BUCZKOWSKI, GRZEGORZ ANDRZEJ. Nestmate recognition and population genetic structure in the Argentine ant, *Linepithema humile*. (Under the direction of Jules Silverman)

The Argentine ant, *Linepithema humile*, is a widespread invasive species characterized by reduced intraspecific aggression within its introduced range. Given the lack of intraspecific aggression typical of introduced populations of Argentine ants and its potential role in their success, it is of great interest to determine the mechanisms responsible for nestmate recognition in this species. To gain an understanding of mechanisms underlying nestmate recognition in Argentine ants, I studied its population genetic structure and the role of genetic vs. environmental cues on intraspecific aggression.

Chapter I reviews the ecology of the Argentine ant and recent literature on nestmate recognition in social insects.

In Chapter II, I examined Argentine ant population genetic structure and intercolony aggression in two portions of its introduced range in the United States: California and the southeastern U.S. My results show that the southeastern *L. humile* population has higher genotypic variability and high intercolony aggression relative to the California population. In the California population, I discovered 23 alleles across seven polymorphic microsatellite loci and no intercolony aggression. However, the southeastern U.S. population has 47 alleles and aggression between neighboring colonies is high. I suggest that distinct colonization patterns for California and the Southeast may be responsible for the striking asymmetry in the genetic diversity of introduced populations. Southeastern colonies may have descended from multiple, independent introductions from the native range, undergoing a single bottleneck at each introduction. In contrast, the California supercolony may have originated

from one or more colonies inhabiting the southeastern U.S., thus experiencing a double bottleneck. I suggest that differences in present-day colonization patterns between California and the Southeast are most likely a result of low winter temperatures in the Southeast and/or competition with another successful and widely distributed ant invader; the red imported fire ant (*S. invicta*).

In Chapter III, I examined changes in intraspecific aggression among colonies of Argentine ants in various discrimination contexts. I determined that aggression occurs at higher rates when either nestmates or familiar territory indicate nest proximity, but is greatly diminished where social context is absent. Context-dependent aggression in the Argentine ant appears to result from a shift in acceptance threshold in response to fitness costs associated with accepting non-kin. Isolated nest referents (familiar territory, conspecific brood, or single familiar nestmates) had no effect on aggression thresholds. I also tested whether nest status of individual ants (residents vs. intruders), rather than genetic similarity is primary in determining the identity of the aggressor in intruder introductions to resident colony. My results show that in the context of nest defense genetically more diverse colonies will initiate attacks on colonies with lower genetic diversity. Finally, I reconciled differences between earlier reports on the use of exogenous nestmate discrimination cues by Argentine ants by providing diminished intraspecific aggression following colony maintenance under uniform conditions in assays providing a social and/or ecological context, but not where this context was absent.

In Chapter IV, I examined the impact of different diet-derived hydrocarbons on intraspecific aggression in the Argentine ant and the potential of shared, diet-derived hydrocarbons to produce colony uniformity where intercolony genetic and/or environmental

differences exist. I determined the baseline level of aggression in pairs of field-collected colonies and discovered that ants showed either high or mild aggression. I then tested the effect of three diets: two hydrocarbon-rich insect prey, *Blattella germanica* and *Supella longipalpa* and an artificial, insect-free diet on aggression loss in both highly and mildly aggressive colony pairs. Results of behavioral assays show that diet-derived hydrocarbons alter nestmate recognition, mask inherent between colony distinctions, and allow non-nestmate acceptance where intercolony differences exist. Mildly aggressive colony pairs experienced a significant reduction in injurious aggression, although aggression was not eliminated completely. In contrast, highly aggressive colony pairs maintained high levels of injurious aggression throughout the study. Results of cuticular hydrocarbon analysis demonstrate that each diet altered the composition of the hydrocarbon profile by contributing unique, diet-specific cues. My results suggest that colony fusion due to common environmental factors may be an important mechanism contributing to the evolution of unicoloniality in introduced populations of the Argentine ant.

In Chapter V, I examined the role of environmental cues (derived from diet) on nestmate recognition in two populations of the Argentine ant. I discovered that the two populations differ in their response to environmentally-derived nestmate recognition cues. Ants belonging to the California supercolony are strongly affected by the imposition of prey-derived hydrocarbons and colony fragments raised in isolation on different prey (*B. germanica* or *S. longipalpa*) display high intracolony aggression when reunited. In contrast, colonies of Argentine ants from the southeastern U.S. show little or no aggression when subjected to the same treatment. Results of cuticular hydrocarbon analysis show that colonies from both regions initially possess *Supella*-specific hydrocarbons and acquire

additional hydrocarbons from both prey. Lack of aggression induction in Argentine ants from the southeastern U.S. may be a result of higher initial levels of *Supella*-specific hydrocarbons, relative to ants from California.

In Appendix I, I used the Argentine ant to compare four aggression bioassays for consistency between replicates, similarity between assays, and ability to predict whole colony interactions. The assays included were 1 live-1 dead ant interactions, live 1-1 battles, live 5-5 battles, and 1 ant introduced to a foreign colony. I tested six ant colonies in all pairwise combinations using four different assays and two to three scoring methods per assay. I also conducted a colony merging experiment to determine which assays were capable of predicting this ecologically important event. I found that scoring methods within assays yielded very similar results, however, assays differed greatly in their consistency between replicates. Assays that utilized the greatest number of live ants were the most likely to reveal high levels of aggression. I found relatively low consistency between trials using the live 1-1 assay, but found that with sufficient replication its results were highly correlated with the assays using more interacting ants. I suggest that isolated aggressive acts in assays do not necessarily predict whole colony interactions: some colonies that fought in bioassays merged when the entire colonies were allowed to interact.

NESTMATE RECOGNITION AND POPULATION GENETIC STRUCTURE IN THE ARGENTINE ANT, *LINEPITHEMA HUMILE.*

by

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BIOGRAPHY

Grzegorz Andrzej Buczkowski was born in Wroclaw, Poland on April 2, 1972 to Jadwiga and Andrzej Buczkowski. In 1989 Grzegorz's family moved to the United States when his father accepted a position at North Carolina State University as a visiting scientist. In 1991, Grzegorz graduated from high school and enrolled at North Carolina State University. He earned a Bachelor of Science degree in Zoology in 1995. After graduation, Grzegorz continued his studies at North Carolina State University as a Postbaccalaureate Student in the Department of Toxicology (1995-1996). Between 1997 and 1998, Grzegorz worked as a research assistant for Rhône-Poulenc Ag. Co. (now Bayer CropScience) in Research Triangle Park. In 1998 Grzegorz began his graduate studies in the Department of Entomology at North Carolina State University on a graduate student assistantship supported in part by a grant from Rhône-Poulenc Ag. Co., the Blanton J. Whitmire Endowment, a scholarship from the North Carolina Pest Control Association, and a scholarship from Phi Chi Omega. In 2000, Grzegorz earned a Master of Science degree in Entomology under the direction of Dr. Coby Schal and continued his studies in the Department of Entomology as a Ph.D. student with Dr. Jules Silverman on a graduate assistantship supported by the Blanton J. Whitmire Endowment. In the fall of 2003, Grzegorz will begin work as a Postdoctoral Researcher at Ohio State University. He will work on the evolutionary biology of slavemaking ants with Dr. Joan Herbers.

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CHAPTER I

Nestmate recognition in the Argentine ant, *Linepithema humile* (Mayr)

Nestmate recognition in ants – complexity and dynamic nature of the system.

Social insects live in colonies and maintain territorial boundaries aimed at preventing heterospecifics as well as conspecifics from invading and exploiting the colony's nest. In order to recognize colony members from non-members, individuals recognize each other through a learned chemical label or "colony odor". Cuticular hydrocarbons have long been implicated as important mediators of nestmate recognition in ants (Vander Meer and Morel 1998) and recent studies provide direct evidence for the role of hydrocarbons in nestmate recognition in several species (Obin 1986; Lahav et al. 1999; Thomas et al. 1999; Boulay et al. 2000; Liang and Silverman, 2000).

The signals used in nestmate recognition may have a genetic or environmental origin, and the hydrocarbon profile is usually composed of a blend of inherited and environmentally-derived hydrocarbons. Environmentally derived cues, which may be derived from nesting material (Gamboa et al. 1986; Breed 1983; Stuart 1987) or diet (Jutsum et al. 1979; Crosland 1989; Le Moli et al. 1992; Obin and Vander Meer 1988) change depending on the ant's living environment and diet. The relative contribution of each source to the overall hydrocarbon profile remains unknown. Irrespective of the source, workers within a colony must learn the cues specific to their nest and they must be able to properly evaluate cues present on newly encountered workers. Nestmate recognition in ants involves the use of template (Waldman et al. 1988) and recognition cues are evaluated by comparing newly encountered odors with the ones that were encountered in the past. Conspecifics bearing a foreign label or lacking an appropriate one are attacked and often killed. Recognition cues are dynamic and may change

throughout the life of the colony (Provost et al. 1993; Vander Meer et al. 1989) and may exhibit seasonal variation (Ichinose 1991). Temporal variation in reproductive phenology of colonies (Passera and Aron 1993), changes in worker density and/or queen number, and variation in intracolony patterns of relatedness may also influence nestmate recognition behavior. Furthermore, the enormous variation and complexity in colony structure and mating systems exhibited by many social insects (polygyny, polyandry, polydomy) may affect the spatial and temporal distribution of kin and further decrease the likelihood that the invader's cues will match the template of the resident. Such variation in genetic and environmental parameters dictates that colony members continually obtain and process information about their changing ecological and social environment and act accordingly, and necessitates that the action component of nestmate recognition be plastic in order to minimize the chance of recognition errors.

The Argentine ant – ecological importance and pest status.

Native to South America, the Argentine ant has been introduced in many parts of the world where it has become a serious ecological and agricultural pest. In its introduced range, the Argentine ant displaces native arthropods (Human and Gordon 1997, 1999; Holway 1998) and it causes problems in agricultural systems by tending honeydew producing pest insects (Newell and Barber 1913; Smith 1936; Bartlett 1961). In addition, the Argentine ant is often an urban pest (Knight and Rust 1990). Tsutsui et al. (2000) and Holway et al. (1998) have demonstrated that reduced genetic variation and reduced intraspecific aggression were responsible for the success of introduced populations of the

Argentine ant. In its native range, the Argentine ant is multicolonial, territorial boundaries between colonies are well-defined and nests are aggressively defended from conspecifics. There is no evidence that this ant negatively affects the native arthropod fauna (Suarez et al. 1999). In its introduced range, however, the ant is unicolonial where intraspecific aggression is reduced, colony boundaries are not defined, and large supercolonies are formed. Previous work has demonstrated that absence of intraspecific aggression leads to increased colony size by reducing the costs associated with territoriality (Holway et al. 1998). The apparent difference between native and introduced populations in both aggressive behavior and colony structure suggests that investigation of the causes of such differences will uncover factors responsible for the Argentine ant's success as an invasive species.

Nestmate recognition in the Argentine ant.

The nestmate recognition system in the Argentine ant consists of genetic (Tsutsui et al. 2000) and environmental (Chen and Nonacs 2000; Liang and Silverman 2000; Suarez et al. 2002) cues and the hydrocarbon profile is composed of a blend of inherited and environmentally derived hydrocarbons. Holway et al. (1998) and Suarez et al. (2002) reported that aggression persisted between *L. humile* colonies despite maintenance under uniform rearing conditions, while Chen and Nonacs (2000) observed a decrease in *L. humile* intercolony aggression following 2 months of lab rearing. Although Tsutsui et al. (2000) demonstrated a significant inverse relationship between *L. humile* genetic similarity

and intercolony aggression, the colony pairs in Holway et al. (1998), Suarez et al. (2002), and Chen and Nonacs (2000) were not subjected to genetic analysis. Therefore, the observed changes (or lack of thereof) in aggression levels may have resulted from different degrees of relatedness between pairs and/or different degrees of distinction in environmentally derived hydrocarbons. In addition, each group employed a different aggression assay making comparisons across studies difficult.

The Argentine ant is one of several invasive ant species in which the relative absence of territoriality is thought to be responsible for its invasion success. In the Argentine ant, neighboring nests are not mutually aggressive, and nest boundaries vanish into a single supercolony comprising all individuals of the local population. Unicoloniality, which is thought to be a relatively recent event and a relatively unstable strategy (Passera 1994), gives Argentine ants enormous advantages in competition with native ant species (Holway et al. 1998), which usually have discrete and much smaller colonies. While it has been suggested that genetic factors are mainly responsible for the unicolonial behavior in the Argentine ants, the role of diet in promoting unicolonial behavior remains unknown. Diet may play a role in producing colony uniformity where intercolony genetic differences exist. Shared dietary hydrocarbons between colonies displaying low intercolony genetic differentiation, may mask subtle inherent between-colony distinctions and allow adjacent colonies to merge, thereby creating a supercolony. Liang and Silverman (2000), Silverman and Liang (2001), and Liang et al. (2001) investigated the role of dietary hydrocarbons on nestmate recognition in the Argentine ant and demonstrated that hydrocarbons acquired from prey were incorporated into the ant's intrinsic cuticular hydrocarbon profile and altered nestmate recognition.

The Argentine ant-prey hydrocarbon nestmate recognition system may be an excellent model for studying problems related to transition from multicoloniality to unicoloniality in invasive ants. Liang and Silverman (2000) and Silverman and Liang (2001) demonstrated the potential of prey hydrocarbons to alter nestmate recognition in the Argentine ant, whereby feeding on distinct diets induced intracolony aggression. Based on this evidence, we believe that exogenous cues (e.g. diet derived hydrocarbons) may have a role in producing colony uniformity where between-colony genetically and/or environmentally based differences exist. Shared diet-derived hydrocarbons between colonies displaying low intercolony variability may mask inherent between-colony distinctions and allow adjacent colonies to merge, thereby creating a supercolony. Introduced populations of *L. humile* are polygyne and contain hundreds to thousands of queens (Markin 1968, 1970). This presents a problem for the maintenance of social coherence where queens are not significantly related and nestmate relatedness is reduced, relative to single queen colonies (Hamilton 1972; Keller and Passera 1989; Keller and Passera 1993; Krieger and Keller 2000). By examining the role of environment-derived cues on nestmate recognition in *L. humile*, we hope to explore the mechanisms of cooperation within polygynous colonies where intranest relatedness is low.

This dissertation is composed of five related chapters that investigate nestmate recognition and population genetic structure in the Argentine ant. In Chapter II, I examine population genetic structure and intercolony aggression in two portions of the Argentine ant introduced range: California and the southeastern U.S. I describe factors that may have contributed to the present-day differences in genetic diversity between these two regions and I also suggest ecological factors that may have contributed to regional differences in current

distribution patterns of the Argentine ant. In Chapter III, I examine the context-dependency of nestmate discrimination in the Argentine ant and the effect of action thresholds on exogenous cue recognition. I also test hypotheses that explain a change in action thresholds as well as factors that affect action thresholds. I examine changes in intraspecific aggression among colonies of Argentine ants in various discrimination contexts. I test the importance of isolated nest referents (familiar territory, conspecific brood, or single familiar nestmates) on aggression thresholds. I also test whether nest status of individual ants (residents vs. intruders), rather than genetic similarity is primary in determining the identity of the aggressor in intruder introductions to a resident colony. In Chapter IV, I examine the impact of different diet-derived hydrocarbons on intraspecific aggression in the Argentine ant and the potential of shared, diet-derived hydrocarbons to produce colony uniformity where intercolony genetic and/or environmental differences exist. In Chapter V, I examine the role of environmental cues (derived from diet) on nestmate recognition in two populations of the Argentine ant. I test the hypothesis that there is geographical variation in the response of Argentine ant populations to nestmate recognition cues derived from hydrocarbon-rich prey. In Appendix I, I use the Argentine ant to compare four aggression bioassays for consistency between replicates, similarity between assays, and ability to predict whole colony interactions. I test four different aggression assays with two or three scoring methods per assay. I also determine whether isolated aggressive encounters can predict whole colony interactions.

References Cited

Bartlett, B. R. 1961. The influence of ants upon parasites, predators, and scale insects. Ann. Entomol. Soc. Amer. 54: 543–550.

Boulay R., A. Hefetz, V. Soroker, and A. Lenoir. 2000. *Camponotus fellah* colony integration: worker individuality necessitates frequent hydrocarbon exchanges. Anim. Behav. 59: 1127–1133.

Breed, M D. 1983. Nestmate recognition in honey bees. Anim. Behav. 31:86-91.

Crosland, M.W. J. 1989. Kin recognition in the ant *Rhytidoponera confusa*. I. Environmental odour. Anim. Behav. 37: 912–919.

Chen J. S. C., and P. Nonacs. 2000. Nestmate recognition and intraspecific aggression based on environmental cues in Argentine ants (Hymentoptera: Formicidae). Ann. Entomol. Soc. Am. 93: 1333–1337.

Gamboa, G. J., H. K. Reeve, and D.W. Pfennig. 1986. The evolution and ontogeny of nestmate recognition in social wasps. Ann. Rev. Entomol. 31: 431–454.

Holway, D.A., A. V. Suarez, and T. J. Case. 1998. Loss of intraspecific aggression in the success of a widespread invasive social insect. Science. 282: 949–952.

Human, K. G., and D.M. Gordon. 1997. Effects of Argentine ants on invertebrate biodiversity in northern California. Conser. Biol. 11: 1242–1248.

Human, K., and D. M. Gordon. 1999. Behavioral interactions of the invasive Argentine ant with native ant species. Ins. Soc. 46: 159–163.

Ichinose, K. 1991. Seasonal variation in nestmate recognition in *Paratrechina flavipes* (Smith) worker ants (Hymenoptera: Formicidae). Anim. Behav. 41: 1–6.

Jutsum, A.R., T. S. Saunders, and J. M. Cherrett. 1979. Intraspecific aggression in the leaf-cutting ant Acromyrmex octospinosus. Anim. Behav. 27: 839–844.

Knight, R. L., and M. K. Rust. 1990. The urban ants of California with distribution notes of imported species. Southwest Entomol. 15: 167–178.

Lahav, S, V. Soroker., A. Hefetz , and R. K. Vander Meer. 1999. Direct behavioral evidence for hydrocarbons as ant recognition discriminators. Naturwissenshaften. 86: 246–249.

Le Moli, F., A. Mori , and D. A. Grasso. 1992. Nestmate and conspecific non-nestmate recognition in *Formica cunnicularia* Latr.: the effect of diet difference. pp. 161–165 in Biology and Evolution of Social Insects (J. Billen, ed.), Leuven University Press, Leuven (Belgium).

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

Liang, D., G. J. Blomquist, and J. Silverman. 2001. Hydrocarbon-released nestmate aggression in the Argentine ant, *Linepithema humile*, following encounters with insect prey. Comp. Biochem. Physiol. 129: 871–882.

Markin, G. P. 1968. Nest relationship of the Argentine ant, *Iridomyrmex humilis* (Hymenoptera: Formicidae). J. Kans. Entomol. Soc. 41: 511–516.

Newell W., and T. C. Barber. 1913. The Argentine ant. USDA Bureau of Entomology Bulletin 122.

Markin, G. P. 1970. The seasonal life cycle of the Argentine ant, *Iridomyrmex humilis* (Hymenoptera: Formicidae) in southern California. An. Ent. Soc. Am. 63: 1238–1242.

Obin, M. S. 1986. Nestmate recognition cues in laboratory and field colonies of Solenopsis invicta Buren (Hymenoptera: Formicidae): Effect of environment and the role of cuticular hydrocarbons. J. Chem. Ecol. 12: 1965–1975.

Obin, M.S., and R. K. Vander Meer. 1988. Source of nestmate recognition cues in the imported fire ant *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Anim. Behav. 36:1361–1370.

Silverman, J., D. Liang. 2001. Colony disassociation following diet partitioning in a unicolonial ant. Naturwissenshaften, 88: 73–77

Smith, M. R. 1936. Distribution of the Argentine ant in the United States and suggestions for its control or eradication. US Dep. Agric. Circ. 387: 1-39.

Stuart, R. J. 1987. Transient nestmate recognition cues contribute to a multicolonial population structure in the ant, *Leptothorax curvispinosus*. Behav. Ecol. Sociobiol. 21: 229–235.

Suarez, A. V, N. D. Tsutsui, D. A. Holway, and T. J. Case. 1999. Behavioral and genetic differentiation between native and introduced populations of the Argentine ant. Biological Invasions. 1: 43–53.

Suarez, A. V., D. A. Holway, D. Liand, N. D. Tsutsui, and T. J. Case. 2002. Spatio-temporal patterns of intraspecific aggression in the invasive Argentine ant. Anim. Beh. 64: 697–708.

Thomas, M. L., L. J. Parry, R. A. Allen, and M. A. Elgar. 1999. Geographic affinity, cuticular hydrocarbons and colony recognition in the Australian meat ant *Iridomyrmex purpureus*. Naturwissenschaften 86: 87–92.

Tsutsui, N.D., A. V. Suarez, D. A. Holway, and T. J. Case. 2000. Reduced genetic variation and the success of an invasive species. Proc. Nat. Acad. Sci. 97: 5948–5953.

Vander Meer, R. K., D. Saliwanchik, and B. Lavine. 1989. Temporal changes in colony cuticular hydrocarbon patterns of *Solenopsis invicta*: Implications for nestmate recognition. J. Chem. Ecol. 15: 2115–2125.

Vander Meer, R. K., and L. Morel. 1998. Nestmate recognition in ants. pp. 79–103 *In* Pheromone Communication in Social Insects (Vander Meer, Breed, Espelie, and Winston, eds), Westview Press, Boulder.

Waldman, B., P. C. Frumhoff, and W. P. Shermam. 1988. Problems of kin recognition. Trends in Ecology and Evolution. 3: 8–13.

Ward, P. S. 1987. Distribution of the introduced Argentine ant (*Iridomyrmex humilis*) in natural habitats of the lower Sacramento Valley and its effects on the indigenous ant fauna. Hilgardia. 55 :1–16.

CHAPTER II

The diminutive supercolony: the Argentine ants of the southeastern U.S. [1]

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[1] Buczkowski, G., E. L. Vargo, and Silverman, J. The diminutive supercolony: the Argentine ants of the southeastern U.S. (as submitted to the journal *Evolution*).

Abstract. Upon its introduction into the western United States, the invasive Argentine ant, (*Linepithema humile*) experienced a genetic bottleneck resulting in diminished intraspecific aggression and the formation of ecologically damaging supercolonies. We examined population genetic structure and intercolony aggression in two portions of the introduced range of this species in the United States: California and the southeastern U.S. Our results show that the southeastern *L. humile* population displays elevated genotypic variability and high intercolony aggression relative to the California population. In the California population, intercolony aggression was absent and 23 alleles were found across seven polymorphic microsatellite loci. However, in the Southeast, aggression between neighboring colonies was high and 47 alleles were present across the same seven loci. The level of intercolony aggression and genetic diversity of Argentine ants in the Southeast, is similar to that found in the native range. We suggest that distinct colonization patterns for California and the Southeast acted as a possible mechanism responsible for the striking asymmetry in the genetic diversity of introduced populations. Southeastern colonies may have descended from multiple, independent introductions from the native range, undergoing a single bottleneck at each introduction. In contrast, the California supercolony may have originated from one or more colonies inhabiting the southeastern U.S., thus experiencing a double bottleneck. The differences in present-day colonization patterns between California and the Southeast are most likely a result of low winter temperatures and/or competition with another successful and widely distributed ant invader; the red imported fire ant (*S. invicta*).

Keywords: Argentine ant, invasive ants, microsatellites, nestmate recognition, unicoloniality.

Introduction

Genetic drift involves changes in gene frequencies due to sampling error in finite populations. One important cause of genetic drift is the founder effect, which causes loss of genetic variation, and produces genetically atypical populations (Nei et al. 1975; Chakraborty and Nei 1977). This occurs when a population, started from a small number of pioneer individuals of a source population, moves to a new area, with a concomitant loss in genetic diversity from the original population. The allele frequencies in the genetically bottlenecked colonizers may be very different from the allele frequencies in the native population. In several social insects, such changes in genetic composition have been accompanied by dramatic changes in social organization of the founding population (Ross and Keller 1995; Ross et al. 1993, 1996; Tsutsui et al. 2000).

Native to South America, the Argentine ant (*Linepithema humile*) has become established in many parts of the world (Suarez et al. 2001), where it is a serious ecological, agricultural, and urban pest (Newell and Barber 1913; Bartlett 1961; Erickson 1971; Knight and Rust 1990; Human and Gordon 1996, 1997, 1999; Holway 1998). Recent evidence from behavioral and population genetics studies indicates that in its native range the Argentine ant is multicolonial, whereby territorial boundaries between colonies are well defined, nests are aggressively defended against conspecifics, and colonies are genetically differentiated (Suarez et al. 1999; Tsutsui et al. 2000). Moreover, there is no indication that this ant negatively impacts the native ant fauna in Argentina (Suarez et al. 1999).

