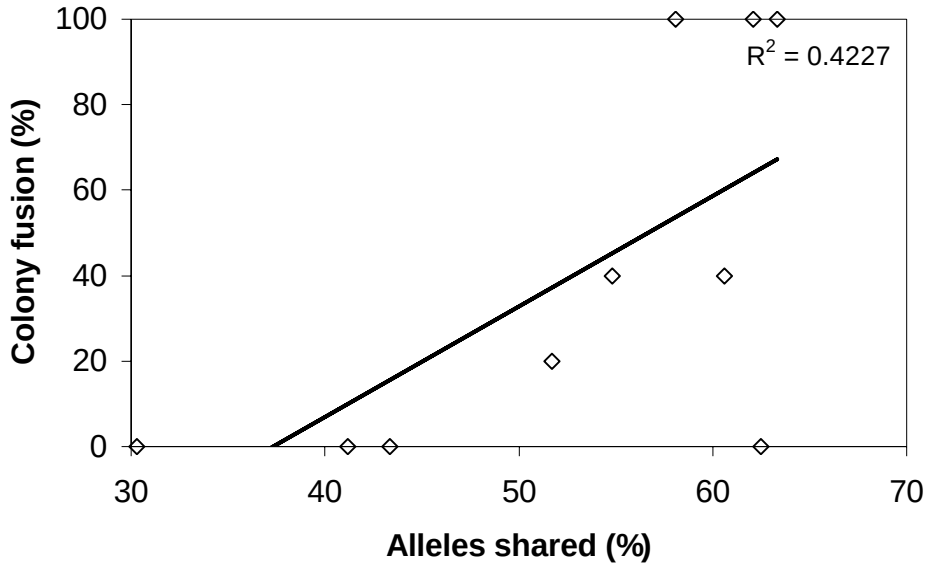


Figure 5. Relationship of pairwise F_{ST} (A) and % alleles shared (B) between colonies vs. colony fusion at 6 mo.

CHAPTER III

Colony fusion and its fitness implications in the invasive Argentine ant

Abstract. The ecological success of invasive ants has been linked to their ability to form vast and dominant supercolonies. In introduced populations of the Argentine ant, *Linepithema humile*, supercolonies may arise via fusion of non-aggressive and genetically homogenous colonies as a consequence of a population bottleneck, or alternatively, through selective mixing of initially aggressive and unrelated colonies harboring common nestmate recognition cues in the absence of ecological constraints preventing their encounters. Individuals within supercolonies mix freely among separated nests and behave altruistically towards all colony members regardless of a low level of within-colony relatedness. It has been suggested that fitness benefits from augmented colony size may offset the dilution of relatedness in genetically heterogeneous social groups, which could relate to the cohesion of *L. humile* supercolonies that through high population densities increase their productivity and attain a superior competitive ability. Here we show that aggressive *L. humile* colony pairs increase in size through colony fusion with enhanced colony productivity and survival. We found that *L. humile* colonies that fused produced more brood (34%) and workers (47%) than aggressive colony pairs that did not fuse under laboratory conditions. Per capita colony productivity (number of brood per queen and worker, number of workers per worker and queen) was generally lower in colony pairs than unpaired control colonies. Queens regardless of colony-origin contributed equally to worker pupae production in all fused replicates, indicating that fusion could lead to changes in colony worker genotypic composition. By demonstrating that fusion of unrelated *L. humile* colonies increases colony fitness benefits through higher brood and worker production we suggest that selective fusion of unrelated colonies may be a

mechanism by which introduced populations increase in size and become ecologically successful.

Key words: Argentine ant, *Linepithema humile*, unicoloniality, nestmate recognition, aggression, colony size, colony fusion.

Introduction

Group living and cooperation among conspecifics result in a variety of fitness benefits and costs for several animal species (Alcock 1998). Fitness benefits include improved foraging efficiency, improved territory and food defense, better care of offspring through communal feeding and protection, and more effective detection or repulsion of enemies. In contrast, sociality may also lead to increased competition for food or mates within the group, increased risk of disease infection, and increased risk of exploitation of or interference with parental care by other group members (Alexander 1974). Consequently, social living is adaptive when benefits counterbalance its unavoidable costs (Gross and MacMillan 1981).

Social insects are extreme exponents of sociality with individuals displaying altruistic behavior and forming colonies in which sterile members (neuters or workers) help raise the offspring of close relatives (sexuals or reproductives) as to gain indirect fitness benefits (Hamilton 1964). Most social insect colonies are composed of close relatives (Crozier and Pamilo 1996), which favors altruism as predicted by kin selection theory (Hamilton 1972). However, the evolution of eusociality is influenced not only by genetic factors affecting relatedness (r) but also by ecological and ergonomic factors that promote reproductive altruism by their effects on the benefit (b) and cost (c) terms in Hamilton's rule ($rb - c > 0$), including factors promoting sociality such as defense against enemies, resource patchiness, nest site shortage, and variance in reproductive success (Strassman and Queller 1989, Bourke and Franks 1995).

In species of social Hymenoptera where relatedness between individuals that cooperate at colony foundation is low (Bernasconi and Strassman 1999, Queller et al. 2000) advantages to group nesting should be great enough to compensate for the risk of not becoming the egg-layer (Strassmann 1989). Also, species with multiple or multiply mated queens exhibit low progeny relatedness (Bourke and Franks 1995, Crozier and Pamilo 1996) that results in reduced indirect fitness benefits to workers rearing the brood, therefore, fitness benefits such as high-egg laying rates to sustain large colonies, escape from predation, and successful nest founding might offset these costs (Ross 2001). For example, colony fitness benefits including increased colony size, growth rates, and survival have been observed in multiply mated and multiple queen ant colonies (Cole and Wiernasz 1999, Elmes and Keller 1993). Unicoloniality, an unusual social organization characterized by mixing of individuals among separated nests in the absence of colony boundaries (Hölldobler and Wilson 1977, 1990), represents an extreme case of altruism in the absence of high levels of within-nest relatedness (Bourke and Franks 1995). The ability of unicolonial ants to form vast and ecologically dominant colonies suggests that ecological factors may be causing a high benefit:cost ratio that favors altruism when relatedness is greater than zero (Foster et al. 2006), or weak altruism in the absence of relatedness and under strong group selection (Wilson 1990). Alternatively, workers cooperate in the absence of other options, especially since workers in unicolonial species seem to be totally sterile and therefore incapable to respond to nest kin-structure by laying male eggs (Bourke and Franks 1995).

Hypotheses that explain cooperation among unrelated individuals assume fitness benefits based on the increased genotypic diversity in heterogenous groups. The disease-

resistance hypothesis posits that genetic diversity improves parasite and pathogen resistance (Sherman et al. 1988, Liersch and Schimd-Hempel 1998, Schimd-Hempel and Crozier 1999), while the task-efficiency hypothesis postulates that specialization in the performance of key colony-maintenance or brood-rearing tasks results in enhanced growth and reproduction (Kukuk et al. 1998, Crozier and Fjerdingstad 2001). Evidence to support the disease-resistance hypothesis in social Hymenoptera is found in experimental studies (Baer and Schmid-Hempel 2001, Tarpy 2003, Hughes and Boomsma 2004), while higher genetic diversity decreases colony level variance in task performance in honeybees (Page et al. 1995) or has no effect on short-term task efficiency in ants (Rosset et al. 2005). Additionally, when examining colony performance in ants and bees, it was found that colonies with low relatedness and that are more genetically diverse individuals could have increased growth rates and survival (Wiernasz et al 2004), while colony size and productivity were not always associated with genetic diversity (Sundström and Ratnieks 1998, Rosset et al. 2005, Oldroyd et al. 1992). Similarly, individual growth rate and survival were not correlated with genetic diversity in tent caterpillars (Costa and Ross 2003). Overall, the importance of genetic diversity in colony productivity and survival seems to vary across taxa, and may only partially explain cooperation among unrelated individuals in social insect colonies. Therefore, factors other than increased genetic diversity may also favor intraspecific cooperation among unrelated individuals in insect societies.

Colony size plays a key role in determining social complexity, within-group conflict, colony productivity, behavioral flexibility and colony organization in social insects (Bourke 1999, Karsai and Wenzel 1998). Insect societies with large colonies

benefit from increased defense, greater homeostasis and work ability, greater ability to manipulate the surrounding environment, higher sexual production and survival, better fighting and competitive ability, and enhanced resistance to seasonal climate (Adams 1990, Bourke 1999 and references cited therein). Moreover, increased colony size by group merging can improve colony performance in cases where unrelated groups merge, suggesting that fitness benefits from augmented colony size may be offsetting the dilution of relatedness (and reduced indirect fitness benefits) in these heterogeneous social groups (Costa and Ross 2003). This could be relevant to unicolonial ant species that commonly attain high population densities (Hölldobler and Wilson 1977, Porter and Savignano 1990, Abbott 2005) that are responsible for their increased competitive ability and ecological dominance (Holway et al. 2002).

In the unicolonial Argentine ant, *Linepithema humile,* colony size, specifically high worker number, has been linked to its ecological success in the introduced range (Holway et al. 1998, Holway and Suarez 2004). Large worker populations may result from a lack of intraspecific aggression thereby allowing colonies to fuse (Holway et al. 1998), and low aggression has been linked to reduced levels of genetic diversity, a consequence of a genetic bottleneck which reduced the phenotypic variability of nestmate recognition cues (Tsutsui et al 2000). Alternatively, increased colony growth and size may result from fusion of colonies sharing the most common recognition alleles when populations are introduced into new habitats with relaxed ecological constraints (Giraud et al 2002). Overall, both explanations for increased colony size leading to supercolony (or unicolonial population) formation invoke a reduction of genetic diversity at nestmate recognition loci. However, recent evidence suggests that loss of

genetic diversity has not played a role in the evolution of unicoloniality since native *L. humile* populations also form supercolonies containing unrelated individuals (Pedersen et al. 2006), and therefore ecological conditions rather than genetic factors may promote unicoloniality in introduced *L. humile*, although the conditions or factors favoring this change in size remain unknown. Even if loss of genetic diversity is not the primary factor leading to vast supercolonies (Tsutsui et al. 2000, Giraud et al. 2002), colony fusion seems to be a plausible mechanism allowing small colonies to increase in size, which is certainly key to *L. humile* success (Holway and Case 2001, Holway and Suarez 2004). Moreover, increased colony size and its selective advantages could also be important factors promoting cooperation among unrelated *L. humile* workers (Krieger and Keller 2000, Tsutsui and Case 2001, Ingram and Gordon 2003) in the absence of indirect fitness benefits. Therefore, studies exploring the factors promoting colony fusion and its fitness consequences will shed light on the role that this process plays in the formation of large and dominant supercolonies and its adaptive significance in this and other unicolonial ants.

The Argentine ant is a widespread invasive species that has become established in regions with a Mediterranean or subtropical climate all over the world (Suarez et al. 2001) where different degrees of unicoloniality and colony size have been observed (Tsutsui et al. 2000, Giraud et al. 2002, Buczkowski et al. 2004, Heller 2004). In the U.S., differences in colonization patterns might explain variation in genetic diversity, intraspecific aggression, and colony size in *L. humile* across regions. In the southeastern U.S., colonies occupy relatively small territories and have relatively high genotypic variability and strong intercolony aggression (Buczkowski et al. 2004). Using

southeastern *L. humile* colonies we were able to determine that in the absence of barriers to intercolony encounters, aggressive colony pairs can fuse, and that fusion is correlated with intraspecific aggression levels and the degree of genetic similarity between colonies (Chapter 2). These findings suggest that the likelihood of fusion may increase with greater levels of similarity in genetically-based recognition cues. Therefore, we considered that fusion between unrelated colonies may have resulted from recognition errors, which warrants an investigation of colony fitness consequences of fusion to reveal its adaptive or maladaptive significance. Fusion may confer fitness benefits such as increased colony productivity, increased survivorship, and superior competitive ability via larger colony size. In *L. humile,* high worker densities are key to the superior competitive ability allowing invasive populations to monopolize resources and dominate entire habitats (Holway 1999, Holway and Case 2001). Hence, we propose that colony fusion is a mechanism by which initially aggressive *L. humile* colonies can increase in size and become ecologically successful.

In this study we investigated whether fusion of unrelated southeastern *L. humile* colonies increases worker and queen numbers, and higher brood production by comparing the total number of workers, brood and queens recorded in colony pairs that fused vs. colony pairs that did not fuse. Fusion should result in larger and more productive colonies mostly due to increased worker number since size of worker force rather than queen number determines colony growth and productivity in social Hymenoptera (Michener 1964, Oster and Wilson 1978). Additionally, we estimated the proportion of brood and workers per queen (B/Q and W/Q, respectively), and brood and workers per worker (B/W and W/W, respectively), and compared these per capita values

across all colony pairs, although we did not expect per capita values to be higher in fused colonies because per capita productivity in social insects tends to decrease as colonies grow (Michener 1964). Reproductive skew in *L. humile* colonies is low (Keller 1988, Founier and Keller 2001), however, these results relate to reproduction partitioning among nestmate queens. Therefore, we investigated if queens in our fused colonies contributed equally to new worker production by genotyping queens and worker pupae to determine their pedigree relationship. Results from this study support the idea that fusion of unrelated *L. humile* colonies increases colony fitness benefits through higher brood and worker production. In addition to reduced genetic diversity and low aggression promoting the formation of expansive supercolonies, we suggest that, in the absence of ecological constraints, supercolonies in the introduced range can result from fusion of small and unrelated colonies into larger and genotypically distinct colonies.

Materials and Methods

Collection and Rearing of Laboratory Colonies

We collected colonies of Argentine ants (*Linepithema humile*) from five locations 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 9.7 km (CAR – RTP) to 402.3 km (CAR – COC). In a colony fusion laboratory assay we tested all 10 colony pair combinations with 5 replicates per colony pair and five colony pairs per trial: CAR-CHH, CAR-COC, CAR-RTP, CHH-FOR, and CHH-RTP in trials 1 and 2; and CAR-FOR, CHH-COC, COC-FOR, COC-RTP, and FOR-RTP in trial 3. Eight colony pairs were highly aggressive: CAR-CHH, CAR-COC, CAR-FOR, CHH-COC, CHH-FOR, COC-FOR, COC-RTP and FOR-RTP, and two colony pairs were moderately aggressive: CAR-RTP and CHH-RTP (see Chapter 2). Levels of worker genetic similarity (% alleles shared) ranged from 30.3% – 62.1% in highly aggressive pairs, and from 58.1% – 63.3 % in moderately aggressive pairs. Experimental colonies from each location consisted of 5 queens of unknown age, ca. 100 pieces of brood (eggs, larvae, and worker pupae), and 500 workers. For each colony pair, all queens and 50 workers from each colony were marked on the thorax and abdomen, respectively, with either pink (colony 1) or yellow (colony 2) water-based paint (Apple Barrel Colors®, Plaid Enterprises Inc., Norcross Georgia, USA) using a 10/0 brush to monitor individuals mixing and determine colony fusion. Colonies were maintained in individual Fluon™-coated trays (17 x 25 x 11 cm) and provided either plastic petri dishes (9 cm diameter) filled with moist grooved

Castone® dental plaster or foil-covered glass tubes half-filled with water and stopped with cotton as artificial nests. Containers were connected via a 12-cm long vinyl tube, with soft earplugs initially inserted at each end to prevent contact between colonies. Each colony was provided with 25% sucrose solution and artificial diet (Bhatkar and Whitcomb, 1970) *ad libidum* during a 24-hour acclimation period. Pairs were provided with 25% sucrose solution, artificial diet *ad libidum*, three freshly-killed *Blattella germanica* adults once a week, and a water source throughout the six-month fusion assay. Controls consisted of unpaired experimental colonies (5 queens, ca 100 pieces of brood, 500 workers) from each location (5 replicates per location per trial). All colonies were maintained at 25 ± 1°C and 50 ± 15 % RH, on a 12:12 h light:dark cycle.

Colony Fusion, Total Colony Size, and Per Capita Brood and Worker Values

We conducted a colony fusion laboratory experiment to determine if colonies that fused and those that did not differed in colony size and per capita productivity. After a 24-hour acclimation period, paired experimental colonies were allowed to interact by unblocking the connecting tubing. We recorded rates of mixing between colonies for 6 hours, at 24 hours, daily for 30 days, and monthly, from month 2 - month 6. Fusion was defined as the presence of all queens and brood in the same nest and the mixing of marked workers without fighting, in contrast to the elimination of one of the colonies that usually occurred when pairs did not fuse. We recorded the total number of workers, brood and queens in each container at 24 h and every month from month 1 – month 6. Data were analyzed as the total number of brood, workers, queens, and proportion of queens surviving (number of queens alive per initial queen number) recorded monthly, and as per

capita values (number of brood and workers per queen and per worker, respectively). Total numbers are useful to determine differences in colony size, while per capita values allow an easy comparison of colony growth rates and caste ratios among colonies with different sizes. Monthly per capita values were obtained by dividing the number of brood by the number of workers (B/W) or queens (B/Q) recorded in the same month, the number of workers divided by the number of queens recorded in the same month (W/Q), and the number of workers recorded each month divided by worker total number recorded in the previous month (W/W). Trials were conducted from August 2003 through February 2004, from May through November 2004, and from March through September 2005.

Microsatellite Analysis of Queens and Offspring in Fused Colonies

We assessed the contribution of individual queens to offspring production in experimental colonies that fused in the first trial (CAR-RTP, CHH-RTP, and CAR-CHH) by genotyping mixed queens and a sample of worker pupae taken 6 months after the fusion assay started. Pupae are expected to be offspring of the genotyped queens based on the time for egg development into workers in this species (Newell and Barber 1913, Markin 1970). Genomic DNA was extracted from all queens (3 – 7) and 10 worker pupae per replicate (59 queens and 110 pupae total) using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) and analyzed at eight microsatellite loci: Lhum-11, Lhum-13, Lhum-19, Lhum-28, Lhum-35, Lhum-39 (Krieger and Keller, 1999), Lihu-M1 and Lihu-T1 (Tsutsui et al., 2000). PCR reactions were performed as described by Buczkowski et al. (2004). Products were separated on 6.5% KB^{Plus} polyacrylamide

sequencing gels using a 4300 LI-COR DNA analyzer. Microsatellite alleles were scored using GeneImagIR software (Scanalytics Inc., Billerica, MA, USA). For each replicate, we compared pupae genotypes with those of queens to examine the pedigree relationship between individual pupae and queens, and determine if observed genotype frequencies were different from expected genotype frequencies for equal offspring production. Also, we compared the allele composition of offspring in fused colonies with that of workers and queens sampled from each of the source colonies at the beginning of the fusion assay and determined if only alleles shared between colonies were present in mixed offspring.

Statistical Analyses

All analyses were conducted using SAS 8.2 statistical software (SAS 2000). Number of workers, brood, queens and queen survival recorded in all colony pairs were first analyzed using PROC MIXED with pair, day (sampling date), and pair by day as fixed factors in the model, trial nested within pair, replicate nested within trial by pair, and trial by day nested within pair as random variables, and the number of workers, brood, queens (after square root transformation) and proportion of queens surviving (arcsine transformed) at 24 h and monthly throughout 6 months as dependent variables. Controls were compared separately with an analysis of variance (ANOVA) using PROC GLM with trial, day by trial, replicate nested within trial, and day by replicate nested within trial as additional factors in the model described for colony pairs, and with appropriate TEST statements. Colony pair and control means were separated with least squares means (LSMeans) and a least significant difference test (LSD), respectively. Both analyses showed that trial effects were negligible for queens and workers, but colony pair

effects for brood seemed to be influenced by trial (MIXED: Trial nested within pair covariance parameter estimate = 0.6378; GLM: Trial by pair $F = 3.00$, df = 8, 48, $p = 0.0082$). Consequently, brood number was subjected to ANOVA in two separate analyses (trial 1 and 2 combined and trial 3) to account for the effect of trial on differences among pairs. Differences in the proportion of queens surviving among colony pairs were also analyzed on each sample date with PROC MIXED using pair and run nested within pair as fixed and random factors, respectively, and mean separation by LSMeans. Queen survival on each sampling date in controls was compared with PROC GLM using trial, replicate, and colony as factors in the model, followed by mean separation by LSD. Queen survival in fused and non-fused pairs was compared to queen survival in controls using paired *t*-tests.

The proportion of brood and workers per queen (B/Q and W/Q) and per worker (B/W and W/W) estimated for colony pairs and controls were compared with PROC MIXED using the same fixed factors as in the model for total numbers, with trial, trial by pair, trial by day, and replicate nested within trial by pair as random variables, and B/Q, W/Q, B/W, and W/W (logarithm transformed) as dependent variables. Colony pairs and control means recorded on day 30, 90, and 180 were compared with LSMeans and ESTIMATE statements were used to determine differences in B/Q, W/Q, B/W, and W/W between each colony pair and their respective controls. Only B/W seemed to be influenced by trial as shown by the ANOVA performed in controls using replicate nested within pair by trial as the appropriate error term for trial effects (Trial by pair $F = 3.00$, df = 8, 60, $p = 0.0025$), consequently, data from trial 1 and 2, and trial 3 were also analyzed

separately. Means for fused and non-fused colony pairs were compared with paired *t*-tests.

Total number of workers, brood, and queens (square root transformed) recorded on day 30 and 180 were compared between all replicates that fused and those that did not across all colony pairs and runs using PROC MIXED with fusion (yes or no) and pair nested within fusion as fixed factors, and run by pair by fusion as a random interaction. Differences between replicates that fused and those that did not within the same colony pair were compared using PROC MIXED with fusion by pair as a fixed factor and run nested within pair as a random variable, followed by mean separation by LSMeans. Similar analyses were carried out for B/Q, B/W, W/Q and W/W estimated on days 1, 90, and 180. All means reported are followed by standard errors.

Results

Colony Fusion, Total Colony Size, and Per Capita Brood and Worker Values

Fusion of Argentine ant colonies varied across colony pairs. At 24 h, 100% fusion was recorded in CAR-RTP and COC-FOR (10/10 or 5/5 replicates, respectively), while 50% and 20% of the replicates fused in CHH-RTP (5/10) and CAR-FOR (1/5), respectively. CAR-CHH, CAR-COC, CHH-COC, CHH-FOR, COC-RTP, and FOR-RTP did not fuse within the first 24 hours, however, unlike all other pairs, CHH-FOR workers mixed in all replicates while all queens from either one of the colonies were killed. Rates of fusion increased after a month in CHH-RTP (100%) and CAR-FOR (40%), while some replicates in two colony pairs that did not fuse at 24 h, CAR-CHH (2/10) and FOR-RTP (2/5), fused after two and one month, respectively. Queen composition was not skewed in fused pairs as revealed by the identity of queens from each of the colonies forming a pair at 6 months, averaging 2.57 ± 0.28 colony 1 vs. 2.19 ± 0.31 colony 2 queens (t = 0.34, df = 8, p = 0.3721), except for CHH-RTP that had 2.30 ± 0.33 CHH queens vs. 3.30 ± 0.30 RTP queens (t = 2.30, df = 18, p = 0.0170). In contrast, the identity of queens surviving after 6 months in pairs that did not fuse indicated that colonies did not have an equal chance to win the battles (50.00%) and colony survival was generally biased in favor of one of the colonies (X^2 =128.44 > $X^2_{0.05,13}$ = 22.36), with COC winning very few battles (20.74 ± 0.74%) when paired with CAR, CHH, or RTP, and FOR winning most of them (66.67 ± 0%) when paired with CAR or RTP. Only FOR and CAR had equal chances of winning when paired with CHH (50.00 ± 0% and 58.34 ± 8.34%, respectively).