In its introduced range in California, Europe, and Chile however, the Argentine ant is unicolonial, whereby it forms large supercolonies with poorly defined boundaries (Suarez

et al. 1999; Tsutsui et al. 2000; Tsutsui and Case 2001; Giraud et al. 2002). Within colonies, ants occupy multiple nests (polydomy) and have many functional queens (polygyny). Both workers and queens move freely between nests (Newell and Barber 1913; Markin 1968, 1970). Such unicolonial behavior promotes efficient food discovery, defense, and retrieval (Human and Gordon 1996; Holway et al. 1998; Holway 1999) that may give the Argentine ant an advantage over native species (Holway and Case 2001). In California and southern Europe, the majority of the introduced populations exist as a single colony, with intraspecific aggression being very rare (Tsutsui et al. 2000; Giraud et al. 2002). Tsutsui et al. (2000) proposed that the formation of the California supercolony was a direct consequence of a genetic bottleneck that reduced the genetic diversity of introduced populations. Reduced genetic diversity resulted in low intraspecific aggression among spatially isolated colonies, and led to the formation of a dominant supercolony. Furthermore, Tsutsui et al. (2003) demonstrated that the establishment and maintenance of the large supercolony is due to asymmetrical aggression, whereby workers from genetically diverse colonies are selected against by workers from genetically less diverse colonies. By contrast, Giraud et al. (2002) hypothesized that the genetic bottleneck experienced by colonies introduced into southern Europe was weak, relative to colonies introduced into California, and played only a minor role in the formation of the European supercolony. Instead, Giraud et al. (2002) proposed that introduction of colonies into a new habitat combined with relaxed ecological constraints resulted in accelerated colony growth and expansion. Rapid colony expansion resulted in increased competition and encounter rate between colonies. These authors suggested that during aggressive encounters, colonies possessing rare recognition alleles were eliminated and colonies harboring the most

common recognition alleles gained a selective advantage. Such colonies survived and dominated the landscape.

In contrast to *L. humile* populations reported from California, southern Europe, and Chile our surveys in North Carolina revealed that Argentine ant colonies from the southeastern U.S. were small and patchily distributed. These diminutive supercolonies were almost always restricted to relatively small patches of urban landscapes (100–300m^2) and two of the largest supercolonies covered an area of approximately one city block. This is in direct contrast to supercolonies in California, Europe, and Chile which cover thousands of square kilometers. In preliminary trials, we recorded high intraspecific aggression between southeastern colonies over relatively small spatial scales, which suggested that the expansive supercolonies reported earlier for introduced populations (Tsutsui et al. 2000 and Giraud et al. 2002) are not universal and may depend on genetic factors and/or regional environmental characteristics. To investigate more closely possible regional variation in Argentine ant population structure, we compared aggressive behavior and the genetic diversity among colonies from sites across similar spatial scales in California and the southeastern U.S.

Material and Methods

Study Sites and Rearing Procedures

To investigate geographic variation in genetic structure and intraspecific aggression in introduced North American populations of the Argentine ant, we collected ants from along two 700 km transects. One transect was through an area of a supercolony in California (37.8N – 33.0N and 122.2W – 117.0W) previously described by Tsutsui et al. (2000), the other spanned across three southeastern states: North Carolina, South Carolina, and Georgia (33.0N – 36.1N and 72.0W – 84.4W). We sampled 16 sites in California: Berkeley, Cambria, Corona, Escondido, Guadalupe, King City, Lompoc, Monterey, Ojai, Pleasanton, Point Piedras Blancas, Refugio State Park, Riverside, San Luis Obispo, San Mateo, and Santa Barbara. All sites sampled in California were different from those in Tsutsui et al. (2000). We did not know apriori whether ants collected in California belonged to the main supercolony or some of the smaller supercolonies. We therefore refer to each of the collection sites as colonies, rather than nests, although some of the collection sites may have belonged to the same supercolony. In the southeastern U.S. we sampled 16 sites: North Carolina (9); Chapel Hill, Emerald Isle, Greenville, Holden Beach (2 sites), Jacksonville, Research Triangle Park, Shallotte, and Winston-Salem; South Carolina (3): Greenville and Greer (2 sites); and Georgia (4): Barnesville, Fayetteville, Gainesville, and Griffin. In both ranges, ants were collected from a wide variety of habitats. In California, typical nesting sites included citrus groves, urban areas, and riparian woodlands. In the Southeast, ants were collected mainly from landscaped residential lots, city parks, or sandy beaches.

For each location, we established a single large laboratory colony consisting of 10,000-15,000 workers, a few hundred queens, and numerous brood. Colonies were maintained in soil-free, Fluon™-coated trays. Nests were plastic dishes filled with moist grooved plaster. Colonies were provided with 25% sucrose solution *ad libidum*, hard-boiled egg once a week, and artificial diet (Bhatkar and Whitcomb 1970). For microsatellite genotyping, ants were placed in 95% ethanol at the time of collection and stored at –20ºC until DNA extraction.

Aggression Tests (Nestmate Recognition Bioassays)

We assessed intraspecific aggression with a colony introduction assay that measured the level of aggression in single worker introductions into a foreign colony (Roulston et al. 2003, Appendix 1). Randomly selected "intruder" workers were introduced into rearing trays containing "resident" ants. Both intruder and resident workers were from recently collected field colonies and all tests were conducted within a week of the collection. For each test, we allowed the intruder up to 25 encounters with resident ants. Each instance of direct physical contact between the intruder and any of the residents was regarded as an encounter. Tests were terminated early if the intruder and the residents engaged in a highly aggressive behavior (level 4) for more than 10 sec. Aggressive behaviors were scored on a 1–4 scale described by Suarez et al. (1999). Ten replicates per colony pair were performed: each colony served as residents five times, and five workers from that colony served as intruders. For each of the two geographic regions, we tested all 16 colonies in all possible pairwise colony combinations (120 intercolony pairings per region). The maximum score per trial was used in the analysis.

Molecular Techniques

Genomic DNA was extracted from 15 workers from each site using the DNeasy Tissue Kit (Qiagen, Valencia, CA) and analyzed at seven polymorphic microsatellite loci: *Lhum-11, Lhum-13, Lhum-19, Lhum-28, Lhum-35, Lhum-39* (Krieger and Keller 1999) and *Lihu-T1* (Tsutsui et al. 2000). The PCR product was labeled with an IRD dye using the method of Oetting et al. (1995), in which the forward primer in each primer pair had the first 19bp of the M13 forward sequence attached to the 5'-end, and IRD-labelled M13 forward primer was included in the PCR. PCR reactions were multiplexed and amplification products were separated on 6.5% KB^{Plus} polyacrylamide sequencing gels using a 4000L Li-Cor DNA sequencer. Microsatellite alleles were scored using RFLPScan software (Scanalytics, Billerica, MA).

Data Analysis

Genetic diversity measures, including the number of observed alleles per locus (A_O) and the expected heterozygosity (H_E, unbiased for sample size), were calculated using the program Genetic Data Analysis (GDA; Lewis and Zaykin 1999). For all analyses, worker genotypes in each of the two regions were pooled across colonies and analyzed as a single reference population (P*). Private alleles, defined as those occurring in only one population, were identified in pairwise population comparisons.

The structure of genetic variability in each of the two regions was estimated using Wright's (1951, 1965) *F*-statistics. *F*-statistics were calculated in Genetic Data Analysis according to

Weir and Cockerham's (1984) method, the significance of which was based on 95% confidence intervals determined by bootstrapping across loci (10,000 permutations).

All pairwise F_{ST} values calculated between colonies were plotted against distance to test for genetic isolation by distance. Distance between collection sites was calculated as the shortest aerial distance. The significance of the regression coefficient was tested by Mantel's 1967 tests in GENEPOP (version 3.3, updated version of Raymond and Rousset 1995) using 10,000 permutations. The regression between the genetic differentiation matrix and the geographic distance matrix is reported as r^2 and one-tailed *P*-values for positive or negative matrix correlations are provided. The average relatedness among nestmates (*r*) for each region was estimated using RELATEDNESS 5.0.7 (Queller and Goodnight 1989). The relationship among all possible pairwise combinations of three variables - intraspecific aggression, genetic differentiation, and geographic distance - was examined in GENEPOP by estimating the Spearman Rank correlation coefficient, using $F_{ST}/(1- F_{ST})$ for the measure of genetic differentiation. The significance of the correlation coefficient in all of the above correlations was tested by Mantel's tests as described above.

Estimates of *F*-statistics and the relatedness coefficient (*r*) were conducted using a reduced set of loci for each region. In California, we excluded *Lhum-28* and *Lihu-T1* from the analysis because they were monomorphic. In the Southeast, we excluded *Lhum-39* and *Lihu-T1* because they showed large differences between expected and observed heterozygosities, possibly due to the presence of a null allele or other scoring anomalies.

Results

Geographic variation in genetic diversity

Relative to Argentine ants from California, ants from the Southeast are genetically more diverse both in terms of allele numbers and heterozygosity (Table 1). Across the seven loci examined, the southeastern populations had a total of 47 alleles, whereas only 23 alleles were detected in the California populations. All 23 alleles found in California were a subset of those found in the Southeast. Similarly, the average expected multilocus heterozygosity in the Southeast was 33 % higher relative to that found in California. On average, colony pairs in California shared 75% of alleles (range 50–95%, median 76%). In contrast, colony pairs in the Southeast shared only 57% of alleles (range 30–77%, median 58%, t-test, P <0.0001). A total of five private alleles were observed in the Southeast (mean frequency = 0.11), whereas no private alleles were observed in California.

A comparison of the genetic structure in the two ranges revealed that the degree of genetic differentiation between colonies was not significantly different between the ranges based on 95% CI's. The fixation index values (F_{ST}) were 0.126 (0.106 – 0.142) for the Southeast and 0.194 (0.107 – 0.330) for California, suggesting moderate to strong differences among sites within each range. Relatedness coefficients for nestmates in the Southeast r = 0.314 (0.204 – 0.424) and in California r = 0.329 (0.169 – 0.489) were also very similar.

The relationship between pairwise F_{ST} and geographic distance was not significant either in California (r^2 = 0.099, P = 0.974) or in the Southeast (r^2 = 0.004, P = 0.732) (Fig. 1A and B). We also tested for genetic isolation by distance by using Slatkin's (1993) M, an estimator of Nm. The relationship between M and the geographic distance was not

significant either in California (r^2 = 0.063, P = 0.889) or in the Southeast (r^2 = 0.091, P = 0.065).

Geographic variation in intraspecific aggression

The results of behavioral assays revealed striking disparities in intraspecific aggression among colonies in the two regions. In contrast to California, where intraspecific aggression was absent, Argentine ants in the Southeast showed extremely high levels of aggression even at relatively small geographic scales (Fig. 2A, Fig. 3). The average (± S.E.) level of aggression across all population pairs was 3.49 ± 0.08 in the Southeast, and 0.05 ± 0.02 in California (*t*-test, P = <0.0001). In the Southeast, 85.8% of all intercolony encounters resulted in fighting (aggression level 3 or higher, Fig. 3). In California, none of the encounters resulted in fighting and level 2 was the highest level of aggression reached between any of the colony pairs. Aggression between regions was always high (average = 3.99 ± 0.01). Relationships between aggression and genetic similarity of colonies (Fig. 2C) revealed that in the Southeast, high aggression was present even between colonies that shared relatively high proportions of alleles. In contrast, in California aggression was absent irrespective of the proportion of alleles shared.

Discussion

Our results indicate profound genetic and behavioral differences between introduced North American populations of the Argentine ant. Most notably, the introduced population in the Southeast shows a higher level of genetic diversity and intercolony aggression relative to the introduced population in California. In many respects, the behavioral and genotypic differences observed between the southeastern U.S. population and the California population mirror those between native populations and the California population (Suarez et al. 1999; Tsutsui et al. 2000; Tsutsui and Case 2001). We discovered 47 alleles in the southeastern population and 23 alleles in California across 7 polymorphic loci (0.49 ratio). Tsutsui et al. (2000) reported 59 alleles for native populations, and 30 alleles for the introduced California population (0.51 ratio) across 7 polymorphic loci. Although some of the loci in Tsutsui et al. (2000) were different from those in our study, a comparison between the two ratios can be made and the magnitude of genetic differentiation between introduced southeastern and California populations is similar to that between the California population and native populations. In addition to large genotypic differences between native populations and the introduced California population, there are also considerable differences in intraspecific aggression between the two ranges (Tsutsui et al. 2000). In its native range, the Argentine ant is multicolonial, territorial boundaries between colonies are well defined and nests are aggressively defended from conspecifics. In the introduced California population however, the ant is unicolonial where intraspecific aggression is absent across large spatial scales (~1,000 km), colony boundaries are not defined, and a large supercolony is formed. Rarely, smaller supercolonies, which are aggressive toward the large supercolony, can be found in

California (Suarez et al. 1999; Tsutsui et al. 2000; Tsutsui and Case 2001). In the southeastern U.S., unlike in California, intraspecific aggression between colonies is high. Therefore, not all introduced populations within the U.S. are genetically and behaviorally uniform.

The finding that all 23 alleles found in California were a subset of those found in the Southeast, and the finding that five private alleles were observed in the Southeast while none were found in California may help reveal patterns of colonization following the introduction of Argentine ants into the United States. The fact that all alleles in California are a subset of those found in the Southeast suggests that the California population was derived from the southeastern population. It also suggests that the separation happened recently, so that no new mutations have arisen in California. In the United States, the Argentine ant was first detected in 1891 in New Orleans, and by 1907 it was present in California (Newell and Barber 1913). While our study supports previously published reports demonstrating that the Argentine ant experienced a severe bottleneck during its introduction to California (Suarez et al. 1999; Tsutsui et al. 2000; Tsutsui and Case 2001), it adds an important dimension to the genetic bottleneck hypothesis, suggesting that the California supercolony may result from a double bottleneck; a minor one upon entry into the United States, and a major one while being transported from New Orleans to California. One or possibly a few introductions may have reached California from the Southeast, with a single successful introduction spreading inside California by human mediated "commercial jumps" without significant additional introductions of ants from the Southeast.

The high genetic diversity of Argentine ants in the Southeast, relative to that found in California may reflect a minor bottleneck during entry to the United States. Perhaps over the

decades of trade between South and North America, multiple introductions have resulted in the southeastern U.S. receiving much of the genetic variation present in the native range, with subsequent genetically diverse populations distributed throughout the Southeast by human-mediated jump dispersal, the primary mode of long-distance spread for Argentine ants (Suarez et al. 2001). Another factor that may contribute to the high level of genetic diversity in the Southeast, relative to California, is the absence of positive frequency-dependent selection against individuals from high diversity colonies as described by Tsutsui et al. (2003). In California, polarized aggression between colonies acts to maintain and further decrease the low levels of genetic diversity. The requirement for such selection process is direct interaction between colonies. In the Southeast colonies are relatively small and disjointed, which effectively prevents positive frequency-dependent selection from occurring. In contrast to California, where Argentine ants largely dominate areas that provide suitable nesting and dietary requirements, colonies in the Southeast cover relatively small areas and are spatially isolated from each other, despite an abundance of apparent suitable nest sites and food sources in human-disturbed habitats. Our preliminary surveys in the Southeast have uncovered Argentine ants only in urban areas, although we would expect that the movement of people and commodities would broadly distribute reproductively competent colony fragments. In laboratory studies, Argentine ants were very efficient colonizers, and propagules as small as a single queen and 10 workers survived and reproduced (Hee et al. 2000).

Several factors could contribute to the slow spread and the patchy distribution of Argentine ants in the portions of the southeastern U.S. that we sampled. Low winter temperatures may not only delay the spread of existing colonies, but more importantly, may

severely limit the survival of newly transplanted colony fragments. Small colonies, which contain few reproductive queens in comparison to mature colonies, may be especially vulnerable to queen loss and colony extinction due to harsh environmental conditions. We compared average low winter temperatures for the month of January, by averaging temperatures across 22 cities from this study (www.worldclimate.com). In California, the average low winter temperature was 4.0ºC; in the Southeast it was -1ºC (t = -8.747, df = 10, P = 0.0001). Furthermore, the average low winter (January) temperature calculated for the cities in southern Europe harboring the large supercolony described by Giraud et al. (2002) was 3.8ºC, which is not significantly different from that in California (t = 1.812, df = 10, P = 0.413). We hypothesize that this difference of approximately 5ºC between the southeastern U.S. and the geographic regions where supercolonies form may affect the survival of Argentine ants, especially since the average low temperature in the southeastern locations is below freezing, whereas in California and southern Europe it is above freezing. In California, Argentine ants still forage at 5ºC (Markin 1970). Subfreezing temperatures in the Southeast may, in contrast, severely limit the foraging efficiency and survival of small, isolated colony fragments. Argentine ants in the Southeast display very characteristic winter aggregation behavior. In November, when temperatures start declining, small colonies from exposed areas aggregate to form larger colonies in protected areas. During winter months, large aggregates are very pronounced, while small colonies are very rare (Newell and Barber 1913). Therefore, it is quite likely that small, newly translocated colony fragments which cannot aggregate to form larger winter colonies have a smaller chance of survival in the Southeast, relative to California. Temperatures above freezing may promote colony survival

and expansion, leading to high nest density and increased rates of encounters between colonies.

An additional factor that may limit range expansion of *L. humile* in the Southeast is competition with another introduced invasive species; the red imported fire ant, *Solenopsis invicta*. The red imported fire ant is now widely distributed in the Southeast (Vinson 1986), and it may compete with the Argentine ant for nesting sites and access to nutritional sources. According to Wilson (1950), the red imported fire ant has previously displaced Argentine ants from parts of the Southeast. We believe that both relatively low winter temperatures and interaction with imported fire ants could act independently or in concert to restrict the spread of Argentine ants in the Southeast.

Argentine ants in their native range coexist with other ants and there is no evidence that this ant negatively affects the native ant fauna (Suarez et al. 1999). By contrast, in its introduced range, the Argentine ant displaces native ant species (Crowell 1968; Erickson 1971; Williams 1994; Human and Gordon 1996; Suarez et al. 1999; Holway 1999). According to Suarez et al. (1999), Argentine ants on average coexist with only three species of native ants in California but in Argentina they occur together with 25 species. Our visual and bait surveys of the invaded areas in the Southeast (Buczkowski, unpublished data), verify that several native and introduced ant species, including *Tapinoma sessile*, *Monomorium minimum*, *Pheidole* spp., *Solenopsis invicta*, *Crematogaster* spp., and *S. molesta* frequently coexist with Argentine ants on small spatial scales and often compete for and dominate various types of food. It appears, therefore, that in the southeastern U.S. the effect of *L. humile* on the native ant fauna is less disruptive compared with that in California. Highly effective exploitative and interference competition by *L. humile* through numerical

dominance due to the presence of supercolonies has been demonstrated to play a key role in the Argentine ant's ecological success (Human and Gordon 1999; Holway et al. 1999). Perhaps colonies making up the diminutive supercolonies in the southeastern U.S. have not reached a critical size needed for efficient competitive displacement of native ants. In the native range, the Argentine ant coexists with endemic ant species that possibly limit its spread through interspecific competition. In the southeastern U.S., the effect of native ants on the spread of *L. humile* is unknown. Therefore, studies of ant species richness and diversity in the Southeast are needed to determine impacts of other species on the spread of the Argentine ant. Furthermore, competition studies between *S. invicta* and *L. humile* - two highly prolific invasive species are needed, as they might help reveal the events that shaped the present-day distribution patterns of both invaders and may predict future distribution patterns.

Native populations of the Argentine ant display a pattern of genetic isolation by distance, whereas this pattern is absent in introduced California populations (Tsutsui and Case 2001). We did not detect a pattern of isolation by distance in either of the two ranges studied, despite large genotypic variation and high intraspecific aggression in the southeastern populations. This may be due to differences in the mode of dispersal of Argentine ants in the native versus introduced ranges. In the native range, Argentine ants are rarely associated with urban habitats and gene flow, mediated almost exclusively by male flight and short-range colony dispersal, is relatively limited. In contrast, in introduced ranges, gene flow occurs not only on a local scale but also through long-distance jumps, which are human-mediated (Suarez et al. 2001). Indeed, the rate of spread by long-range dispersal is three orders of magnitude higher than that due to budding (Suarez et al. 2001).

Long-range dispersal most likely acts to disrupt the pattern of isolation by distance in the introduced populations.

Relatedness among nestmate workers within colonies in the native range is fairly high (~0.4), relative to colonies within 1–10 km (Tsutsui and Case 2001), suggesting close family relationships among nestmates in introduced populations. In the present study, nestmate relatedness coefficients for the Southeast (r = 0.314) and California (r = 0.329) were also high as were those previously reported by Tsutsui and Case (2001), who found relatedness values of ~ 0.3 at spatial scales of 100–1,000 km, a much larger area than used in the native range for estimating r. However, unlike in the native range, the high relatedness in the introduced populations where queen number is very high is most likely due to significant higher level genetic structure which inflates values of nestmate relatedness (Ross 2001).

Colony pairs in California shared, on average, 75% of alleles, while colony pairs in the Southeast shared only 57% of alleles. Tsutsui et al. (2000) reported allele sharing in the range of 75–100% for nest pairs in California, whereas nest pairs in the native range shared only 17–63% of alleles. A comparison between allele sharing and average level of intraspecific aggression (Fig. 2C) revealed differences between the two populations. Namely, certain colonies from the Southeast and California shared the same percentage of alleles, but showed markedly different aggression levels. This is especially evident in the 50–70% allele sharing range, where there is much overlap in allele sharing between the two geographic regions, yet aggression levels for each remain distinctly divergent. Tsutsui et al. (2000) reported that nests sharing 60% or fewer alleles displayed moderate to high interspecific aggression. Nests that shared >75% alleles, however, were non-aggressive. We did not discover a clear relationship between the degree of genetic similarity and the

level of intraspecific aggression. In California, colony pairs were always non-aggressive, even when they shared a relatively low (~50%) proportion of alleles. In contrast, in the Southeast colony pairs were almost always aggressive despite sometimes high allele sharing (70%). The observed differences may be due to population-specific differences and not related to the percent allele sharing. Therefore, it is difficult to pinpoint a threshold level for the lack of intraspecific aggression in the Argentine ant, and, if such a threshold exists, it is likely to vary among populations.

The present results have some bearing on the mechanisms underlying the formation of supercolonies in the Argentine ant. Tsutsui et al. (2000) and Giraud et al. (2002) propose dissimilar mechanisms for the evolution of unicoloniality. According to Tsutsui et al. (2002), a population bottleneck has reduced the genetic diversity of introduced population(s). This loss is associated with reduced intraspecifc aggression and leads to the formation of supercolonies. In contrast, Giraud et al. (2002) suggested a process whereby introduction into new habitats with relaxed ecological constraints leads to high population density, which in turn causes increased rates of encounters between workers from aggressive colonies. These authors propose that fights between aggressive colonies lead to selection against rare recognition alleles and give colonies with the most common alleles a selective advantage. Our results, which indicate that there may have been one or only a few introductions of Argentine ants into California, support the genetic bottleneck model of Tsutsui et al. (2000), at least in this region. In addition, Argentine ants introduced into the Southeast experienced a relatively minor bottleneck. Tsutsui et al. (2000) reported a 48% loss of allelic diversity in the California supercolony. Giraud et al. (2002) reported a 28% loss in the European

supercolony, but these authors likely underestimated the genetic diversity in the native range (Tsutsui et al. 2003).

Unlike prior reports of extreme unicoloniality within the Argentine ants' introduced range, we have identified a geographic region where colonies occupy relatively small territories and display high levels of intraspecific aggression and genetic variability. Given that Argentine ants were introduced into the Southeast before they were introduced into California, it is evident that certain factors are either slowing down or possibly even preventing the evolution of a supercolony in the Southeast. Additional studies are needed to follow the spread of the Argentine ant in the Southeast to examine how intrinsic (high genotypic variability) and extrinsic (temperature, competing species, and interaction with humans) factors interact to shape the evolution of *L. humile* social organization in the Southeast. The investigation of population structure of Argentine ants in and around New Orleans might offer a unique insight into the role of these factors and possible interaction between them. On the one hand, mild winter temperatures in New Orleans might promote the spread of colonies. Such spread might lead to polarized aggression between adjoining colonies, selection against genetically diverse colonies, and ultimately formation of large supercolonies (Tsutsui et al. 2003). On the other hand, resource competition with *S. invicta* in this area might severely impede such spread. Investigations of other introduced populations, including New Orleans, that differ in levels of genetic diversity, winter temperatures, and degree of competition with other ants should provide valuable insights into the evolution of supercolonies in this invasive species.

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References Cited

Bartlett, B. R. 1961. The influence of ants upon parasites, predators, and scale insects. Ann. Entomol. Soc. Amer. 54: 543–550.

Bhatkar, A. P. and W. H. Whitcomb. 1970. Artificial diet for rearing various species of ants. Florida Entomol. 53: 229–232.

Chakraborty, A. B., and M. Nei. 1977. Bottleneck effect on average heterozygosity and genetic distance with the stepwise mutation model. Evolution. 31: 34 –356.

Cole, F. R., A. C. Medeiros, L. L. Loope, and W. W. Zuehlke. 1992. Effects of the Argentine ant on arthropod fauna of Hawaiian high-elevation shrubland. Ecology 73: 1313–322.

Crowell, K. L. 1968. Rates of competitive exclusion by the Argentine ant in Bermuda. Ecology. 49: 551–555.

Erickson, J. M. 1971. The displacement of native ant species by the introduced Argentine ant *Iridomyrmex humilis* Mayr. Psyche. 78: 257–266.

Giraud, T., J. S. Pedersen, and L. Keller. 2002. Evolution of supercolonies: the Argentine ants of southern Europe. Proc. Natl. Acad. Sci. 99: 6075–6079.

Harned, R. W., and M. R. Smith. 1922. Argentine ant control campaigns in Mississippi. J. Econ. Entomol. 15: 261–264.

Hee, J. J., D. A. Holway, A. V. Suarez, and T. J. Case. 2000. Role of propagule size in the success of incipient colonies of the invasive Argentine ant. Conserv. Biol. 14: 559–563.

Holway, D. A. 1998. Effect of Argentine ant invasions on ground-dwelling arthropods in northern California riparian woodlands. Oecologia 116: 252–258.

Holway, D. A. 1999. Competitive mechanisms underlying the displacement of native ants by the invasive Argentine ant. Ecology. 80: 238–251.

Holway, D. A., and T. J. Case. 2000. Mechanisms of dispersed central-place foraging in polydomous colonies of the Argentine ant. Anim. Behav. 59: 433–441.

Holway, D. A., and T. J. Case. 2001. Effects of colony-level variation on competitive ability in the invasive Argentine ant. Anim. Behav. 61: 1181–1192.

Holway, D. A., A. V. Suarez, and T. J. Case. 1998. Loss of intraspecific aggression in the success of a widespread invasive social insect. Science 282: 949–952.

Human, K. G., and D. M. Gordon. 1996. Exploitation and interference competition between the invasive Argentine ant, *Linepithema humile*, and native ant species. Oecologia 105: 405–412.

Human, K. G., and D. M. Gordon. 1997. Effects of Argentine ants on invertebrate biodiversity in northern California. Conserv. Biol. 11: 1242–1248.

Human, K., and D. M. Gordon. 1999. Behavioral interactions of the invasive Argentine ant with native ant species. Ins. Soc. 46: 159–163.

Knight, R. L. and M. K. Rust. 1990. The urban ants of California with distributional notes of imported species. Southwestern Entomol. 15: 167–178.

Krieger, M. J. B., and L. Keller. 2000. Low polymorphism at 19 microsatellite loci in a French population of Argentine ants (*Linepithema humile*). Mol. Ecol. 8: 1075–1092.

Lewis, P. O., and D. Zaykin. 2001. Genetic Data Analysis: Computer program for the analysis of allelic data. Version 1.0 (d16c). Free program distributed by the authors over the internet from http://lewis.eeb.uconn.edu/lewishome/software.html.

Mantel, N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Res. 27: 209–220.

Markin, G. P. 1968. Nest relationship of the Argentine ant, *Iridomyrmex humilis* (Hymenoptera: Formicidae). J. Kans. Entomol. Soc. 41: 511–516.

Markin, G. P. 1970. The seasonal life cycle of the Argentine ant, *Iridomyrmex humilis* (Hymenoptera: Formicidae) in southern California. Ann. Entomol. Soc. Am. 63: 1238–1242.

Nei, M., T. Maruyama, and R. Chakraborty. 1975. The bottleneck effect and genetic variability in populations. Evolution. 29: 1–10.

Newell W., and T. C. Barber. 1913. The Argentine ant. USDA Bureau of Entomology Bulletin 122.

Obin, M. S. 1986. Nestmate recognition cues in laboratory and field colonies of *Solenopsis invicta* Buren (Hymenoptera: Formicidae): Effect of environment and the role of cuticular hydrocarbons. J. Chem. Ecol. 12: 1965–1975.

Oetting, W. S., H. Lee, D. Flanders, G. Wiesner, T. Sellers, and R. A. King. 1995. Linkage analysis with multiplex short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. Genomics. 30: 450–458.

Queller, D. C., and K. F. Goodnight. 1989. Estimating relatedness using genetic markers. Evolution. 43: 258–275.

Raymond, M. and F. Rousset. 1995. An exact test for population differentiation. Evolution. 49 : 1280–1283.

Ross, K. G. 2001. Molecular ecology of social behaviour: analyses of breeding systems and genetic structure. Mol. Ecol. 10: 265–284.

Ross, K. G., and L. Keller. 1995. Ecology and evolution of social organization: insights from fire ants and other highly eusocial insects. Annu. Rev. Ecol. Syst. 26: 631–656.

Ross, K. G., E. L. Vargo, L. Keller, and J. C. Trager. 1993. Effect of a founder event on variation in the genetic sex-determining system of the fire ant *Solenopsis invicta*. Genetics. 135: 843–854.

Ross, K. G., E. L. Vargo, and L. Keller. 1996. Social evolution in a new environment: the case of introduced fire ants. Proc. Natl. Acad. Sci. 93: 3021–3025.

Roulston, T. H., G. Buczkowski, and J. Silverman. 2003. Nestmate discrimination in ants: effect of bioassay on aggressive behavior. Ins. Soc. 50: 151–159.

Slatkin, M. 1993. Isolation by distance in equilibrium and non-equilibrium populations. Evolution. 47: 264–279.

Smith, M. R. 1936. Distribution of the Argentine ant in the United States and suggestions for its control or eradication. U.S. Dep. Agric. Circ. 387: 1–39.

Suarez, A. V, N. D. Tsutsui, D. A. Holway, and T. J. Case. 1999. Behavioral and genetic differentiation between native and introduced populations of the Argentine ant. Biol. Inv. 1: 43–53.

Suarez, A. V, D. A. Holway, and T. J. Case. 2001. Patterns of spread in biological invasions dominated by long-distance jump dispersal: Insights from Argentine ants. Proc. Natl. Acad. Sci. 98: 1095–1100.

Tsutsui, N. D., A. V. Suarez, D. A. Holway, and T. J. Case. 2000. Reduced genetic variation and the success of an invasive species. Proc. Natl. Acad. Sci. 97: 5948–5953.

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

Tsutsui, N. D., A. V. Suarez, and R. K. Grosberg. 2003. Genetic diversity, asymmetrical aggression, and recognition in a widespread invasive species. Proc. Natl. Acad. Sci. 100: 1078–1083.

Vinson, S. B. 1997. Invasion of the red imported fire ant (Hymenoptera:Formicidae): spread, biology, and impact. Am. Entomol. 43: 23–39.

Ward, P. S. 1987. Distribution of the introduced Argentine ant (*Iridomyrmex humilis*) in natural habitats of the lower Sacramento Valley and its effects on the indigenous ant fauna. Hilgardia 55 : 1–16.

Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38: 1358–1370.

Williams, D. F., ed. 1994. Exotic ants: biology, impact, and control of introduced species. Westview Press, Boulder, CO.

Wilson, E. O. 1951. Variation and adaptation in the imported fire ant. Evolution. 5: 68–79.

Wright, S. 1969-1978. Evolution and the genetics of populations. 4 Vols. Univ. of Chicago Press, Chicago.

Wright, S. 1951. The genetical structure of populations. Ann. Eugenics. 15: 395–420.

Wright, S. 1965. The interpretation of population structure by F-statistics with special regards to systems of mating. Evolution. 19: 395–420.

Table 1. The observed number of alleles (A_O) and the expected heterozygosity (H_E) for Southeastern (North Carolina, South Carolina, Georgia) and Californian populations of *Linepithema humile*.

	Southeast (n=240)		California (n=240)	
Locus	A_O	H_E	A_O	H_E
Lhum-11	8	0.806	4	0.641
Lhum-13	6	0.729	4	0.727
Lhum-19	7	0.736	4	0.660
Lhum-28	2	0.407	1	0.000
Lhum35	13	0.815	6	0.696
Lhum-39	6	0.554	3	0.326
Lihu -T1	5	0.497	1	0.000
Total	47		23	
Mean (SE)		0.649 (0.061)		0.436 (0.123)

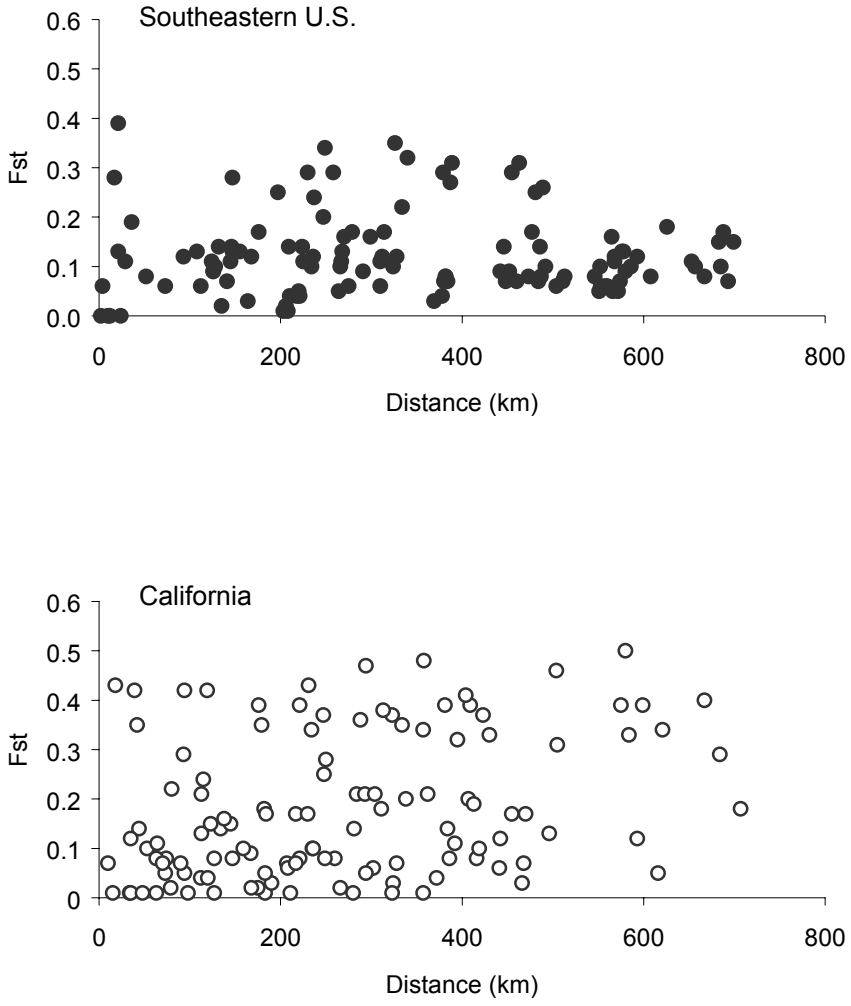

Fig. 1. Relationship between pairwise F_{ST} between colonies plotted against geographic distance in the Southeast (●) and in California (○). Significance was tested using a Mantel test (Southeast: $r^2 = 0.004$, $P = 0.732$; California: $r^2 = 0.099$, $P = 0.974$).

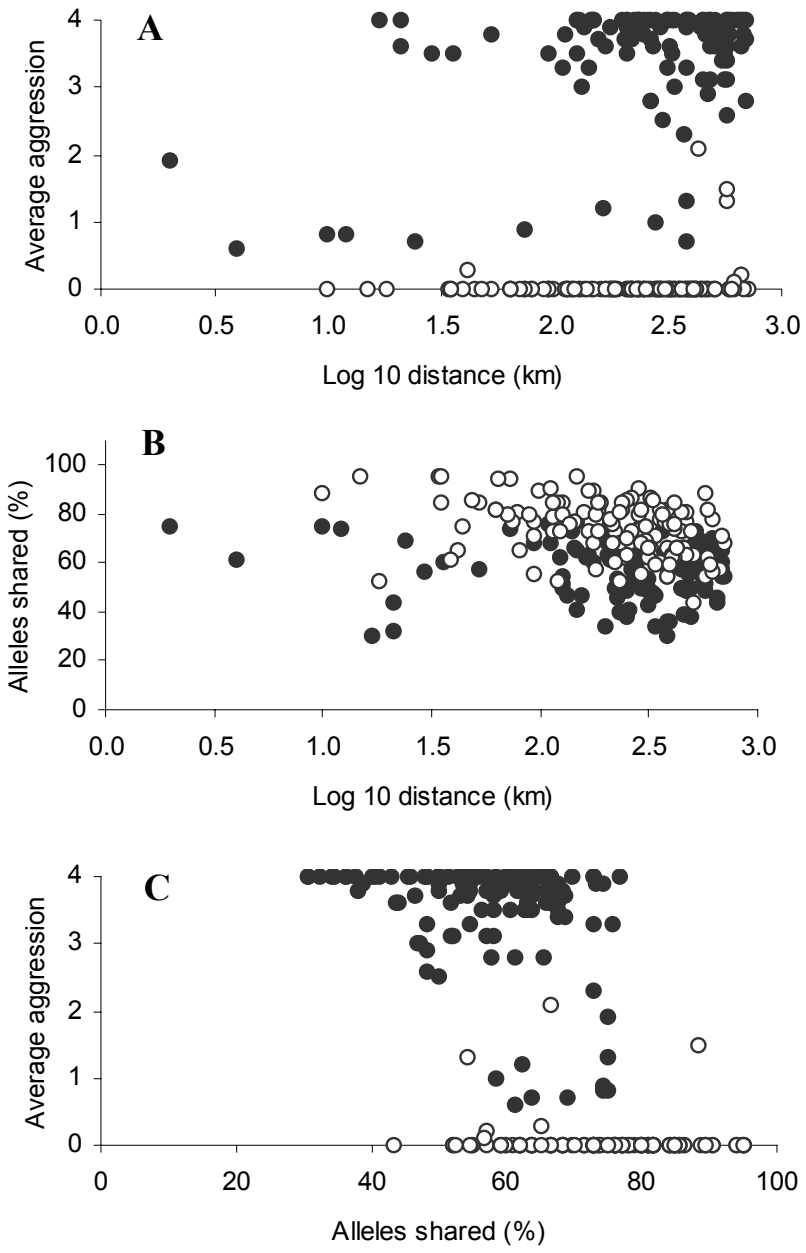

Fig. 2. Relationships among intraspecific aggression, geographic distance, and genetic similarity in the Southeast (●) and in California (○). (A) Relationship between intraspecific aggression and distance between colonies. (B) Relationship between genetic similarity between colony pairs (% alleles shared) and geographic distance. (C) Relationship between intraspecific aggression and genetic similarity of colonies.

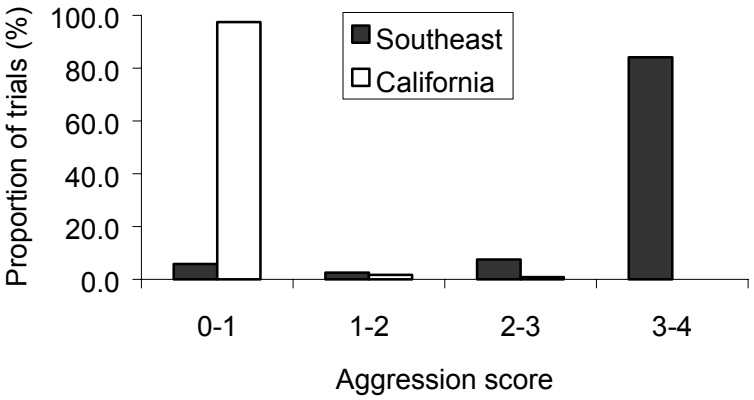

Fig. 3. Proportion of aggressive encounters that resulted in one of the four levels of aggressive behavior (0-1 = ignore, 1-2 = touch, 2-3 = aggression (including lunging, and brief bouts of biting), 3-4 = fighting (including prolonged aggression, abdomen curling and apparent attempts to spray defensive compounds)).

CHAPTER III

Context-dependent nestmate discrimination and the effect of action thresholds on exogenous cue recognition in the Argentine ant. [1]

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[1] to be submitted to *Animal Behaviour*

Abstract. Reeve's (1989) optimal acceptance threshold model predicts that kin/nestmate discrimination is context dependent and that in a fluctuating environment the action component of nestmate discrimination is plastic, rather than static. By examining changes in intraspecific aggression among colonies of Argentine ants (*Linepithema humile*) in various discrimination contexts, we determined that aggression occurs at higher rates when either nestmates or familiar territory indicate nest proximity, but is greatly diminished where social context is absent, thereby providing additional support for the optimal acceptance threshold model. Context-dependent aggression in the Argentine ant appears to result from a shift in acceptance threshold in response to fitness costs associated with accepting non-kin. Starks's et al. (1998) nest indicator and the cost minimizer hypotheses explained to some degree the change in the action component of Argentine ant nestmate discrimination, however, isolated nest referents (familiar territory, conspecific brood, or single familiar nestmates) had no effect on aggression thresholds. We provide mixed support for Tsutsui et al. (2003) proposition that workers from genetically less diverse colonies attack workers from more diverse colonies. We found that in the context of nest defense genetically more diverse colonies will initiate attacks on colonies with lower genetic diversity. Therefore, the role of asymmetrical aggression in reducing genetic diversity within introduced populations of *L. humile* remains unknown and other extrinsic factors such as nest status and/or colony size may affect the outcome of aggressive interactions in the field. Finally, we reconciled differences between earlier reports on the use of exogenous nestmate discrimination cues by Argentine ants by providing diminished intraspecific aggression following colony maintenance under uniform conditions in assays providing a social and/or ecological context, but not where this context was absent.

Keywords: acceptance threshold model, aggression, Argentine ant, social context, invasive ants, nestmate recognition.

Introduction

Kin recognition, defined as the ability to identify relatives, is an adaptive behavior that has evolved independently in many animal taxa (Fletcher and Michener 1987). In social insect societies where individuals frequently live in large and complex colonies, the effect of relatives on the evolution of behavior has been especially profound. The constant communication and exchange of information through an array of sophisticated social behaviors acts to synchronize interactions between individuals, thereby promoting efficient foraging, reproduction, and defense. Eusocial insects maintain territorial boundaries aimed at preventing heterospecifics, as well as conspecifics, from invading and exploiting the colony's nest. Individuals inhabiting a nest are identified through a process of nestmate recognition, whereby nestmates display differential treatment towards conspecifics according to their nest of origin (Gamboa et al. 1986b). In most groups of social insects, nestmates are usually kin and nestmate and kin recognition are generally assumed to be equivalent and involve similar modes of discrimination (Gamboa et al. 1991a).

The nestmate recognition system of social insects is thought to consist of three distinct components: (1) expression, (2) perception, and (3) action (Sherman and Holmes 1985; Gamboa et al. 1986b; Waldman 1987; Reeve 1989). The expression component can be any aspect of the phenotype that signifies membership reliably, as well as the mechanisms involved in cue production and acquisition. The perception component involves the development of the recognition template, the processing of that cue once perceived, and the algorithm used to match a perceived cue with the template. Finally, the action component describes the process of cue-template matching, whereby the phenotype of an encountered

individual is matched with a memory of a set of acceptable cues (the template) and an action is taken.

The unpredictable and ever changing nature of the expression component has a profound effect on the evolution of social behavior. Recognition cues are dynamic and may change throughout the life of the colony (Provost et al. 1993; Vander Meer et al. 1989) and may exhibit seasonal variation (Ichinose 1991). Temporal variation in reproductive phenology of colonies (Passera and Aron 1993), changes in worker density and/or queen number, and variation in intracolony patterns of relatedness may also influence nestmate recognition behavior. Furthermore, the enormous variation and complexity in colony structure and mating systems exhibited by many social insects (polygyny, polyandry, polydomy) may affect the spatial and temporal distribution of kin and further decrease the likelihood that the invader's cues will match the template of the resident. Such variation in genetic and environmental parameters dictates that colony members continually obtain and process information about their changing ecological and social environment and act accordingly, and necessitates that the action component of nestmate recognition be plastic in order to minimize the chance of recognition errors.

Reeve (1989) developed the optimal acceptance threshold model to describe the plasticity of kin discrimination mechanisms in social insects. According to the model, the acceptance threshold varies according to context, to balance the fitness costs of accepting non-kin and rejecting kin. Specifically, this model predicts that as the cost of accepting non-kin increases, the acceptance threshold is lowered. Starks et al. (1998) contrasted the optimal acceptance threshold hypothesis with the fixed error hypothesis. Occasional errors in discrimination are expected (Reeve 1989) and the fixed error hypothesis states that a

certain frequency of discrimination errors will be observed. This frequency, however, is not dependent on the context.

The recognition cues expressed, perceived, and acted upon are chemical in nature (Hepper 1991). In order to recognize colony members from non-members, individuals recognize each other through a learned chemical label or "colony odor". The signals used in nestmate recognition may have a genetic or environmental origin, and these signals are generally thought to be cuticular hydrocarbons (e.g., Obin 1986; Bonavita-Cougourdan et al. 1987,1989; Howard 1993). Environmentally derived cues, which may originate from nesting material (Breed 1983; Gamboa et al. 1986b; Stuart 1987) or diet (Jutsum et al. 1979; Crosland 1989; Obin and Vander Meer 1988; Le Moli et al. 1992) change depending on the ant's living environment and diet. The relative contribution of each source to the overall hydrocarbon profile remains unknown. Irrespective of the source, workers within a colony must learn the cues specific to their nest and they must be able to properly evaluate cues present on newly encountered workers. Conspecifics bearing a foreign label or lacking an appropriate one are attacked and often killed.

The Argentine ant, *Linepithema humile*, is an invasive species native to South America. In its native range, *L. humile* is multicolonial maintaining distinct territorial boundaries with aggression between colonies occurring across relatively small spatial scales (Suarez et al. 1999; Tsutsui et al. 2000). Within introduced populations, however, *L. humile* is unicolonial, producing large multiple-queen colonies that lack clear boundaries due to a general absence of intraspecific aggression (Markin 1968; Newell and Barber 1913; Suarez et al. 1999; Tsutsui et al. 2000; Giraud et al. 2002). It has been suggested that unicoloniality appeared in introduced populations as a result of a genetic bottleneck at the time of

introduction (Tsutsui et al. 2000) and was further strengthened by polarized aggression by individuals from less genetically diverse colonies toward individuals from more genetically diverse colonies (Tsutsui et al. 2003). Argentine ants respond to both genetically based cues (Tsutsui et al. 2000; Suarez et al. 2002) and cues derived from the environment (Chen and Nonacs 2000; Liang and Silverman 2000). The relative importance of genetic and environmental sources for nestmate recognition in the Argentine ant was addressed in laboratory and field studies, where the environmental component was kept constant and changes in aggression patterns between individuals from different colonies were assessed. Chen and Nonacs (2000) recorded a complete loss of aggression in colonies reared for 2 months under uniform laboratory conditions, suggesting a primary role for environmentally-derived cues. In contrast, Suarez et al. (2002) and Holway et al. (1998) detected little change in the pattern of intraspecific aggression over time and concluded that the role of exogenous cues was minor, relative to inherited cues. Possible explanations for the disparate conclusions include distinct laboratory rearing regimes (e.g. dietary and nesting conditions) as well as the length of time during which the colonies were subjected to laboratory rearing conditions (3 weeks to > 1 year). Also, different source colonies and collection times as well as distinct aggression tests to estimate changes in patterns of nestmate discrimination may have differentiated the study results.

While differences in colony source and phenology may account for the relative contributions of heritable and exogenous nestmate recognition cues, we suspect that the social context in which aggression behavior was examined strongly affected the action component of discrimination as per Reeve's (1989) model, with conspecific discrimination being sensitive to experimental conditions. The choice of aggression assay can significantly

affect estimates of intercolony aggression, with assays utilizing the greatest number of ants being most likely to reveal aggression (Roulston et al. 2003, Appendix 1). Herein, we attempt to reconcile differences between earlier conclusions regarding the relative contribution of heritable and environmental nestmate recognition cues in *L. humile* by examining intraspecific aggression between identical source colonies in different social contexts raised under uniform laboratory conditions. In addition, we test two hypotheses proposed by Starks et al. (1998): (1) the nest indicator hypothesis, which predicts that the presence of nestmates indicates nest proximity and denotes a fitness payoff for active defense, and (2) the cost minimizer hypothesis, which predicts that nestmates are willing to share the cost of nest defense in groups, but not singly.