Total worker number differed among colony pairs when averaging worker number across all sampling dates ($F = 10.92$, df = 9, 75.1, $p < 0.0001$) with no pair by day interaction ($F = 0.92$, df = 54, 31, $p = 0.6198$). Two of the fused pairs, CAR-RTP and CHH-RTP, and one of the non-fused pairs, CHH-FOR, had the highest worker number (Figure 1A). Although no differences in total brood number were found among colony pairs ($F = 1.8$, df = 9, 5, $p = 0.2677$) across all three trials (Figure 1B), number of brood differed among pairs in trials 1 and 2 ($F = 9.90$, df = 4, 6, $p = 0.0237$) with CHH-RTP, CHH-FOR and CAR-RTP having 2.1 to 1.7 times more brood than CAR-COC ($p < 0.05$, LSD), while no differences in brood number were found between pairs in trial 3 ($F = 0.15$, df = 4, 16, $p = 0.9601$). Total queen number differed among pairs ($F = 5.32$, df = 9, 70.9, $p < 0.0001$) with the fused pairs CAR-RTP, CHH-RTP, and COC-FOR having higher queen number than all other pairs (Figure 1C), with a considerable pair by day interaction ($F = 2.24$, df = 54, 31.5, $p = 0.0085$) mostly due to differences at 24 h ($F = 22.47$, df = 9, 5, $p = 0.0016$), and on days 30 and 120 ($F = 10.32$, df = 9, 5, $p = 0.0096$, and $F = 5.05$, df = 9, 5, $p = 0.0446$). Although COC-FOR had high queen number, brood and worker production was lower than in other fused pairs with high queen number, which could be associated with a trade-off between sexual brood production (numerous male larvae and pupae, 3-12 adult males produced) and new worker production in this fused pair (day 120). Male production was also observed in some CAR-FOR replicates (4 - 8), two COC-RTP (2, 23), and one FOR-RTP (4) replicates on day 120. Sexual production can be related to differences in source colony phenology since COC, FOR, and RTP controls also produced adult males (6 - 31) and male larvae and pupae on day 120 but only in trial 3, while no sexuals were produced in trial 1 in neither controls nor

pairs, and only one new queen was produced in one CHH-FOR replicate on day 150 in trial 2 probably due to its queenless status. Also, a pair by day interaction was found for proportion of queens surviving ($F = 2.52$, df = 54, 31.7, $p = 0.0032$), with greater differences at 24 h ($F = 13.48$, df = 9, 5, $p = 0.0053$), and on days 120 and 150 ($F = 5.44$, df = 9, 5, $p = 0.0383$, and $F = 5.32$, df = 9, 5, $p = 0.0402$, respectively) and with greater queen survival in fused pairs (Table 1).

Total worker and brood production averaged across sampling dates (227.85 ± 42.53 and 177.67 ± 14.92, respectively) did not differ among controls ($F = 3.25$, df = 4, 8, $p = 0.0730$, and $F = 0.22$, df = 4, 8, $p = 0.9202$, respectively). Two colony pairs that fused, CAR-RTP and CHH-RTP, produced 1.9 times more workers than controls ($t = 2.75$, df = 75, $p = 0.0037$ and $t = 2.80$, df = 75, $p = 0.0032$), while the colony pair that did not fuse but where workers mixed, CHH-FOR, had 2.1 times more workers than controls ($t = 3.42$, df = 75, $p = 0.0005$). Most of the pairs that did not fuse produced the smallest number of brood (Figure 1B), however, only CAR-COC produced significantly fewer brood than controls ($t = -2.04$, df = 75, $p = 0.0224$). Queen number did not differ among controls when averaged across sampling dates (4.47 ± 0.14) ($F = 3.39$, df = 4, 8, $p = 0.0665$), and three colony pairs that fused, CAR-RTP, CHH-RTP, and COC-FOR, had 1.6 times more queens than controls ($t = 3.68$, df = 75, $p = 0.0002$, $t = 4.09$, df = 75, $p < 0.0001$, and $t = 2.83$, df = 40, $p = 0.0037$). Also, we found a significant colony by day interaction for differences in queen number among controls ($F = 1.79$, df = 24, 48, $p = 0.0423$) probably due to variation in queen survival across time, with FOR and CHH having a lower proportion of queens surviving than the other control colonies on day 120 and 150 ($F = 2.11$, df = 24, 48, $p = 0.0140$). Queen survival averaged across all controls

at 24 hours (0.99 ± 0.01) and 150 days (0.83 ± 0.02) was higher than queen survival averaged across all fused replicates at 24 hours (0.97 ± 0.01) and 150 days (0.59 ± 0.03) (t = 2.23, df = 104, p = 0.0138 and t = 6.30, df = 102, p < 0.0001, respectively). Non-fused replicates had an even lower queen survival than controls at 24 hours (0.85 ± 0.02) and day 150 (0.27 ± 0.02) (t = 5.56, df = 117, p < 0.0001 and t = 17.41, df = 115, p < 0.0001, respectively).

We found a day effect on differences in the proportion of brood per queen (B/Q) among controls and colony pairs (F = 2.59, df = 84, 776, p < 0.0001). Overall, B/Q was higher for controls than pairs on day 30 and 90 (F = 8.40, df = 14,13, p = 0.0002 and F = 6.27, df = 14,13, p = 0.0010, respectively). No differences between controls and pairs were found on day 180 (F = 2.22, df = 14,13, p = 0.0797), however, B/Q was lower in colony pairs that fused vs. those that did not (t = -2.85, df = 13, p = 0.0136). We found a day effect on differences in the proportion of brood per worker (B/W) in trials 1 and 2 (F = 2.45, df = 54, 520, p < 0.0001), and also in trial 3 (F = 3.93, df = 54, 237, p < 0.0001). Although B/W did not differ among controls and pairs on days 30 and 90 (F = 81.87, df = 14,13, p = 0.1337 and F = 1.82, df = 14,13, p = 0.1435, respectively), controls had a higher B/W than pairs on day 180 (F = 3.19, df = 14,13, p = 0.0219). Differences in the proportion of workers per queen (W/Q) and workers per worker (W/W) were influenced by sampling date (F = 5.47, df = 84, 780, p < 0.0001, and F = 3.79, df = 84, 789, p < 0.0001). W/Q differed among controls and pairs on days 30 and 90 (F = 5.43, df = 14,13, p = 0.0021 and F = 4.26, df = 14,13, p = 0.0065, respectively) with a higher W/Q in fused pairs than non-fused ones on day 30 (t = 2.93, df = 13, p = 0.0116). Similarly, differences in W/Q were found on day 180 (F = 3.91, df = 14,13, p = 0.0094) with controls having a

lower W/Q than pairs. Colony pairs and controls did not differ in W/W on days 30, 90, and 180 (F = 1.95, df = 14,13, p = 0.1187, F = 1.35, df = 14,13, p = 0.2988, and F = 1.80, df = 14,13, p = 0.1498, respectively), although fused pairs had a higher W/W than non-fused pairs on day 180 (t = 2.82, df = 13, p = 0.0144).

Although controls did not differ in total worker and brood numbers, we found differences in B/Q and W/Q among controls (F = 2.71, df = 4, 60, p = 0.0384, and F = 5.92, df = 4, 60, p = 0.0004, respectively). Similarly, B/W differed among controls across all trials (F = 3.43, df = 4, 60, p = 0.0137) being highly significant in trial 3 (F = 9.07, df = 4, 20, p = 0.0002), in contrast to W/W where no differences were found (F = 0.44, df = 4, 60, p = 0.7827). Consequently, differences in per capita brood and worker production were compared between individual pairs and their respective controls on days 30, 90 and 180 to account for colony intrinsic differences (Figure 2). B/Q was generally higher in controls than colony pairs on day 30, however these differences decreased throughout the experiment and by day 180 B/Q did not differ between pairs and controls, while two pairs (CHH-FOR and FOR-RTP) had higher B/Q than their controls (Figure 2A). Although B/W did not differ greatly between controls and colony pairs on day 30, it was lower in fused pairs than controls on days 90 and 180, and in one non-fused pair (CHH-FOR) on day 180 (Figure 2B). W/Q was significantly lower in all non-fused pairs than controls except for CHH-FOR on day 30, whereas most fused and non-fused pairs had a higher W/Q than their controls on day 180 (Figure 2C). W/W did not generally differ between pairs and controls, but was higher in CAR-FOR on day 30 and CAR-RTP on day 90, and lower in FOR-RTP on day 180 (Figure 2D).

Total brood number did not differ between replicates that fused and those that did not across all pairs on day 30 ($t = 0.28$, df = 6.43, $p = 0.6163$), but by day 180 fused replicates produced 1.3 times more brood than non-fused ones ($t = 5.59$, df = 62, $p = 0.0212$) (Figure 3A). Differences on day 180 were even greater ($t = 8.40$, df = 53, $p = 0.0055$) when CHH-FOR (colony pair where workers but not queens mixed) was not included in the analyses with fused replicates producing 1.7 times more brood than non-fused ones. Also, we found a pair by fusion interaction on total brood production on day 180 ($F = 2.79$, df = 12, 62, $p = 0.0042$), with fused CAR-CHH and FOR-RTP replicates producing more brood (217.00 ± 141.00 and 205.00 ± 87.00, respectively) than their non-fused replicates (107.38 ± 23.36 and 23.00 ± 14.64, respectively) ($t = 2.24$, df = 8, $p = 0.0275$, and $t = 9.63$, df = 3, $p = 0.0012$, respectively). Worker number was higher in fused vs. non-fused replicates on both day 30 and 180 ($t = 15.23$, df = 62, $p = 0.0002$, and $t = 7.40$, df = 9.6, $p = 0.0223$, respectively) (Figure 3A) with fused replicates having 1.5 times more workers than non-fused ones by the end of the experiment. Similarly when CHH-FOR was not included in the analysis, fused replicates had 1.9 times more workers than non-fused ones ($t = 10.21$, df = 7.5, $p = 0.0138$). A significant influence of differences among pairs was found on days 30 and 180 ($F = 24.41$, df = 12, 62, $p < 0.0001$ and $F = 3.68$, df = 12, 7, $p = 0.0464$, respectively), with fused FOR-RTP replicates producing more workers than non-fused ones (553.00 ± 67.00 vs. 33.33 ± 18.33) on day 180 ($t = 21.96$, df = 3, $p = 0.0001$). Higher queen number occurred in fused replicates on day 30 ($t = 20.65$, df = 9.38, p = 0.0013) and day 180 ($t = 6.30$, df = 8.16, p = 0.0358) (Figure 3B) with a strong colony pair effect on day 30 ($F = 11.06$, df = 12, 7.08, $p = 0.0019$) with CAR-FOR and FOR-RTP fused replicates having more queens

(9.00 ± 0.00 and 7.00 ± 2.00) than replicates that did not fuse (3.33 ± 1.20 and 4.67 ± 0.33) (t = 3.65, df = 3, p = 0.0177 and t = 5.42, df = 3, p = 0.0062, respectively). Queen survival in replicates that fused was higher than in replicates that did not fuse at 24 h and at 150 days (t = 2.99, df = 73, p = 0.0019, and t = 8.41, df = 73, p < 0.0001, respectively). The proportion of workers per worker (W/W) was the only colony growth rate estimate that differed between fused and non-fused replicates on days 1 (t = 8.87, df = 9.12, p = 0.0153) and 90 (t = 6.38, df = 8.22, p = 0.0347) (Figure 4). However, we found a fusion by colony pair interaction for B/Q and W/Q on day 180 (F = 3.3, df = 12, 57, p = 0.0011 and F = 3.96, df = 12, 6.34, p = 0.0465, respectively) due to higher brood number per queen in fused FOR-RTP replicates (157.80 ± 134.20) vs. non-fused ones (8.33 ± 4.67) (t = 24.80, df = 3, p < 0.0001), and higher number of workers per queen in fused FOR-RTP replicates (358. 60 ± 261.40) than non-fused ones (11.94 ± 5.74) (t = 46.78, df = 3, p < 0.0001).

Microsatellite Analysis of Queens and Offspring in Fused Colonies

Queens contributed equally to worker pupae production in all fused replicates as determined by observed vs. expected offspring genotype frequencies (highest X^2 = 5.40 < $X^2_{0.05,6}$ = 22.36). In replicates where the identity of all queens was known, we found a worker pupae composition equivalent to queen composition. For example, in the CAR-CHH replicate, 50% of the genotyped worker pupae were either CHH or CAR, corresponding to two CHH and two CAR queens present. Similarly, one CAR-RTP replicate with four CAR and three RTP queens had 60% CAR pupae and 40% RTP pupae, while in one CHH-RTP replicate with one CHH and four RTP queens, 20% were

CHH and 80% RTP worker pupae suggesting that even in mixed groups with asymmetries in queen composition, worker offspring production was not completely limited to the colony with higher queen number. Moreover, colony 1 queen:colony 2 queen ratios estimated at 6 months and averaged across all fused colonies was 1.07:1, which did not differ from the expected 1:1 ratios in groups with symmetric queen composition ($t = 0.1173$, df = 5, $p = 0.4556$), therefore, we would expect a proportional worker pupa composition in most fused replicates. Also, we found that fused colonies possessed not only alleles shared by both sources but also private alleles from each source colony (Tables 2 and 3).

Discussion

We demonstrated that fusion of aggressive *L. humile* colonies can be adaptive through increased brood and worker production, and consequently fusion of unrelated colonies may be a mechanism by which introduced populations expand their range. Greater brood production and larger worker populations were recorded in non-aggressive *L. humile* colonies that fused (Holway et al. 1998), highlighting the contribution of reduced intraspecific aggression to increased population densities with enhanced performance against native ant competitors (Holway and Suarez 2004). Here, we explored a possible mechanism involved in the formation of expansive *L. humile* populations by examining the likelihood of fusion between colonies displaying varying levels of aggression and genetic similarity (Chapter 2). Unlike previous findings (Holway et al. 1998, Holway and Suarez 2004, Thomas et al. 2005), we found that aggressive *L. humile* colonies fused and produced larger new colonies, suggesting that colony fusion is not just a by-product of inaccuracies in nestmate recognition. Instead, increased colony productivity in fused colonies can produce fitness benefits, and is thereby adaptive.

The selective fusion observed between aggressive colony pairs points out the importance of the genetic relationship between colonies. Colony pairs that fused were genetically more similar than colony pairs that did not fuse (Chapter 2) even when pairs were formed from colonies up to 289 km apart. It has been proposed that if genetic similarity is used as a proxy for relatedness, cooperative behaviors towards genetically similar but distantly related individuals may arise (Tsutsui and Case 2001), which offers a mechanistic explanation for fusion between unrelated but genetically similar colonies. In

addition, variation in the likelihood and time of fusion between replicates of the same pair, suggests that factors such as differences in colony genetic composition (queen and worker genotypes) and/or physiological and behavioral traits (queen reproductive status, worker age, proportion of aggressive worker phenotypes) may further influence this process. Interestingly, in colony pairs that did not fuse, chances to win battles were not equal between colonies, which was previously hypothesized to be related to polarized aggression due to asymmetries in genetic diversity with attackers that have low genetic diversity more likely to win fights (Tsutsui et al 2003). However, we found that the least genetically diverse colony (Table 2) lost most of the encounters, while colonies with equal levels of genetic diversity had equal chances of winning. This is in line with previous findings where genetically more diverse colonies initiated attacks on colonies with lower genetic diversity (Buczkowski and Silverman 2005). In addition, differences in colony phenology (age, reproductive stage, worker composition) may also influence the odds of winning interspecific battles. Overall, the identity of the interacting colonies and their composition (colony intrinsic traits), seem to be important in determining the outcome of intraspecific interactions between colonies.

Aggressive *L. humile* colonies that fused produced more brood (34%) and workers (47%) after 180 days than aggressive colony pairs that did not fuse. However, greater differences in brood and worker production were found between non-aggressive colonies that fused and aggressive colonies that did not fuse after 70 days, with non-aggressive colonies producing 75% as much brood and 50% as many workers than aggressive ones (Holway et al. 1998). Worker number was also higher (52%) in non-aggressive vs. aggressive *L. humile* pairs when reared with *Forelius mccooki* (Holway

and Suarez 2004). Differences between studies may be due to higher worker mortality and small worker numbers in aggressive pairs that ultimately fused at the beginning of our experiment, which may have affected brood production (Oster and Wilson 1978). Also, by comparing the number of brood produced by controls between studies, we found that colonies in our study were not as productive as in Holway et al. (1998). Therefore, differences may be due to greater brood production in colonies with higher worker number (Hee et al. 2000) and fewer queens (Keller and Vargo 1993) since colonies in our study had lower caste ratios (worker:queen, worker:brood). Even with less productive colonies, we were able to detect differences in brood and worker production between fused and non-fused aggressive colonies. Moreover, replicates that fused vs. those that did not fuse within the same colony pair produced up to 10 and 16 times more brood and workers, respectively.

Although most colony pairs that fused had greater worker and brood numbers than non-fused pairs, one pair that did not fuse, FOR-CHH, did as well. Low worker mortality in combination with high queen mortality may result in a better provision by workers with subsequent higher queen productivity. Low worker production in one fused pair (COC-FOR) may have been balanced by increased production of haploid males. The proportion of haploid eggs laid by *L. humile* queens is affected by seasonal differences or the social environment (Aron et al. 1994, Keller et al. 1996), which could explain colony disparities in male production. Additionally, variation in colony genetic and phenotypic composition and other intrinsic colony traits (queen age and reproductive status, age of worker) could explain the variation in total brood and worker numbers among fused and non-fused aggressive pairs. As expected, queen survival in non-fused pairs was lower

than in control colonies due to high aggression between colonies. The lower queen survival in fused pairs than controls could reflect elimination of the least productive queens to optimize the colony worker to queen ratio and increase colony productivity (Reeve and Ratnieks 1993).

In social insects, as colonies grow per capita productivity decreases (Michener 1964), an apparent strategy capitalizing on rapid growth of a large work force thereby decreasing variance in colony performance (Karsai and Wenzel 1998). As predicted, *L. humile* colonies that fused had lower per capita brood production than unpaired and smaller control colonies. In *L. humile*, high queen number per colony tends to lower individual queen productivity (Keller 1988), which could explain the initial lower brood numbers per queen observed in fused pairs vs. controls, although no differences were detected by the end of the experiment (180 days) probably due to a reduction in queen number in fused colonies. Lower brood per queen in non-fused colonies may reflect large worker reductions with concomitant less efficient rearing of offspring (Hölldobler and Wilson 1990). The range of number of brood per queen found in all colony pairs and controls falls within the ratios reported for nests in introduced *L. humile* populations (Ingram 2002). Although the proportion of brood per worker was lower in fused pairs than controls, it was near the higher values of the brood per worker proportion range found in natural conditions (Markin 1970). The lower number of workers per queen in non-fused pairs probably reflects the high worker mortality in these pairs at the beginning of the experiment, however, after 180 days numbers of workers per queen were higher in most fused and non-fused pairs than in controls probably due to reduction in queen number in fused colonies or reduced task efficiency in small colonies in non-fused pairs.

Interestingly, the number of new workers per initial worker number did not differ between pairs and controls, suggesting a similar worker replacement rate. Overall, lower per capita brood production in non-fused pairs may reflect a reduced offspring production in colonies with small worker numbers that provide poorer care due to an atypical worker composition (e.g. number of young vs. older workers). In contrast, the lower per capita brood production in fused colonies is expected in small colonies as they increase their worker numbers (Michener 1964). This effect has also been observed in small *L. humile* propagules (10-1000 workers), although in larger colonies (>1000 workers) per capita brood production was unrelated to worker number (Hee et al. 2000). Interestingly, the only per capita value that differed between fused and non-fused replicates was the number of workers per initial worker, implying that the rate of new worker production is greater than the rate at which workers die in larger colonies, which may correlate with more efficient brood rearing. Similarly, per capita worker productivity was shown to increase with colony size in *L. humile* laboratory colonies that also produced more workers, queens and males as colony size increased (Rosset et al. 2005).

Direct fitness benefits of merging between unrelated social groups may arise from increased genotypic diversity (i.e improved task performance, increased disease resistance) or, alternatively, from increased group size regardless of genetic variation if it improves resource exploitation, defensive behaviours, or overall colony performance (Costa and Ross 2003). For example, merging of incipient *Solenopsis invicta* nests through brood raiding in the field results in very large nests with higher chances to win contests (Tschinkel, 2006). In *L. humile*, larger colony size rather than increased genetic diversity has been found to improve short-term task efficiency (food collection, territory

exploration) and colony productivity (worker larvae and sexual production) (Rosset et al. 2005). Similarly, we found that larger colonies produced higher numbers of brood and workers, which could translate into more successful colonies since larger groups of *L. humile* workers retrieve more food, are better in competitive exploitation, and have a greater ability to monopolize resources (Human and Gordon 1996, Holway and Case 2001, Holway and Suarez 2004). Colonies with high worker numbers have more efficient task allocation among castes, improved defense against predators and competitors, better prey detection, superior nest construction and offspring rearing, and an increased ability to dominate and exploit resources (Oster and Wilson 1978, Sudd and Franks 1987, Herbers 1984, Herbers 1993). Also, in *S. invicta*, worker numbers determine brood production, colony growth, and survival in incipient colonies (Tschinkel 2006), and larger colonies produce more sexuals earlier and can undergo greater worker losses and still recover (Vargo 1988). Although not tested in this study, it is possible that increased sexual production may also occur in fused *L. humile* colony pairs since the high efficiency and productivity observed in social insect colonies with high worker numbers generally leads to higher rates of sexual production (Michener 1964).

In ants, variation in reproduction among nestmate queens is extensive, with queens sharing reproduction relatively equally in some species where in others a single queen can monopolize reproduction (Keller and Vargo 1993). In *L. humile*, reproductive skew for egg and sexual production is low (Keller 1988, Fournier and Keller 2001). Similarly, we found that unrelated queens in colony pairs that fused contributed equally to worker pupae production, which is in accord with the truly polygynous nature of this

species and further suggests the lack of within-colony conflicts for worker production in unrelated fused colonies. Whether reproductive skew for sexual production, and therefore queen direct fitness, is high in these unrelated fused colonies remains to be determined, although it seems unlikely as suggested by the low reproductive skew found between nestmate *L. humile* (Fournier and Keller 2001). The finding that worker force in the newly fused colony possessed alleles from both original colonies suggests that colony fusion may allow gene flow between genetically distinct colonies, leading to changes in colony genetic structure. These changes may result in a broader recognition template through an increase in phenotypic variability of genetically-derived recognition cues in the fused colony, thereby forming more open colonies that may accept individuals from other unrelated colonies. Previous studies have found little to no gene flow between established introduced supercolonies (Jaquiéry et al. 2005, Thomas et al. 2006) suggesting that colony fusion is unlikely, however high levels of genetic differentiation reported between these supercolonies may have prevented exchange of individuals. Higher levels of genetic similarity between fused groups in our study may reduce the cost of fusion with indirect fitness benefits arising from workers raising unrelated but genetically similar offspring.

Previous studies examining interspecific and intraspecific competition in *L. humile* failed to detect fusion between aggressive colony pairs that consistently had reduced numbers of eggs, brood, and workers (Holway et al. 1998, Holway and Suarez 2004, Thomas et al. 2005). Discrepancies between our results and those from previous studies may be due to methodological differences including experimental set-up, duration of the experiment, colony size, and caste ratios, however, regional differences between

populations may better explain these different outcomes. The high genotypic variability in the southeastern *L. humile* population results in a wider range of intraspecific aggression and genetic similarity levels between colonies, and may explain why we detected selective fusion between aggressive colonies. Previous exposure has been shown to increase intraspecific aggression in *L. humile* (Thomas et al. 2005), therefore, if experimental colonies used in previous studies originated from neighboring colonies, high intraspecifc aggression could have prevented fusion. Although colony pairs tested in our study experienced no previous exposure, levels of aggression did not increase but remained either high in non-fused pairs or decreased in fused pairs (Chapter 2), further suggesting that the outcome of intraspecific interactions in *L. humile* are strongly regulated by colony phenotypic composition.