Using neutral arena behavioral tests (dyad interactions) Tsutsui et al. (2003) demonstrated polarized aggression, whereby individuals from genetically less diverse colonies attack individuals from more diverse colonies. Our preliminary observations indicated that in intruder introductions the residents are protective of their territory and often initiate attacks on the intruders. The importance of a familiar territory for defense of social insect societies has been explicitly demonstrated in numerous studies (e.g., Hölldobler 1976; Hölldobler and Lumsden 1980; Gordon 1989). Therefore, we hypothesized that in intruder introductions nest status of individual ants (residents vs. intruders), rather than the level of genetic disparity between individuals is of primary importance in determining the identity of the aggressor. To test this hypothesis we compared colonies with different levels of genetic diversity (microsatellite alleles) and performed intruder introduction tests between low and high diversity colonies.

Materials and Methods

Collection and Maintenance of Laboratory Colonies

We used six colonies of Argentine ants (*Linepithema humile*) collected in North Carolina: Chapel Hill (CHH), Emerald Isle (EMI), Greenville (GNC), Jacksonville (JAC), Research Triangle Park (RTP), and Winston-Salem (FOR) and one colony from California: Pleasanton (PLS). For each location, we established a single colony consisting of 15,000–20,000 workers, a few hundred queens, and numerous brood. Colonies were maintained in soil-free, Fluon™-coated trays containing nests comprised of plastic dishes filled with moist grooved plaster. Colonies were provisioned with 25% sucrose solution and artificial diet (Bhatkar and Whitcomb, 1970) *ad libidum* and hard-boiled egg once a week. All colonies were maintained at 24 ± 1ºC, 50 ± 10% RH, and a 12:12 L:D cycle.

Aggression Tests

We assessed the initial level of aggression between 16 colony pairs: GNC–FOR, GNC–EMI, EMI–JAC, FOR–JAC, GNC–CHH, CHH–FOR, EMI–CHH, JAC–CHH, CHH–RTP, EMI–RTP, PLS–CHH, PLS–FOR, PLS–GNC, PLS–EMI, PLS–RTP, PLS–JAC using three different aggression bioassays: worker dyad interactions within a neutral arena, group worker interactions in a neutral arena, and intruder introductions into an established resident territory. Detailed descriptions of each assay are presented below. We then compared the results of each of these bioassays, each designed to measure context-dependent changes in aggression. For each assay, the observer who recorded worker aggression level did not know the identity of the interacting colonies, and was unfamiliar with the hypothesis being tested.

Individual ants were not used in more than one trial and estimates of initial aggression levels were determined within a week of collection. The same colony pairs were evaluated four months later for changes in aggression.

Assay 1: intruder introduction to resident territory

Relative to the other two assays, the nestmate recognition context in this assay is apparent, with resident workers discriminating within a familiar territory and in close proximity to the nest. Non-nestmates represent a usurpation pressure and nestmates may be more aggressive towards non-nestmates, the high worker density indicating that the nest is nearby. Individual intruder workers were collected on a toothpick and introduced into rearing trays (52 by 38 cm) containing a resident colony (~ 10, 000 workers). The level of aggression was scored using the 0–4 aggression scale of Suarez et al. (1999). For each test, we followed the intruder through as many as 25 encounters with residents: direct physical contact between intruder and any resident was considered an encounter. The intruder was discarded after each trial, and subsequent trials were conducted when the residents were no longer visibly agitated (5-10 min). Ten replicates per colony pair were performed; five replicates with colony 1 as the resident and five replicates with colony 1 as the intruder. Data were analyzed as the maximum score per trial (Roulston et al. 2003, Appendix 1).

Assay 2: symmetrical group interactions in a neutral arena

This bioassay was conducted in a neutral arena where worker cohorts interacted apart from their nests. Although without the nest, workers may be less inclined to attack non-nestmates when there were no resources to protect, we predict that workers would be aggressive toward

non-nestmates because increasing worker density indicates nest proximity. Furthermore, we predict that the presence of familiar nestmates would facilitate aggression toward non-nestmates because the cost of defense is shared with other nestmates. We followed the protocol of Chen and Nonacs (2000). Twenty randomly selected workers were transferred to a plastic, fluoned container (10 cm diameter, 5 cm high). Similarly, 20 workers were placed in a plastic fluoned arena (30 by 17.5 by 8.25 cm high). Both groups of ants were allowed to calm for 5 minutes, after which the two groups of workers were combined by gently emptying ants from the smaller tray into the arena. We performed 10 replicates per pairing. The number of ants involved in fighting (aggression level 3 or above) was recorded for 15 minutes. For analyses we used the highest number of simultaneous fights observed in 15 min.

Assay 3: dyad interactions

In dyad interactions, the context of colony defense is absent as workers are isolated from both nestmates and a familiar territory and the threat of resource usurpation is low, therefore we predict that the chance of detecting aggression will be less than in the above contexts. Two workers, selected at random from each of two stock colonies, were collected on a toothpick and placed sequentially into a glass vial (2-dram). Vials were fluoned within 1 cm of the bottom thereby restricting ants to a small area and maximizing encounters. Ant interactions were scored on a 0–4 scale (Suarez et al. 1999) with highest aggression observed during 5 continuous minutes recorded, and analyzed as the maximum score per trial.

Response of Same Individual in Different Social Contexts

In contrast to dyadic interactions, assays that utilized greater numbers of individuals were more likely to reveal high aggression (Roulston et al. 2003). To further examine the context-dependency of aggression we measured the aggressiveness of the same individual by placing it in two different contexts: as a member of a dyadic pair and as an intruder.

Using two colonies, Chapel Hill (CHH) and Pleasanton (PLS), which in earlier tests displayed aggression we first assessed the level of aggression in dyadic interactions (60 replications). To distinguish workers from the two colonies during trials, PLS workers were marked by feeding them a small amount of dilute sugar water, which does not affect ant discriminatory capacity (Tsutsui et al. 2003). In instances where the two ants did not display aggression within 5 min, the marked workers were removed from the vial and introduced into a rearing tray containing the opposing colony. Behavioral interactions between the intruder and the resident ants were scored again as described in "Assay 1". To examine the change in behavior in ants that did not show aggression in dyadic tests we noted whether the intruder or the resident initiated aggression.

Effect of Familiar Territory, Familiar Nestmates and Brood on Aggressive Interactions

Starks et al. (1998) reported that in *Polistes dominulus* aggression toward non-nestmates increased in the presence of either familiar nestmates (cost minimizer hypothesis) or a familiar nest fragment (nest indicator hypothesis). We measured aggression between two *L. humile* colonies, Cary (CAR) and Winston-Salem (FOR) in dyad interactions (Assay 1) and intruder introductions (Assay 3) and also modified these assays to evaluate relative importance of nestmates or brood. We determined the effect of nest and/or familiar territory by measuring aggression towards intruders by workers that were or were not isolated from

their nest for one week. Approximately ~2,000 workers from a laboratory colony were transferred to a clean tray without queens or brood and were provisioned with 25% sucrose solution and a water vial stoppered with cotton, to prevent ants from using the vial as a nest. To test whether modifying the social context increases aggression between worker dyads we included brood, an additional nestmate (triad) or colony-specific odor. Brood (two late instar larvae and two pupae) were transferred into the vial with a brush, followed by a nestmate worker. After one minute, we added a non-nestmate worker and noted the highest aggression score in 5 min and the number of workers that carried brood at any time during the trial. To precondition the vials with colony-specific odor, we first fluoned the vials and then placed them in stock colonies for 9 d. In the Argentine ant, marking of home ranges and/or territories has not been reported. However, home range and territory marking has been described in several other ants (Cammaerts et al. 1977; Hölldobler and Wilson 1977; Jaffe et al. 1979). We observed Argentine ants visiting objects that had not been previously exposed to the colony, possibly depositing chemicals that may aid in territory recognition. Aggression in tests with conditioned vials and triads was measured as described in Assay 3. Fifty replicates were performed for each assay.

Territory Defense and its Effect on the Polarity of Aggression

We performed intruder introduction tests (Assay 1) between colonies having different numbers of microsatelite alleles to test whether nest status of individual ants (residents vs. intruders), rather than the direction of allelic diversity asymmetry is of primary importance in determining the identity of the aggressor. For microsatellite analysis, genomic DNA was extracted from 15 workers from each site using the DNeasy Tissue Kit (Qiagen, Valencia,

CA) and analyzed at seven polymorphic microsatellite loci: *Lhum-11, Lhum-13, Lhum-19, Lhum-28, Lhum-35, Lhum-39* (Krieger and Keller 1999) and *Lihu-T1* (Tsutsui et al. 2000). PCR reactions were multiplexed and amplification products were separated on 6.5% KB^{Plus} polyacrylamide sequencing gels using a 4000L Li-Cor DNA sequencer. Microsatellite alleles were scored using RFLPScan software (Scanalytics, Billerica, MA). Two colonies, PLS and CHH, 17 and 16 alleles, respectively were paired up against the remaining five colonies having 21 to 29 alleles. An observer, blind to the identity of the colonies and the hypothesis being tested, recorded the frequency of initiation of aggression by residents, intruders, or both. There were 20 replicates per colony pair, 10 replicates with the lower diversity colony acting as the resident and 10 with the higher diversity colony acting as the resident. The frequency of trials in which the intruder and the resident attacked simultaneously was relatively low (8 out of 200). Therefore, such trials were disregarded and replaced with a new replicate.

Statistical Analyses

We determined the effect of exogenous cues on nestmate recognition by measuring the change in aggression within each of the three social contexts in each of two aggression categories: mild (n=4) and high (n=12) using the PROC UNIVARIATE procedure (Signed Rank test) in SAS 8.1 (SAS 2002). This procedure was also used to examine changes in aggression in assays that examined the relative importance of additional nest referents on intercolony aggression. To detect possible differences in aggression change between mildly and highly aggressive colony pairs within each aggression bioassay, we used the PROC NPAR1WAY procedure (Wilcoxon rank-sum test, assuming a t approximation). In all

analyses, absolute rather than relative aggression loss values were used. The polarity of aggression between residents and intruders in dyadic interactions was tested using Wald's chi-square test.

Results

Initial and four month intercolony aggression levels are presented in Table 1 and Fig. 1. We detected 12 pairings with high initial aggression only with assays that permitted group interactions (symmetrical group interactions and intruder introductions). By contrast, dyad interactions revealed only low and non-injurious aggression. Dyad interactions and intruder introductions used the same scoring scale (0–4), therefore permitting a direct comparison of aggression scores. In the most aggressive pairs, intruder introductions revealed aggression scores (4.0 ± 0.0) significantly higher than dyadic interactions (1.6 ± 0.2; $P < 0.0001$), demonstrating differences between assays and the context-dependency of *L. humile* aggression. When the same individual worker was placed in different social contexts (dyad interactions vs. intruder introductions), 37 out of 60 dyad interactions revealed no aggression (score of 0) and the average aggression score for all replications was 1.05 ± 0.22 S.E. When workers that did not show aggression in dyadic encounters were introduced into an opposing colony, high aggression ensued between the intruders and the residents (average score = 4.0 ± 0.0 S.E.**;** *t*-test: df = 59, $t = 2.0$, $P < 0.0001$) demonstrating that lack of aggression in dyad interactions is due to the lack of context and not chance selection of non-aggressive phenotypes.

The effect of additional nestmates (triads), familiar territory (nest), or brood on aggressive interactions are presented in Fig. 2. The standard intruder introduction assay (with nest) produced an aggression score of 3.2 ± 0.1 and only 2 out of 50 trials resulted in no aggression (score of 0). In contrast, dyad interactions (without brood) revealed significantly lower aggression, 1.1 ± 0.2, and 36 out of 50 trials resulted in no aggression

(Signed Rank test: df = 49, S = 426, P <0.0001). Aggression was not diminished by isolating workers from their nest and a familiar territory (Signed Rank test: df = 49, S = 19.5, P = 0.669). In dyadic interactions, the addition of brood reduced aggression from 1.1 ± 0.2 to 0.3 ± 0.1 (Signed Rank test: df = 49, S = 68.5, P = 0.008). We observed that workers immediately picked up their own brood and carried them around the vial, usually for the duration of the test. In 20 out of 50 trials both workers carried brood, in 22 trials one of the workers carried brood, and in 8 trials none of the workers carried brood. Dyadic interactions in preconditioned vials were no more aggressive than interactions in clean vials (Signed Rank test: df = 49, S = -25.5, P = 0.617). Similarly, triads were no more aggressive than dyads (Signed Rank test: df = 49, S = 4.0, P = 0.909).

In intruder introductions between colonies having disparate levels of genetic diversity, aggression was polarized relative to both allelic diversity and direction of worker introduction (Table 2; P <0.0001, Wald's χ^2). In colony pairings involving CHH workers, aggression was polarized with respect to nest status, and not genetic diversity. Intruding CHH workers were aggressed upon by the higher diversity resident colonies in 96% of trials (48/50) (P <0.0001, Wald's χ^2). When the roles were reversed, resident CHH workers initiated aggression more often (P <0.0001, Wald's χ^2) attacking the intruders in 84% of trials (42/50). The probability of the intruder attacking when CHH workers were intruders was not different from the probability of the intruder attacking when CHH workers were residents (P = 0.76, Wald's χ^2). In colony pairings involving PLS workers aggression was polarized with respect to genetic diversity, and not nest status. Intruder PLS workers initiated aggression in 74% of trials (37/50), more often than the residents (P <0.0001, Wald's χ^2), while resident PLS workers initiated aggression in 82% of trials

(41/50), more often than the intruder (P <0.0001, Wald's χ^2). The probability of the intruder attacking when PLS workers were intruders was significantly different from the probability of the intruder attacking when PLS workers were residents (P <0.0001, Wald's χ^2).

We recorded changes in temporal patterns of aggression in the Argentine ant, however altered responses were detected only with assays permitting interactions between many individuals (Fig. 2). Changes in aggression were evident in all but the dyad pairings, with significant estimates of aggression loss for both mildly and highly aggressive colony pairings (Table 1). In dyadic interactions, intercolony aggression increased by ~15% in the highly aggressive category (P = 0.28) and decreased by ~15% in the mildly aggressive category (P = 0.25). In symmetrical group interactions and intruder introductions, all colony pairs revealed a loss in aggression irrespective of the level of initial aggression. By contrast, in dyadic interactions, aggression declined in 5 out of 16 (31%) colony pairings, increased in 9 (56%) pairings, and remained unchanged in 2 (13%) pairings.

Comparisons of the magnitude of aggression loss between mildly and highly aggressive categories within each assay produced varied results. Symmetrical group interactions and dyadic interactions revealed no loss in aggression between both aggression categories (Wilcoxon rank-sum test: P = 0.136 and P = 0.181, respectively). However, in intruder introductions the aggression loss experienced by ants in the highly aggressive category was lower relative to the mildly aggressive category (Wilcoxon rank-sum test: P = 0.027).

Average allele sharing between highly aggressive colony pairs was 45% (range 31-68%) and average aggression was 4.0 ± 0.0. Mildly aggressive colony pairs shared on average 60% of alleles (range 50-76%) with an average aggression score of 2.4 ± 0.5.

Discussion

We demonstrate that aggressive interactions between Argentine ant workers are affected by social and/or ecological contexts and that responses to externally derived recognition cues are also context-dependent. We provide additional support for Reeve's (1989) optimal acceptance threshold model, whereby the action component of nestmate discrimination in *L. humile* is flexible rather than static so that as the fitness cost of accepting non-nestmates decreases, the aggression threshold increases, and the probability of rejecting non-nestmates decreases.

We provided social and ecological contexts that varied from minimal (or possibly even absent) to one approximating field conditions. As a consequence, aggression between non-nestmates was high in group interactions where the fitness cost of accepting non-kin was high and low (< 60% of encounters) in dyad pairings, where the cost of accepting non-kin was low.

Starks et al. (1998) offered two hypotheses to explain enhanced aggression towards non-nestmates in the presence of nestmates in *Polistes dominulus* (Hymenoptera: Vespidae), finding strong support for the nest indicator hypothesis, whereby the presence of a nestmate indicates proximity to the nest, and a fitness payoff for active defense, but not the cost minimizer hypothesis where nestmates share the cost of nest defense in groups, but not singly. Context-dependent aggression and the importance of a familiar territory for social organization and defense is evident in various social insects (Hölldobler 1976; Hölldobler and Lumsden 1980; Pfennig and Reeve 1980; Gordon 1989; Downs and Ratnieks 2000). Jaffe (1986) suggested that territory and nestmate recognition cues are

identical and Vander Meer (1988) demonstrated a constant exchange of exogenous and endogenous cues between colony members and the environment. Hubbard (1974) found that *Solenopsis invicta* workers colonized material from their own nests rather than that of other *S. invicta* colonies or clean soil. Also, aggression towards non-nestmates increased with increasing proximity to the nest (Gamboa et al. 1991b; Venkataraman and Gadagkar 1992).

In *L. humile* dyadic encounters, the absence of familiar nestmates may signal that the nest is relatively distant and individuals thereby perceive no threat of colony resource usurpation. Furthermore, the cost of nest defense is not shared with nestmates; consequently individuals may be reluctant to engage in aggressive behavior. Also, we frequently observed workers attempting to escape the vial, indicating that their priorities may switch from guarding the colony to assuring their own safety within an unfamiliar environment. Results from our symmetrical group interactions, where workers from both colonies fought, despite the absence of familiar nest cues, support the cost minimizer hypothesis, with individuals in the presence of nestmates being more aggressive since the cost of nest defense can be shared. High aggression of resident workers toward intruders is consistent with both nest indicator and cost minimizer hypotheses.

Context dependency of aggression in *L. humile* was apparent when the same individual worker was placed in different social contexts (dyad interactions vs. intruder introductions). Workers that did not exhibit aggression in dyadic interactions displayed high aggression in intruder introductions. The general lack of aggression across dyadic encounters may result either from selection of one or two non-aggressive workers or from inhibition of aggression due to the lack of context. Where two randomly selected ants are

non-aggressive, the prospect of detecting aggression is indeed very low. Where one of the ants is non-aggressive, submissive behavior by one worker may not provoke aggression in the other as the behavior of each participant in a dyadic interaction is a function of both the template-cue match and the behavior of the participants (Gamboa et al. 1991a). We show that lack of aggression in *L. humile* dyads is not because of selection of one or two non-aggressive phenotypes, but rather that workers are unwilling to incur the full cost of aggression in the absence of nestmates and away from the nest.

While aggression toward non-nestmate *P. dominulus* increased in the presence of either a familiar nest fragment or familiar nestmates (Starks et al. 1998), we recorded no independent effect of familiar territory, presence of brood, or presence of one additional nestmate on Argentine ant aggression. Social wasps construct and maintain elaborate paper nests throughout an entire season and use cues originating from both the brood and the nest in nestmate recognition (Ross and Gamboa 1981; Shellman 1982; Klahn and Gamboa 1983; Gamboa et al. 1986a; Singer and Espelie1992; Layton and Espiele 1995), therefore factors associated with the nest may be important territorial cues. In contrast, Argentine ants create impermanent nests (Newell and Barber 1913; Markin 1968) moving frequently in response to changes in the physical environmental (Newell and Barber 1913; Markin 1970) and the distribution of food resources (Holway and Case 2000; Silverman and Nsimba 2000). Therefore, the importance of nest material in *L. humile* territorial defense may be negligible.

Contrary to our predictions, the addition of conspecific brood reduced aggression between non-nestmates. Brood carrying behavior was largely responsible for the observed reduction in aggression and the presence of brood may have caused one or both worker's

priorities to switch from fighting rivals to finding a secure place to deposit brood. Also, mandibles grasping brood effectively prevented the workers from engaging in fights.

While we predicted that colony-specific odors would signal the proximity of the nest providing a fitness payoff for protection of relatives, no increase in aggression in preconditioned vials was observed. Although species-specific home range marking occurs in ants (Gordon 1988; Mayade et al. 1993; Jaffe et al. 1979), including the Argentine ant (Aron et al. 1990), colony-specific territory marking observed in various ants (Hölldobler and Wilson 1977**;** Jaffe and Puche 1984**;** Salzemann 1993) has not been reported in the Argentine ant. We also considered that although the appropriate colony specific cues may have been present, Argentine ant worker aggression was low due to the reluctance to engage in territory protection when familiar nestmates were absent.

While support for the cost minimizer hypothesis (Starks et al. 1998) comes from increased aggression in *P. dominulus* triad vs. dyad interactions we observed no elevated aggression in Argentine ant triads. However, elevated *L. humile* aggression in large symmetrical group interactions suggests that there may be a group size threshold required to provoke aggression, possibly related to nest defense.

We provide mixed support for Tsutsui et al.'s (2003) proposition that selection against rare alleles at recognition loci may promote unicoloniality in Argentine ants. Using worker dyads, Tsutsui et al. (2003) reported asymmetrical aggression whereby workers from genetically less diverse colonies attacked workers from more diverse colonies, and attackers were six times more likely to survive agonistic encounters than recipients of aggression. However, in the context of nest defense we found that genetically more diverse colonies initiated attacks on colonies with lower genetic diversity. Surprisingly however,

genetically more diverse colonies initiated attacks only in pairings involving CHH ants, and not ants from PLS. While attackers having low genetic diversity are more likely to win in dyadic fights (Tsutsui et al. 2003), lone intruders introduced into resident colonies are always killed, irrespective of the level of genetic diversity simply because they are outnumbered by the resident workers. Under natural conditions the sizes of competing Argentine ant colonies are likely to be unequal and fights are likely to involve groups of workers rather than dyads. In such situations the odds of winning may be primarily influenced by asymmetry in colony size rather than asymmetry in colony allelic diversity. While further evidence is required to determine the relative importance of territorial status vs. genetic diversity in intraspecific competition, we suggest that more genetically diverse Argentine ant colonies may not be displaced by their less diverse neighbors.

We also demonstrated that cues derived from the Argentine ants' external environment contribute to the overall recognition profile in this species. However, assays employing intercolony dyads may underestimate the importance of exogenous cues in nestmate recognition as acceptance thresholds are not exceeded prior to and after a period of colony maintenance under constant environmental conditions. Thus, the differing conclusions of Chen and Nonacs (2000) and Suarez et al. (2002) regarding the contribution of environmentally-derived cues to nestmate recognition can be reconciled, at least in part, when the context in which intercolony aggression was monitored is considered. Our data from colonies assayed for aggression in different social contexts, support the conclusions of both Chen and Nonacs (2000) and Suarez et al. (2002); that is temporal reduction in aggression occurred when measured in group interactions (Chen and Nonacs 2000), but not in dyadic pairings (Suarez et al. 2002).

The magnitude of aggression loss in *L. humile* depended on the initial aggression level between colony pairs, such that high levels of intraspecific aggression decreased only slightly over the course of the study, suggesting that highly aggressive pairings may possibly be more dissimilar at recognition loci, and consequently exogenous cues have a minor impact on nestmate recognition. In contrast, initially mildly aggressive colony pairs became substantially less aggressive following prolonged laboratory rearing allowing us to ascertain a significant exogenous nestmate recognition cue component. Previously we (Buczkowski et al. unpublished data) determined that colony pairs with similar levels of genetic similarity may display markedly different levels of aggression suggesting a role for environmental factors in nestmate recognition, as direct relationship between intercolony aggression and genetic similarity would be expected when cues are derived strictly from heritable sources. The Argentine ant utilizes both genetic (Tsutsui et al 2000) and environmental nestmate recognition cues (Chen and Nonacs 2000; Liang and Silverman 2000), therefore no relationship between aggression and genetic similarity would be expected.

Our results demonstrate the importance of using appropriate aggression assays for evaluating changes in intercolony aggression patterns. Acceptance thresholds are context specific, which entails that nestmate discrimination is sensitive to experimental conditions. Our results show that aggression is a function of the relative frequency of interactions with conspecifics and the fitness consequences of accepting or rejecting conspecifics. Therefore, we suggest that investigators be alert to the context-dependency of aggressive behavior when investigating conspecific discrimination in neutral experimental arenas. We

recommend the use of assays that involve group fights and present realistic aggression contexts.

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References Cited

Aron, S. J., M. Pasteels, S. Goss, and J. L. Deneubourg. 1990. Self-organizing spatial patterns in the Argentine ant *Iridomyrmex humilis* (Mayr). Pp. 438–451 *In* R. K. Vander Meer, K. Jaffe, and A. Cedeno [Eds.] Applied myrmecology, a world perspective. Westview Press, Boulder, Colorado.

Bhatkar, A. D., and W. H. Whitcomb. 1970. Artificial diet for rearing various species of ants. Florida Entomol.53: 229–232.

Bonavita-Cougourdan, A., J. L. Clement, and C. Lange. 1987. Nestmate recognition: the role of cuticular hydrocarbons in the ant *Camponotus vagus* Scop. J. Entomol.Sci. 22: 1–10.

Bonavita-Cougourdan, A., J. L. Clement, and C. Lange. 1989. The role of cuticular hydrocarbons in recognition of larvae by workers of the ant *Camponotus vagus*: changes in the chemical signature in response to social environment (Hymenoptera: Formicidae). Sociobiology, 16: 49–74.