The dynamics of colony fusion and its effects on colony productivity described in this study may be relevant to incipient *L. humile* colonies or small fragments dispersing by colony budding, and may not extend to larger field colonies in which multiple nests support considerably higher numbers of queens and workers (Newell and Barber 1913, Ingram 2002). Also, colonies in this study may not reflect field colony worker composition since aggressive encounters could have eliminated older and/or more aggressive workers leaving only nurses and young workers that could be less efficient in performing foraging-tasks, therefore, underestimating fitness benefits from fusion. On the other hand, higher growth rates and increased productivity could result from *ad libitum* feeding and controlled laboratory conditions. Nevertheless, our results clearly reflect the role of intrinsic colony traits in regulating interspecific interactions. In addition, growth rates and productivity in small and large ant colonies may differ, as has been seen in *S.*

invicta where small colonies invest mostly in workers to facilitate colony growth, while large colonies alternate investing in reproductives and workers (Tschinkel 2006). Therefore, we suggest that studies examining fusion between larger colonies or colonies of a size that yields the greatest colony productivity, i.e. worker or pupae produced per queen (Sudd and Franks 1987), would shed light on alternative competition strategies and possible fitness consequences in larger colonies. This study investigated intrinsic factors that may be regulating colony fusion, however, ecological factors must be important determinants of fusion in *L. humile,* which is known to occur under certain environmental conditions (Markin 1970). How environmental (nesting site, temperature variation, humidity) and biotic factors (interspecific competitors) affect this process warrants further investigation.

In conclusion, colony fusion between unrelated *L. humile* colonies results in higher brood and worker numbers, which has been associated with *L. humile* ecological success, and is therefore adaptive. Moreover, fusion of unrelated but genetically similar colonies may also underlie supercolony formation in areas of multiple introductions producing new colonies with altered genetic structure. Finally, this study supports the view that augmented group size can produce direct fitness benefits in genetically heterogeneous and unrelated social groups (Sundström 1995, Costa and Ross 2002), given that colony size is an important factor regulating colony productivity and social structure in this and possibly other unicolonial ant species.

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Table 1. Colony fusion during a 6 month period and queen survival at 24 hours, 120 days and 150 days for ten Argentine ant colony pairs.

			Proportion of queens surviving					
Colony pair	Fusion	*n*	24 hours *		120 days		150 days	
CAR-RTP	Y	10	1.00 ± 0.00	a	0.64 ± 0.05	a	0.61 ± 0.04	a
CHH-RTP	Y	10	0.96 ± 0.02	abc	0.65 ± 0.04	a	0.62 ± 0.04	a
COC-FOR	Y	5	0.96 ± 0.02	abc	0.60 ± 0.12	ab	0.58 ± 0.14	ab
CAR-CHH	V	10	0.95 ± 0.03	abc	0.34 ± 0.04	c	0.26 ± 0.04	c
CAR-FOR	V	5	0.84 ± 0.11	c	0.44 ± 0.14	abc	0.40 ± 0.11	abc
FOR-RTP	V	5	0.88 ± 0.06	bc	0.44 ± 0.09	abc	0.40 ± 0.08	abc
CAR-COC	N	10	0.99 ± 0.01	ab	0.33 ± 0.04	c	0.24 ± 0.05	c
CHH-COC	N	5	1.00 ± 0.00	abc	0.40 ± 0.08	bc	0.32 ± 0.10	bc
CHH-FOR	N	10	0.53 ± 0.03	d	0.34 ± 0.04	c	0.31 ± 0.05	c
COC-RTP	N	5	1.00 ± 0.00	abc	0.30 ± 0.04	c	0.26 ± 0.04	bc

CAR: Cary; CHH: Chapel Hill; COC: Greenville; FOR: Winston-Salem; RTP: Research Triangle Park. N = none of the replicates fused; V = some replicates fused; Y = all replicates fused; *n* = number of replicates.

* Means within a column followed by a different letter are significantly different (LSMeans, $P < 0.05$)

Table 2. Allele (alle) frequencies (freq) by locus in colonies used in the fusion assay (first trial) genotyped at eight microsatellite loci.

	CAR (15/7)*		CHH (15/6)		COC (15/0)		FOR (15/0)		RTP (15/17)	
Locus	alle	freq	Alle	freq	alle	freq	alle	freq	Alle	Freq
Lhum-11	-	-	142	.0476	142	.0100	142	.1667	142	.218
	-	-	-	-	144	.5333	144	.1333	144	.046
	146	.4091	146	.0714	146	.3667	146	.3000	146	.187
	148	.0455	148	.0714	-	-	-	-	148	.062
	154	.0227	-	-	-	-	-	-	154	.046
	156	.5000	156	.6905	-	-	156	.2333	156	.421
	158	.0227	158	.1190	-	-	158	.1667	158	.015
Lhum-13	182	.0714	-	-	182	.5000	182	.2000	182	.083
	186	.4048	186	.4211	186	.0333	186	.5000	186	.450
	188	.2143	188	.4737	-	-	-	-	188	.183
	-	-	194	.1053	194	.1000	194	.2667	-	-
	198	.3095	-	-	198	.3667	198	.0333	198	.133
	-	-	-	-	-	-	-	-	202	.150
Lhum-19	174	.2955	174	.0526	174	.2000	174	.1000	174	.265
	-	-	-	-	178	.4667	178	.0667	-	-
	-	-	-	-	-	-	-	-	180	.015
	182	.0227	182	.2105	182	.3000	182	.3333	182	.187
	184	.6818	184	.7368	184	.0333	184	.5000	184	.531
Lhum-28	202	.6429	202	.8571	202	1.000	202	.9667	202	.859
	208	.3571	208	.1429	-	-	208	.0333	208	.140
Lhum-35	134	.0227	134	.5500	-	-	134	.2667	134	.281
	148	.2955	-		148	.3667	148	.0667	148	.296
	-	-	154	.0250	-	-	-	-	-	-
	156	.1136	156	.2250	156	.0333	156	.6000	156	.140
	-	-	-	-	-	-	-	-	158	.046
	160	.5227	-	-	160	.0667	-		160	.078
	-	-	-	-	174	.4000	174	.0333	-	-
	-	-	-		176	.1333	-	-	-	-
	180	.0455	180	.1250	-	-	180	.0333	180	.031
	-	-	182	.0250	-	-	-	-		
	-	-	-	-	-	-	-	-	184	.078
	-	-	-	-	-	-	-	-	186	.046
	-	-	188	.0500	-	-	-	-	-	-
Lhum-39	173	.7500	173	.6190	173	.8667	173	.9615	173	.609
	175	.0682	175	.3810	-	-	175	.0385	175	.250
	-	-	-	-	-	-	-	-	191	.031
	207	.1818	-	-	-	-	-	-	207	.109
	-	-	-	-	213	.1333	-	-	-	-
Lihu- T1	147	.6591	147	.8571	-	-	147	.0769	147	.903
	151	.3409	151	.0238	151	1.000	151	.9231	151	.080
	-	-	-	-	-	-	-	-	153	.016
	-	-	157	.1190	-	-	-	-	-	-
Lihu- M1	-	-	134	.1842	-	-	134	.2857	134	.071
	136	.5714	136	.7632	136	1.000	136	.7143	136	.696
	138	.4286	138	.0526	-	-	-	-	138	.232

*Number in parenthesis indicates number of genotyped individuals.

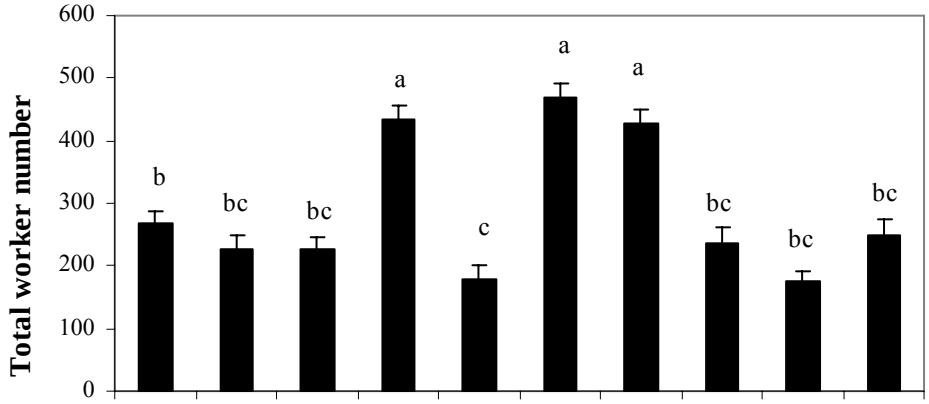
Table 3. Alleles of queens (Q) and worker pupae (P) in fused colony pairs genotyped at eight microsatellite loci.

	CAR-CHH		CAR-RTP		CHH-RTP	
Locus	Q (4)*	P (10)	Q (28)	P (50)	Q (27)	P (50)
Lhum-11	-	-	142	142	142	142
	-	-	144	144	144	144
	146	146	146	146	146	146
	-	-	148	-	148	148
	-	-	-	154	154	-
	156	156	156	156	156	156
	158	158	158	158	158	158
Lhum-13	-	-	182	182	182	182
	-	-	186	186	186	-
	188	188	188	188	188	188
	-	-	-	-	194	194
	-	198	198	198	198	198
	-	202	202	202	202	202
Lhum-19	174	174	174	174	174	174
	-	-	-	180	-	-
	-	-	182	182	182	182
	184	184	184	184	184	184
Lhum-28	202	202	202	202	202	202
	-	-	208	208	208	208
Lhum-35	134	134	134	134	134	134
	148	148	148	148	148	148
	-	-	-	-	154	154
	-	-	156	156	156	156
	160	160	160	160	160	160
	-	--	-	-	180	180
	182	182	-	-	-	-
	-	-	184	184	184	184
	-	-	186	186	186	186
	188	188			188	-
Lhum-39	173	173	173	173	173	173
	-	175	175	175	175	175
	-	-	191	-	-	-
	-	-	193	-	-	-
	207	207	207	207	207	207
Lihu- T1	-	-	-	-	145	-
	147	147	147	147	147	147
	151	151	151	151	151	151
	-	-	153	153	-	-
	157	157			157	157
Lihu- M1	-	-	-	-	134	134
	136	136	136	136	136	136
	138	138	138	138	138	138

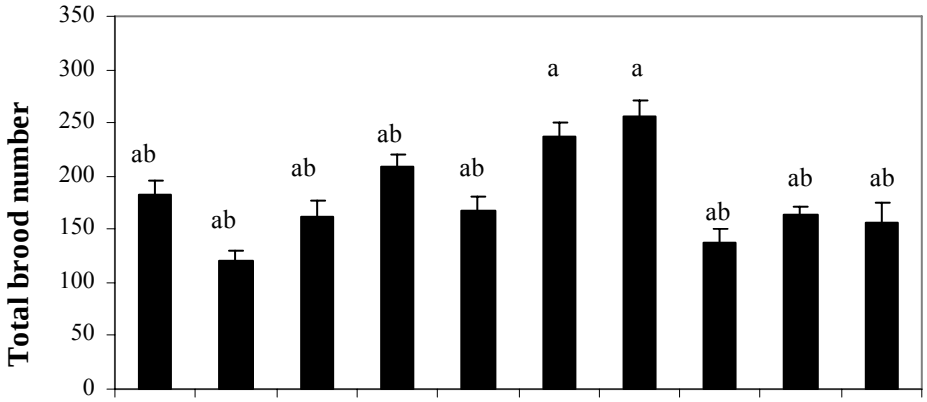
*Number in parenthesis indicates number of genotyped individuals.

Figure 1. Mean (±1 SE) total worker (A), brood (B), and queen (C) number recorded during the 6-month colony fusion assay. Set of bars with different letters indicates significant differences in numbers of workers, brood, or queens among colony pairs (LSMeans, $P < 0.05$). See Table 1 for colony abbreviations.

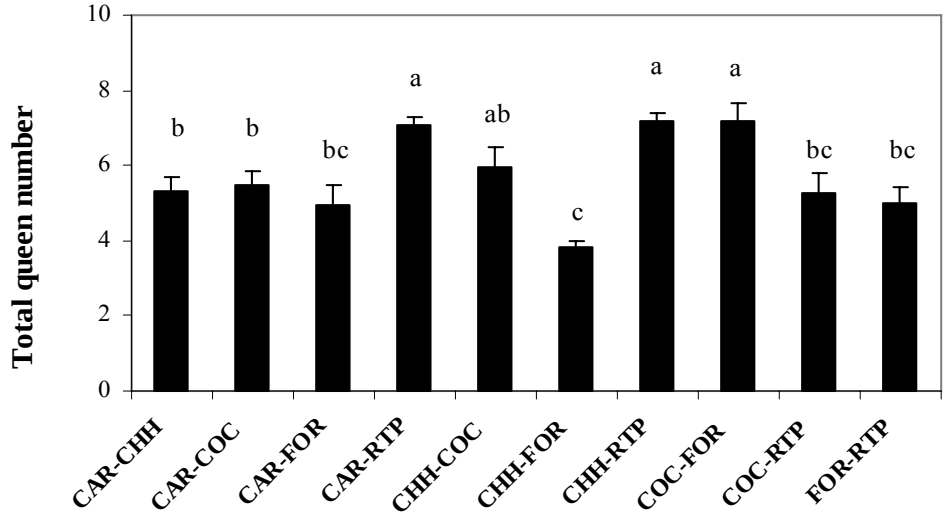
A



B



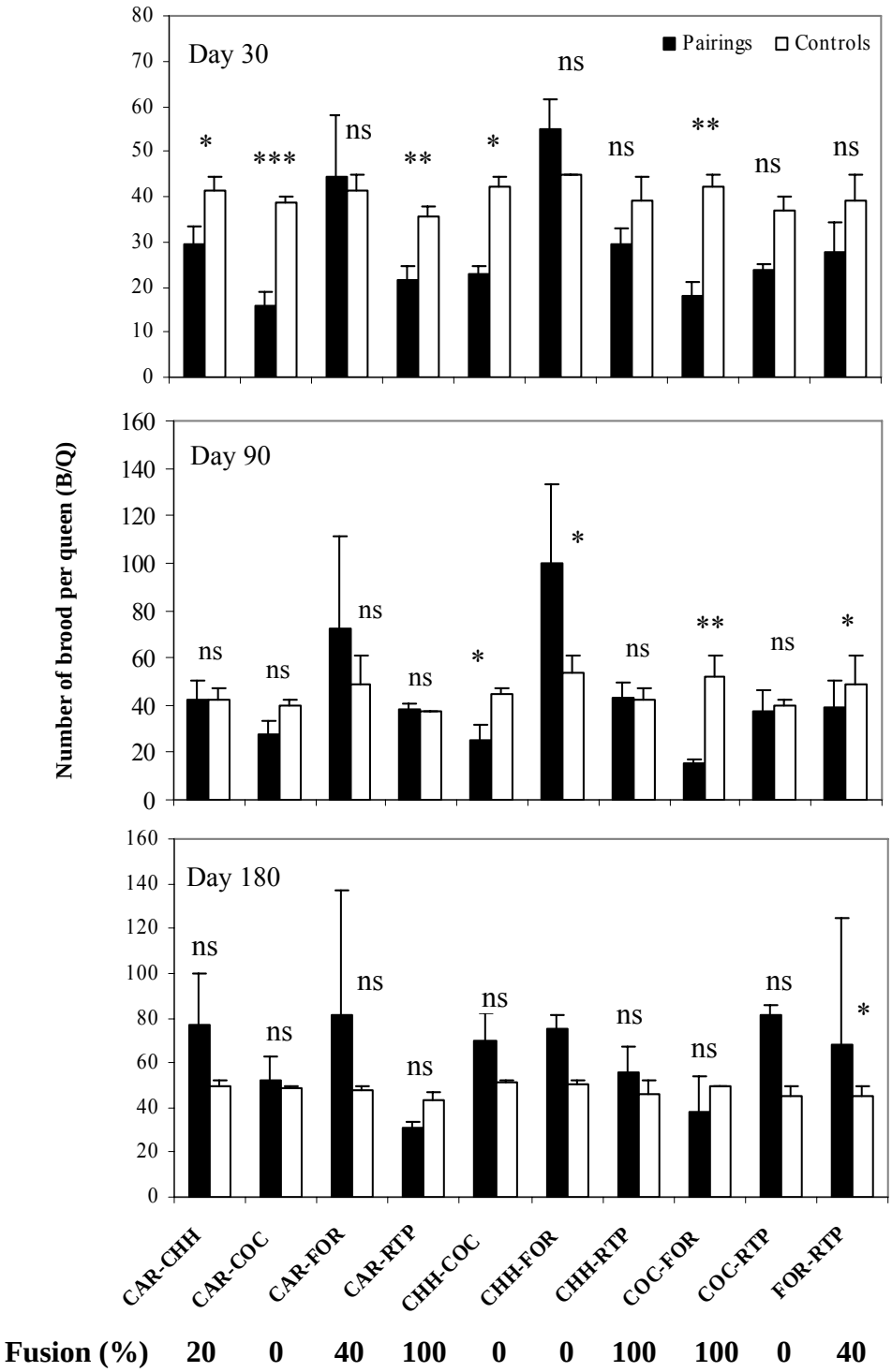
C



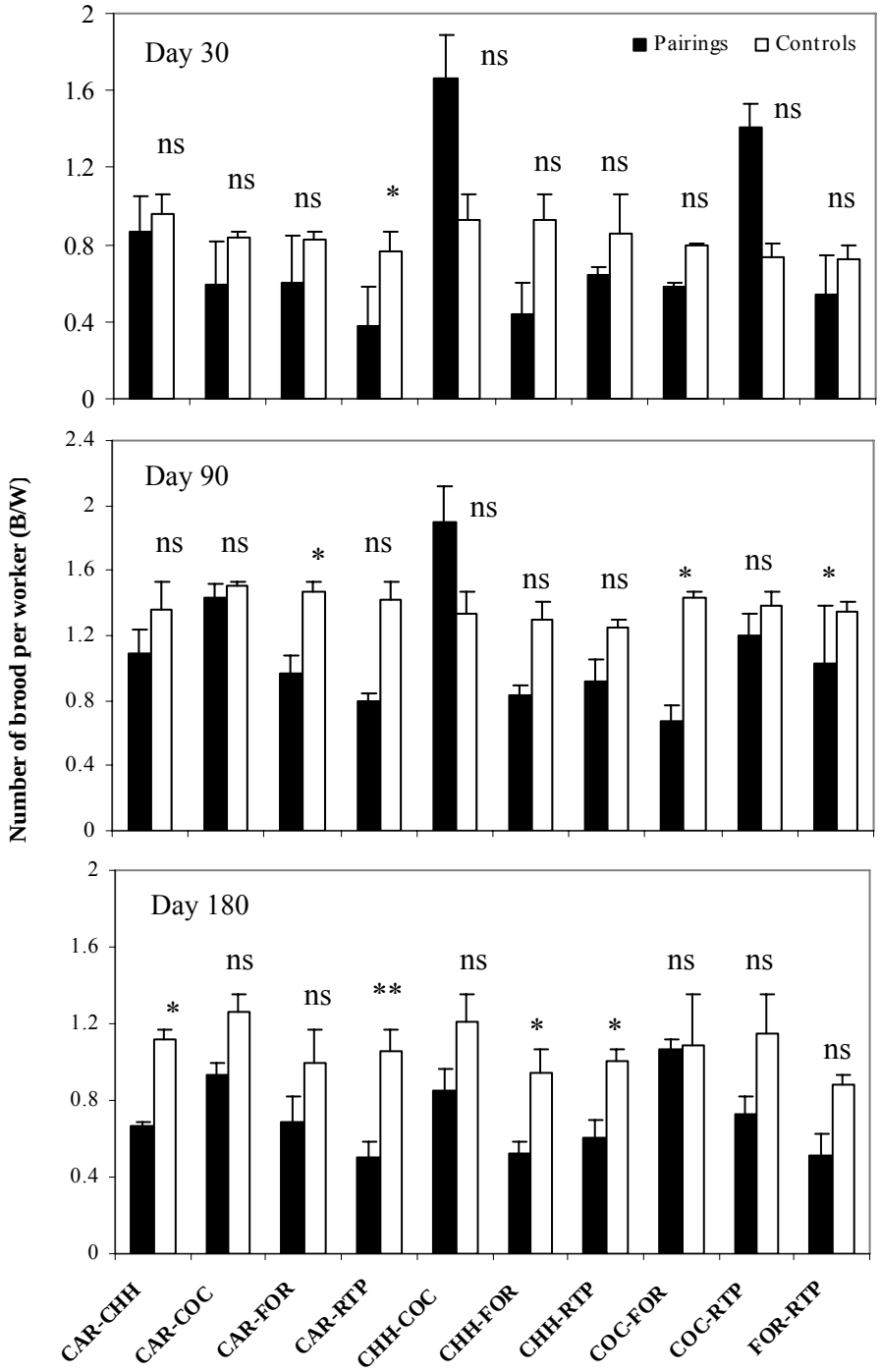
	CAR-CHH	CAR-COC	CAR-FOR	CAR-RTP	CHH-COC	CHH-FOR	CHH-RTP	COC-FOR	COC-RTP	FOR-RTP
Fusion (%)	**20**	**0**	**40**	**100**	**0**	**0**	**100**	**100**	**0**	**40**

Figure 2. Mean (±1 SE) per capita brood production for queens (A) and workers (B), and per capita worker production for queens (C) and workers (D) for colony pairs vs. their combined unpaired controls on days 30, 90, and 180. See Table 1 for colony abbreviations. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0001$; ns = nonsignificant.