Breed, M D. 1983. Nestmate recognition in honey bees. Anim. Behav. 31: 86-91.

Cammaerts, M. C., E. D. Morgan, and R. Tyler. 1977. Territorial marking in the ant *Myrmica rubra* L. (Formicidae). Biology of Behavior, 2: 263–272.

Chen, J. S. C. and P. Nonacs. 2000. Nestmate recognition and intraspecific aggression based on environmental cues in Argentine ants (Hymenoptera : Formicidae). Ann. Entomol. Soc. Am. 93: 1333–1337.

Crosland, M. W. J. 1989. Kin recognition in the ant *Rhytidoponera confusa*. I. Environmental odour. Anim. Behav. 37: 912–919.

Downs, S. G., and F. L. W. Ratnieks. 2000. Adaptive shifts in honey bee (*Apis mellifera* L.) guarding behavior support predictions of the acceptance threshold model. Behav. Ecol. 11: 326–333.

Fletcher, D. J. and C. Michener (eds.). 1987. Kin Recognition in Animals. John Wiley & Sons, New York.

Gamboa, G.J., Reeve, H.K., Ferguson, I.D., Wacker, T.L. 1986a. Nestmate recognition in social wasps: the origin and acquisition of recognition odours. Anim. Behav. 34: 685–695.

Gamboa, G. J., H. K. Reeve, and D.W. Pfennig. 1986b. The evolution and ontogeny of nestmate recognition in social wasps. Ann. Rev. Entomol. 31: 431– 454.

Gamboa, G. J., H. K. Reeve, and W. G. Holmes. 1991a. Conceptual issues and methodology in kin recognition research, a critical discussion. Ethology. 88: 109–127.

Gamboa, G. J., R.L. Foster, J. A. Scope, and A. M. Bitterman. 1991b. Effects of stage colony cycle context and intercolony distance on conspecific tolerance by paper wasps, *Polistes fuscatus*. Behav. Ecol. Sociobiol. 29: 87–94.

Giraud, T., J. S. Pedersen, and L. Keller. 2002. Evolution of supercolonies: the Argentine ants of southern Europe. Proc. Natl Acad. Sci. 99: 6075–6079.

Gordon, D. M. 1988. Group-level exploration tactics in fire ants. Behaviour. 104: 162–175.

Gordon, D. M. 1989. Ants distinguish neighbors from strangers. Oecologia. 81: 198–200.

Hepper, P.G. (ed.). 1991. Kin Recognition. Cambridge Univ. Press, Cambridge.

Hölldobler, B. 1976. Recruitment behavior, home range orientation and territoriality in harvester ants, *Pogonomyrmex*. Behav. Ecol. Sociobiol., 1: 3–44.

Hölldobler, B., and E. O. Wilson. 1977. Colony-specific territorial pheromone in the African weaver ant, *Oecophylla longinoda* (Latreille). Proc. Natl. Acad. Sci. 74: 2074–2075.

Hölldobler, B., and C. J. Lumsden. 1980. Territorial strategies in ants. Science, 210: 732–739.

Holway, D. A., A. V. Suarez, and T. J. Case. 1998. Loss of intraspecific aggression in the success of a widespread invasive social insect. Science, 282: 949–952.

Holway, D. A. and T. J. Case. 2000. Mechanisms of dispersed central-place foraging in polydomous colonies of the Argentine ant. Anim. Behav. 59: 433–441.

Howard, R. W. 1993. Cuticular hydrocarbons and chemical communication, pp.179–226. *In* Stanley-Samuelson, D. and D. Nelson [Eds.], Insect Lipids: Chemistry, Biochemistry, and Biology. University of Nebraska Press, Lincoln, Nebraska.

Hubbard, M. D. 1974. Influence of nest material and colony odor on digging in the ant *Solenopsis invicta* (Hymenoptera: Formicidae). J. Georgia Entomol. Soc. 9: 127–132.

Ichinose, K. 1991. Seasonal variation in nestmate recognition in *Paratrechina flavipes* (Smith) worker ants (Hymenoptera: Formicidae). Anim. Behav., 41: 1–6.

Jaffe, K. 1986. Nestmate recognition and territorial marking in *Solenopsis geminata* and in some Attini, pp. 211–222. *In* Lofgren C. S. and R. K. Vander Meer [Eds.], Fire ants and leaf-cutting ants: biology and management. Westview Press, Boulder, Colorado.

Jaffe, K., and H. Puche. 1984. Colony-specific territorial marking with the metapleural gland secretion in the ant *Solenopsis geminata* (Fabr.). J. Ins. Physiol. 30: 265–270.

Jaffe, K., M. Bazire-Benazet, and P. E. Howse. 1979. An integumentary pheromone-secreting gland in *Atta* sp.: territorial marking with a colony-specific pheromone in *Atta cephalotes*. J. InsPhysiology, 25: 833–839.

Jutsum, A. R., T. S. Saunders, and J. M. Cherrett. 1979. Intraspecific aggression in the leaf-cutting ant *Acromyrmex octospinosus*. Anim. Behav. 27: 839–844.

Klahn, J. E., Gamboa, G. J. 1983. Social wasps: Discrimination between kin and nonkin brood. Science, 221: 482–484.

Krieger, M. J. B., and L. Keller. 2000. Low polymorphism at 19 microsatellite loci in a French population of Argentine ants (*Linepithema humile*). Molec. Ecol. 8: 1075–1092.

Layton, J. M. and K. E. Espelie. 1995. Effects of nest paper hydrocarbons on nest and nestmate recognition in colonies of *Polistes metricus* Say. J. Insect Behav. 8: 103–113.

Le Moli, F., A. Mori , and D. A. Grasso. 1992. Nestmate and conspecific non-nestmate recognition in *Formica cunnicularia* Latr.: the effect of diet difference, pp. 161–165. *In* J. Billen [ed], Biology and Evolution of Social Insects. Leuven University Press, Leuven, Belgium.

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

Markin, G. P. 1968. Nest relationship of the Argentine ant, *Iridomyrmex humilis* (Hymenoptera: Formicidae). J. Kansas Entomol. Soc. 41: 511–516.

Markin, G. P. 1970. The seasonal life cycle of the Argentine ant, *Iridomyrmex humilis* (Hymenoptera: Formicidae) in southern California. Ann. Entomol. Soc. Am. 63: 1238–1242.

Mayade, S., M. C. Cammaerts, and J. P. Suzzoni. 1993. Home range marking and territorial marking in *Cataglyphis cursor* (Hymenoptera: Formicidae). Behav. Proc. 30: 131–142.

Newell, W., and T. C. Barber. 1913. The Argentine ant. USDA Bureau of Entomology Bulletin 122.

Obin, M. S. 1986. Nestmate recognition cues in laboratory and field colonies of *Solenopsis invicta* Buren (Hymenoptera: Formicidae): Effect of environment and the role of cuticular hydrocarbons. J. Chem. Ecol. 12: 1965–1975.

Obin, M. S. and R. K. Vander Meer. 1988. Sources of nestmate recognition cues in the imported fire ant *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Anim. Behav. 36: 1361–1370.

Passera, L. and S. Aron. 1993. Social control over the survival and selection of winged virgin queens in an ant without nuptial flights: *Iridomyrmex humilis*. Ethology, 93: 225–235.

Pfennig, D. W., and H. K. Reeve. 1980. Neighbor recognition and context-dependent aggression in a solitary wasp, *Sphecius speciosus* (Hymenoptera: Sphecidae). Ethology, 80: 1–18.

Provost, E., G. Riviere, M. Roux, E. D. Morgan, and A. G. Bagneres. 1993. Change in the chemical signature of the ant, *Leptothorax lichtensteini* Bondroit with time. Ins. Biochem. Molec. Biol. 23: 945–957.

Reeve, H. K. 1989. The evolution of conspecific acceptance thresholds. Am. Nat. 133: 407–435.

Ross, N. M., Gamboa, G. J. 1981. Nestmate discrimination in social wasps (*Polistes metricus*, Hymenoptera: Vespidae). Behav. Ecol. Sociobiol. 9: 163–165.

Roulston, T. H., G. Buczkowski, and J. Silverman. 2003. Nestmate discrimination in ants: effect of bioassay on aggressive behavior. Ins. Soc. 50: 151–159.

Salzemann, A. 1993. Leaf cutting ant *Atta laevigata* (Formicidae, Attini) marks its territory with a colony specific Dufour gland secretion. Chem. Pharm. Bull. 41: 796.

SAS Institute. 2002. SAS/STAT guide for personal computers, version 8.1. SAS Institute, Cary, NC.

Silverman, J. and B. Nsimba. 2000. Soil-free collection of Argentine ants (Hymenoptera: Formicidae) based on food-directed brood and queen movement. Fl. Entomol. 83: 10–16.

Shellman, J.S. 1982. Nestmate discrimination in social wasps: The role of exposure to nest and nestmates (*Polistes fuscatus*, Hymenoptera: Vespidae). Behav.Ecol. Sociobiol. 11: 51–53.

Sherman, P. W. and W. G. Holmes. 1985. Kin recognition: issues and evidence. Fortschr. Zool. 31: 437–460.

Starks, P. T., D. J. Fischer, R. E. Watson, G. L. Melikian, and S. D. Nath. 1998. Context-dependent nestmate discrimination in the paper wasp, *Polistes dominulus*: a critical test of the optimal acceptance threshold model. Anim. Behav. 56: 449–458.

Stuart, R. J. 1987. Transient nest mate recognition cues contribute to a multicolonial population structure in the ant, *Leptothorax curvispinosus*. Behav. Ecol. Sociobiol. 21: 229–235.

Suarez, A. V, N. D. Tsutsui, D. A. Holway, and T. J. Case. 1999. Behavioral and genetic differentiation between native and introduced populations of the Argentine ant. Biol. Inv. 1: 43–53.

Suarez, A. V., D. A. Holway, D. Liand, N. D. Tsutsui, and T. J. Case. 2002. Spatio-temporal patterns of intraspecific aggression in the invasive Argentine ant. Anim. Behav. 64: 697–708.

Tsutsui, N. D., A. V. Suarez, D. A. Holway, and T. J. Case. 2000. Reduced genetic variation and the success of an invasive species. Proc. Natl. Acad. Sci. 97: 5948–5953.

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

Tsutsui, N. D., A. V. Suarez, and R. K. Grosberg. 2003. Genetic diversity, asymmetrical aggression, and recognition in a widespread invasive species. Proc. Natl. Acad. Sci. 100: 1078–1083.

Vander Meer, R. K., D. Saliwanchik, and B. Lavine. 1989. Temporal changes in colony cuticular hydrocarbon patterns of *Solenopsis invicta*: implications for nestmate recognition. J. Chem. Ecol. 15: 2115–2125.

Venkataraman, A., and R. Gadagkar. 1992. Kin recognition in a semi-natural context: behavior towards foreign conspecifics in the social wasp *Ropalidia marginata* (Lep.) (Hymenoptera: Vespidae). Insect Society, 39: 285–299.

Waldman, B. 1987. Mechanisms of kin recognition. J. Theor. Biol. 128: 159–185.

Table 1. Initial aggression levels and subsequent changes over a 4 month period in mildly and highly aggressive colony pairings in three social contexts.

Aggression bioassay	Aggression levels [a]		Aggression loss		
	Initial	End	Absolute	Relative	*P* [b]
Symmetrical group interactions (high)	83.0 ± 2.2	62.6 ± 2.6	– 20.4	– 24.6 %	0.001
Symmetrical group interactions (mild)	25.2 ± 0.7	11.6 ± 1.9	– 13.6	– 53.8 %	0.009
Intruder introductions (high)	4.0 ± 0.0	3.5 ± 0.1	– 0.5	– 10.7 %	0.0005
Intruder introductions (mild)	2.4 ± 0.1	0.9 ± 0.3	– 1.5	– 61.7 %	0.02
Dyad interactions (high)	1.6 ± 0.2	1.8 ± 0.2	+ 0.2	+ 14.7 %	0.28
Dyad interactions (mild)	1.3 ± 0.1	1.1 ± 0.1	– 0.2	– 15.4 %	0.25

[a] Values reported are mean ± SE.

[b] Absolute aggression loss values were used in statistical analysis. Based on results of a Signed Rank (SAS Institute 2001).

Table 2. The frequency of aggression between intruders and residents in intruder introductions.

Intruder	Resident	Allelic ratio	Attacks by intruders	Attacks by residents	Intruder	Resident	Allelic ratio	Attacks by intruders	Attacks by residents
Chh (16[a])	Emi (26)	0.62	0	10	Emi (26)	Chh (16)	1.63	2	8
Chh (16)	For (21)	0.76	1	9	For (21)	Chh (16)	1.31	0	10
Chh (16)	Gnc (25)	0.64	0	10	Gnc (25)	Chh (16)	1.56	2	8
Chh (16)	Jac (27)	0.59	0	10	Jac (27)	Chh (16)	1.69	1	9
Chh (16)	Rtp (29)	0.55	1	9	Rtp (29)	Chh (16)	1.81	1	9
Total			**2**	**48**				**6**	**44**
Pls (17)	Emi (26)	0.65	7	3	Emi (26)	Pls (17)	1.53	3	7
Pls (17)	For (21)	0.81	7	3	For (21)	Pls (17)	1.24	0	10
Pls (17)	Gnc (25)	0.68	7	3	Gnc (25)	Pls (17)	1.47	2	8
Pls (17)	Jac (27)	0.63	8	2	Jac (27)	Pls (17)	1.59	3	7
Pls (17)	Rtp (29)	0.59	8	2	Rtp (29)	Pls (17)	1.71	1	9
Total			**37**	**13**				**9**	**41**

[a] Numbers in parentheses are the total number of alleles across seven microsatellite loci.

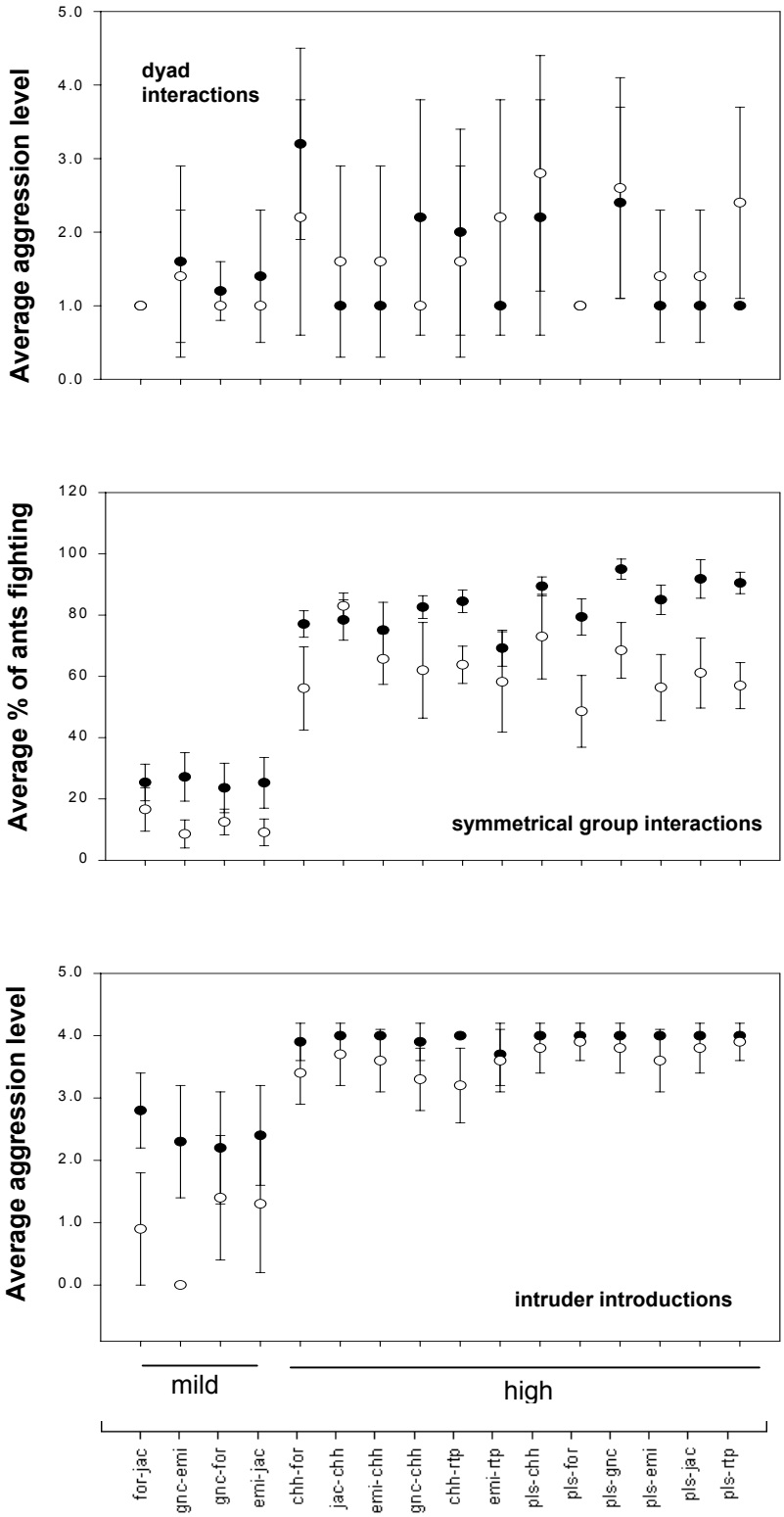

Fig. 1. Mean (± S.E.) aggression levels for mildly and highly aggressive colony pairings at the beginning (●) and at the end of 4 months (○).

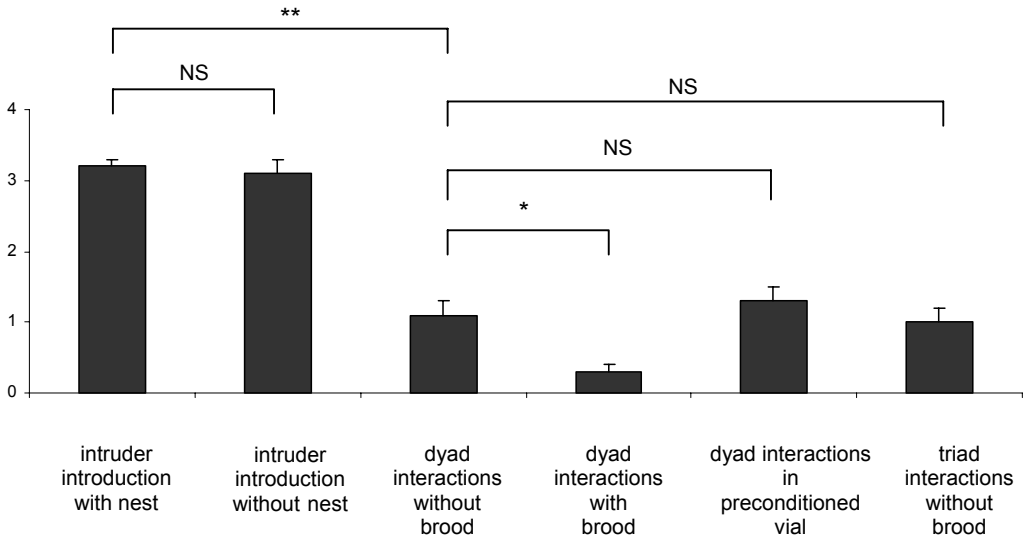

Fig. 2. Mean (± S.E.) aggression levels between non-nestmates in different contexts. $N = 50$. NS: not significant, * $P < 0.01$, ** $P < 0.0001$.

CHAPTER IV

Shared environmental cues diminish intercolony aggression in the Argentine ant (*Linepithema humile*). [1]

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[1] Buczkowski, G., R. Kumar, S. Suib, and J. Silverman. Shared environmental cues diminish intercolony aggression in the Argentine ant (*Linepithema humile*). to be submitted to *Insectes Sociaux*

Abstract. Social insects use both genetic and environmental recognition cues when discriminating between nestmates and non-nestmates. We examined the impact of different diet-derived hydrocarbons on intraspecific aggression in the Argentine ant (*Linepithema humile*) and the potential of shared, diet-derived hydrocarbons to produce colony uniformity where intercolony genetic and/or environmental differences exist. We measured the baseline level of aggression in pairs of field-collected colonies and discovered that ants showed either high or moderate aggression. We then tested the effect of three diets: two hydrocarbon-rich insect prey, *Blattella germanica* and *Supella longipalpa* and an artificial, insect-free diet on aggression loss in both highly and mildly aggressive colony pairs. Results of behavioral assays showed that diet-derived hydrocarbons alter nestmate recognition, mask inherent between colony distinctions, and allow non-nestmate acceptance where intercolony differences exist. Moderately aggressive colony pairs experienced a significant reduction in injurious aggression, although aggression was not eliminated completely. In contrast, highly aggressive colony pairs maintained high levels of injurious aggression throughout the study. Results of cuticular hydrocarbon analysis demonstrated that each diet altered the composition of the hydrocarbon profile by contributing unique, diet-specific cues. Our results suggest that colony fusion due to common environmental factors may be an important mechanism contributing to the evolution of unicoloniality in introduced populations of the Argentine ant. In areas where environmental variation is low and genetic differences between colonies are relatively minor, such process may be important in allowing adjacent colonies to merge, thereby creating a supercolony.

Keywords: Argentine ant, colony odor, cuticular hydrocarbons, invasive ants, nestmate recognition, unicoloniality.

Introduction

Social insects have evolved a highly developed recognition system that forms the basis of social structure and communication. The signals used in nestmate recognition may have a genetic or environmental origin, with exogenous cues derived from nest materials (Gamboa et al. 1986; Stuart 1987) or diet (Jutsum et al. 1979; Le Moli et al. 1992; Obin and Vander Meer 1988; Liang and Silverman 2000). Cuticular hydrocarbons have long been implicated as important mediators of nestmate recognition in ants (Vander Meer and Morel 1998) with recent evidence supporting a direct role (Obin 1986; Bonavita-Cougourdan et al. 1987; Lahav et al. 1999; Liang and Silverman 2000). However, the relative contribution of heritable and environmentally-derived hydrocarbons to the recognition profile is not known. Irrespective of the source, workers must learn colony- specific cues and must be able to properly evaluate cues present on newly encountered workers. Recognition cues are generally dynamic and may change throughout the life of the colony (Vander Meer et al. 1989) and may exhibit seasonal variation (Ichinose 1991). Therefore, a worker must continually update its perception of colony odor in response to changing environmental conditions.

The Argentine ant, *Linepithema humile* is one of several invasive ants in which the relative loss of territorial behavior is thought to be responsible for invasion success. Unicolonial populations of *L. humile*, are generally large and numerically dominant over multicolonial native ant species (Holway et al. 1998). Nestmate recognition in the Argentine ant is influenced by genetic (Tsutsui et al. 2000; Suarez et al. 2002) and environmental (Chen and Nonacs 2000; Liang and Silverman 2000) inputs. Holway et al.

(1998) and Suarez et al. (2002) reported that aggression persisted between *L. humile* colonies despite maintenance under uniform rearing conditions, while Chen and Nonacs (2000) observed a decrease in *L. humile* intercolony aggression following 2 months of lab rearing. Although Tsutsui et al. (2000) demonstrated a significant inverse relationship between *L. humile* genetic similarity and intercolony aggression, the colony pairs used by Holway et al. (1998), Suarez et al. (2002), and Chen and Nonacs (2000) were not subjected to genetic analysis. Therefore, the observed changes (or lack of thereof) in aggression may have resulted from different degrees of relatedness between pairs and/or exposure to distinct environmentally-derived hydrocarbons.

While it has been suggested that genetic factors are mainly responsible for unicoloniality in introduced Argentine ant populations, the role of shared environmental cues such as diet in promoting unicolonial behavior remains unknown. Shared dietary components, specifically hydrocarbons, by colonies displaying low intercolony genetic differentiation, may mask subtle inherent between-colony distinctions and allow adjacent colonies to merge. In the present investigation, we examine the effect of exogenous cues on nestmate reconition in the Argentine ant. We hypothesize that colony pairs that are most genetically dissimilar will be most aggressive prior to laboratory rearing and retain their aggression when maintained under extended constant conditions. We also examine the relationships between genetic similarity (allele sharing), hydrocarbon sharing, and aggression and expect a positive correlation between shared alleles and hydrocarbons and an inverse relationship between shared hydrocarbons and aggression.