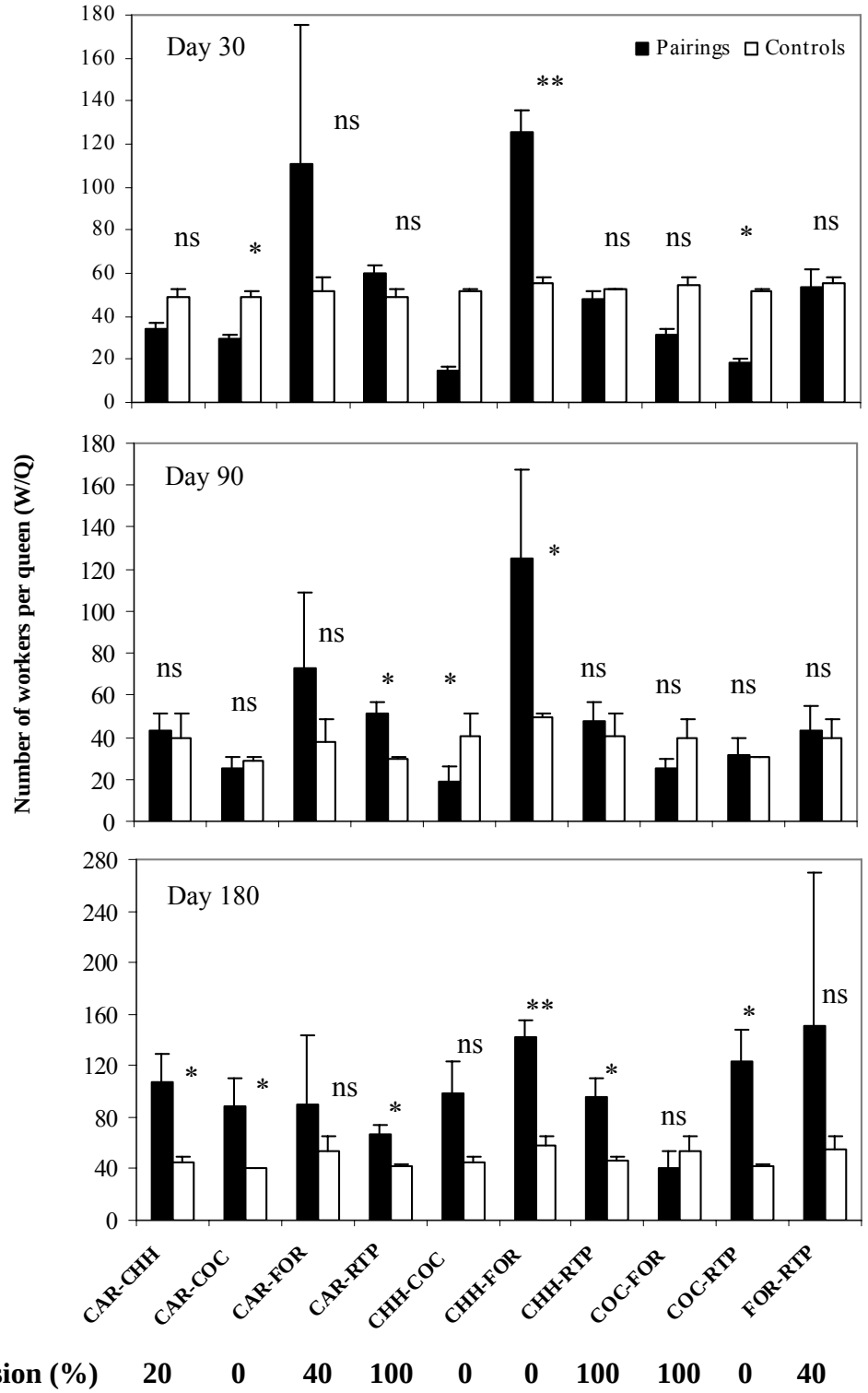
A


Day 30
Pairings
Controls
Day 90
Day 180
Number of brood per queen (B/Q)
CAR-CHH
CAR-COC
CAR-FOR
CAR-RTP
CHH-COC
CHH-FOR
CHH-RTP
COC-FOR
COC-RTP
FOR-RTP
Fusion (%) 20 0 40 100 0 0 100 100 0 40

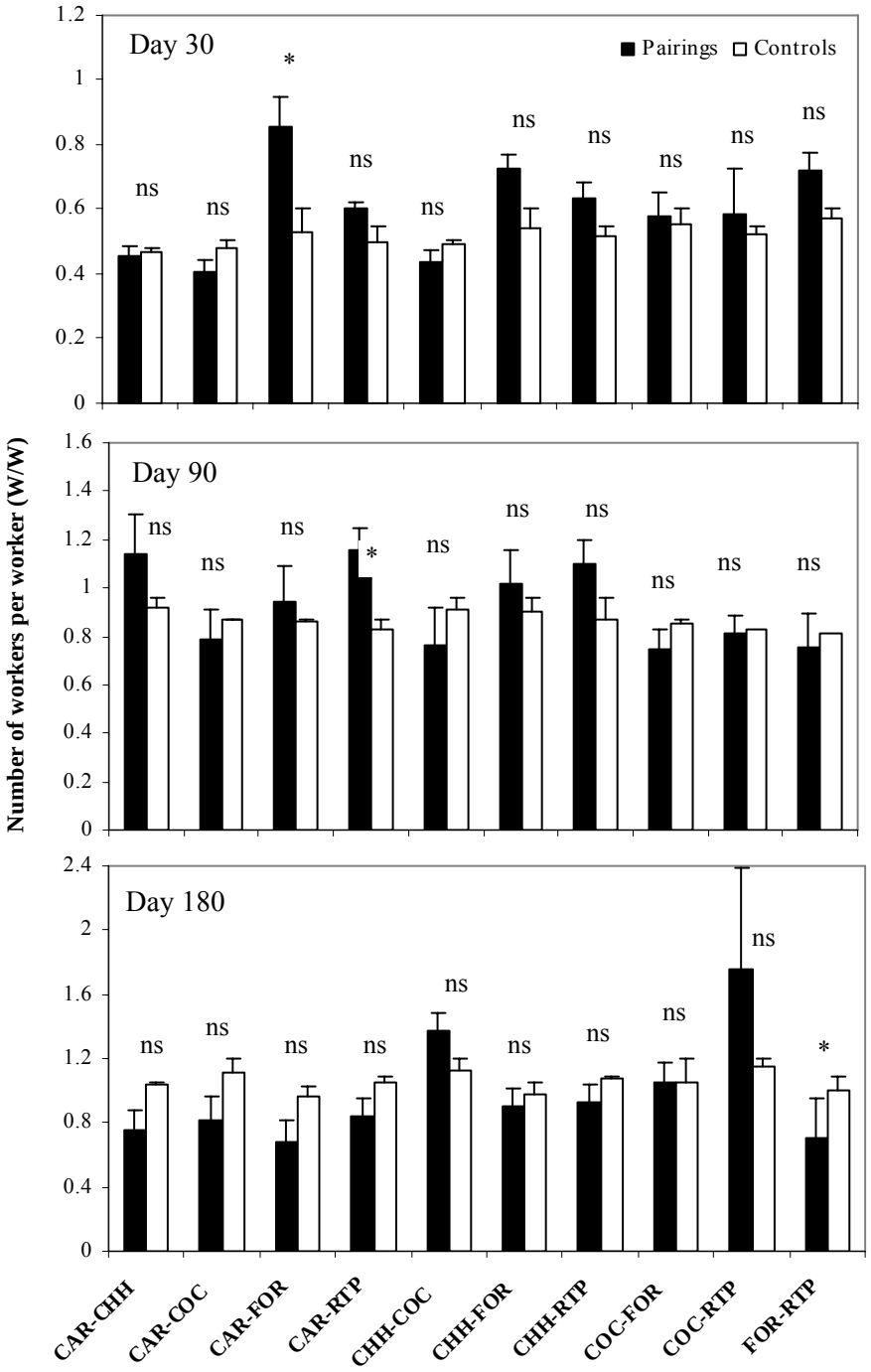
B


Day 30
Pairings
Controls
Day 90
Day 180
Number of brood per worker (B/W)
CAR-CHH
CAR-COC
CAR-FOR
CAR-RTP
CHH-COC
CHH-FOR
CHH-RTP
COC-FOR
COC-RTP
FOR-RTP
Fusion (%) 20 0 40 100 0 0 100 100 0 40

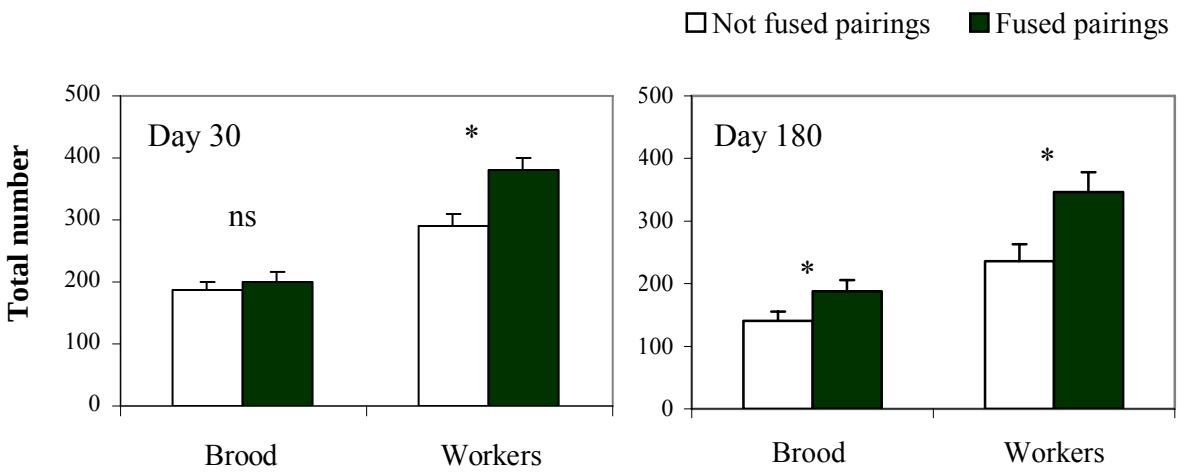
C


Day 30
Pairings
Controls
Day 90
Day 180
Number of workers per queen (W/Q)
CAR-CHH
CAR-COC
CAR-FOR
CAR-RTP
CHH-COC
CHH-FOR
CHH-RTP
COC-FOR
COC-RTP
FOR-RTP
Fusion (%) 20 0 40 100 0 0 100 100 0 40

D


Day 30
Pairings
Controls
Day 90
Day 180
Number of workers per worker (W/W)
CAR-CHH
CAR-COC
CAR-FOR
CAR-RTP
CHH-COC
CHH-FOR
CHH-RTP
COC-FOR
COC-RTP
FOR-RTP
Fusion (%) 20 0 40 100 0 0 100 100 0 40

A



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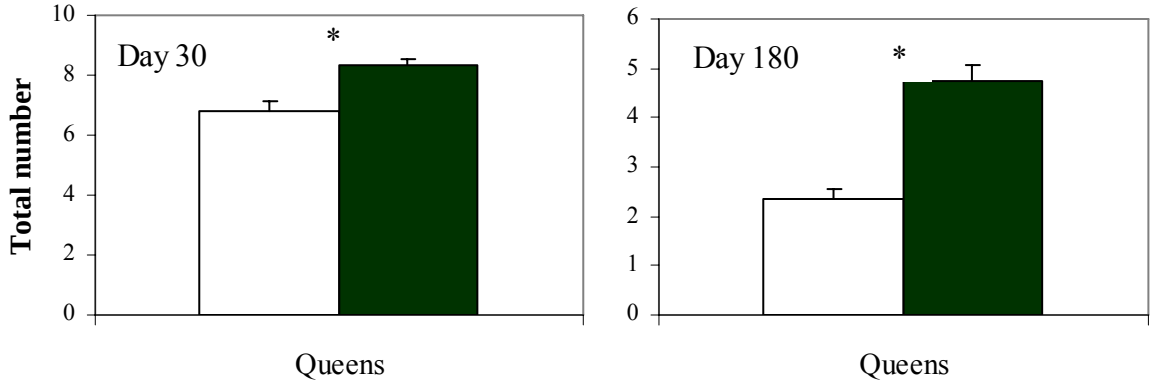

Figure 3. Mean (±1 SE) total brood and worker production (A) and total queen number (B) for all colony pair replicates that fused vs. those that did not on days 30 and 180. *$P < 0.05$; **$P < 0.005$; ns = nonsignificant.

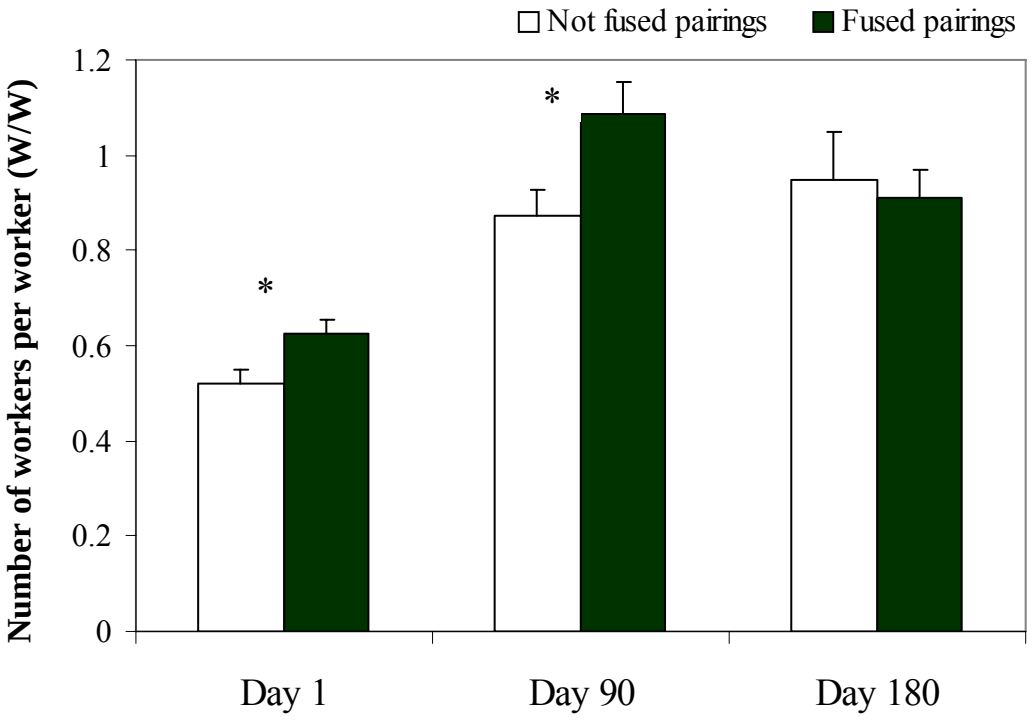

Figure 4. Mean (±1 SE) per capita worker production for workers for all colony pair replicates that fused vs. those that did not on days 1, 90 and 180. *$P < 0.05$; ns = nonsignificant.

CHAPTER IV

Cuticular hydrocarbons as recognition cues in non-nestmate queen adoption and colony fusion in the Argentine ant

Abstract. In social insect species, individuals recognize and behave aggressively towards conspecific non-nestmates to maintain the colony's integrity. This is achieved via a well-developed nestmate recognition system in which recognition cues are genetic and/or environmentally derived. Introduced populations of the Argentine ant, *Linepithema humile* (Mayr), in California, have experienced a loss of genetic diversity that has been linked to reduced diversity at loci encoding for nestmate recognition cues, thereby reducing their ability to distinguish nestmates from non-nestmates. However, introduced *L. humile* populations in the southeastern U.S. are genetically more diverse, show high levels of intraspecific aggression, and are minimally affected by the acquisition of prey-derived cues. To further explore the nestmate recognition system, we compared both worker and queen cuticular hydrocarbon (CHC) profiles between aggressive southeastern *L. humile* colony pairs, and related CHC similarities with two processes that may shape this invasive ant's social structure—non-nestmate queen adoption and colony fusion. We also investigated the relationship between worker CHC similarity and worker-worker aggressive behavior, and estimated levels of both queen and worker genetic similarity between colony pairs to determine whether they were correlated with CHC profile similarity. We found that both non-nestmate queen adoption and colony fusion were associated with similarity of queen CHC profiles, whereas worker CHC profile similarity was strongly inversely associated with intraspecific aggression and positively correlated with colony fusion. We also found an association between similarity of the CHC profiles of both queens and workers and genetic similarity at DNA microsatellite markers. To illustrate the dynamic nature of *L. humile* recognition cues, we examined CHC profiles of non-nestmate queens two weeks after adoption by

queenless colonies, and the profiles of queens and workers in colony pairs that fused and those that did not fuse after six months. Non-nestmate queens that were adopted by queenless colonies did not change their CHC profiles, whereas the hydrocarbons of queens in fused colony pairs were a mix of the two colony phenotypes. When only one of two paired colonies survived, the CHC profiles of the surviving queens did not diverge from those of their unpaired controls. Similarly, workers in non-fused colonies maintained their colony-specific CHC, whereas in fused colonies the worker hydrocarbon profiles were intermediate between the respective unpaired controls. In addition, we treated queens with non-nestmate queen CHC and recorded the responses of nestmate workers towards treated queens. We found that treated queens were readily attacked by nestmate workers, demonstrating that queen CHC are used as queen recognition cues in this species. These findings highlight the complexity and dynamic nature of nestmate recognition in *L. humile*, and shed light on the factors underlying variation in cue expression and perception. Moreover, by examining nestmate discrimination in genetically unrelated aggressive *L. humil*e colonies we provide insight into the role of nestmate recognition in colony interactions and in shaping introduced *L. humile* populations.

Keywords: Argentine ant, *Linepithema humile*, nestmate recognition, cuticular hydrocarbons, intraspecific aggression, non-nestmate queen adoption, colony fusion.

Introduction

In social insects, nestmate recognition allows an individual's integration within a colony and prevents non-colony members—both conspecifics and heterospecifics—from invading and exploiting the colony's resources, with active defensive behaviors initiated when an intruder is recognized as non-nestmate (Hölldobler and Wilson 1990, Vander Meer and Morel 1998). Typically, individuals discriminate colony members from non-members by means of a phenotype matching mechanism in which the phenotype of previously unencountered individuals are compared with the individual's inner learned template (Lacy and Sherman 1983). Recognition of phenotypic cues or traits by allele matching (recognition-allele mechanism), which implies no need for a learned template, also has been demonstrated in an ant species (Keller and Ross 1998). Phenotypic recognition cues must be reliable signals and originate from either environmental (diet, nesting substrate), endogenous sources (genetically determined, acquired from queens or workers), or both (Breed and Bennett 1987, Vander Meer and Morel 1998). The template represents a memory pattern of the colony's recognition cues derived from the environment, the individual's own phenotype, or collectively from all colony members; and the process of cue-template matching guides a behavioral response, resulting in the acceptance or rejection of the encountered individual (Reeve 1989, Gamboa et al. 1986).

Appropriate behavioral responses are guided by recognition decision rules concerning the level of dissimilarity between the template and the phenotypic cues of the encountered individual (Breed and Bennett 1987). Models for decision rules in recognition include an individualistic model in which individuals retain their own cue

integrity and score other individuals by comparison with themselves, accepting them based on genotypic similarity; and a Gestalt model in which cue transfer occurs among colony members resulting in a unique mixture of chemical cues (colony "odor"), and individuals are classified as colony members based upon the degree to which they posses the odor (Crozier and Dix 1979, Getz 1982, Crozier 1987). Self-characteristics are used as a template in the individualistic model while the Gestalt template is based on a combination of the phenotypes of nearby individuals. Also, it has been proposed that decisions are made according to a recognition threshold so that if the template-odor match is greater than a minimum similarity threshold (or below a dissimilarity threshold) the individual is accepted and treated as a nestmate (Gamboa et al. 1986, Reeve 1989). Interaction frequency with foreign conspecifics and the fitness consequences of accepting or rejecting conspecifics determine the optimal acceptance threshold (Reeve 1989), hence, discrimination varies according to the social and ecological context as to balance the fitness costs of accepting non-nestmates and rejecting nestmates. Alternatively, a graded behavioral response depending on the degree of cue and template similarity would suggest a non-threshold model (Vander Meer and Morel 1998).

Nestmate recognition in social insects is adaptive because workers obtain inclusive fitness benefits from aiding nestmates and discriminating against non-nestmates, provided that nestmates are more closely related to one another than to members of other nearby colonies (Hölldobler 1995). Natural selection should favor the use of cues that optimize discrimination because recognition errors—rejecting a desirable conspecific or accepting an undesirable individual—lower the benefits expected from kin interactions (Lehman and Perry 2002). In social insects and other animals these cues are

primarily chemical in nature and perceived by olfaction or contact chemoreception (Hölldobler and Michener 1980, Breed 1998). Chemically-based recognition usually relies on compounds that originally may have had other functions. For example, cuticular lipids serve multiple functions in insects, but primarily they protect insects against desiccation by acting as a water loss barrier; but cuticular lipids also serve as pheromones and are used as kairomones (reviewed by Howard and Blomquist 2005). The role of cuticular hydrocarbons (CHC) as nestmate recognition cues has been evidenced by a number of studies in ants (Lahav et al. 1999, Thomas et al. 1999, Boulay et al. 2000, Liang and Silverman 2000, Ozaki et al. 2005), wasps (Gamboa et al. 1996, Dani et al. 2001, Ruther et al. 2002), and termites (Clèment and Bagnères 1998) by evaluating the effects of either purified fractions of cuticular extracts or individual hydrocarbons on nestmate recognition.

In social insect species with large colonies, queens and workers seem to be labeled by a more or less homogenous recognition odor, or colony gestalt label, where each colony member bears a mixture of cues representative of the variation among members of the colony (Stuart 1988, Errard and Jallon 1987). In ants, this colony odor (CHC) is acquired by all members of the colony through trophallaxis and allogrooming, and the postpharyngeal gland is the organ in which individual ants admix their own CHC with those of nestmates (Soroker et al. 1995, Lahav et al. 1998, Soroker et al. 1998). This gestalt label is expected to prevail in polygynous ant species, although extreme polygyny may limit the creation of unique labels thereby reducing intercolony variation. The lack of distinct intrinsic colony odors may facilitate formation of unicolonial populations in

which colony boundaries are weak or absent, although some odor differences arising from extrinsic factors may still exist (Hölldobler and Wilson 1990).

Introduced populations of the Argentine ant, *Linepithema humile* (Mayr), are highly polygynous and unicolonial (Newell and Barber 1913, Hölldobler and Wilson 1990, Suarez et al. 1999), and exhibit a pronounced variation in intraspecific aggression that stems from diversity in nestmate recognition behavior (Tsutsui et al. 2000, Suarez et al. 2002, Giraud et al. 2002, Buczkowski et al. 2004). These populations are, therefore, useful to examine differential behaviors toward conspecifics, and the effects of social and ecological context on behavioral thresholds (Buczkowski and Silverman 2005). In the widespread invasive *L. humile*, exogenous prey-derived hydrocarbons dramatically influence worker-worker aggression, demonstrating that CHC play a central role in nestmate recognition and that the *L. humile* recognition system includes environmentally-derived cues (Liang and Silverman 2000, Silverman and Liang 2001, Liang et al. 2001). In addition, the inverse relationship between intraspecific aggression and genetic similarity between nests in native and introduced populations suggests that recognition cues are also heritable (Tsutsui et al. 2000). Recently, it has been shown that the contribution of environmentally derived cues to nestmate recognition varies among introduced populations, suggesting that their past phenology and genotypic diversity affect the expression and perception of components of the *L. humile* recognition system (Buczkowski and Silverman 2006). Therefore, examining variation in recognition cue diversity among colonies displaying varying degrees of intraspecific aggression and genetic similarity, and how this variation may be linked to differential responses towards conspecifics at both the individual and group level, may further contribute to our

understanding of the ontogeny of recognition cues, cue perception, and how actions resulting from cue-template matching may have population level implications.

We have previously shown that unrelated *L. humile* colonies from the southeastern U.S. selectively adopt foreign queens and fuse, and that genetic similarity of colony pairs regulates both processes (Chapters 1 and 2). These findings have broad implications in shaping introduced *L. humile* social organization since adoption of foreign queens and colony fusion will likely result in changes in the genetic composition of colonies, thereby affecting nestmate discrimination through their effect on recognition cue expression and template formation, which could further lead to modified social structure. In unicolonial *L. humile* populations that exhibit low variation in genetic-based recognition cues, non-nestmates may be acepted if the template-cue dissimilarity is below a rejection threshold (Starks 2003). Likewise, higher levels of genetic-based recognition cue similarity between colonies in more genetically diverse populations may lead to non-nestmate acceptance. Therefore, supercolony formation in introduced *L. humile* populations may result not only from lower recognition cue diversity due to a loss of genetic diversity, but also from selective mixing of non-nestmates that share higher levels of phenotypic similarity. We investigated similarity of recognition cues between southeastern colony pairs by comparing their CHC profiles. We hypothesize that CHC similarities are correlated with, and likely guide, behavioral interactions both at the individual and group levels. We thus expect that the CHC profiles of adopted non-nestmate queens would be more similar to host colony queens than the CHC profiles of non-adopted queens, and that fusion would occur more between colonies with greater queen and worker CHC profile similarities. Also, we investigated the relationship

between worker recognition cue similarity vs. levels of worker intraspecific aggression and genetic similarity. Previous studies have found an association between aggression and genetic similarity (Tsutsui et al. 2000) and between aggression and CHC similarity (Suarez et al. 2002), therefore, we expect to find a direct correlation between CHC profile similarities and levels of genetic similarity. Also, we examined the relationship between CHC profile similarity of queens and their genetic similarity. An association between phenotypic and genetic similarity under controlled environmental conditions would suggest that nestmate recognition cues are probably genetically derived. We also experimentally manipulated the *L. humile* nestmate recognition cues by applying purified non-nestmate queen CHC onto live queens, recording nestmate behavior and analyzing the treated queen CHC. Because application of prey-derived hydrocarbons onto *L. humile* workers elicits nestmate worker aggression (Liang and Silverman 2000), we expected that application of naturally occurring non-nestmate queen CHC onto queens would also elicit aggressive worker responses. In addition, we examined the chemical profiles of queens adopted by queenless colonies to determine whether queens acquired non-nestmate CHC as a means of colony integration, and the chemical profiles of queens and workers in fused colonies to determine whether homogenization of chemical recognition cues occurs.