Liang and Silverman (2000) and Silverman and Liang (2001) demonstrated the potential of prey hydrocarbons to alter nestmate recognition in the Argentine ant, whereby

feeding on distinct diets induced intracolony aggression. Of the many different prey exposed to *L. humile* workers, contact with the brown-banded cockroach, *Supella longipalpa*, induced the highest level of intracolony aggression (Liang et al. 2001). *Supella longipalpa* contain several hydrocarbons that are identical or similar to those of *L. humile* and may be important in *L. humile* nestmate recognition (Liang et al. 2001). We propose that the opposite process can occur, whereby key *S. longipalpa*-acquired recognition chemicals attenuate differences between *L. humile* colonies thus diminishing intercolony aggression. We compare intercolony aggression levels before and after continuous exposure to diets including *Supella longipalpa*, *Blattella germanica*, and artificial diet and also measure changes in key prey-specific hydrocarbons in *L. humile* hydrocarbon profile. We hypothesize that diets with *S. longipalpa* will diminish aggression the most. By documenting changes in intercolony aggression following exposure to sources of exogenous recognition cues we hope to develop a deeper understanding of the dynamic nature of nestmate discrimination and Argentine ant population structure.

Materials and Methods

Collection and Rearing of Laboratory Colonies

We used 11 colonies of Argentine ants (*Linepithema humile*) from 11 sites in the southeastern U.S.: North Carolina (6); Chapel Hill (CHH), Emerald Isle (EMI), Greenville (GNC), Jacksonville (JAC), Shallotte (SCH), and Winston-Salem (FOR); South Carolina (2): Greenville (HTO) and Greer (GWM); and Georgia (3): Barnesville (BCH), Fayetteville (FAY), and Griffin (GRF). For each location, we established three large colonies consisting of 5,000-10,000 workers, a few hundred queens, and numerous brood. Colonies were maintained in soil-free, Fluon™-coated trays. Nests were plastic dishes filled with moist grooved plaster. Colonies were reared on one of three diets, each of which included a 25% sucrose solution *ad libidum* and hard-boiled eggs once a week: artificial non-insect diet (Bhatkar and Whitcomb, 1970), *Supella longipalpa* male and female adults, or *Blattella germanica* male and female adults. All colonies were maintained at 24 ± 1ºC, 50 ± 10% RH, and 12:12 LD cycle.

Aggression Tests (Nestmate Recognition Bioassay)

We assessed the initial level of aggression between 18 colony pairs (listed below) with an assay that measured the level of aggression in single worker introductions into a foreign colony. This behavioral assay has low variance among replicates within the same colony pairing (Roulston et al. 2003, Appendix 1). The observer who recorded the aggression level did not know the identity of the interacting colonies, and was unfamiliar with the hypothesis being tested. Individual ants were not tested in more than one trial. All assays to estimate

the initial aggression levels were performed within a week since collection and after the extraction of ants from the original nesting substrate. Ten aggression trials per colony pair per time period were conducted. Data were analyzed as the maximum score per trial.

Our preliminary observations indicated a possible relationship between the initial level of aggression displayed by a colony pair and that colony pair losing aggression over time, with pairs having high initial aggression maintaining it over time and colonies with moderate levels of initial aggression becoming non-aggressive. We define moderate aggression as an average score of 3.0 or lower and high aggression as a score of 3.0 or higher, on a 0-4 scoring scale (Suarez et al. 1999). This assignment is based on aggression above level 3 being injurious (biting, stinging), while aggression below level 3 is non-injurious (mutual antennation, avoidance). Eight colony pairs showed moderate aggression: GNC-FAY, FOR-EMI, CHH-BCH, FOR- GNC, CHH-GRF, CHH-HTO, GWM-SCH, GWM-FAY, and 10 colony pairs showed high aggression: JAC-FAY, JAC-SCH, JAC-CHH, EMI-BCH, JAC-HTO, EMI-GRF, EMI-CHH, EMI-HTO, EMI-SCH, JAC-BCH. Aggression assays and hydrocarbon analyses were repeated 140 days later for all 3 dietary regimes to assess changes in nestmate recognition patterns and to determine whether behavioral changes were consistent with hydrocarbon patterns. Aggression test to examine aggression loss were performed again at day 224 to determine whether aggression had further declined with prolonged laboratory rearing.

Extraction, Isolation, and Chemical Analysis of Cuticular Hydrocarbons

Ants were killed by freezing (–20°C) prior to hydrocarbon extraction. External lipids were extracted from the cuticle by immersing ten whole thawed ants in 1 ml hexane for 10 min,

followed by a brief second rinse. The samples were gently shaken for the first and last 20 sec of the soak period. Hexane extracts were concentrated under nitrogen to ~ 100 µl and applied to prewetted (hexane) Pasteur pipette mini-columns filled with 500 mg of silica gel (63-200 mesh size, Selecto Scientific, Georgia, USA). The HC fraction was eluted with 6 ml hexane. Capillary gas chromatography (GC) was carried out using an HP 5890 gas chromatograph equipped with a DB-1 column (30m X 0.25mm X 0.25µm film thickness). Oven temperature was held at 40°C for 2 min, then increased to 200°C at a rate of 20°C/min and finally to 310°C at 40°C/min. Injector and flame-ionization detector were at 270°C and 320°C, respectively. Helium was the carrier gas, and the make-up gas was nitrogen. The extract was resuspended in 5 µl hexane and 1 µl was injected (2 ant equivalents). The quantitative data was obtained by integrating the peaks and calculating the percent area under each peak.

Molecular Techniques

Genomic DNA was extracted from 15 workers from each colony using the DNeasy Tissue Kit (Qiagen, Valencia, CA) and analyzed at seven polymorphic microsatellite loci: *Lhum-11, Lhum-13, Lhum-19, Lhum-28, Lhum-35, Lhum-39* (Krieger and Keller 1999) and *Lihu-T1* (Tsutsui et al. 2000). PCR reactions were multiplexed and amplification products were separated on 6.5% KB^{Plus} polyacrylamide sequencing gels using a 4000L Li-Cor DNA sequencer. Microsatellite alleles were scored using RFLPScan software (Scanalytics, Billerica, MA).

Statistical Analyses

The significance of main effects (diet and initial aggression category) and their interaction was tested by using a mixed model ANOVA (PROC MIXED) in SAS 8.1 (SAS 2002). Upon finding that the effect of diet was not the same in the two aggression categories we tested for the effect of diet on aggression loss within each of the two aggression categories by using the PROC UNIVARIATE procedure. This procedure first performs tests to examine the normality of the data and subsequently performs both parametric (Student's t-test) and non-parametric tests (Sign test and Signed Rank test). The results of parametric and non-parametric tests were similar, thus we report the results of the Student's t-test. The level of aggression loss was estimated for each of the three dietary treatments (*Blattella*, *Supella*, *Artificial*) within each of the 2 aggression categories (moderate and high) by using a mixed model ANOVA (PROC MIXED) with colony pairing treated as random and allowing different error variances within moderate and high aggression categories. ANOVA was then followed by pairwise comparisons of least square means to test for significant differences between the 3 dietary treatments within and across aggression categories. To evaluate the magnitude of aggression loss we used absolute, rather than relative aggression loss values.

To examine divergence patterns between field-collected colonies (Initial) and the same colonies raised on each of the three diets (*Blattella*, *Supella*, Artificial) we used linear discriminant analysis (LDA) (Statgraphics Plus, v. 5.1). The analysis was performed using standardized variables, and a LDA matrix was constructed using 11 colonies, each belonging to each of four treatments (Initial, *Blattella*, *Supella*, Artificial) and their 27 peak percentages of the most abundant cuticular hydrocarbons. Significance tests comparing diets used the MANOVA procedure (PROC GLM). The degree of dispersion around the

centroids (i.e. the degree of differentiation between colonies within a treatment) was calculated by averaging standard deviations for each of the 11 colonies across all 27 hydrocarbons within each treatment. To test whether Argentine ants acquired key prey-specific hydrocarbons we used 11- and 13- and 15-Methylnonacosane and 3-Methylnonacosane from *Blattella* (Jurenka et al. 1989); 15, 19-Dimethylheptatriacontane from Supella (Liang et al. 2001); and n-Heptacosane, n-Nonacosane, and n-Octacosane from the Artificial diet. To compare changes in hydrocarbon levels we used one of two types of *t*-tests, depending on the equality of variances. A parametric *t*-test was used when the variances were homogenous. In cases where the variances were unequal we used the Welch *t*-test with a Satterthwaite correction (Zar 1999).

The relationship among all possible pairwise combinations of three variables - intraspecific aggression, genetic diversity, and hydrocarbon similarity - was examined by estimating Pearson's correlation coefficient. Genetic similarity between colonies was characterized by percent allele sharing. Hydrocarbon similarity was estimated based on pairwise correlations of hydrocarbon profiles and field-collected colonies (prior to lab rearing) were used in the analysis. Regression values are reported as r^2 and one-tailed *P*-values for matrix correlations are provided.

Results

Analysis of the behavioral data revealed that both main effects and their interaction were significant: *diet* (ANOVA, $F_{2, 15.1} = 10.32$, $P = 0.0015$), *aggression* (ANOVA, $F_{1, 18.2} = 52.02$, $P < 0.0001$), *diet*aggression* (ANOVA, $F_{2, 15.1} = 13.38$, $P = 0.0005$) when data from the two aggression categories were pooled and analyzed as a single set. This analysis revealed that diet effects were not the same in the two aggression categories, thus necessitating a separate analysis of diet effects for each of the two aggression categories. Initial aggression levels for moderately and highly aggressive colony pairings and the extent of aggression loss at 140 d under each of the three dietary treatments are presented in Table 1 and Fig. 1. Under controlled laboratory conditions, mutually aggressive colony pairs in both aggression categories lost a significant portion of their initial aggression, irrespective of the diet. In the moderately aggressive category colonies raised on either of the two cockroach diets lost ~ 40% of their initial aggression. The difference in the amount of aggression loss between the *Blattella* and the *Supella* diet was not significant ($P = 0.910$, Table 2). Ants raised on the Artificial diet, however lost ~70% of initial aggression and this loss was significantly higher than that experienced by ants raised on either *B. germainca* ($P = 0.0007$) or *S. longipalpa* ($P = 0.0009$). Argentine ants displaying high initial aggression experienced relatively low aggression loss, approximately 8% for each of the three dietary regimes. This decrease, although relatively low, was statistically significant for each of the three diets (Table 1). The magnitude of aggression loss did not differ between dietary categories (Table 2; ANOVA, $F_{2, 18} = 0.72$, $P = 0.498$). A comparison of the magnitude of aggression loss between the aggression categories revealed that moderately aggressive colony pairs lost a

significantly higher proportion of their initial aggression across all dietary treatments, relative to colony pairs showing high initial aggression (Table 2). Results of aggression tests performed 84 d after the first testing revealed no further aggression loss in any of the aggression/diet categories ($P > 0.05$).

To provide another means of assessing the magnitude of aggression loss in both aggression categories, we focused on changes in the proportion of injurious/non-injurious encounters between colony pairs. Our initial aggression classification (moderate vs. high) was based on a distinction between injurious and non-injurious aggression. We classified aggression above level 3 as injurious (biting, stinging), and aggression below level 3 as non-injurious (mutual antennation, avoidance). Although the magnitude of aggression loss was statistically significant in each of the two aggression categories, our results indicated that in contrast to the moderately aggressive category, the incidence of injurious fights in the highly aggressive category remained high. Therefore, we question the biological importance of the statistically significant aggression loss in the highly aggressive category. In the moderately aggressive category, initially 69 of 80 (86%) encounters resulted in an aggression score of 3 or 4 (3 out of 80 had an aggression score of 4). At the end of the study only 36% of *Supella*-fed ants displayed level 3 aggression (corresponding to a 58% reduction in the number of injurious encounters), 36% of *Blattella*-fed ants (58% reduction), and 19% of ants on the artificial diet (78% reduction). In contrast, in the highly aggressive category, initially 100% of encounters (100/100) had a score of 4. At the end of the study 96% of *Supella*-fed ants still engaged in injurious aggression (2% reduction), 97% of *Blattella*-fed ants (3% reduction), and 98% of ants on the artificial diet (2% reduction). The incidence of level 4 aggression remained high with 71% of *Supella*-fed ants, 78% of *Blattella*-fed ants, and 79%

of ants on the artificial diet displaying level 4 aggression at the end of the study. Therefore, we believe that the statistically significant aggression loss results for the highly aggressive category are not biologically important.

The results of the hydrocarbon analysis revealed that Argentine ants acquired significant levels of dietary hydrocarbons (Fig. 2): *Blattella*: 11- and 13- and 15-Methylnonacosane ($P < 0.0001$), 3-Methylnonacosane ($P < 0.0001$); Supella: 15, 19-Dimethylheptatriacontane ($P < 0.0001$). Moreover, Argentine ants on the Artificial diet acquired significant levels of n-Heptacosane ($P = 0.001$), n-Nonacosane ($P < 0.0001$), and n-Octacosane ($P < 0.0001$), although it remains unknown where these hydrocarbons originated from (diet vs. other materials used in the rearing process). Furthermore, the results of discriminant analysis revealed marked divergence in cuticular hydrocarbon composition between field-collected colonies (Initial) and colonies raised on either *B. germanica*, *S. longipalpa*, or the Artificial diet (Fig. 3). The results of MANOVA showed that these changes were statistically significant: Initial vs. *Blattella* (MANOVA, Wilk's lambda = 0.0028, $F_{26, 5} = 67.04$, $P < 0.0001$), Initial vs. *Supella* (Wilk's lambda = 0.010, $F_{26, 5} = 18.16$, $P = 0.002$), Initial vs. Artificial (Wilk's lambda = 0.010, $F_{26, 5} = 18.94$, $P = 0.002$). The divergence between *Blattella* and the *Supella* diets was also significant (Wilk's lambda = 0.0066, $F_{26, 5} = 28.88$, $P = 0.0007$). Estimates of intracolony variability within treatments revealed that field-collected colonies (Initial) had the lowest variance (1.029), followed by colonies on the Artificial diet (1.035), *Supella* (1.147), and *Blattella* (1.209).

The relationship between hydrocarbon similarity and either genetic similarity or intercolony aggression was not significant ($P > 0.1$, Fig. 4A and C). The relationship

between intercolony aggression and genetic similarity was also not significant ($P > 0.1$, Fig. 4B).

Discussion

Our results demonstrate that shared, environment-derived hydrocarbons influence intraspecific aggression patterns in *L. humile*. Acquisition of similar cues can override relatively minor behavioral differences between colonies of *L. humile*. Liang and Silverman (2000) and Liang et al. (2001) determined that hydrocarbons of *B. germanica* and *S. longipalpa* were acquired by *L. humile* and Silverman and Liang (2001) reported that isolation for 28 days or more between colony fragments fed different prey caused hostility between these fragments. These experiments demonstrated that in the unicolonial *L. humile* exogenous hydrocarbons can affect colony integrity and may induce a multicolonial colony structure. The aim of the present study was to test the role of prey hydrocarbons in causing a reverse process, namely in inducing unicoloniality where between colony genetically and/or environmentally determined differences exist. We determined that the magnitude of aggression loss in *L. humile* depends on the initial aggression level between colony pairs. When initially present at high levels, intraspecific aggression decreased only slightly over the course of the study. Aggression loss was negligible in highly aggressive colony pairs, despite the fact that ants raised on each of three diets acquired statistically significant amounts of diagnostic hydrocarbons and the divergence of hydrocarbon profiles over time was significant among dietary categories. Although the decrease in aggression was statistically significant, the biological importance of such decrease remains uncertain. Our results demonstrate that the frequency of injurious aggression remained high, thus suggesting that the statistical significance of aggression loss in the highly aggressive category may not be biologically meaningful. Other studies have demonstrated that aggression between colonies is maintained or can even increase (Le Moli et al. 1992; Heinze et al. 1996; Holway

et al. 1998; Stuart and Herbers 2000; Suarez et al. 2002). Lack of change in the pattern of intraspecific aggression in the highly aggressive pairings suggests that external recognition cues did not override intrinsic cues. In contrast, moderately aggressive Argentine ant colonies were strongly affected by the imposition of diet-based cues as evidenced by reduced aggression across all three diets. Reduced intraspecific aggression in colonies acquiring similar dietary cues has also been reported in other taxa (*Acromyrmex*, Jutsum et al. 1979; *Polistes*, Gamboa et al. 1986; *Solenopsis*, Obin and Vander Meer 1988). Moreover, our results indicate that cues originating from the artificial diet alter nestmate recognition more profoundly, relative to cues from *B. germanica* or *S. longipalpa*. This in contrary to our prediction that the *S. longipalpa* diet will diminish aggression the most. The results of the hydrocarbon analysis indicated that ants raised on the artificial diet acquired significant amounts of normal straight chain alkanes: n-heptacosane, n-nonacosane, and n-octacosane. These hydrocarbons were present in relatively low amounts in field-collected colonies (3.3%, 3.4%, and 0.1% respectively). Our laboratory raised colonies however, showed significantly elevated levels of each of these hydrocarbons. The ants have most likely acquired these hydrocarbons by contact with the polyethylene rearing trays and/or other plastic material used in the rearing process, such as polystyrene Petri dishes (nests) and polypropylene vials (sucrose feeding stations and diet dispensers). All three hydrocarbons are low molecular weight analogs of polyethylene and they may have been derived from the original polyethylene or as degradation products. Our results suggest that these normal straight chain alkanes may affect nestmate recognition in the Argentine ant and promote aggression loss between mutually aggressive colonies. Liang et al. (2001) demonstrated that hydrocarbons that released nestmate aggression occurred within a narrow range of C35 to C37 in chain

length and were derived from the prey (*S. longipalpa*). Here we show that shorter chain hydrocarbons may also play an important role in the nestmate recognition in the Argentine ant. Since all colonies in this study, including ants fed *B. germanica* and *S. longipalpa*, were maintained in plastic polyethylene trays, it is difficult to separate the relative role of hydrocarbons originating from the rearing environment versus those originating from the prey in promoting aggression loss. Colonies raised on *B. germanica* or *S. longipalpa* acquired significant amounts of diagnostic hydrocarbons from both prey as well as hydrocarbons originating from the rearing environment, yet they lost less aggression relative to colonies raised on the artificial diet alone. It appears therefore that straight chain alkanes originating from the rearing environment may be of primary importance in promoting aggression loss. Colonies raised on either of the two cockroach diets lost ~ 40% of their initial aggression and the difference in the amount of aggression loss between the two diets was not significant. This result is rather unexpected, given that Liang et al. (2001) have reported that long chain hydrocarbons from *S. longipalpa* (33 carbons or more) played an important role in affecting nestmate recognition in the Argentine ant. Other prey items from diverse insect taxa (including *B. germanica*), which had less or none of the long chain hydrocarbons failed to alter the nestmate recognition process and induced only slight or no aggression. Perhaps the duration of the experiment (140 d) permitted enough time for colonies raised on both *B. germanica* and *S. longipalpa* to reach a maximum level of aggression loss possible. It appears that given enough time, both prey can cause equal levels of aggression loss in the Argentine ant. Furthermore, the finding that no additional decrease in aggression occurred after the initial 140 d of the study suggests that such threshold is ~40%.

In our study, the *S. longipalpa* diet especially, was intended to introduce key unifying nestmate recognition cues and cause a reduction in intraspecific aggression. We showed that the role of *Supella*-derived cues in reducing intercolony aggression is significant, yet no greater than the role of *Blattella*-derived cues or those originating from the artificial diet. This suggests that the role of prey hydrocarbons in promoting intercolony unity may not be the same as their role in inducing intracolony aggression. Perhaps the difference in the way prey hydrocarbons alter nestmate recognition in *L. humile* is related to quantitative differences in hydrocarbon levels necessary to observe a certain amount of change in nestmate recognition behavior. While the addition of a certain amount of prey hydrocarbons to the ant's intrinsic hydrocarbon profile may be sufficient to change ant behavior in one direction (for example from no aggression to aggression), addition of an equal amount of the same hydrocarbons may not be sufficient to cause an equivalent change in behavior in the opposite direction. In a system where two colonies are created by splitting a single colony into two colony fragments, the between-fragment correlation in hydrocarbon profile similarity is high as workers share genetically and/or environmentally derived cues. The acquisition of foreign cues (e.g. *S. longipalpa)* by one of the fragments results in aggression between the fragments. In cases where the hydrocarbon profile similarity between two colonies is lower, the acquisition of foreign prey hydrocarbons may not be sufficient to override genetically-based aggression completely.

A comparison between the degree of genetic similarity and the level of intraspecific aggression (Fig. 4B) revealed no clear relationship between the two variables. By contrast Tsutsui et al. (2000) reported that Argentine ant nests belonging to a California population sharing 60% or fewer alleles displayed moderate to high interspecific aggression. Nests that

shared >75% alleles, however, were non-aggressive. In our study, which involved colonies from southeastern U.S., colony pairs displayed either high or moderate aggression and within each aggression category colony pairs with markedly different levels of genetic similarity showed similar levels of aggression. We recently examined the relationship between intraspecific aggression and genetic similarity in introduced populations in California and the southeastern U.S. (Chapter III) and discovered no clear relationship between the degree of genetic similarity and the level of intraspecific aggression in either population. Specifically, in the southeastern U.S. population, aggression between colonies remained high along a continuum of allele sharing ranging from ~ 40 to 80%. In California, colony pairs were always non-aggressive, even when they shared a relatively low (~50%) proportion of alleles. In the present study, average allele sharing for highly aggressive colony pairs was 55% (range 34-70%) and average aggression was 4.0. Moderately aggressive colony pairs shared on average 56% of alleles (range 47-73%) and average aggression was 2.8 ($P = 0.803$). These results support our previous findings and indicate that colony pairs with similar levels of genetic similarity may display markedly different levels of aggression and suggest that environmental factors play an important role in nestmate recognition. A relationship between intercolony aggression and genetic similarity would only be expected when cues used in nestmate recognition are derived strictly from heritable sources. Alternatively, neutral genetic markers may not represent recognition loci. The Argentine ant utilizes both genetic (Tsutsui et al 2000) and environmental cues (Chen and Nonacs 2000; Liang and Silverman 2000). When environmental factors influence nestmate recognition no relationship between aggression and genetic similarity would be expected. In other studies spatial distance between nests was a primary factor affecting aggression between colonies

and the role genetic factors was secondary (Heinze et al. 1996; Stuart and Herbers 2000; Pirk et al. 2001). Nestmate recognition in the Argentine ant may further be complicated by its polydomous nature, whereby a single colony occupies multiple and often distant nests. As a result, relatives may live in places that provide different environmental cues. Stuart and Herbers (2000) reported that in *Leptothorax longispinosus* which consist of either polydomous/polygynous or monodomous/monogynous colonies, the importance of exogenous cues depends on the population's social and nest structure. In areas where polydomy is widespread genetic factors affect intercolony aggression. By contrast, in areas where monodomy is more common, both genetic similarity and spatial distance affect nestmate recognition.

Although differences in cuticular hydrocarbon composition among field-collected colonies were observed, these differences were inadequate predictors of behavioral response by resident workers toward intruders. We detected no significant relationship between intercolony aggression and similarity in cuticular hydrocarbon composition (Fig. 4C). Numerous studies demonstrate that cuticular hydrocarbons are responsible for nestmate recognition in social insects (Howard et al. 1982; Bonavita-Cougourdan et al. 1987; Vander Meer and Morel 1998) and intercolonial aggression in ants correlates with variation in cuticular hydrocarbon profiles (Bonavita-Cougourdan et al. 1987; Nowbahari et al. 1990; Suarez et al. 2002). Other studies, however, have found no significant relationship between the two variables (Obin 1986; Stuart and Herbers 2000). Lack of a negative relationship between hydrocarbon similarity and intercolony aggression suggests one of two possibilities. First, hydrocarbons other than or in addition to those analyzed in this study provide nestmate recognition cues. Alternatively, the major cuticular

hydrocarbons analyzed here do provide nestmate recognition cues but chemicals other than hydrocarbons also play a role. Vander Meer and Morel (1998) suggested that nestmate recognition in social insects might be affected by cues other than cuticular hydrocarbons. Such cues might include various exocrine gland secretions and excretory products. Due to their chemical nature and/or high volatility such compounds might be difficult to isolate and/or detect by gas chromatography. Even if amenable to detection by gas chromatography, standard protocols for cuticular hydrocarbon extraction, purification, and analysis might preclude identification of other classes of compounds. Similarly, Morel et al. (1988) investigated nestmate recognition in *Camponotus floridanus* and concluded that while cuticular hydrocarbons may dominate nestmate recognition in this species, they do not wholly regulate the nestmate recognition process and other classes of compounds may also have behavioral properties. A comparison between Fig. 4B and C revealed that certain highly aggressive colony pairings with relatively high degree of genetic and hydrocarbon similarity displayed high aggression. For example, EMI and HTO shared 70% of alleles and had similar hydrocarbon profiles (Pearson's correlation: $r = 0.938$). Despite genetic and phenotypic similarities the two colonies always displayed high aggression (ave. = 4.0 ± 0.0). It remains unclear what exogenous and/or endogenous factors might be responsible for the discrepancy between the analytical and the behavioral data. However, these results indicate that factors other than cuticular hydrocarbons may affect nestmate recognition in *L. humile*.

The relationship between allele sharing and cuticular hydrocarbon profile similarity (Fig. 4A) was not significant. Colony pairs almost always shared a high proportion of hydrocarbons (ave. $r = 0.88 \pm 0.1$; range 0.65-0.97) despite sometimes low allele sharing

(30-60%). The average correlation coefficient for hydrocarbon similarity for moderately aggressive colony pairs was $r = 0.89$ and $r = 0.87$ for highly aggressive pairs (*t*-test, $P > 0.1$). This indicates that colonies with similar cuticular hydrocarbon profiles may show hostile behavior. A relationship between cuticular hydrocarbon similarity and genetic similarity would only be expected when cuticular hydrocarbons are derived strictly from heritable sources. When environmental components (e.g. food, nesting material) contribute substantially to the colony odor no relationship between hydrocarbon similarity and genetic similarity would be expected.

The results of our study may provide insights into the origin and evolution of unicoloniality in the Argentine ant and may help discern evolutionary mechanisms leading to the transition from multicoloniality to unicoloniality. Tsutsui et al. (2000) and Giraud et al. (2002) have proposed dissimilar mechanisms to explain the transition from multicoloniality to unicoloniality in *L. humile*. Tsutsui et al. (2000) emphasized the role of genetic factors (i.e. reduction of heterozygosity due to genetic drift) as pivotal in initiating the transition to unicoloniality. The role of ecological factors, namely colony range expansion to allow interactions between colonies and ultimately colony fusion was secondary. Furthermore, Tsutsui et al. (2003) demonstrated that asymmetrical aggression, whereby individuals from less genetically diverse colonies attack individuals from more diverse colonies can maintain or further decrease the low levels of genetic diversity that underlies unicoloniality. By contrast, Giraud et al. (2002) proposed a mechanism where the role of ecological factors, specifically introduction into new habitats with relaxed ecological constraints played a primary role in driving the transition to unicoloniality. According to Giraud et al. (2002) the genetic bottleneck experienced by colonies introduced into southern Europe was relatively

insignificant and played only a minor role in the formation of the European supercolony. Both studies emphasized the role of behavioral factors, namely selection against rare recognition alleles. While Tsutsui et al. (2000, 2003) and Giraud et al. (2002) demonstrated the importance of genetic, ecological, and behavioral factors in the transition from multicoloniality to unicoloniality our study highlights the role of environmentally derived recognition cues in possibly promoting unicoloniality where between colony differences exist. Shared dietary hydrocarbons between colonies displaying aggression may mask inherent between-colony distinctions, allowing adjacent colonies to merge. The requirement for this process, is that neighboring colonies obtain similar recognition cues through shared use of the environment. Competing Argentine ant colonies will most likely interact with each other at advancing invasion fronts where they are likely to compete for nesting sites and/or food resources that provide similar nestmate recognition cues. In areas where mutually aggressive colonies exploit a common locally abundant food source, two independent mechanisms may work either individually or in concert to promote fusion of neighboring colonies: diet sharing and intraspecific dear enemy phenomenon whereby competing animals respond less aggressively to threats by neighbors than strangers (Temeles 1994; Heinze et al. 1996; Langen et al. 2000). Diet sharing through cooperative use of locally abundant food sources may provide sufficient levels of critical hydrocarbons to alter nestmate recognition and promote unicolonial behavior. Argentine ants, like most ants, are generalist feeders (Newell and Barber 1913; Markin 1970) primarily tending homoptera, and scavenging living and dead insects. Alternatively, uniform cues originating from nesting material may promote unicolonial behavior. The dear enemy phenomenon may also play a role in the Argentine ant's transition from multicoloniality to unicoloniality. An increase in

the frequency of encounters between aggressive colonies may decrease the frequency of aggression between them, especially in areas where food is abundant and intraspecific competition is limited (Foitzik and Heinze 1998). Furthermore, Stuart and Herbers (2000) reported that repeated interactions in the field are essential for maintaining recognition among spatially isolated nests in the polydomous *L. longispinosus*. Non-aggressive field colonies became hostile after three months of laboratory rearing indicating a possible role for common environment-derived cues and/or contact between colonies in maintaining unicolonial population structure. In areas where Argentine ants interact and compete for resources that provide similar recognition cues, such process may be important in promoting cooperation among nests. Over time, spatially isolated nests may acquire similar cues and display progressively lower rates of intercolony aggression. In turn, widespread cooperation among nests may lead to colony fusion which may be an important mechanism contributing to the evolution of unicoloniality in introduced populations of the Argentine ant.

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References Cited

Bhatkar, A. D., and W. H. Whitcomb. 1970. Artificial diet for rearing various species of ants. Fl. Entomol. 53: 229–232.

Bonavita-Cougourdan, A., J. L. Clement, and C. Lange. 1987. Nestmate recognition: the role of cuticular hydrocarbons in the ant *Camponotus vagus*. Scop. J. Entomol. Sci. 22: 1–10.

Buczkowski, G., E. L. Vargo, and J. Silverman. 2003. The dimunitive supercolony: The Argentine ants of the southeastern U.S. Evolution, (submitted).

Chen, J. S. C. and P. Nonacs. 2000. Nestmate recognition and intraspecific aggression based on environmental cues in Argentine ants (Hymenoptera : Formicidae). Ann. Entomol. Soc. of Am. 93: 1333–1337.

Foitzik S, and J. Heinze. 1998. Nestsite limitation and colony take over in the ant, *Leptothorax nylanderi*. Behav. Ecol. 9: 367–375.

Gamboa, G. J., H. K. Reeve, I. D. Ferguson, and T. L. Wacker. 1986. Nestmate recognition in social wasps: the origin and acquisition of odours. Anim. Behav. 34: 685–695.

Giraud, T., J. S. Pedersen, and L. Keller. 2002. Evolution of supercolonies: the Argentine ants of southern Europe. Proc. Natl. Acad. Sci. 99: 6075–6079.

Heinze, J., S. Foitzik, A. Hippert, and B. Hölldobler. 1996. Apparent dear-enemy phenomenon and environment-based recognition cues in the ant *Leptothorax nylanderi*. Ethology, 102: 510–522.

Hölldobler, B., and C. D. Michener. 1980. Mechanisms of identification and discrimination in social hymenoptera. In: Evolution of Social Behaviour (Ed. by H. Markl), pp. 35–58. Weinheim: Verlag Chemie.

Hölldobler, B. and E. O. Wilson. 1990. The Ants. Cambridge, Massachusetts: Harvard University Press.

Holway, D. A., A. V. Suarez, and T. J. Case. 1998. Loss of intraspecific aggression in the success of a widespread invasive social insect. Science, 282: 949–952.

Howard, R. W, C. A. McDaniel, and G. J. Blomquist. 1982. Chemical mimicry as an integrating mechanism: cuticular hydrocarbons of a termitophile and its host. Science, 210: 431–433.

Ichinose, K. 1991. Seasonal variation in nestmate recognition in *Paratrechina flavipes* (Smith) worker ants (Hymenoptera: Formicidae). Anim. Behav. 41: 1–6.

Jutsum, A. R., T. S. Saunders, and J. M. Cherrett. 1979. Intraspecific aggression in the leaf-cutting ant *Acromyrmex octospinosus*. Anim. Behav. 27: 839–844.

Jurenka, R. A., C. Schal, E. Burns, J. Chase, and G. J. Blomquist. 1989. Structural correlation between cuticular hydrocarbons and female contact sex pheromone of German cockroach *Blattella germanica* (L.). J. Chem. Ecol. 15: 939–949.

Krieger, M. J. B., and L. Keller. 1999. Low polymorphism at 19 microsatellite loci in a French population of Argentine ants (*Linepithema humile*). Mol. Ecol. 8: 1075–1092.

Lahav, S, V. Soroker., A. Hefetz , and R. K. Vander Meer. 1999. Direct behavioral evidence for hydrocarbons as ant recognition discriminators. Naturwissenshaften, 86: 246–249.

Langen, T. A., F. Tripet, and P. Nonacs. 2000. The red and the black: habituation and the dear-enemy phenomenon in two desert *Pheidole* ants. Behav. Ecol. Sociobiol. 48: 285–292.

Le Moli, F., A. Mori , and D. A. Grasso. 1992. Nestmate and conspecific non-nestmate recognition in *Formica cunnicularia* Latr.: the effect of diet difference, pp. 161–165. *In* J. Billen [ed], Biology and Evolution of Social Insects. Leuven University Press, Leuven, Belgium.

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

Liang, D., G. J. Blomquist, and J. Silverman. 2001. Hydrocarbon-released nestmate aggression in the Argentine ant, *Linepithema humile*, following encounters with insect prey. Comp. Biochem. Physiol. 129: 871–882.

Markin, G. P. 1970. The seasonal life cycle of the Argentine ant, *Iridomyrmex humilis* (Hymenoptera: Formicidae) in southern California. Ann. Entomol. Soc. Am. 63: 1238–1242.

Morel, L., R. K. Vander Meer, and B. K. Lavine. 1988. Ontogeny of nestmate recognition cues in the red carpenter ant (*Camponotus floridanus*): behavioral and chemical evidence for the role of age and social experience. Behav. Ecol. Sociobiol. 22: 175–183.

Nowbahari, M. and A. Lenoir. 1989. Age related changes in aggression in ant *Cataglyphis cursor* (Hymenoptera, Formicidae): Influence on intercolonial relationships. Behav. Proc. 18: 173–181.

Obin, M. S. 1986. Nestmate recognition cues in laboratory and field colonies of *Solenopsis invicta* Buren (Hymenoptera: Formicidae): Effect of environment and the role of cuticular hydrocarbons. J. Chem.Ecol. 12: 1965–1975.

Obin, M. S. and R. K. Vander Meer. 1988. Sources of nestmate recognition cues in the imported fire ant *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Anim. Behav. 36: 1361–1370.

Pirk, C. W., P. Newmann, R. F. A. Moritz, and P. Pamilio. 2001. Intranest relatedness and nestmate recognition in the meadow ant *Formica pratensis* (R.). Behav. Ecol. Sociobiol. 49: 366–374.

Roulston, T. H., G. Buczkowski, and J. Silverman. 2003. Nestmate discrimination in ants: effect of bioassay on aggressive behavior. Ins. Soc. 50: 151–159.

SAS Institute. 2002. SAS/STAT guide for personal computers, version 8.1. SAS Institute, Cary, NC.

Silverman, J. and D. Liang. 2001. Colony disassociation following diet partitioning in a unicolonial ant. Naturwissenschaften, 88: 73–77.

Stuart, R. J. 1987. Transient nest mate recognition cues contribute to a multicolonial population structure in the ant, *Leptothorax curvispinosus*. Behav. Ecol. Sociobiol. 21: 229–235.

Stuart, R. J., and J. M. Herbers. 2000. Nest mate recognition in ants with complex colonies: within- and between-population variation. Behav. Ecol. 11: 676–685.

Suarez, A. V, N. D. Tsutsui, D. A. Holway, and T. J. Case. 1999. Behavioral and genetic differentiation between native and introduced populations of the Argentine ant. Biol. Inv. 1: 43–53.

Suarez, A. V., D. A. Holway, D. Liand, N. D. Tsutsui, and T. J. Case. 2002. Spatio-temporal patterns of intraspecific aggression in the invasive Argentine ant. Anim. Behav. 64: 697–708.

Temeles, E. J. 1994. The role of neighbors in territorial systems: when are they "dear enemies"? Anim. Behav. 47: 339–350.

Tsutsui, N. D., A. V. Suarez, D. A. Holway, and T. J. Case. 2000. Reduced genetic variation and the success of an invasive species. Proc. Natl. Acad. Sci. 97: 5948–5953.

Vander Meer, R. K., D. Saliwanchik, and B. Lavine. 1989. Temporal changes in colony cuticular hydrocarbon patterns of *Solenopsis invicta*: implications for nestmate recognition. J. Chem. Ecol. 15: 2115–2125.

Vander Meer, R. K., and L. Morel. 1998. Nestmate recognition in ants. In: Pheromone Communication in Social Insects (Ed. by R. K. Vander Meer, M. D. Breed, K. E. Espiele, and M. L. Winston), pp. 79–103. Boulder, Colorado:Westview Press.

Waldman, B., P. C. Frumhoff, and P. W. Sherman. 1988. Problems of kin recognition. Trends in Ecology and Evolution, 3: 8–13.

Wilson, E. O. 1971. The Insect Societies. Cambridge, Massachusetts: Harvard University Press.

Zar, J. H. 1999. Biostatistical Analysis. New Jersey, Prentice Hall.

Table. 1. Initial aggression levels and aggression loss at 140 d in moderately and highly aggressive colony pairings under three dietary regimes.

Aggression category	Initial aggression level [a]	Aggression loss				
		Diet	End	Absolute	Relative	*P* [b]
Moderate	2.79 ± 0.08 (*n*=8)	Supella	1.7 ± 0.2	1.1 ± 0.2	39.5 ± 7.1 %	< 0.0001
		Blattella	1.8 ± 0.2	1.0 ± 0.2	36.7 ± 5.7 %	0.0001
		Artificial	0.8 ± 0.2	2.0 ± 0.2	73.0 ± 7.0 %	< 0.0001
High	4.00 ± 0.00 (*n*=10)	Supella	3.7 ± 0.1	0.3 ± 0.1	8.3 ± 2.2 %	0.002
		Blattella	3.8 ± 0.1	0.2 ± 0.1	5.5 ± 2.1 %	0.009
		Artificial	3.8 ± 0.1	0.2 ± 0.1	5.8 ± 2.4 %	0.018

[a] Initial aggression levels are between pairs of field-collected colonies. Values reported are mean ± SE.

[b] Based on results of ANOVA (SAS Institute 2001).

Table. 2. Comparison of aggression loss within and among aggression categories [1]

Diet	Aggression	Diet	Aggression	*P*
Supella	Moderate	Blattella	Moderate	0.910
Supella	Moderate	Artificial	Moderate	0.0009
Blattella	Moderate	Artificial	Moderate	0.0007
Supella	High	Blattella	High	0.417
Supella	High	Artificial	High	0.251
Blattella	High	Artificial	High	0.726
Supella	High	Supella	Moderate	0.001
Blattella	High	Blattella	Moderate	0.0006
Artificial	High	Artificial	Moderate	< 0.0001

[1] Least square analysis (PROC MIXED).

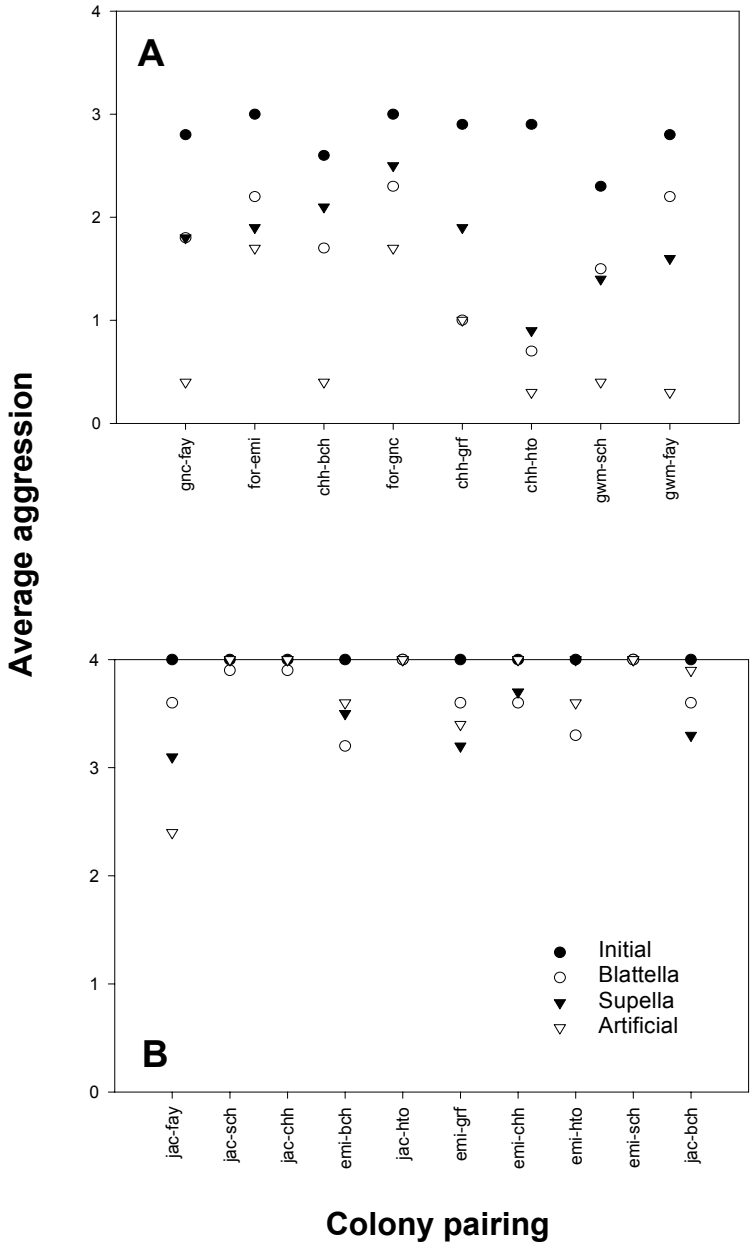

Fig. 1. Changes in intraspecific aggression in moderately (A) and highly (B) aggressive colony pairings. Field-collected colonies (Initial, closed circles) raised on one of three diets: *Blattella* (open circles), *Supella* (closed triangles), Artificial (open triangles). Mean values are reported (*n*=10) with error bars omitted for clarity.

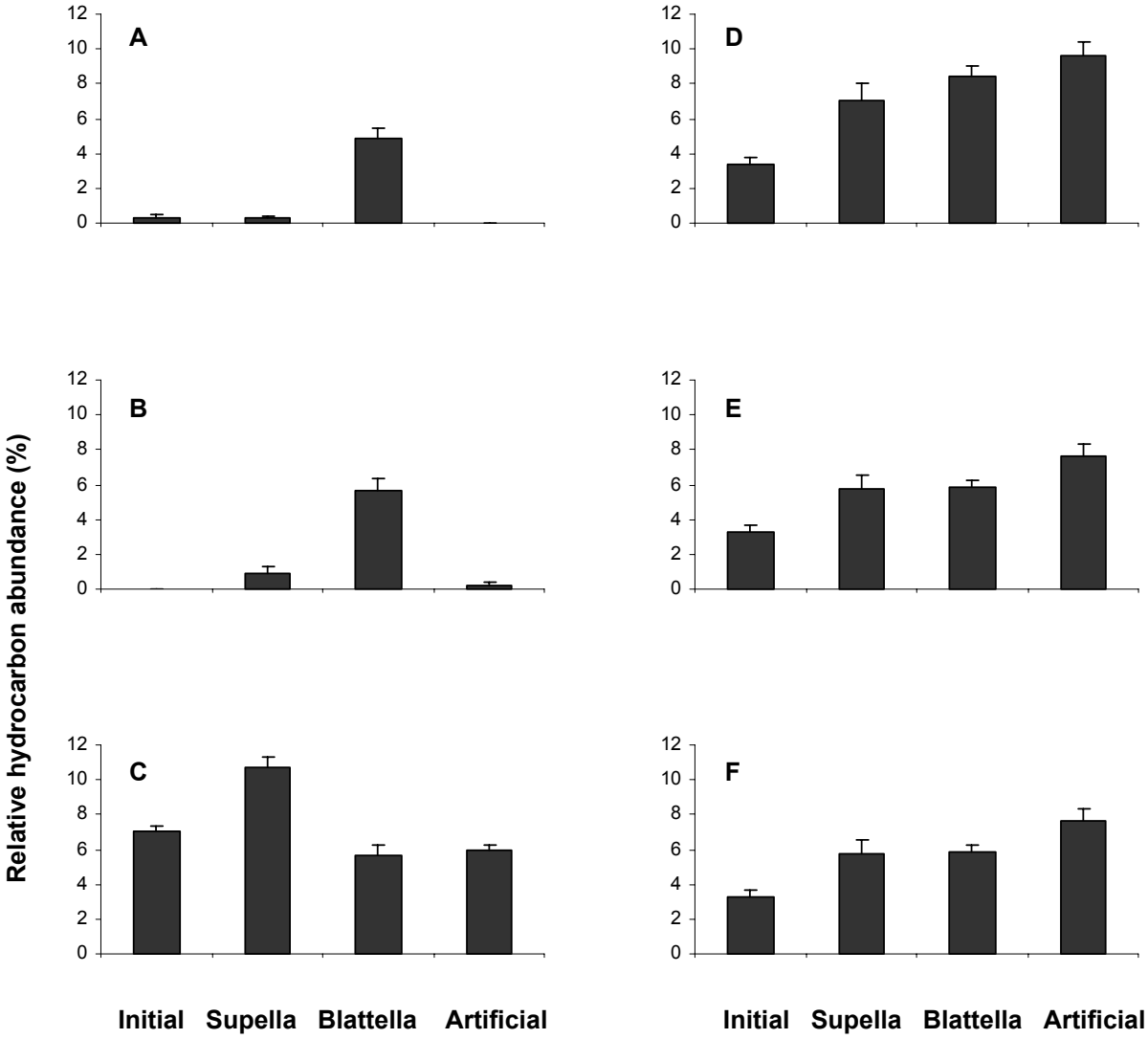

Fig. 2. Relative abundance (% total area) of key diet-derived hydrocarbons in field-collected colonies of *L. humile* (Initial) and colonies provisioned with one of three diets: *S. longipalpa* (*Supella*), *B. germanica* (*Blattella*), and Artificial diet (Artificial). Hydrocarbons in Fig. 2 (A) and (B) are *Blattella*-derived, (C) *Supella*-derived, and (D), (E) and (F) are Artificial diet. (A) 11-and13- and 15-Methylnonacosane (B) 3-Methylnonacosane (C) 15, 19-Dimethylheptatriacontane (D) n-Nonacosane (E) n-Heptacosane (F) n-Octacosane. Means for 11 colonies and standard errors are presented.

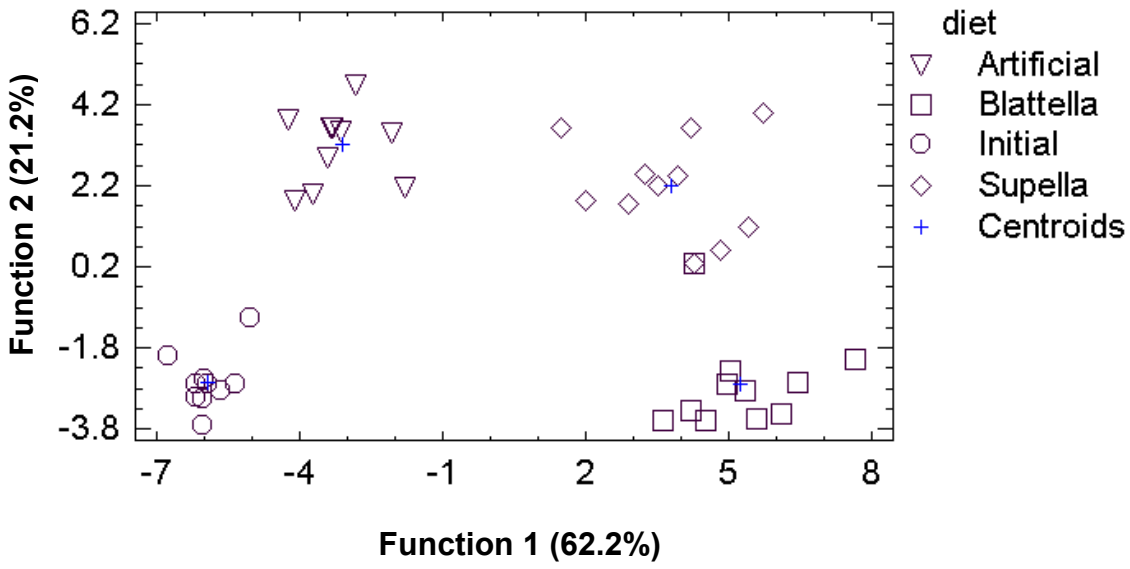

Fig. 3. Linear discriminant analysis of the 27 predictor variables (relative proportions of HCs) for 11 colonies of *L. humile* each provided a unique diet (*B. germanica*, *S. longipalpa*, Artificial).