Materials and Methods

Experimental Colonies

We used colonies of Argentine ants (*Linepithema humile*) collected from five 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 9.6 km (CAR – RTP) to 402.3 km (CAR – COC). Ants collected from these sites were genetically differentiated with colony pairs sharing different levels of genetic similarity (Chapters 1 and 2). Experimental and source colonies used in both queen adoption and colony fusion assays were set up and maintained as described in Chapters 1 and 2, respectively.

Behavioral Assays and Sampling of CHC

Application of Non-Nestmate Queen CHC on Queens and Nestmate Worker Aggression

To test if CHC are used as cues in *L. humile* nestmate queen recognition we compared worker aggressive behavior towards nestmate queens treated with nestmate and non-nestmate queen CHC. We set up three RTP multiple-queen experimental colonies as described in the queen adoption assay in Chapter 1. RTP queens were treated with purified CHC extracts of FOR or RTP queens, or with hexane as control. Purified CHC from 6 queens (cuticular lipid extraction and CHC isolation procedures detailed below) were resuspended in 100 µl hexane, applied to the inside surface of a 12 x 32 mm glass vial, and the solvent allowed to evaporate. Three vials were coated per treatment and each vial was used to treat three individual queens. Each queen was anesthetized by brief

exposure to CO_2, placed individually in a treated vial, rotated gently for 3 min, allowed 15-30 sec to recover and then introduced to one of the experimental colonies. Each experimental colony received a total of three queens per treatment. Worker behavior was scored as non-aggressive (antennation, queen moving into nest without being attacked) or aggressive (biting, pulling, lunging, gaster flexion) during a 3-min period by an observer blinded to the type of treatment applied to queens and unfamiliar with the hypothesis being tested. All tested queens were killed by freezing (-20˚C) and their cuticular lipids extracted and analyzed as described below to determine if CHC profiles differed between treatments, and between attacked and non-attacked queens.

Non-Nestmate Queen Adoption

To examine *L. humile* non-nestmate queen adoption we introduced queens into queenless and queenright CHH, COC, FOR, and RTP colonies, intermittently recorded worker behavior towards the introduced queen for 24 h and estimated percentage of queens adopted at 24 h as described in Chapter 1. Queen adoption was also examined by introducing queens into queenless and queenright FOR and RTP colonies for two weeks as described in the adopted queen fecundity assay in Chapter 1.

We collected 10 queens from each of the source colonies used in the 24-h adoption assay for CHC analysis. Queens were placed individually in 3.7 ml glass vials and stored at -20˚C until extraction. Source queen CHC profiles were compared to determine queen CHC similarities between colonies, and to relate similarities of queen CHC to worker response, percent queen adoption at 24 h, and levels of genetic similarity between introduced and resident queens. We developed, validated and employed a non-

destructive CHC sampling method to sample all queens (96) in the 2-week adoption assay 24 h prior to introduction and a group of 46 queens adopted by queenless colonies two weeks after introduction. A hexane-extracted air-dried cotton ball (2 mm diameter) held by a pair of hexane-rinsed forceps was gently stroked for 3 min over the cuticular surface of a queen's abdomen and stored at -20˚C. CHC profiles of adopted queens were compared before and after introduction with those of nestmate control queens to determine if changes in CHC occurred after adoption.

Colony Fusion

To determine if unrelated *L. humile* colonies would fuse we recorded marked queens and workers mixing without fighting at 24 h and monthly throughout 6 months for 10 pairwise colony combinations (CAR-CHH, CAR-COC, CAR-FOR, CAR-RTP, CHH-COC, CHH-FOR, CHH-RTP, COC-FOR, COC-RTP, FOR-RTP); levels of worker-worker aggression were measured following Roulston et al. (2003) as described in Chapter 2.

We collected workers from each source colony in groups of ten, placed them in glass vials (nine vials per colony), and stored at -20˚C. Source worker CHC profiles were compared to determine similarities of worker CHC between colony pairs, and to relate CHC similarities to worker aggression, colony fusion, and genetic similarity. After six months, we collected queens (4–11 individuals) and workers (8-10 samples, 10 workers/sample) from each colony pair tested in the first trial (CAR-CHH, CAR-COC, CAR-RTP, CHH-FOR, CHH-RTP), and queens (6–10 individuals) and workers (6 samples, 10 workers/sample) from unpaired control replicates. We compared queen and

worker CHC profiles from colony pairs with their respective unpaired control colonies. In addition, CHC of queens and workers from control colonies were compared to determine the relationship between CHC similarities between colonies and colony fusion.

Extraction, Isolation, and Chemical Analysis of Cuticular Hydrocarbons

Cuticular lipids of thawed queens, cotton samples, and workers collected in all behavioral assays were extracted by immersion in 1 ml hexane for 10 min, followed by a brief second rinse in 100 µl hexane. Samples were lightly shaken for the first and last 15-20 sec of the immersion period. The solvent was removed under a gentle stream of N_2, the vial rinsed with two 100-µl hexane and the concentrated extract (200 µl) was applied to a hexane-prewetted Pasteur pipette mini-column filled with 500 mg of silica gel (100-200 mesh). The hydrocarbon fraction was eluted with 6 ml hexane. Capillary gas chromatography (GC) was carried out using a HP5890 gas chromatograph equipped with a DB-XLB column (30m x 0.25mm x 0.25µm film thickness) for analyses of CHC of source queens and workers from the 24-h queen adoption and colony fusion assay, respectively, and a DB-5 (30m x 0.25mm x 0.5 µm) for analyses of CHC of workers and queens collected at 6 months in the colony fusion assay and cotton samples taken in the 2-week queen adoption assay. Extracts were introduced into a split-splitless injector operated at 300˚C in splitless mode (2 min purge) and helium was the carrier gas at an average linear velocity of 30 cm/sec. Oven temperature was held at 80˚C for 2 min, increased to 270˚C at a rate of 20˚C/min, then to 310˚C at 3˚C/min and held at 310˚C for 20 min. The flame-ionization detector was operated at 310˚C with nitrogen make-up gas at 30 ml/min. Whole queen extracts were resuspended in 20 µl hexane and 0.5 µl was

injected (0.025 queen equivalents); cotton sample extracts were resuspended in 4 µl of octane and 2 µl were injected (0.5 queen equivalents); and worker extracts were resuspended in 10 µl hexane and 2 µl were injected (2 worker equivalents). Quantitative data were obtained by integrating the area under each peak and calculating its percentage of the total CHC; only peaks with a mean percent area across all colonies of 1% or higher were used for data analysis. All selected peak areas were standardized to 100%. The identity of discriminating peaks was determined by matching *L. humile* normal-alkanes with external hydrocarbon standards (n-C23 – n-C36) and diagnostic peaks were confirmed by GC-MS with those from previous studies (Liang et al. 2001, de Biseau et al. 2004).

Genetic Similarities between Colony Pairs

We assessed genetic similarity between queens tested in the 24-h queen adoption assay (CHH, COC, FOR, RTP) and between workers from source colonies used in the fusion assay (CAR, CHH, COC, FOR, RTP) using microsatellite markers. Genomic DNA was extracted from 40-46 queens and 15 workers from each of the colonies using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) and analyzed at eight microsatellite loci: Lhum-11, Lhum-13, Lhum-19, Lhum-28, Lhum-35, Lhum-39 (Krieger and Keller 1999), Lihu-M1 and Lihu-T1 (Tsutsui et al. 2000). PCR reactions were performed as described by Buczkowski et al. (2004). Products were separated on 6.5% KB^{Plus} polyacrylamide sequencing gels using a 4300 LI-COR DNA analyzer. Microsatellite alleles were scored using GeneImagIR software (Scanalytics Inc., Billerica, MA, USA). Genetic differentiation (F_{ST}) between Argentine ants from different locations was estimated with the program FSTAT v.2.9.3.2 (Goudet 1995). Levels of genetic similarity between

colonies were estimated based on the percentage of alleles shared between these groups (Tsutsui et al. 2000).

Statistical Analyses

Data analyses were performed using SAS 8.2 statistical software (SAS 2000). We performed a Multivariate Analysis of Variance (MANOVA) on the quantitative CHC data using PROC GLM to identify variables (GC peaks) that differed significantly among groups of queens or workers in each behavioral assay. Peak areas were transformed following Aitchison's formula: $Z_{ij} = \ln[Y_{ij}/g(Y_j)]$, where Z_{ij} is the standardized peak area *i* for individual *j*, Y_{ij} is the peak area *i* for individual *j*, and $g(Y_j)$ is the geometric mean of all peaks for individual *j*. The homogeneity of variance of these variables was tested with Brown and Forsythe's test. MANOVA was performed on transformed variables that met the assumptions of homogeneity of variance. We performed a canonical discriminant analysis (DA) on transformed variables that were significantly different among groups according to MANOVA using PROC CANDISC to determine whether the predefined groups (colonies or treatments) could be discriminated on the basis of their chemical profiles. We also conducted a stepwise discriminant analysis (stepwise DA) using PROC STEPDISC on transformed variables used in MANOVA followed by DA on the selected peaks. Pairwise generalized square distances between groups and classification error rates were calculated using PROC DISCRIM. Distances between group means (centroids) were used as an estimate of the degree of CHC differentiation between colonies or treatments. Error rates for group classification were compared across all DA analyses. To determine changes in queen and worker CHC profiles of fused and non-fused colonies

vs. their respective controls we first estimated the linear discriminant function coefficients only for control colonies using PROC DISCRIM and then computed the linear discriminant function for fused and non-fused colony pairs using these coefficients.

Correlations were performed using Pearson correlation coefficients, and included non-nestmate queen adoption and queen-queen genetic similarity versus queen CHC similarities; colony fusion vs. queen and worker CHC similarities; and levels of worker-worker aggression and genetic similarity versus worker CHC similarities.

Results

Queens Treated with Non-nestmate Queen CHC: Chemical Profiles and Worker Aggression

We found that CHC profiles of RTP queens treated with nestmate RTP queen CHC, non-nestmate FOR queen CHC, or hexane differed as shown by the DA performed on seven peaks selected based on significance by MANOVA (Wilks' $\lambda = 0.23$, $F = 2.62$, DF = 14, 34, $P = 0.0109$) with all variance in the data set explained by the first two discriminant functions (72.1% and 27.9%, respectively) (Figure 1A). When 30 peaks were used in stepwise DA, we identified five peaks that distinguished all three groups (Wilks' $\lambda = 0.19$, $F = 4.88$, DF = 10, 38, $P = 0.0002$) with function 1 (explaining 60.8% of variance) differentiating FOR-treated from RTP-treated queens and function 2 (explaining 39.2% of variance) distinguishing FOR-treated queens from solvent-treated ones (Figure 1B). The DA with five peaks permitted the correct classification of 96.2% of the individuals, better than the 73.1% correct classification obtained using seven peaks (significant in MANOVA) in DA. These five discriminating peaks were 5-methylnonacosane (5-MeC29), 5-methyldotriacontane (5-MeC32), tritriacontene (xC33:1), and two unidentified compounds.

The proportion of queens attacked by RTP workers was higher for RTP queens treated with FOR queen CHC (0.56) than for RTP queens treated with RTP queen CHC or solvent control (0.22) ($t = 2.45$, N = 4, $P = 0.0352$). CHC profiles of the solvent-treated queens that were attacked were more similar to the CHC profiles of queens treated with FOR CHC than other solvent-treated queens, while profiles of RTP CHC-treated

queens that were attacked were less similar to solvent-treated queens (controls) than the other RTP CHC-treated queens (Figure 1B). The DA using six peaks (significantly different between groups according to MANOVA) for attacked vs. not-attacked queens was not able to distinguish the two groups (Wilks' $\lambda = 0.71$, $F = 1.32$, DF = 6, 19, $P = 0.2955$) and only 73.1% of the queens were correctly classified (Figure 2A). However, the DA using four peaks selected from a total of 31 peaks by stepwise DA distinguished these two groups (Wilks' $\lambda = 0.43$, $F = 6.83$, DF = 4, 21, $P = 0.0011$) with 100% of variance explained by function 1 (Figure 2B) and 84.7% of the queens classified to the correct group. These four discriminating peaks were identified as n-heptacosane (n-C27), 5-MeC32, 5-methlytetratriacontane (5-MeC34), and xC33:1.

CHC Profiles of Queens in the Queen Adoption Assay

Queens from source colonies used in the queen adoption assay were distinguished by DA after data transformation using 16 peaks that differed among groups according to MANOVA with function 1 and function 2 explaining 81.0% and 11.2% of the total variation in the analysis (Wilks' $\lambda < 0.01$, $F = 7.23$, DF = 48, 63.3, $P < 0.0001$); all queens were correctly classified in this analysis. The stepwise DA on 24 transformed peaks selected 12 variables that clustered all queens according to their colony of origin (Wilks' $\lambda < 0.01$, $F = 13.11$, DF = 36, 74.6, $P < 0.0001$) with function 1 (86.1% of variation) separating CHH and RTP from both COC and FOR, and function 2 (9.5%) further separating CHH and RTP (Figure 3); all queens were correctly classified in this stepwise DA. Discriminating compounds selected in the stepwise DA were identified as 5-MeC29, xC33:1, and three unidentified compounds.

CHC Profile Similarities versus Queen Adoption and Queen Genetic Similarity

We found different levels of queen CHC profile similarities between colonies as indicated by generalized square distances between colony means (centroids) on DA canonical variables obtained using 12 transformed peaks identified by stepwise DA (Table 1). CHC similarities between colonies were positively associated with recipient colony response (queens adopted, attacked or killed) in queenless (R = 0.86, $P = 0.0276$) and single-queen host colonies (R = 0.90, $P = 0.0154$) with non-nestmate queens more likely to be attacked and killed with increasing distances between queen CHC profiles (Figure 4). In contrast, we found a weak association between CHC similarities and recipient response in multiple queen colonies (R = 0.66, $P = 0.1504$). Also, percent of non-nestmate queens adopted (averaged across queenless and queenright recipients) was associated with queen CHC profile similarities (R = -0.87, $P = 0.0228$).

We found a relationship between levels of queen-queen genetic similarity and queen CHC similarities between colonies (R = -0.85, $P = 0.0333$) with more genetically similar queens having greater CHC similarities (Figure 5A). Also, a positive yet not significant correlation (R = 0.79, $P = 0.0643$) was found between queen pairwise F_{ST} values and CHC similarities (Figure 5B).

Queen Adoption and Changes in Queen CHC

DA of cuticular lipids sampled by the non-destructive method showed that all queens could be distinguished and correctly classified into their colony of origin based on 13 peaks selected out of 29 peaks by stepwise DA, with function 1 (64.8% of variation)

separating COC and FOR from CHH and RTP, while function 2 (26.5% of variation) distinguished RTP from CHH (Wilks' $\lambda < 0.01$, $F = 13.95$, DF = 39, 62.9, $P < 0.0001$) (Figure 6). Squared distances between colony means obtained by DA of these 13 discriminating peaks (Table 1) were positively yet not significantly associated with those obtained for queens from sources used in the 24 h adoption assay and extracted by solvent ($R = 0.78$, $P = 0.0689$), even though queens were collected in different years. Moreover, identified discriminating peaks by stepwise DA included compounds selected when hexane-extracted queen CHC were analyzed (xC33:1, 5-MeC34, and three unidentified compounds).

The DA analysis of CHC of non-nestmate COC and nestmate FOR queens sampled 24 h before and two weeks after adoption by FOR colonies showed that queens could be distinguished based on eight peaks that differed among groups according to MANOVA (Wilks' $\lambda = 0.06$, $F = 4.37$, DF = 24, 61.5, $P < 0.0001$) and six peaks selected by stepwise DA (Wilks' $\lambda = 0.15$, $F = 8.51$, DF = 9, 63.4, $P < 0.0001$); the DA analyses showed that COC and FOR queens were correctly classified before introduction but an adopted FOR queen was classified as a COC queen before adoption; all adopted COC queens were correctly classified. Also, the distance between centroids for COC queens before and after adoption (0.20) using peaks selected by stepwise DA was not greater than for FOR queens before and after adoption (9.27), suggesting that COC queens did not change their profiles so as to resemble more those of FOR queens (Figure 7). However, after adoption FOR and COC queens were less dissimilar than before adoption as indicated by a reduction in the distance between centroids of these two colonies (from 19.11 to 5.52).

CHH and RTP queens could also be distinguished based on five peaks that were significantly different according to MANOVA (Wilks' $\lambda = 0.06$, $F = 4.45$, DF = 15, 39.1, $P < 0.0001$) and 11 discriminating peaks selected by stepwise DA were able to differentiate queens among these four groups (Wilks' $\lambda < 0.01$, $F = 15.38$, DF = 33, 24.3, $P < 0.0001$). These discriminating peaks classified all queens into their corresponding group, even after queen adoption. Unexpectedly, however, the distance between centroids of adopted CHH and RTP queens increased (Figure 8), possibly because few CHH queens were analyzed. These results relate to flexibility of queen CHC composition in cases in which foreign *L. humile* queens are adopted by queenless colony fragments, while results from the colony fusion assay illustrate queen CHC profile variation in groups with unrelated mixed queens.

CHC Profiles of Workers in the Colony Fusion Assay

Workers from source colonies used in the colony fusion assay were distinguished by 28 peaks significantly different between colonies according to MANOVA (Wilks' $\lambda < 0.01$, $F = 82.76$, DF = 112, 54.2, $P < 0.0001$), with function 1 explaining 88.3% of the total variation and function 2 accounting for 5.2% of the variation. The stepwise DA selected 18 variables that grouped all workers according to their colony of origin (Wilks' $\lambda < 0.01$, $F = 151.23$, DF = 72, 92.8, $P < 0.0001$) with functions 1 and 2 explaining 18.5% and 26.2% of the total variation (Figure 9); as before, DA analyses classified all individuals to the correct colony. Among the discriminating peaks selected in stepwise DA we identified 3-MeC29, 3-MeC31, dimethylhentriacontane (x,y-diMeC31), and four unidentified compounds.

Worker CHC Profile Similarities versus Worker-Worker Aggression, Colony Fusion, and Genetic Similarity

The levels of similarity of source worker CHC profiles varied between colony pairs as indicated by generalized square distances between colony centroids obtained using the 18 peaks identified by stepwise DA (Table 2). We found a positive relationship between worker CHC similarities between colonies and levels of worker-worker aggression (R = 0.66, $P = 0.0375$) with aggression increasing with greater worker CHC profile dissimilarities between colonies (Figure 10). We also found a strong relationship between similarities of worker CHC and both the percentage of alleles shared (R = -0.82, $P = 0.0038$) and pairwise F_{st} (R = 0.73, $P = 0.0163$) (Figure 11A,B), suggesting that these sets of compounds not only distinguished workers from different colonies but may also be linked to levels of worker genetic similarity between colonies. In contrast, we did not find an association between source worker CHC similarities and colony fusion either at 24 h (R = -0.40, $P = 0.2493$) or at 6 months (R = -0.52, $P = 0.1192$) (Figure 12A). However, we found a correlation between control worker CHC similarities (Table 2) and colony fusion at 24h (R = -0.78, $P = 0.0075$) and 6 months (R = -0.79, $P = 0.0061$) (Figure 12B). Similarly, CHC of queens from control colonies (Table 2) were associated with colony fusion at 24 h (R = -0.69, $P = 0.0280$) and 6 months (R = -0.75, $P = 0.0116$).

Colony Fusion and Changes in Queen and Worker CHC

Queens from control colonies were distinguished by DA using 20 peaks that were significantly different among colonies based on MANOVA with function 1 and function

2 explaining 78.0% and 14.8% of the total variation (Wilks' $\lambda < 0.01$, $F = 7.24$, DF = 80, 89.2, $P < 0.0001$). The stepwise DA performed on 25 peaks selected 9 peaks that distinguished queens from different colonies (Wilks' $\lambda < 0.01$, $F = 13.63$, DF = 36, 125.4, $P < 0.0001$) with function 1 and 2 explaining 83.6% and 11.3% of the total variation (Figure 13); one CHH queen was misclassified. All peaks shown to differ among colonies included the same discriminating compounds identified in the queen adoption assay but nonacosene (xC29:1) and n-triacontane (n-C31) were also identified.

When we compared control queen CHC with those of five colony pairs (CAR-CHH, CAR-COC, CAR-RTP, CHH-FOR, CHH-RTP) we found that 21 out of 30 peaks differed significantly among groups ($F = 3.85$, DF = 189, 534.1, $P < 0.0001$), although 19.6% of the control queens were misclassified. The stepwise DA selected 12 peaks that distinguished COC, FOR, CHH-FOR and CAR-COC queens from all the other groups (Wilks' $\lambda < 0.01$, $F = 5.81$, DF = 108, 529.4, $P < 0.0001$) with function 1 explaining 79.6% of variation and function 2 explaining 6.5% of variation (Figure 14). However, some control queens were classified into either other control groups or colony pairs (23.9% misclassified queens). To better examine queen CHC profiles of colony pairs and controls, we computed discriminant functions for each colony pair using discriminant function coefficients estimated from 9 discriminating peaks for control colonies. When we plotted CHC profiles of each colony pair with profiles of their respective unpaired controls we found that CHC profiles of queens in colony pairs that fused (CAR-RTP, CHH-RTP and one CAR-CHH replicate) were found scattered throughout the control groups (Figure 15) indicating that mixed queens could not be distinguished according to colony of origin, as for example in the CAR-RTP pair where CHC profiles of queens of

known identity (5 CAR and 6 RTP) were not more similar to those of their respective controls. Similarly, CHC profiles of CAR and CHH queens in CAR-CHH were more similar to CHH control queens suggesting that CAR queens may have acquired CHH hydrocarbons, although in the CHH-RTP pair queens of known identity seemed not to have changed their CHC drastically. CHC of queens matched those of their respective unexposed control in colony pairs that did not fuse (CAR-COC and CHH-FOR) (Figure 16), as for example in CHH-FOR where all queens were known to be FOR and their CHC profiles matched those of the FOR control group.

CHC profiles of workers from all five control colonies and five colony pairs were distinguished by DA using 21 peaks significantly different based on MANOVA (Wilks' $\lambda < 0.01$, $F = 3.14$, DF = 189, 384.4, $P < 0.0001$) with function 1 (54.1% of variation) clearly distinguishing COC and FOR from CAR, CHH, and RTP, while function 2 (16.0 % of variation) separated RTP and four colony pairs from CAR and CHH, although one CARY queen was classified as CHH. Stepwise DA performed on 26 variables identified 11 peaks that distinguished workers among these ten groups (Wilks' $\lambda < 0.01$, $F = 5.09$, DF = 99, 392.3, $P < 0.0001$) with function 1 and 2 explaining 52.7% and 14.7% of the total variation (Figure 17), but one CHH and one RTP worker replicate were misclassified. When we plotted CHC profiles of each colony pair with profiles of their respective unpaired controls using 9 discriminating peaks, we found that worker CHC profiles in colony pairs that fused (CAR-RTP, CHH-RTP and one CAR-CHH replicate) were very similar to those of their unexposed controls although they were not scattered throughout control CHC profiles as did queen CHC profiles, probably because each sample consisted of a mixture of 10 workers of unknown identity (Figure 18). However,

we can infer that the average CHC profiles of workers in fused colonies reflect a homogenized CHC composition of both colonies. In the non-fused CAR-COC, samples were taken from replicates that were either CAR or COC and workers matched CHC profiles of CAR or COC controls (Figure 19). Interestingly, in CHH-FOR pairings all CHH queens were killed, but CHH and FOR workers mixed, which could partially explain the distinct CHC profile of CHH-FOR workers.

Discussion

Our findings contribute to our understanding of the *L. humile* nestmate recognition system. We demonstrate that CHC are used as queen recognition cues by *L. humile*, that southeastern colonies of this invasive ant can be distinguished based on unique CHC profiles, and that similarity of CHC profiles of unrelated colonies is positively associated with non-nestmate queen adoption and colony fusion in *L. humile*. Previous studies have demonstrated that CHC are used as nestmate recognition cues in this species and that they modulate intraspecific worker-worker aggression (Liang and Silverman 2000, Liang et al. 2001, Suarez et al. 2002, Buczkowski et al 2005, Buczkowski and Silverman 2006), with one study examining the effect of prey-derived CHC on interactions between colonies (Silverman and Liang 2001). Our findings confirm that these chemical cues modulate the intensity of worker-worker aggression, and reveal that similarity of CHC profiles is inversely related to aggression and positively associated with worker genetic. Similarly, we found a relationship between similarity of queen CHC and levels of queen-queen genetic similarity. Together, these results suggest that the CHC element of the *L. humile* nestmate recognition system has an important genetic component. Also, the CHC patterns of fused colonies suggest that changes in colony genotypic composition can lead to changes in recognition cues among colony members with subsequent recognition template expansion and lower aggression, leading to more open colonies that accept non-nestmates matching a certain level of cue-template similarity. Overall, we suggest that discrimination abilities are important in structuring *L. humile* societies, and further

support the idea that expansive colonies in the introduced range can result from mixing of unrelated colonies that share a certain level of phenotypic similarity.

Nestmate Recognition, Queen Adoption, and Queen CHC

The aggressive responses of workers towards nestmate queens that had been treated with CHC purified from non-nestmate queens support our first conclusion, that CHC are important cues in nestmate queen recognition. Queens were distinguished based upon their CHC profiles, with most queens that were treated with non-nestmate CHC grouping together. The few unexpectedly attacked queens (treated with nestmate CHC or solvent only) were either more similar to the non-nestmate CHC-treated queens or less similar to the solvent control than other queens in the group, suggesting that the gentle rotation of ants in glass vials could have affected CHC profiles (e.g., of solvent-treated controls accidentally removing some CHC). Alternatively, physiological or behavioral variability among queens within a colony might have been responsible. These concerns could be addressed in future experiments by testing queens of known age, reducing the time of exposure to minimize unintended CHC removal, working with more inert substrates (e.g., silanized glass) or by direct application of precise CHC quantities to queens. Nevertheless, as indicated by the distinct CHC profiles of attacked and non-attacked queens, changes in the relative proportions of queen CHC are associated with worker aggressive response. Interestingly, compounds that appear to be associated—at least statistically—with worker behavior towards queens were mono-methyl alkanes and alkenes that are either absent or occur in considerably lower quantities in the CHC of workers. Different classes of CHC may have different roles in ants, as for example in

Pachycondyla villosa where internally branched mono- and di-methylalkanes and monomethylalkenes may constitute the colonial signature, while n-alkanes and externally branched monomethylalkanes seem to be involved in waterproofing (Lucas et al. 2004).

Similarity of the CHC of introduced and resident queens appeared to guide the responses of workers (queens adopted, attacked or killed) in resident queenless and single-queen colonies. The selective responses of workers suggest that they recognize nestmate queens by matching the recognition cues of the newly encountered queen with their internal template. This memorized template may also allow workers in queenless colonies to recognize non-nestmate queens even in the absence of resident queens. Thus, if the match is below a dissimilarity threshold, queens are accepted regardless of their colony of origin. However, the behavioral responses of workers in multiple queen colonies were more independent of the similarity of the CHC of introduced and resident queens, suggesting that the acceptance threshold is influenced by the social context. It is possible that the slight heterogeneity of CHC among multiple queens relaxes the stringency of the workers' internal template, thus lowering the queen acceptance threshold. Queen presence affects worker aggressive behavior in other ants (Vienne et al. 1998, Provost 1989, Boulay et al. 2003), and it is possible that *L. humile* queen pheromones influencing other aspects of recognition, including aggression towards female sexual larvae (Passera et al. 1995), may also affect nestmate recognition. 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 if a colony's survival is at high risk a reduction in the cost of accepting foreign conspecifics is expected (Sudd and Franks 1987). The positive relationships

between queen adoption and similarity of queen CHC, and between CHC similarity and overall queen-queen genetic similarity, suggest that queen recognition cues have a genetic component, implying that rejection of genetically similar yet unrelated queens might have been favored by the high cost of erroneously rejecting nestmate queens combined with the low cost of accepting non-nestmate queens (Reeve 1989).

Non-destructive CHC sampling allowed us not only to record differences in CHC profiles between colonies, but also to detect slight temporal changes in these patterns. Analysis of relative proportions of queens CHC before and after adoption showed that non-nestmate adopted queens did not change their CHC profiles more than nestmate adopted queens, and that they maintained their colony-characteristic CHC profiles, suggesting that queens may not acquire CHC from workers. Interestingly, profiles of *L. humile* queens have been shown not only to differ significantly from worker CHC, but also to be dynamic, changing quantitatively and qualitatively according to the queen's ovarian activity (de Biseau et al. 2004). Mated egg-laying queens have predominantly monomethylalkanes (5-MeC27 to 5-MeC34) and alkenes (C29:1, C31:1, C33:1) (de Biseau et al. 2004), while workers are largely represented by dimethylalkanes and trimethylalkanes (diMe- and triMeC33, C35 and C37) (Liang et al. 2001). These qualitative differences could result from selective biosynthesis of CHC, with shorter chain monomethylalkanes predominantly produced by queens through enzymes that regulate the generation of hydrocarbons of different chain length (Blomquist et al. 1998), or by selective transfer of CHC from oenocytes to the cuticle via lipophorin (Schal et al. 2003). The lack of cue exchange between queens and workers could be related to the distinct CHC profiles of these two castes. In our assays, queens acquired queen CHC

mechanically from glass surfaces. Similarly, when workers of this species were exposed to large quantities of exogenous CHC they incorporated long-chain CHC (C35-C37) within the range of their intrinsic CHC (Liang et al. 2001). It is not known, however, whether in natural interactions queens or workers would selectively acquire more queen or worker CHC. It has been suggested that unlike other ant species where colony odor is derived from the queen (Carlin and Hölldobler 1986) or transferred from worker to queen (Lahav et al. 1998), *L. humile* represents an alternative model for colony odor formation since reproductives and non-reproductives have very different CHC profiles (de Biseau et al. 2004). Therefore, *L. humile* colonies appear to lack a unified colony gestalt odor, and have instead two subsets of odors, one originating from queens and another from workers. Therefore the lack of queen CHC change in our assays could be because *L. humile* queens do not to contribute to the worker gestalt and vice versa. Similarly, queens appear not to be important contributors to the colony Gestalt having queen-specific profiles in other ants (Boulay et al. 2003, Dahbi and Lenoir 1998, Dietemann et al. 2003) as well. As already suggested, the slight divergence in the proportions of CHC of adopted nestmate and non-nestmate queens after two weeks may relate to changes in their social environment (e.g., no contact with other queens) or physiological changes (e.g., increased egg-laying rates in the absence of other queens).

Intraspecific Aggression, Colony Fusion, and CHC Profile Flexibility

By examining worker CHC profiles we found that quantitative variation of CHC reflects colony identity, and that not all but a statistically-derived subset of variable compounds could mediate colony discrimination. Worker CHC profile similarity of was inversely

correlated with worker-worker aggression and positively correlated with overall genetic similarity of colony pairs, which further supports the view that the *L. humile* recognition system has an important genetic component (Tsutsui et al. 2000, Suarez et al. 2002), and contrary to the view that there is little association between genetic variability at neutral markers and at loci involved in nestmate recognition cue expression (Giraud et al. 2002). In *L. humile*, genetically-based cues appear to play a major role in nestmate discrimination in genetically diverse populations (Buczkowski and Silverman 2006), whereas in populations with reduced genetic variability environmentally (prey)-derived CHC appear to be important contributors to *L. humile* worker recognition. Genetically based recognition systems are important in other ant species (Beye et al. 1998, Stuart and Herbers 2000, Pirk et al. 2001) as well as in termites where genetic relationships among colonies reflect CHC variation (Kaib et al. 2004, Dronnet et al. 2006).

In contrast to the weak positive association between similarity of source worker CHC and colony fusion, CHC similarity between workers from control colonies was correlated with colony fusion. Temporal variation in worker CHC profiles may explain these results. Workers from source colonies sampled at the beginning of the experiment may have both exogenous and endogenous derived CHC while profiles of workers from control colonies may mostly reflect intrinsic CHC as they were sampled 6 months after the start of the experiment. The weak association between source worker CHC and fusion may also suggest that factors other than recognition cue phenotypic similarity (e.g. colony phenology, caste ratios, worker age) govern the outcome of group interactions. We found that in some colony pairs, queen CHC profiles were more similar than the CHC profiles of workers, suggesting that while workers may not be aggressive toward

foreign queens they might be aggressive to workers from the same foreign colony. Therefore, the outcome of group interactions may not exclusively reflect worker discrimination capability, or individual worker interests, but that of the whole group.

We found that colony fusion led to changes in queen and worker CHC profiles. Profiles of individual queens in fused colony pairs were found scattered throughout the profiles of both source colonies, and not as a distinct group resembling either the parent colony or an intermediate CHC profile. This, together with the observation that queens could be distinguished according to their colony of origin in colony pairs that did not fuse, suggests that by exchanging CHC, queens match phenotypes in both colonies that ultimately fuse. We examined changes in CHC profiles of groups of 10 workers, not of individual workers, in fused colony pairs. However, the collective worker CHC composition suggests that a mixture of CHC between colonies could have occurred. Alternatively, individual workers may have retained their own CHC and instead the template changed to accommodate other CHC phenotypes. Transfer of CHC between individuals of the same colony, between mixed species, and in dulotic and inquiline species has been well documented (Soroker et al. 1994, Howard et al. 1980, Vander Meer and Wojcik 1982, Kaib et al. 1993). Cue exchange within castes could have occurred through direct body contact, grooming and trophallaxis in the same way interactions with adult workers allow newly eclosed ant workers (callows) to acquire colony's odor (Vander Meer and Morel 1998) or interactions with heterospecifics result in mixed hydrocarbon profiles in ants (Vienne et al. 1995).

The homogenization of colony CHC between fused colony pairs may explain the reduced aggression observed towards both unpaired control colonies (Chapter 2), while

the high aggression observed between non-fused colony pairs (winning colony) and their respective unpaired controls (defeated colony) after 6 months may be explained by maintenance of colony chemical signature. Worker CHC profile similarity between field and laboratory *L. humile* colonies has been associated with levels of intraspecific aggression (Suarez et al. 2002). In addition, we found that changes in worker and queen CHC profiles are linked to reduced aggression in fused colony pairs and that fusion is the process mediating these changes. Variation in colony genotypic composition through mixed workers and queens may lead to the formation of a new colony odor, implying that an updated recognition template must also be learned. It has been proposed that the greater the dissimilarity in CHC profiles between ant species that mix experimentally in the lab, the lower the aggression towards other ant species due to a broader template (Errard et al. 2006). Similarly, increased phenotypic cue diversity in fused colonies should result in a much broader template thereby, having implications at the population level since changes in social structure may arise from changes in recognition cue diversity and/or template formation. Therefore, by increasing colony phenotypic diversity, fusion between unrelated colonies may be a proximate mechanism involved in the formation of expansive *L. humile* supercolonies in the introduced range.

CHC and Argentine ant Colony Signature

We have shown that by direct manipulation of CHC we can affect aggression behavior in *L. humile* workers, but whether all or only some of the CHC are important in nestmate recognition remains unknown. Based on our findings, however, we suggest that alkenes and monomethylalkanes are important queen discriminators, while dimethyl- and

trimethylalkanes and other unidentified long chain CHC are important in worker recognition. Methyl-branched alkanes, n-alkanes, and an alkene/n-alkane mixture have been shown to be important colony recognition cues in wasps (Dani et al. 1996, Gamboa et al 1996), and in ants methyl-branched CHC are more colony-specific than n-alkanes (Bonavita-Cougourdan et al. 1987, Provost et al. 1992, Astruc et al. 2001), although dimethyl alkanes seem not to be important in nestmate recognition in *Cataglyphis* species (Dahbi et al. 1996). These findings reflect considerable variation in the role of specific compounds or chemical classes as nestmate recognition cues among social insects. We cannot rule out that additional recognition-active compounds other than those that seem to be linked to the colony's chemical profile specificity may also be important. Therefore, chemical supplementation studies testing these presumably important CHC structural classes or the compounds individually or in mixtures, and at different concentrations, would corroborate our findings.

In a Gestalt model, chemical cues are transferred among workers resulting in a mixture of cues unique to the colony, recognition cues are learned by nestmates forming a gestalt template that represents the colony's composition, and individuals make acceptance decisions according to a certain level of deviation from the gestalt (Crozier and Dix 1979, Breed and Bennett 1987). A graded recognition response (or non-threshold response) may suggest a colony odor Gestalt given that change in colony composition could lead to a variety of responses to levels of template-cue similarity. Although *L. humile* seems to recognize nestmates by means of phenotype matching, the distinct CHC profiles of queens and workers and the statistical identification of different chemical classes distinguishing queens and workers from different colonies suggest that *L. humile*

does not form a unique colony odor distributed among all colony members (castes), and that individuals may have two kinds of recognition templates (reproductives and sterile workers) that may constitute a more complex template. A similar mechanism of template formation has been suggested for mixed-species groups in which individuals seem to learn and memorize allospecific cues early in adult life (Errard 1994). We cannot exclude the possibility that individuals bear their own endogenous cues and that these are matched with a learned Gestalt-type template. Alternatively, CHC present in both queens and workers, although in different relative proportions, could be used as colony recognition cues, thus, a single mean template would suffice. In *Camponotus vagus*, dimethylalkanes are present across all castes and are thought to be the colony chemical cues, while specific n-alkanes and monomethylalkanes characterize larvae, workers, sexuals, and queens (Bonavita-Cougourdan et al. 1993). If queen CHC signaling ovarian activity are present in such large quantities that variation in CHC proportions for other minor compounds in queens is difficult to detect, it is possible that our statistical analyses may reflect variation in reproductive status rather than colony membership. Therefore, studies examining the role of caste-specific vs. colony-specific cues may further clarify the nature of the nestmate recognition cues in this species.

This study provides insights into *L. humile* expression, perception and action components of recognition (Sherman and Holmes 1985, Waldman 1987, Reeve 1989, Gamboa et al. 1991). We show that cuticular hydrocarbons are used as queen recognition cues, and that the discriminating compounds are different from those of workers, suggesting a high level of recognition cue complexity as they may convey information regarding both ovarian activity and colony membership. We demonstrate that unrelated

colonies can fuse given certain level of chemical recognition cue similarity tightly linked to the overall genetic similarity, and that fused colonies posses an odor reflecting the phenotypic variability of both colonies, suggesting the formation of a broader template based on recognition cues from mixed individuals. We also provide evidence for the role of the social context in modulating the perception of recognition cues, with more permissive thresholds for non-nestmate queen acceptance in queenless conditions. Our combined behavioral, chemical, and genetic data shed light on the dynamics and complexity of nestmate recognition in *L. humile* and the effects of interspecific variation in CHC on colony-level consequences: Argentine ant colonies showed varying levels of cuticular hydrocarbon similarities, the degree of cuticular hydrocarbon similarities modulated levels of intercolony intraspecific aggression and queen adoption, and consequently, colonies either fused to form new colonies with more diverse recognition cues and a broader template, or fought until colony elimination. We suggest that a better understanding of the recognition process in this and other invasive ants would greatly contribute to elucidation of the factors responsible for changes in their social organization and ecological success.

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Table 1. Generalized squared distances between colony means (centroids) calculated by discriminant analysis of CHC of queens from colonies used in the queen adoption assay extracted by solvent or sampled using a non-disruptive method.

	Solvent extraction		Non-destructive sampling	
Colony pair	16 variables[a]	12 variables[b]	10 variables[a]	13 variables[b]
CHH-COC	92.92	129.03	26.50	136.47
CHH-FOR	64.97	85.48	14.25	68.86
CHH-RTP	17.46	13.23	12.32	29.09
COC-FOR	22.43	30.16	21.60	69.85
COC-RTP	105.97	155.67	28.99	95.56
FOR-RTP	69.68	100.46	16.60	70.62

[a] Transformed variables selected based on MANOVA.

[b] Transformed variables selected using stepwise discriminant analysis.

Table 2. Generalized squared distances between colony means (centroids) calculated by discriminant analysis of CHC of workers and queens from colonies used in the fusion assay.

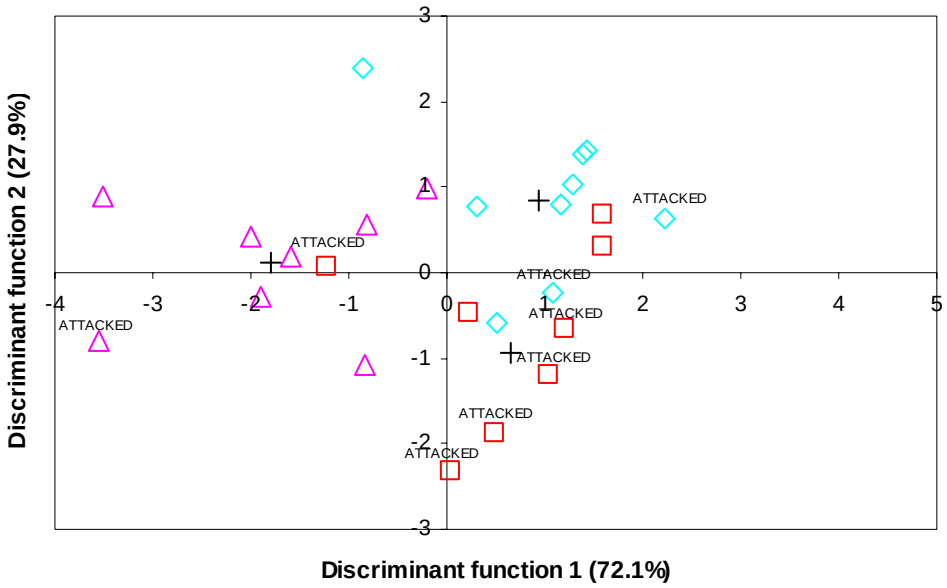
Colony pair	Workers		Queens	
	18 variables[b,c]	10 variables[b]	20 variables[a]	9 variables[b]
CAR-CHH	1026.00	120.96	27.38	8.52
CAR-COC	4373.00	243.19	196.03	123.19
CAR-FOR	1934.00	174.85	208.51	105.36
CAR-RTP	184.01	24.83	13.08	7.50
CHH-COC	4461.00	140.56	193.07	122.32
CHH-FOR	741.82	98.24	186.74	96.46
CHH-RTP	1094.00	51.06	17.21	8.09
COC-FOR	2746.00	46.71	79.28	36.64
COC-RTP	4521.00	205.91	164.30	111.14
FOR-RTP	2101.00	144.86	196.96	103.63

[a] Transformed variables selected based on MANOVA.

[b] Transformed variables selected using stepwise discriminant analysis.

[c] Distances between workers from source colonies sampled at the beginning of the experiment. All other values are for workers and queens from control colonies.

A



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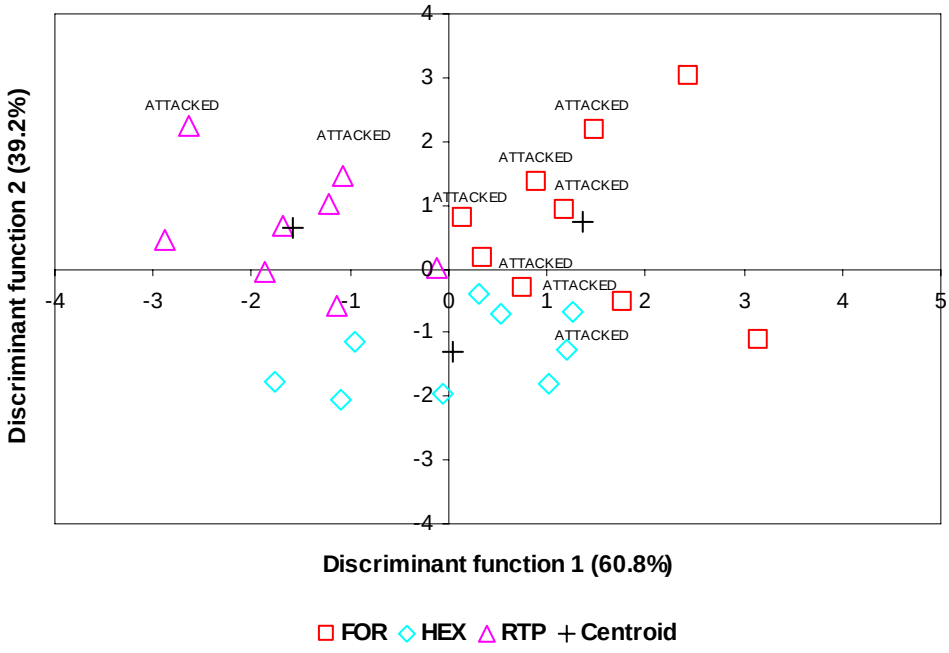
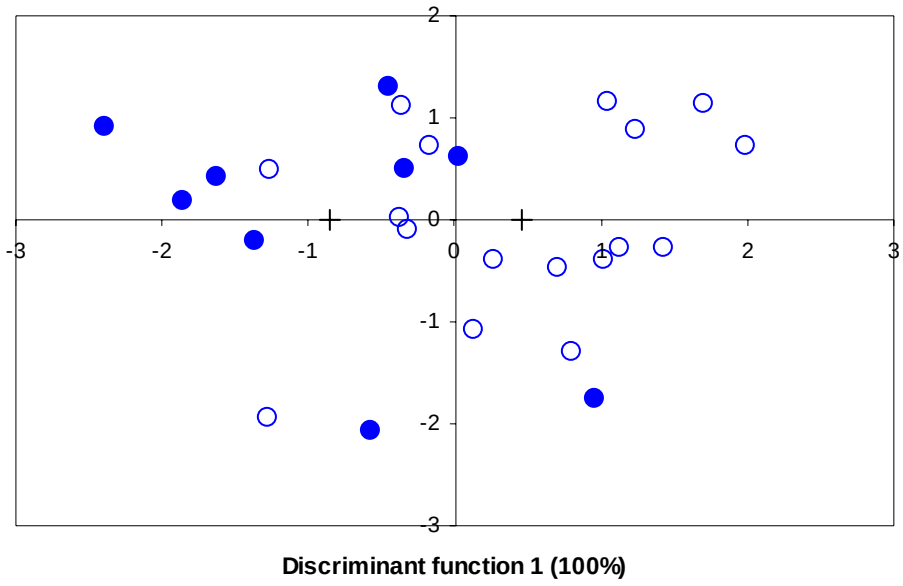


Figure 1. Discriminant analyses of seven (A) and five (B) variables (relative proportions of cuticular hydrocarbons) selected from MANOVA and by stepwise discriminant analysis, respectively, for three groups of *L. humile* queens each treated with nestmate queen hydrocarbons (RTP), non-nestmate queen hydrocarbons (FOR), and hexane (HEX). The centroid of each group is marked by a +.

A



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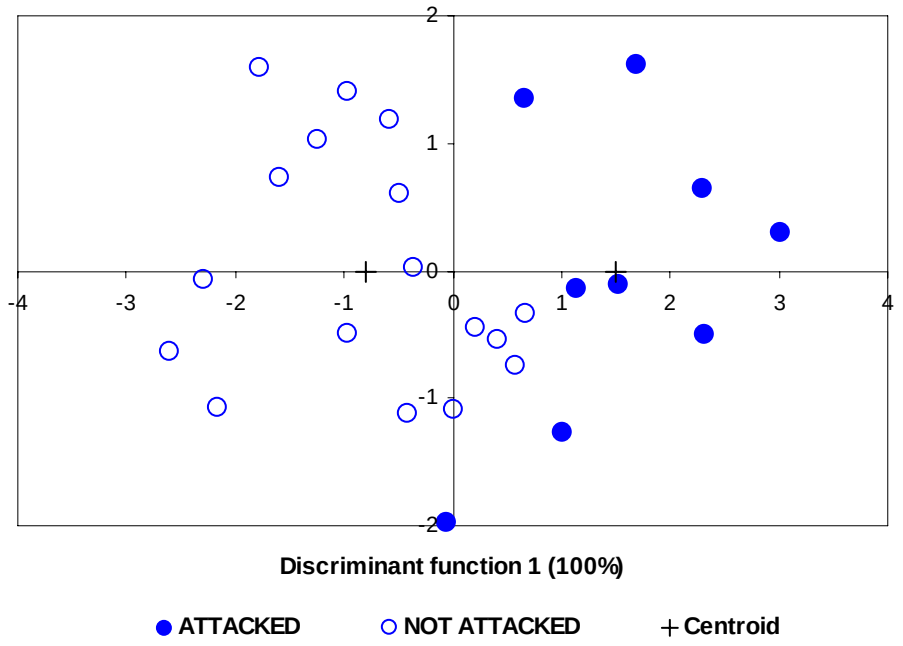


Figure 2. Discriminant analyses of six (A) and four (B) variables selected from MANOVA and by stepwise discriminant analysis, respectively, for two groups of treated *L. humile* queens that were either attacked or not attacked by nestmate workers. The centroid of each group is marked by a +.

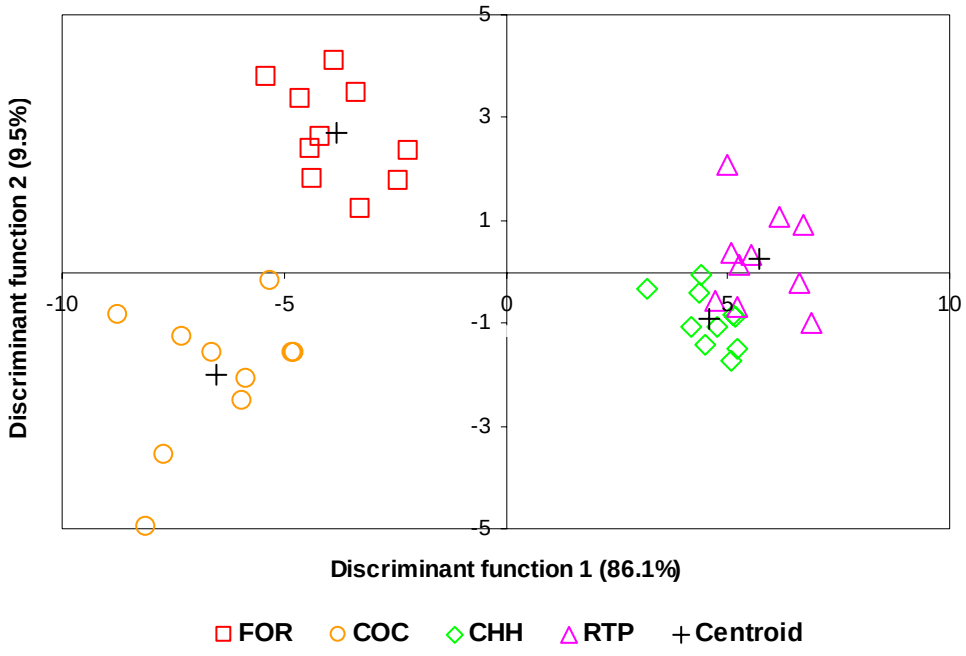


Figure 3. Discriminant analysis of 12 variables selected by stepwise discriminant analysis for queens from four *L. humile* colonies (CHH, COC, FOR, RTP). The centroid of each group is marked by a +.

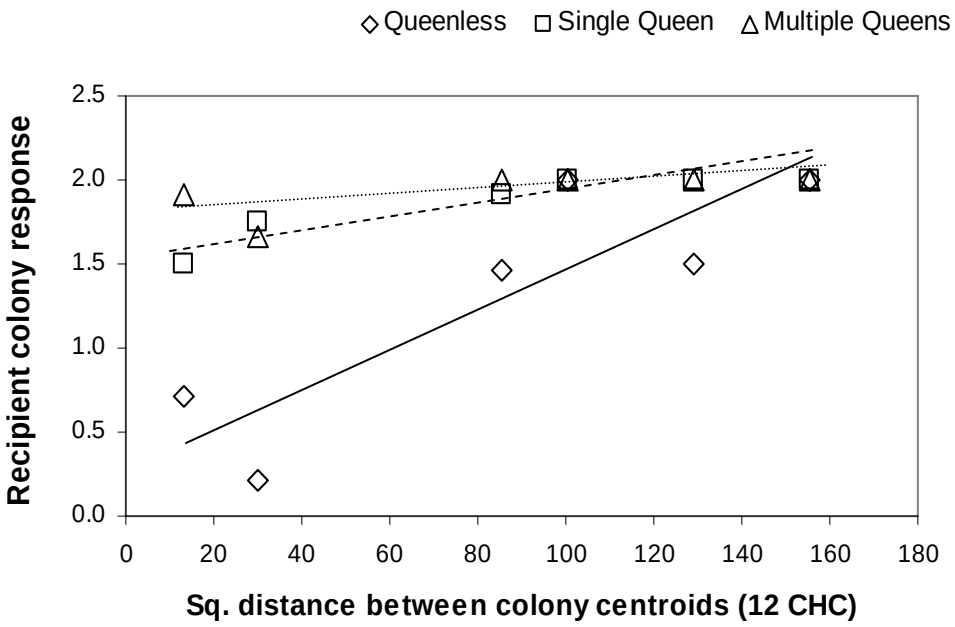
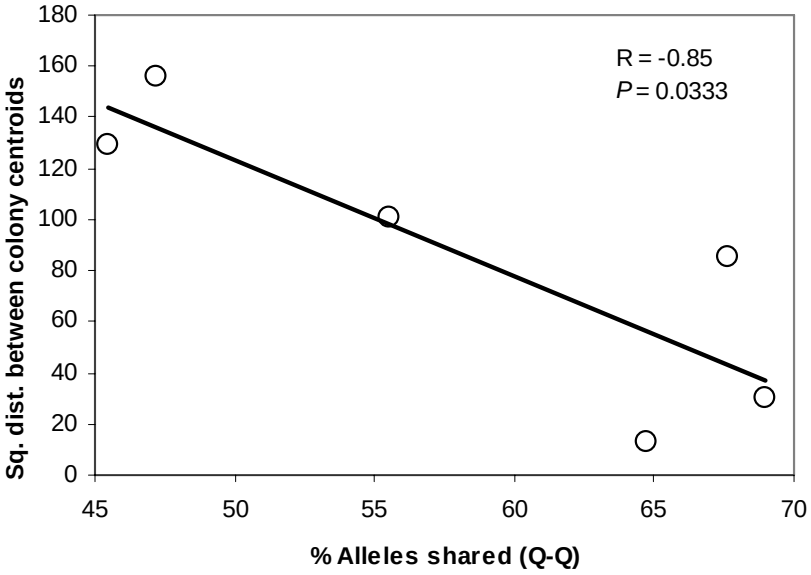


Figure 4. Relationship between queen cuticular hydrocarbon profile similarities (generalized square distance between colony centroids) based on 12 variables and recipient colony response (0 = adoption, 1 = physical attack, 2 = intruder killed) to non-nestmate queens introduced in queenless (◇), single queen (□), and multiple queen (△) colonies.

A



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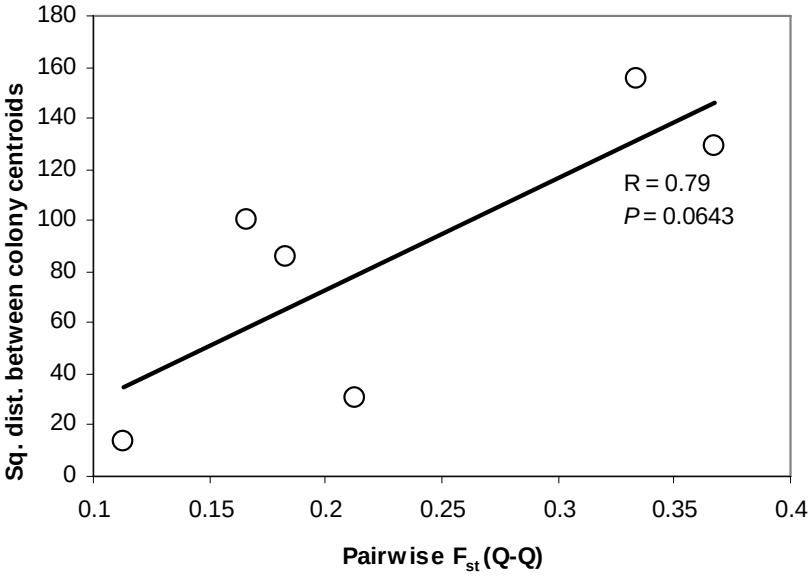


Figure 5. Relationship of queen cuticular hydrocarbon profile similarities based on 12 variables vs. % alleles shared (A) and pairwise F_{st} (B).

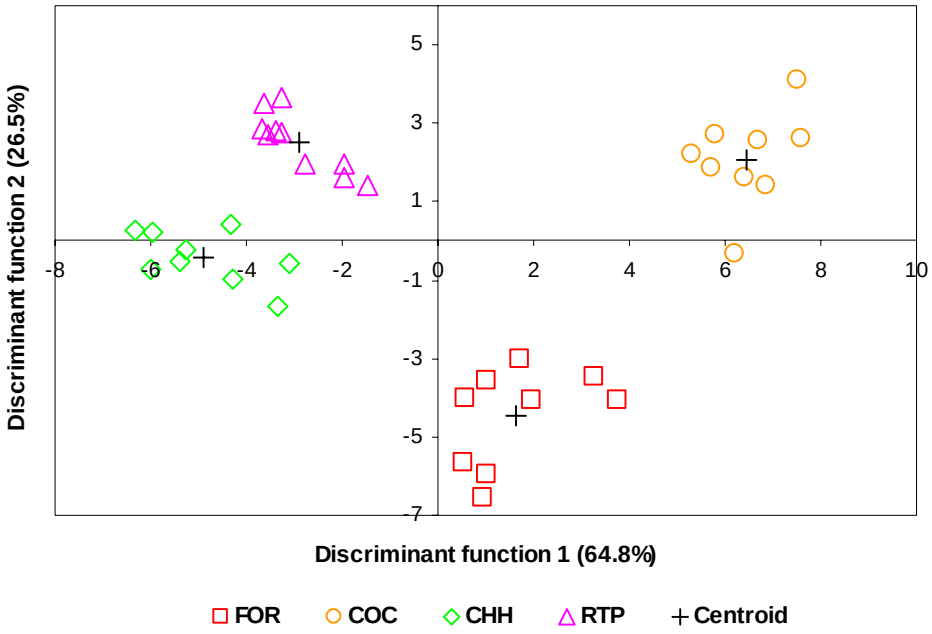


Figure 6. Discriminant analysis of 13 variables selected by stepwise discriminant analysis for queens from four *L. humile* colonies (CHH, COC, FOR, RTP). Cuticular hydrocarbons were sampled using a non-destructive method. The centroid of each group is marked by a +.

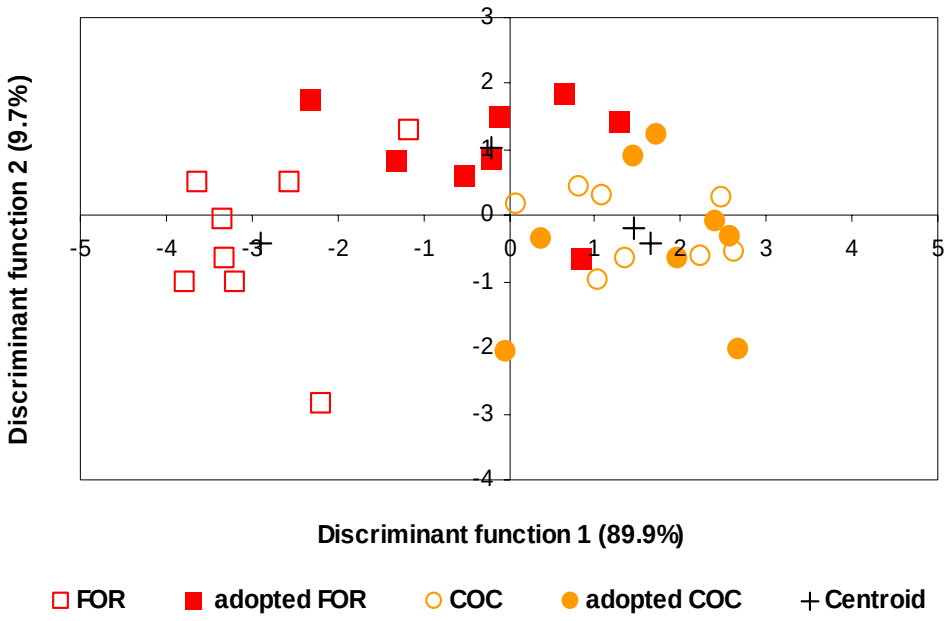


Figure 7. Discriminant analysis of three variables selected by stepwise discriminant analysis for queens from two *L. humile* colonies (COC and FOR). Cuticular hydrocarbons of queens were sampled 24 h before introduction and 2 weeks after adoption by a FOR queenless recipient colony using a non-disruptive method. The centroid of each group is marked by a +.

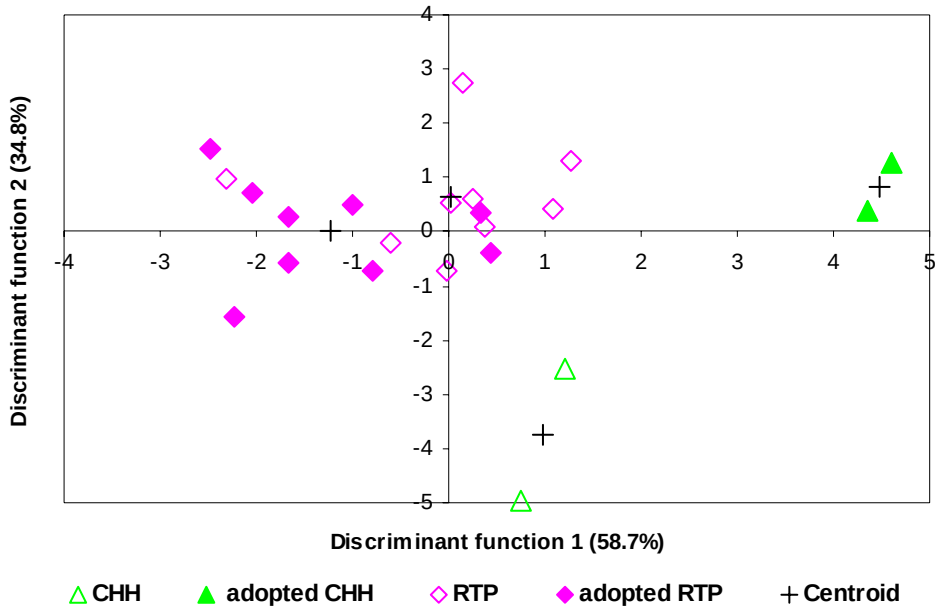


Figure 8. Discriminant analysis of 11 variables selected by stepwise discriminant analysis for queens from two *L. humile* colonies (CHH and RTP). Cuticular hydrocarbons of queens were sampled 24 h before introduction and 2 weeks after adoption by a RTP queenless recipient colony using a non-destructive method. The centroid of each group is marked by a +.

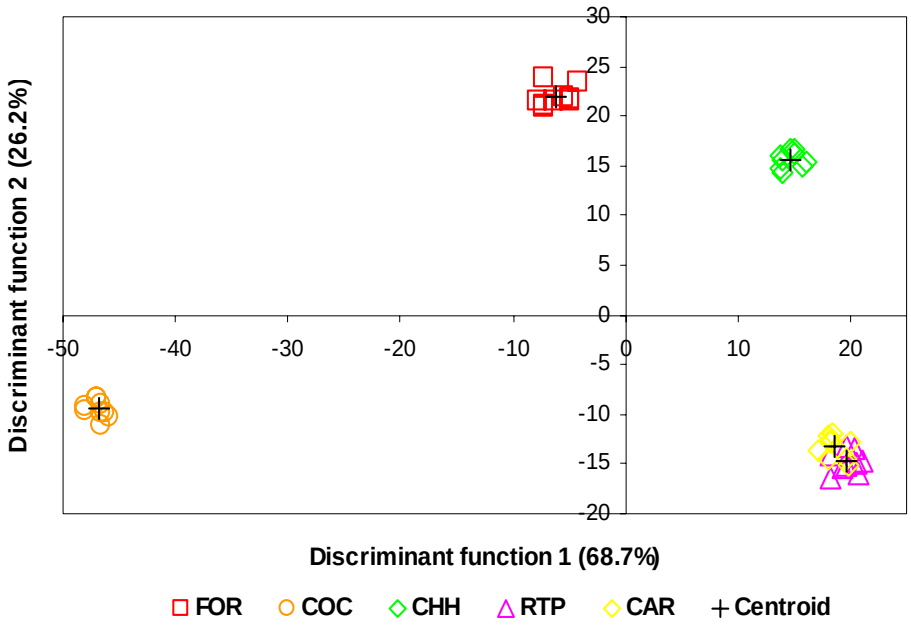


Figure 9. Discriminant analyses of 18 variables selected by stepwise discriminant analysis for workers from five *L. humile* colonies (CAR, CHH, COC, FOR, RTP). The centroid of each group is marked by a +.

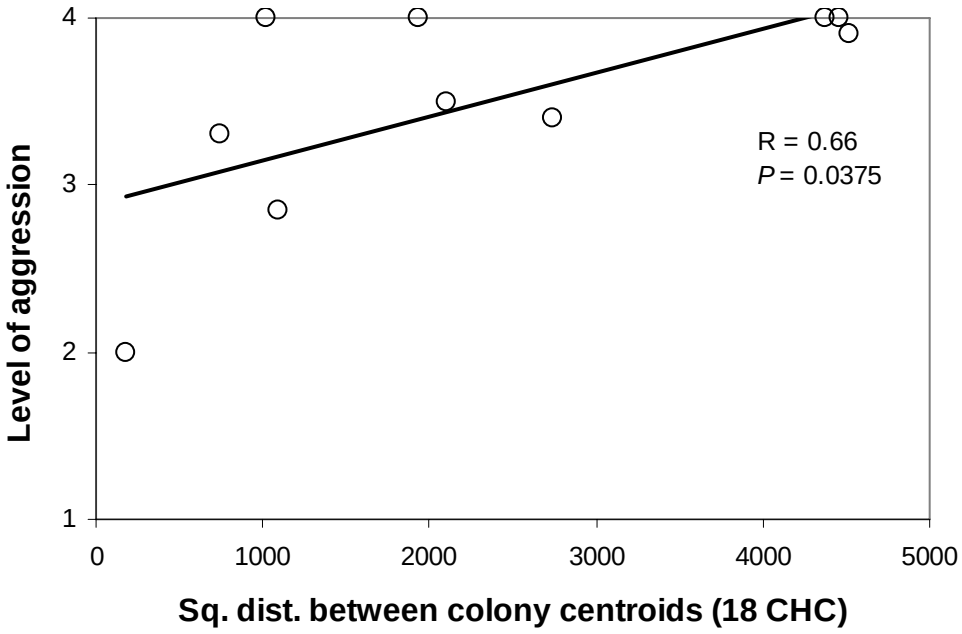
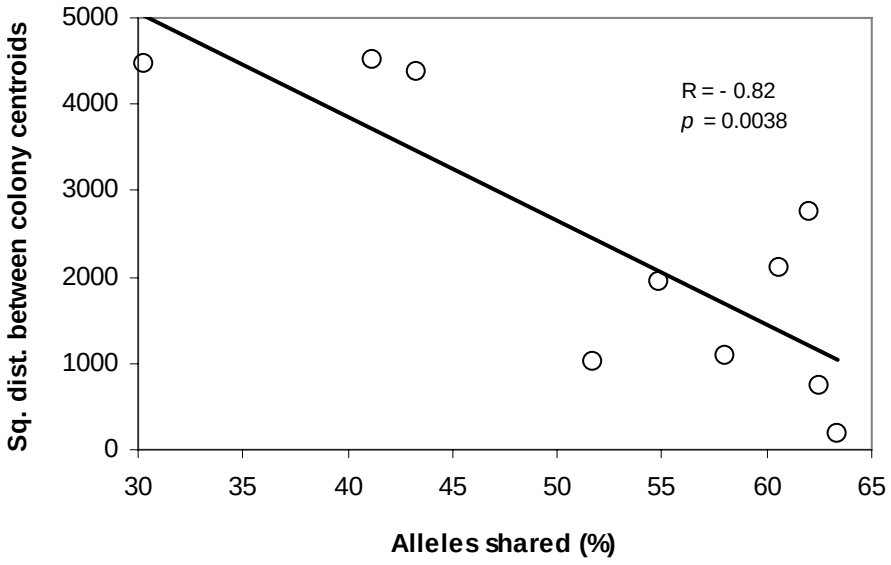


Figure 10. Relationship between worker cuticular hydrocarbon profile similarities based on 18 variables and levels of worker intraspecific aggression.

A



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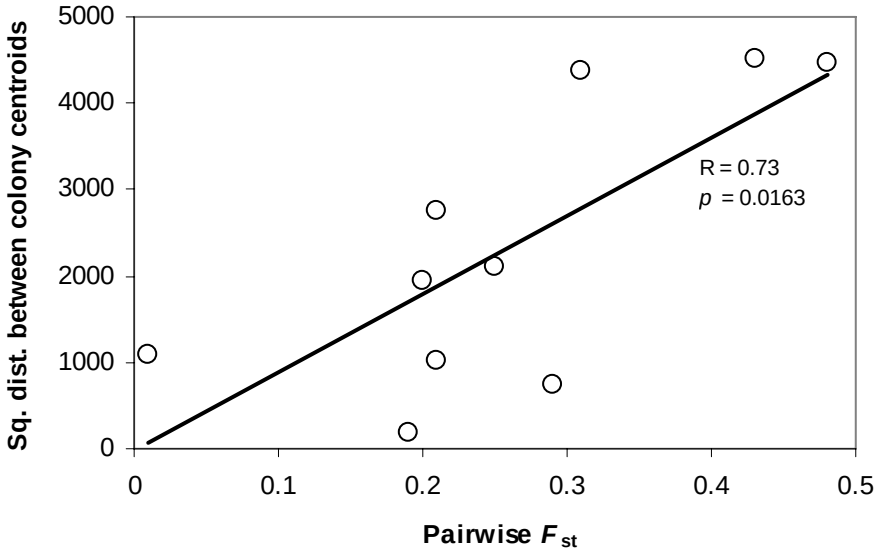
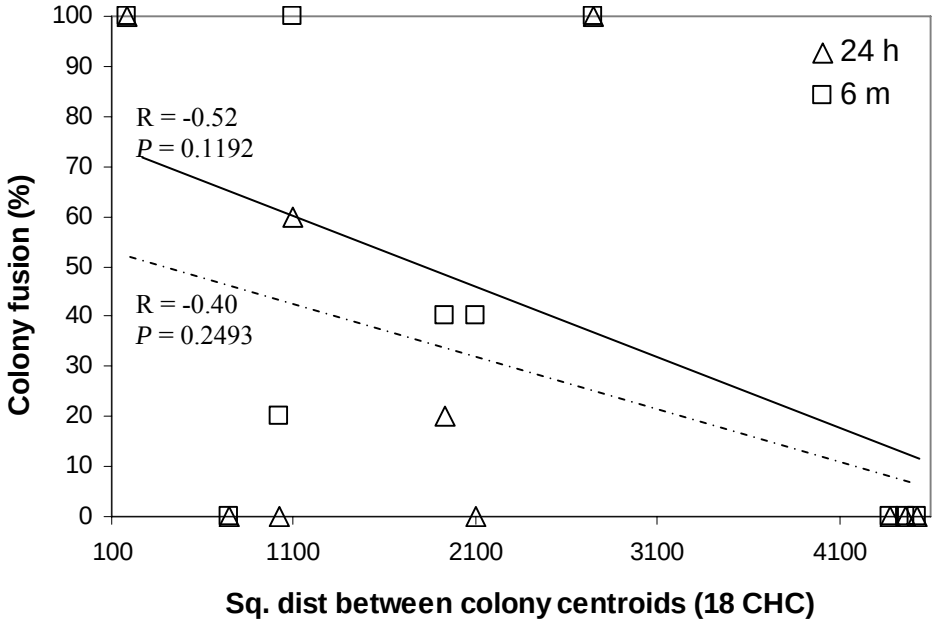


Figure 11. Relationship of worker cuticular hydrocarbon profile similarities based on 18 variables vs. % alleles shared (A) and pairwise F_{st} (B).

A



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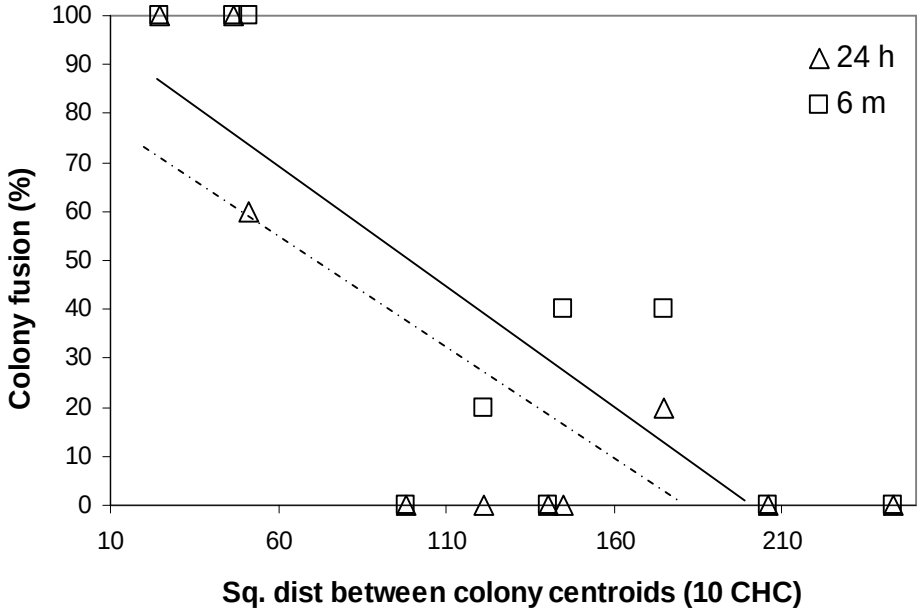

Figure 12. Relationship between source (A) and control (B) worker cuticular hydrocarbon profile similarities based on 18 and 10 transformed variables, respectively, and colony fusion at 24 h (△) and six months (□).

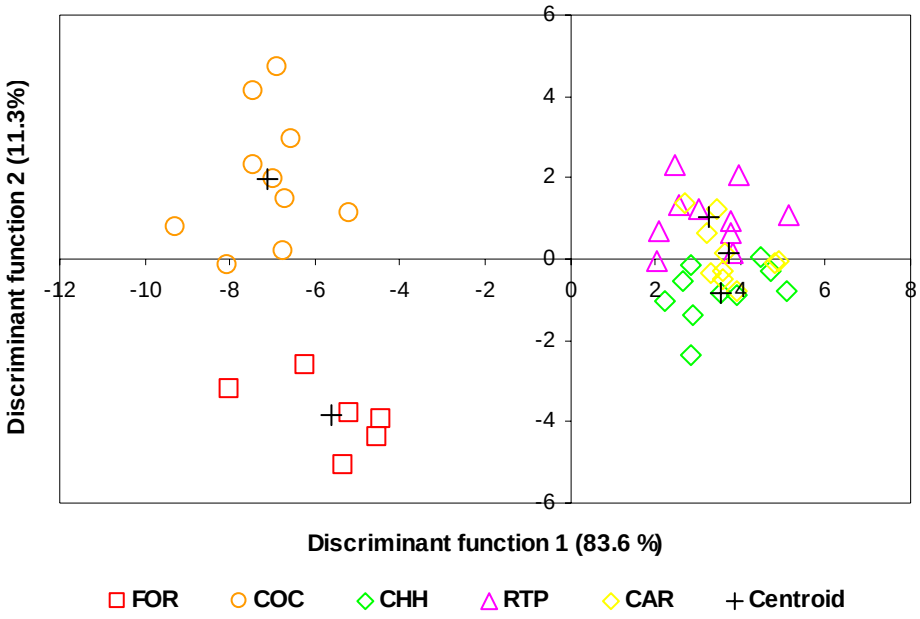

Figure 13. Discriminant analysis of nine variables selected by stepwise discriminant analysis for queens from five *L. humile* colonies (CAR, CHH, COC, FOR, RTP). The centroid of each group is marked by a +.

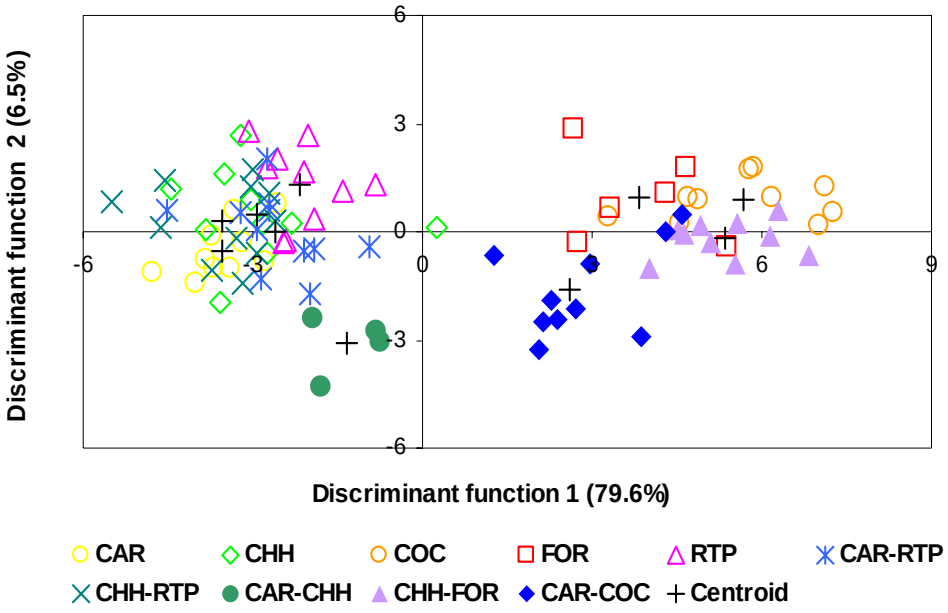

Figure 14. Discriminant analysis of 12 variables selected by stepwise discriminant analysis for queens from five *L. humile* colonies (CAR, CHH, COC, FOR, RTP) and five *L. humile* colony pairs (CAR-CHH, CAR-COC, CAR-RTP, CHH-FOR, CHH-RTP). The centroid of each group is marked by a +.

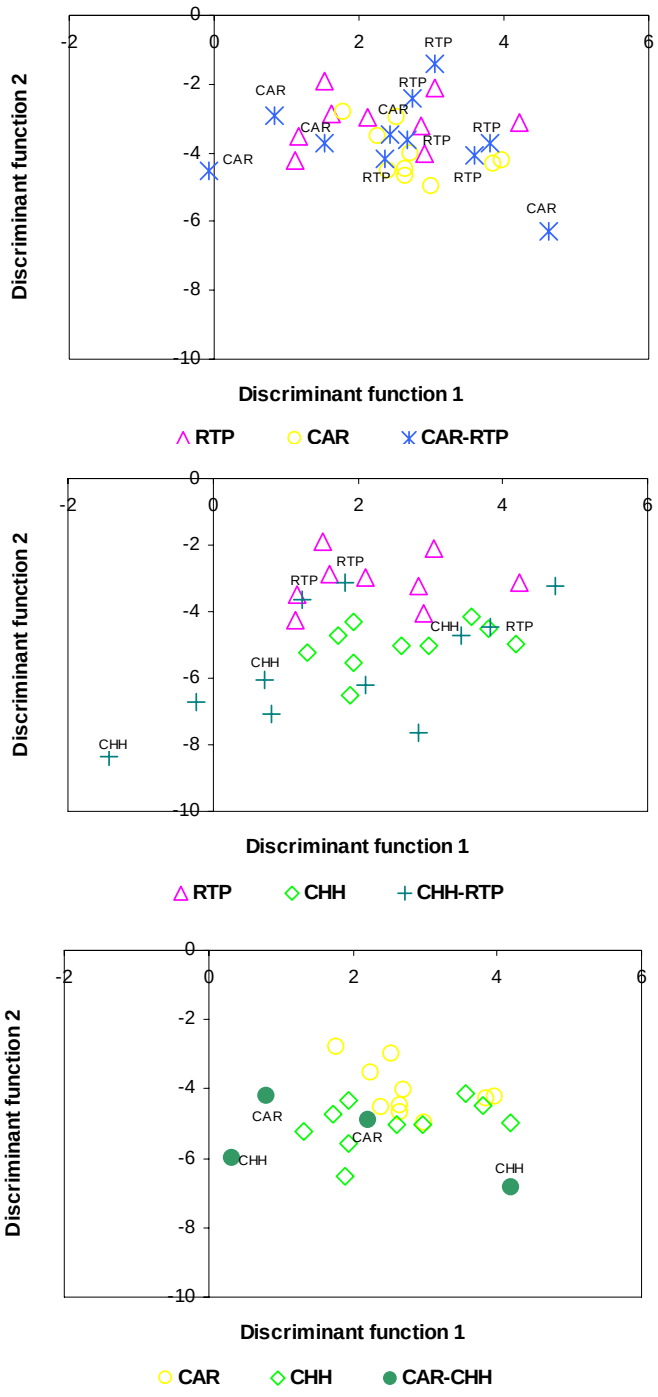

Figure 15. Linear discriminant functions plotted for queens of fused colony pairs (CAR-CHH, CAR-RTP, CHH-RTP) and their respective unpaired controls (CAR, CHH, RTP) based on nine variables.

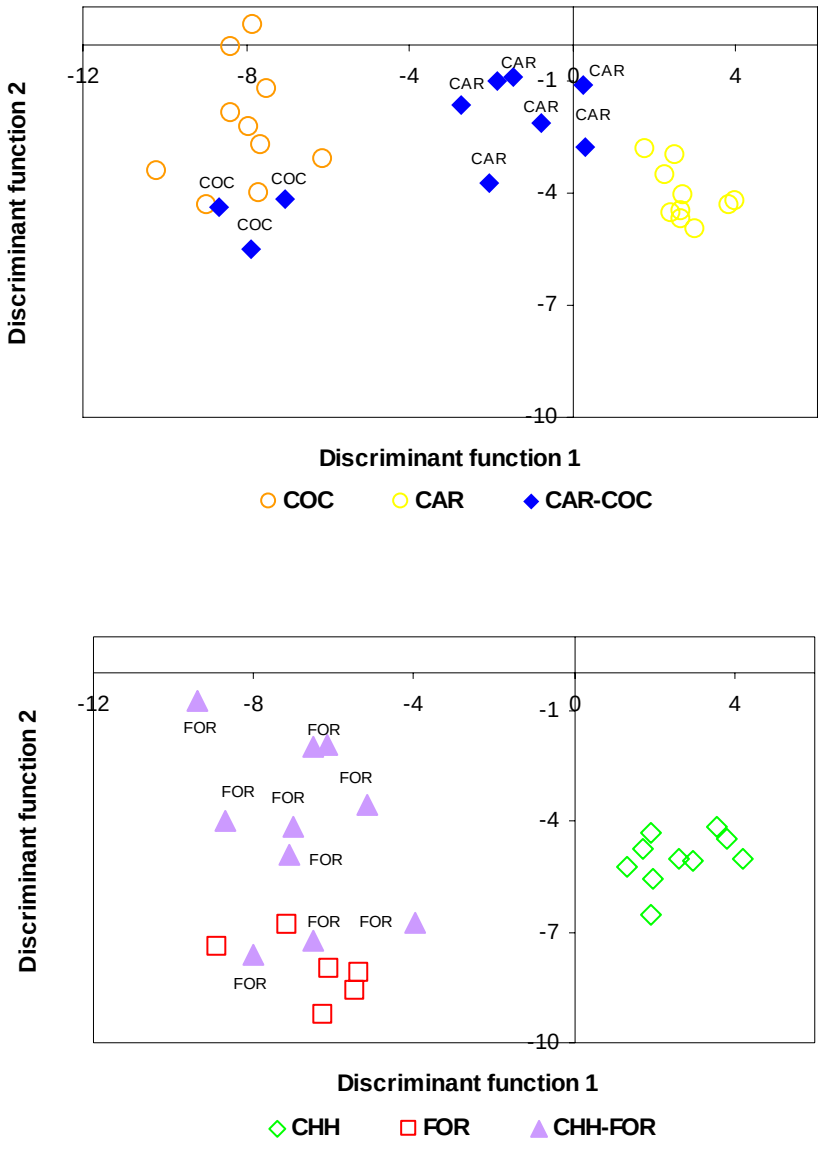

Figure 16. Linear discriminant functions plotted for queens of non-fused colony pairs (CAR-COC, CHH-FOR) and their respective unpaired controls (CAR, CHH, COC, FOR) based on nine variables.

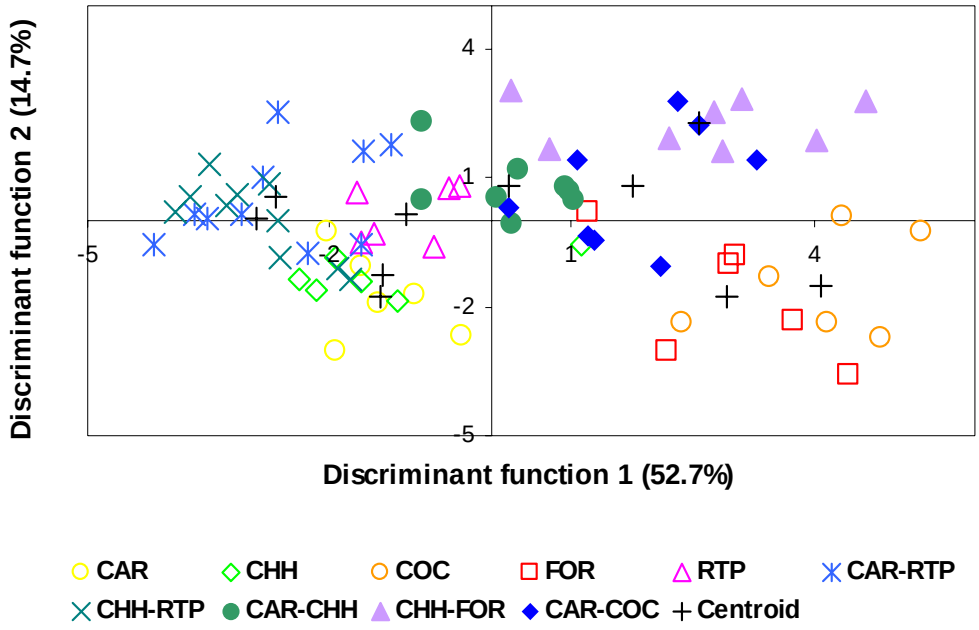

Figure 17. Discriminant analysis of 11 variables selected by stepwise discriminant analysis for workers from five *L. humile* colonies (CAR, CHH, COC, FOR, RTP) and five *L. humile* colony pairs (CAR-CHH, CAR-COC, CAR-RTP, CHH-FOR, CHH-RTP). The centroid of each group is marked by a +.

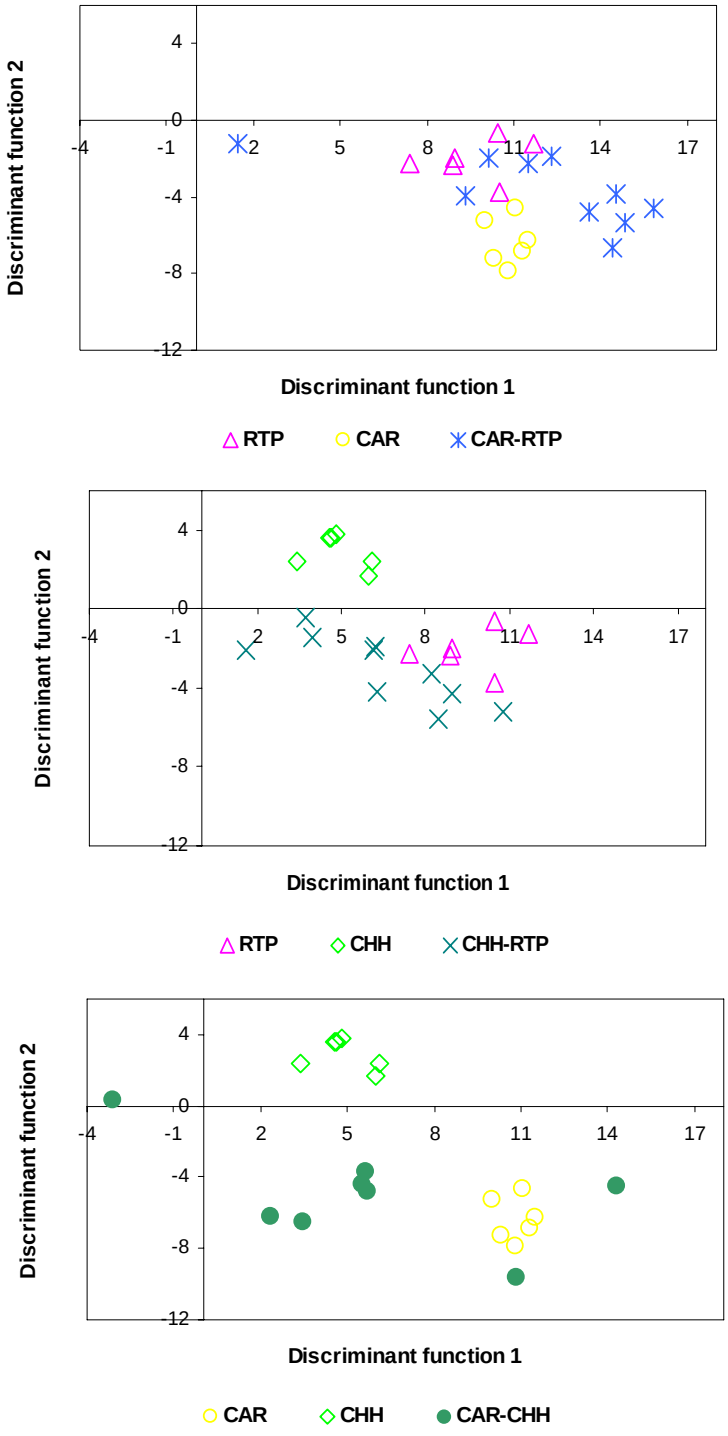

Figure 18. Linear discriminant functions plotted for workers of fused colony pairs (CAR-CHH, CAR-RTP, CHH-RTP) and their respective unpaired controls (CAR, CHH, RTP) based on 10 variables.

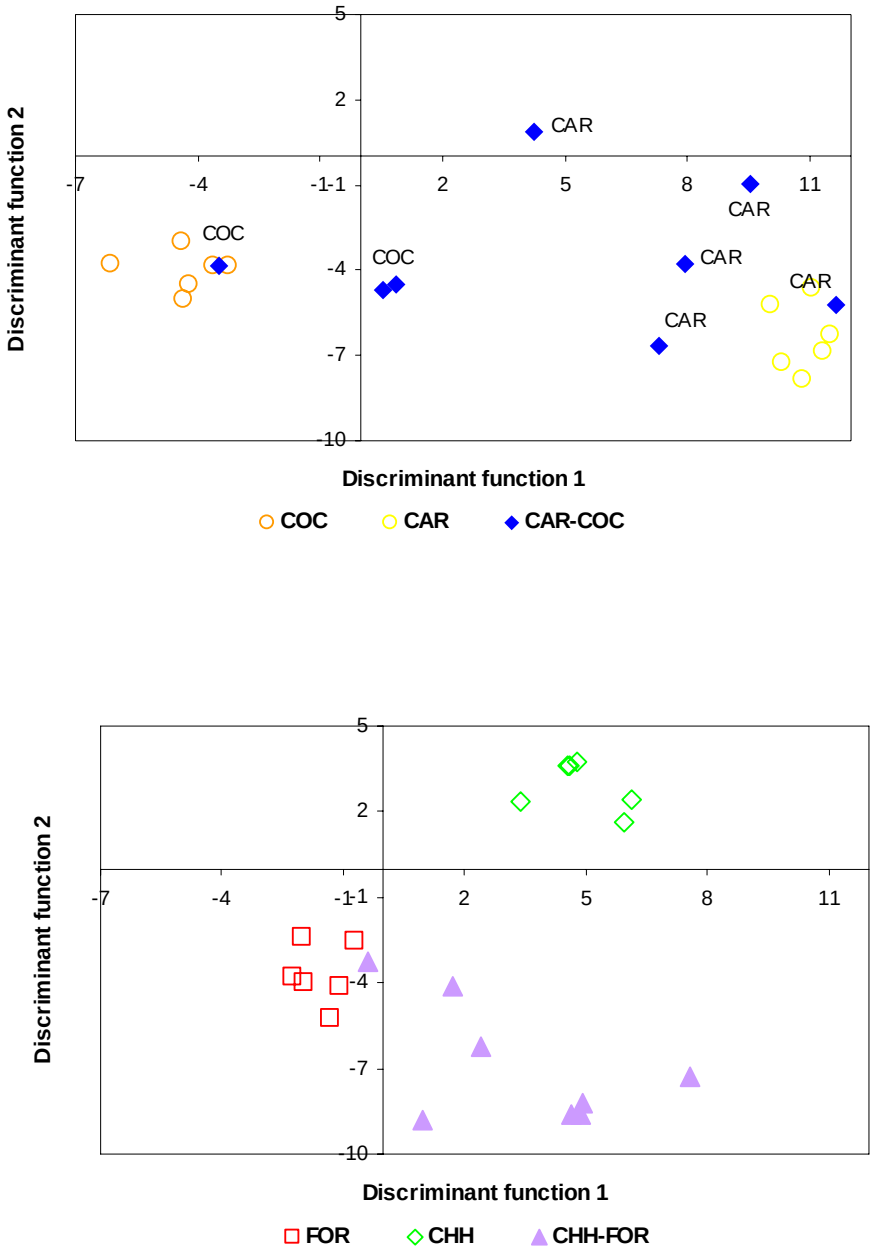

Figure 19. Linear discriminant functions plotted for workers of non-fused colony pairs (CAR-COC, CHH-FOR) and their respective unpaired controls (CAR, CHH, COC, FOR) based on 10 variables.