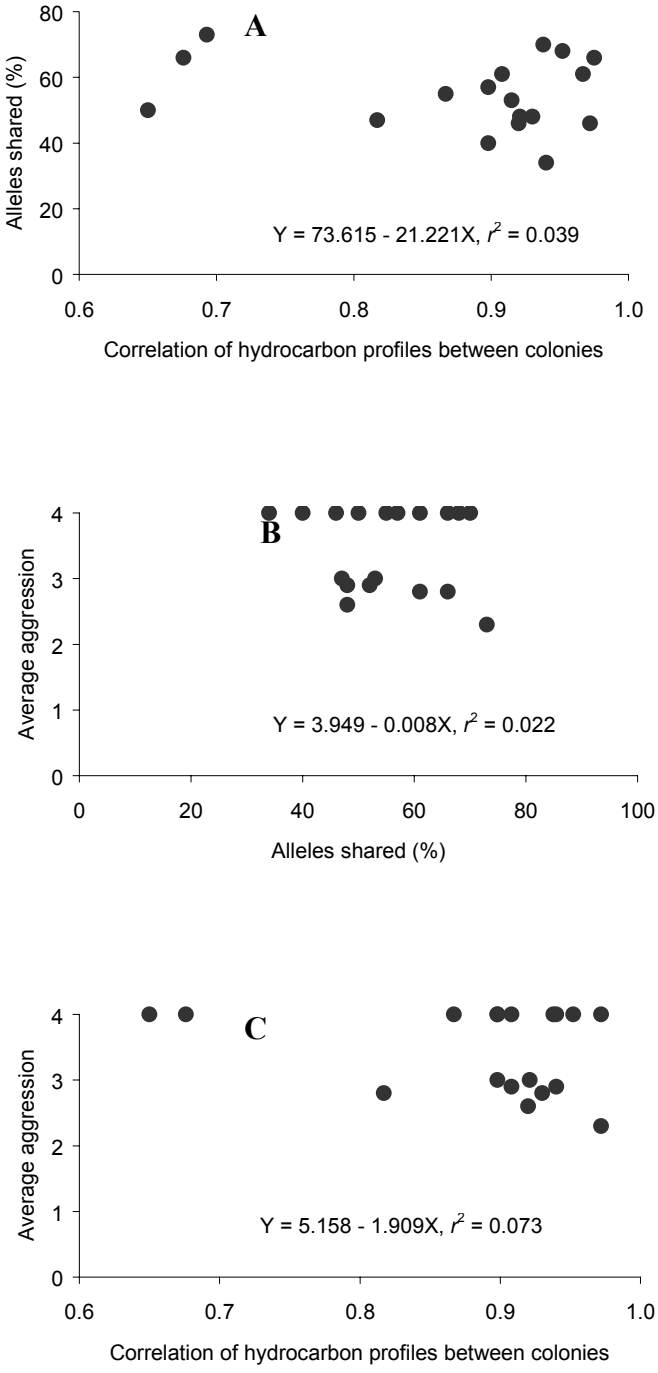

Fig. 4. Relationships among intraspecific aggression, genetic similarity, and hydrocarbon similarity. (A) Relationship between genetic similarity between colony pairs (% alleles shared) and hydrocarbon similarity (Pearson's *r*) (B) Relationship between genetic similarity and intraspecific aggression (C) Relationship between intraspecific aggression and hydrocarbon similarity of colonies.

CHAPTER V

Geographic variation in nestmate recognition behavior in the Argentine ant: the effect of past phylogenies and current selection pressures. [1]

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[1] manuscript in preparation.

Abstract. Social insects use both genetic and environmental recognition cues when making nestmate discriminatory decisions. We examined the role of environmental cues (derived from diet) on nestmate recognition in two populations of the Argentine ant, *Linepithema humile*. We discovered that the two populations differ in their response to environmentally-derived nestmate recognition cues. Ants belonging to the California supercolony are strongly affected by the imposition of prey-derived hydrocarbons and spatially-isolated colony fragments provided with different prey (*B. germanica* or *S. longipalpa*) display high intracolony aggression when reunited. In contrast, colonies of Argentine ants from the southeastern U.S. show little or no aggression when subjected to the same treatment. Our results indicate that field-collected colonies of *L. humile* already possess hydrocarbons in the range of those provided by *S. longipalpa*, with colonies from the southeastern U.S. having significantly higher initial levels of *Supella*-shared hydrocarbons. When raised on prey diets, Argentine ants from both regions acquired additional amounts of *Supella*-specific and *Blattella*-specific hydrocarbons. In both populations, the increase in the level of *Supella*-specific hydrocarbons was not significant, while the increase in the level of *Blattella*-specific was significant. Lack of aggression induction in Argentine ants from the southeastern U.S. may therefore result from higher initial levels of *Supella*-specific hydrocarbons, relative to ants from California. *Blattella*-fed ants from the southeastern U.S. probably recognized former nestmates raised on the *S. longipalpa* diet, since ants raised on both diets had high initial levels of *Supella*-specific hydrocarbons. Similarly, ants raised on *B. germanica* did not attack former nestmates raised on *S. longipalpa* since ants from both colony fragments had relatively high initial levels of Supella-specific hydrocarbons. In California, intracolony

aggression was induced, most likely as a result of low initial levels of *Supella*-specific hydrocarbons.

Keywords: Argentine ant, cuticular hydrocarbons, invasive ants, nestmate recognition.

Introduction

Nestmate recognition, a mechanism that evolved in social organisms as defense against predation and enslavement, is largely mediated by chemical signals (Hepper 1986, 1991). In order to recognize colony members from non-members, individuals recognize each other through a learned chemical label or "colony odor". In the recognition process, individuals discriminate colony members from nonmembers by a multicomponent process called "phenotype matching" (Lacy and Sherman 1983; Sherman and Holmes 1985; Gamboa et al. 1986b). Phenotypic recognition cues can be any aspect of an organism's phenotype that signifies colonial membership reliably. Chemical cues, however, are widely used because they are potentially information-rich, while requiring little energy to produce, as when they are metabolic by-products or when they are passively acquired from the environment. Cuticular hydrocarbons have long been implicated in nestmate recognition in ants (Vander Meer and Morel 1998) and recent evidence from several species of ants indicates that cuticular hydrocarbons are the chemical signals used (Lahav et al. 1999; Thomas et al. 1999; Boulay et al. 2000; Liang and Silverman 2000).

Phenotypic recognition labels may have an endogenous and/or an exogenous origin (Crozier and Dix 1979; Gamboa et al. 1986a) and to gain accurate information about colony membership social insects have evolved to use cues originating from either source (Gamboa et al. 1986b; Ratnieks 1990). The relative importance of genetic and environmental sources for nestmate recognition is often addressed in laboratory studies, where the environmental component is kept constant and the importance of the genetic vs. environmental recognition cues is then estimated based on changes in aggression patterns between individuals. In

ants, numerous studies have reported decreases in intraspecific aggression in colonies reared under uniform laboratory conditions (Gamboa et al. 1986a; Obin 1986; Stuart 1987; Crosland 1989; Boulay et al. 2000; Chen and Nonacs 2000). In contrast, other studies have demonstrated that aggression between colonies is maintained or can even increase (Le Moli et al. 1992; Heinze et al. 1996; Holway et al. 1998; Stuart and Herbers 2000; Suarez et al. 2002). A significant decrease in aggression suggests that nestmate discrimination is largely influenced by environmental cues and maintenance of aggression implies a strong genetic component. Gamboa et al. (1986b), however, caution that a demonstration of dominance of genetic cues in the laboratory does not provide evidence that genetic cues are more important than environmental factors in field contexts. The relative importance of genetic versus environmental factors ultimately depends on the organism's genetic structure, ecology, and context. Genetic cues should be most useful for groups of organisms having high within-group relatedness and occurring in relatively homogenous environments. Environmentally based cues, on the other hand, should be most useful in areas where the chance of encounters between unrelated individuals is high and the environment is more heterogenous. Despite these theoretical predictions, the relative importance of genetic and environmental components for social insects remains largely undetermined (reviews by Hölldobler and Michener 1980; Jaisson 1985; Breed and Bennett 1987; Gamboa et al. 1991b; Vander Meer and Morel 1998).

The Argentine ant nestmate recognition system integrates both genetic (Tsutsui et al. 2000; Suarez et al. 2002; Tsutsui et al. 2003) and environmental (Chen and Nonacs 2000; Liang and Silverman 2000) cues. Tsutsui et al. (2000) found a negative relationship between the level of intraspecific aggression and the degree of genetic similarity, which

suggests that nestmate recognition signals are heritable. Others have demonstrated that environmental factors can profoundly affect the discriminatory ability in the Argentine ant (Chen and Nonacs 2000; Liang and Silverman 2000; Suarez et al. 2002). Liang and Silverman (2000) examined Argentine ant nestmate recognition and its modification by diet and demonstrated that hydrocarbons acquired from *Supella longipalpa* and *Blattella germanica* prey were incorporated into the ant's intrinsic cuticular hydrocarbon profile and altered nestmate recognition. Colony fragments raised in isolation on different diets displayed high aggression when reunited. Furthermore, Silverman and Liang (2001) demonstrated that isolating Argentine ant colony fragments for as few as 28 days and feeding them different prey (either *B. germanica* or *S. longipalpa*) was sufficient to prevent re-establishment of internest communication.

In our ongoing efforts to understand the role of prey-acquired hydrocarbons on nestmate recognition and colony dynamics in the Argentine ant we attempted to induce intracolony aggression within a colony of *L. humile* collected from Winston-Salem, North Carolina by following the methodology of Liang and Silverman (2000). Despite prolonged (4 mo.) rearing on distinct prey diets, we were unable to induce high aggression in this colony. Former nestmates reared on different prey showed only slight and non-injurious aggression (level 1 or 2 on a scale of 0-4, Suarez et al. 1999) and colony fragments merged readily when reunited. This preliminary observation suggested that results obtained by Liang and Silverman (2000) and Silverman and Liang (2001) for a single colony, from a single location (California), at one time cannot be universally extended to all populations of *L. humile*. Thus, we hypothesized that Argentine ant response to environmental cues may be related to regional differences in Argentine ant's population structure.

We have recently examined Argentine ant population structure and intercolony aggression in two portions of its introduced range: California and the southeastern United States. We discovered that colonies in the southeastern U.S. display high genetic diversity and high intercolony aggression relative to the California population (Buczkowski, Vargo, Silverman, unpublished data), and the genotypic variability in the Southeast is twice that in California (47 vs. 23 alleles at seven microsatellite loci). Moreover, intercolony aggression is absent in California, while in the Southeast aggression among colonies is high. Here we hypothesize that the striking genetic and behavioral asymmetries observed in introduced populations of *L. humile* may manifest themselves in differences in nestmate discrimination between the two populations. The objective of this project is to screen multiple colonies from California and the southeastern U.S. to investigate more closely possible regional variation in Argentine ant's utilization of environmental cues in nestmate discrimination. The comparison of *L. humile* colonies from both populations will increase our understanding of the interplay between genes and environment, it will offer insights into mechanisms of invasions, and possibly even initial stages of speciation.

Materials and Methods

Collection and Rearing of Laboratory Colonies

To investigate geographic variation in Argentine ant's response to prey derived hydrocarbons, we collected ants from along two 700 km transects. One transect was through an area approximating that of a supercolony in California (38.3N–33.0N and 122.2W–117.0W) previously described by Tsutsui et al. (2000), the other spanned across three Southeastern states: North Carolina, South Carolina, and Georgia (33.0N – 36.1N and 72.0W – 84.4W). We sampled seven sites in California: Berkeley, Corona, Davis, Escondido, Ojai, Pleasanton, and Riverside. In the Southeastern USA we sampled 11 sites: North Carolina (6): Chapel Hill, Emerald Isle, Greenville, Jacksonville, Shallotte, and Winston-Salem; South Carolina (2): Greenville and Greer; and Georgia (3): Barnesville, Fayetteville, and Griffin. For each location, we established two colony fragments each consisting of ~5,000 workers, several dozen queens, and numerous brood. Colonies were maintained in soil-free, Fluon™-coated trays. Nests were plastic dishes filled with moist grooved plaster. For each location, colony fragments were reared on one of two prey diets, each of which included a 25% sucrose solution *ad libidum* and hard-boiled eggs once a week: *Supella longipalpa* male and female adults, or *Blattella germanica* female adults. The cockroaches were presented live, but injured to facilitate prey handling by the ants. Colonies were raised under a 12:12 LD cycle, 75ºF, and 55% RH.

Aggression Tests (Nestmate Recognition Bioassay)

We tested the effect separation between fragments of the same colony on aggression by rearing fragments in isolation. We divided a colony (Winston-Salem) containing approximately 20,000 workers into two colony fragments and raised them under identical environmental and dietary conditions for six months. Subsequently, we performed 60 worker introductions between the two fragments; 30 with workers from one colony acting as intruders, and 30 with workers from the other colony acting as intruders.

We assessed changes in intracolony communication patterns with an assay that measured the level of aggression in single worker introductions into a foreign colony. This behavioral assay has low variance among replicates within the same colony pairing (Roulston et al. 2003). Ten aggression tests per colony per time period were conducted; 5 with *Blattella*-fed workers acting as intruders, and 5 with *Supella*-fed workers acting as intruders. For each test, we allowed the intruder up to 25 encounters with resident ants. Each instance of direct physical contact between the intruder and any of the residents was regarded as an encounter. Individual ants were not tested in more than one trial. Data were analyzed as the maximum score of 25 encounters. If the intruder and the resident ants engaged in a highly aggressive behavior (level 4) for more than 10 sec., a fluoned ring was placed around the fighting individuals, so that exactly seven resident ants were enclosed with the intruder. The ring was fluoned on both sides in order to prevent possible escape by the intruder, as well as to prevent additional residents from entering the fighting arena. The trial was permitted to continue for 2h and the physical condition of the intruder was examined every 1-4 min. Aggression assays were performed 12, 22, 32, 42, 52, 82, 112, 132, and 162 days since the colonies were first provisioned with prey.

Extraction, Isolation, and Chemical Analysis of Cuticular Hydrocarbons

Ants were killed by freezing at –20°C prior to hydrocarbon extraction. External lipids were extracted from the cuticle by immersing ten whole thawed ants in 1 ml hexane for 10 min, followed by a brief second rinse. The samples were gently shaken for the first and last 20 sec of the soak period. Hexane extracts were concentrated under nitrogen to ~ 100 µl and applied to prewetted (hexane) Pasteur pipette mini-columns filled with 500 mg of silica gel (63-200 mesh size, Selecto Scientific, Georgia, USA). The HC fraction was eluted with 6 ml hexane. Capillary gas chromatography (GC) was carried out using an HP 5890 gas chromatograph equipped with a DB-1 column (30m X 0.25mm X 0.25µm film thickness). Oven temperature was held at 40°C for 2 min, then increased to 200°C at a rate of 20°C/min and finally to 310°C at 40°C/min. Injector and flame-ionization detector were at 270°C and 320°C, respectively. Helium was the carrier gas, and the make-up gas was nitrogen. Quantitative data was obtained by integrating the peaks and calculating the percent area under each peak.

Data analysis

All behavioral and hydrocarbon data analyses were performed using SAS 8.1 statistical software (SAS 2002). Aggression scores, instances of injurious aggression (score ≥ 3), and the number of workers that died as a result of diet-induced aggression were each averaged in the following order: (1) over replicates within a colony (n=10), (2) over colonies within a time period (California: n=7; southeastern U.S.: n=11), and (3) over time periods (n=9). To examine regional differences in the level of aggression induction we compared aggression levels on day 12, day 162, and an average of all time periods using PROC TTEST. The

PROC TTEST procedure examines the equality of variances and we report the results of one of two types of *t*-tests, depending on the equality of variances. Results of a Student's *t*-test are reported when the variances were homogenous. In cases where the variances were unequal, we used the Welch *t*-test with a Satterthwaite correction (Zar 1999). Next, we determined the slope for aggression increase over time for each colony (PROC REG) and tested the hypothesis that the slope for each region (averaged over colonies) is not different from zero (PROC UNIVARIATE). Subsequently, we compared regression slopes between regions using PROC TTEST. To compare the incidence of injurious aggression and the number of dead workers between regions we used values averaged over all nine time periods.

To examine cuticular hydrocarbon composition in populations of *L. humile* we first identified key hydrocarbons provided by each prey. For *B. germanica* we selected two hydrocarbons: 11- and 13- and 15-Methylnonacosane and 3-Methylnonacosane. Both hydrocarbons are relatively abundant in adult female *B. germanica,* comprising approximately 14.5% and 10.3% of the total hydrocarbons, respectively (Jurenka et al. 1989). Furthermore, our preliminary analysis indicated that both hydrocarbons were readily acquired by Argentine ants. For *S. longipalpa* we selected four hydrocarbons: 15, 19-Dimethylpentatriacontane, 5,9- and 5,11-Dimethylpentatriacontane, 13- and 15- and 17- and 19-Methylheptatriacontane, and 15,19- and 17,21-Dimethylheptatriacontane. These hydrocarbons are present in *S. longipalpa* at 19.0, 9.1, 8.5, and 25.7% respectively and each is acquired by Argentine ants from *S. longipalpa* prey (Liang and Silverman 2000; Liang et al. 2001).

We hypothesized that population-specific differences in response to environmental hydrocarbons may result from differences in the initial levels of hydrocarbons that are within

the range of those provided by the prey (i.e. there would be a negative relationship between the level of aggression induced and the level of hydrocarbons similar to those provided by the prey). Therefore, we examined colonies in both regions for the presence of *Blattella*-specific and *Supella*-specific hydrocarbons and compared the initial levels of those hydrocarbons between regions (PROC TTEST). To test whether Argentine ants acquired significant amounts of prey hydrocarbons following different diet provisions we examined changes in levels of prey-specific hydrocarbons by comparing their initial levels (field-collected colonies; d 0) to levels on d 52 of the study by performing analysis of variance (ANOVA) using PROC GLM. We chose to examine hydrocarbon profiles on d 52 arbitraily. Our objective was to allow enough time for ants to acquire prey hydrocarbons and our preliminary analysis of cuticular hydrocarbon profile indicated that ants acquired hydrocarbons from both prey within a few days. To examine possible regional differences in prey hydrocarbon acquisition we compared the relative amounts of prey-specific hydrocarbons acquired by Argentine ants in California vs. the southeastern U.S. (PROC GLM). All analyses were performed for each diet in two ways: (1) by considering each hydrocarbon separately, and (2) by combing diet-specific hydrocarbons into a single group.

Results

Intracolony aggression

Separation of colony fragments did not result in aggression between the fragments and workers were never attacked (0/60). In addition, Liang and Silverman (2000) reported lack of aggression in self-introductions in a colony from California. Our examination of intraspecific geographic variation in Argentine ant nestmate recognition behavior revealed a differential response to environmental cues between California and southeastern U.S. populations. Colonies comprising the California population were affected by the imposition of prey-derived hydrocarbons and colony fragments raised in isolation on either *B. germanica* or *S. longipalpa* displayed high aggression when reunited (Fig 1 and 2). By contrast, colonies belonging to the southeastern U.S. population displayed only moderate aggression. The average level of aggression (averaged over all colonies and all time periods) was 3.22 ± 0.06 in California (range over time periods: 2.91 – 3.67; range over colonies: 3.02 – 3.42) and 1.68 ± 0.10 for colonies from the Southeast (range over time periods: 1.26 – 2.10; range over colonies: 1.22 – 2.37, $P < 0.0001$). A considerable difference in the average level of aggression induction between regions was already evident by d 12 (California 2.91 ± 0.09) vs. Southeast (1.78 ± 0.20, $P = 0.002$). Over time, the difference between regions grew larger and on d 162 California colonies were highly aggressive (3.67 ± 0.13) while southeastern U.S. colonies showed relatively low aggression (1.60 ± 0.17, $P < 0.0001$).

The incidence of injurious aggression in California was relatively high and in 25 encounters, we recorded 14.6 ± 1.2 (range across time periods: 12.0 – 20.1) instances of aggression ≥ 3 (Fig. 2). In the Southeast, we recorded only 2.7 ± 0.7 (range across time

periods: 1.4 – 4.7) instances of injurious aggression ($P < 0.0001$). We recorded 2.2 ± 0.3 (range across time periods: 1.0 – 4.0) dead workers per ten intruder introductions in fights involving ants from California. No mortality was observed when ants from the southeastern U.S. interacted ($P < 0.0001$).

Regression analysis revealed that from d 12 to d 162 the slope of aggression increase over time was not significant in either California ($P = 0.108$) or the Southeast ($P = 0.260$). The difference between slopes was also not significant ($P = 0.058$). These results indicate that colonies from both regions reached maximal aggression by d 12 and maintained it throughout the study, despite slight fluctuations between time periods.

Comparison of hydrocarbon profiles

Our results indicate that field-collected colonies of *L. humile* already possessed hydrocarbons in the range of those provided by both insect prey (Table 1). Colonies in California and the southeastern U.S. initially had relatively low levels of *Blattella*-specific hydrocarbons (<1% of total hydrocarbons) with no difference between regions ($P = 0.837$). In comparison to *Blattella* hydrocarbons, Argentine ants in both regions had higher initial levels of *Supella* hydrocarbons (~ 4–9% of total hydrocarbons). Argentine ants from California had lower initial levels of *Supella* hydrocarbons (total of 23.55 ± 1.32 %) relative to colonies from the Southeast (28.60 ± 0.87 %; $P = 0.016$). The difference between Californian and the southeastern populations was especially pronounced with respect to two hydrocarbons: 15,19-Dimethylpentatriacontane (4.17 vs. 6.78%, respectively; $P < 0.0001$) and 13- and 15- and 17- and 19-Methylheptatriacontane (4.41 vs. 6.10%, respectively; $P = 0.005$). The levels of the other two *Supella*-specific hydrocarbons were not different between regions.

Changes in cuticular hydrocarbon composition in *L. humile* raised on *B. germanica* and *S. longipalpa* are presented in Table 2. Argentine ants fed *B. germanica* acquired significant levels of *Blattella*-derived hydrocarbons in both California ($P < 0.0001$) and the southeastern U. S. ($P < 0.0001$). In each region, the increase was significant for both 11- and 13- and 15-Methylnonacosane and Methylnonacosane. Analysis of *Supella*-derived hydrocarbons gave mixed results as the levels of some hydrocarbons increased, while the levels of others decreased. In California, the levels of three hydrocarbons increased: 13- and 15- and 17- and 19-Methylheptatriacontane ($P = 0.024$), 15,19-Dimethylpentatriacontane ($P = 0.009$) and 5, 9- and 5, 11- Dimethylpentatriacontane ($P = 0.038$), and the level of 15, 19- and 17, 21- Dimethylheptatriacontane decreased ($P = 0.027$). In Argentine ant colonies from the Southeast the level of 15,19-Dimethylpentatriacontane and 15, 19- and 17, 21-Dimethylheptatriacontane increased ($P = 0.102$ and $P = 0.0002$, respectively). At the same time, the level of 13- and 15- and 17- and 19-Methylheptatriacontane and 5, 9- and 5, 11-Dimethylpentatriacontane decreased ($P = 0.003$ and $P = 0.028$, respectively). When all four *Supella*-derived hydrocarbons were analyzed as a single group, Argentine ant colonies in California acquired more *Supella*-derived hydrocarbons relative to ants from the southeastern U.S. (4.96 vs. 2.01%). However, the level of *Supella*-specific hydrocarbons did not significantly increase in either California ($P = 0.093$) or the Southeast ($P = 0.246$). Furthermore, *L. humile* raised on *B. germanica* lost a significant proportion of *Supella*-specific hydrocarbons in both California ($P = 0.0002$) and the southeastern U.S. ($P < 0.0001$). In Argentine ants raised on *S. longipalpa* the levels of *Blattella*-specific hydrocarbons increased slightly though not significantly in either California ($P = 0.096$) or the Southeast ($P = 0.1$).

Comparisons of changes in hydrocarbon levels revealed no regional differences when prey-specific hydrocarbons were analyzed as a single group (Table 3). Argentine ants raised on *B. germanica* did not differ in either the amount of *Blattella*-specific cues they acquired ($P = 0.207$) or in the amount of *Supella*-specific cues they lost ($P = 0.983$). Similarly, ants raised on *S. longipalpa* did not differ in the amount of *Blattella*- or *Supella*-specific cues they acquired ($P = 0.764$ and $P = 0.313$, respectively). However, when cuticular hydrocarbons were analyzed individually, differences between regions were evident. Considerable differences between regions were evident especially with respect to the amount of *Supella*-specific hydrocarbons acquired by colonies raised on *S. longipalpa*. Most notably, for three out of four hydrocarbons, changes in hydrocarbon levels occurred in opposite directions, possibly further contributing to behavioral differences between regions.

Aggression between colony fragments raised on distinct prey diets is most likely caused by two factors pertaining to changes in the levels of key nestmate recognition hydrocarbons. First, net diet-specific changes in the levels of prey-specific hydrocarbons are probably important. Second, the difference in the level of prey-specific hydrocarbons between diets is also important. Ultimately, intraspecific aggression most likely results from a combination of both factors. We compared final levels of hydrocarbons between diets for each of the two regions (Table 4). Our results showed significant differences between diets for all hydrocarbons in each of the two regions. Liang et al. (2001) suggested that certain *Supella*-derived hydrocarbons, especially 15,19-Dimethylpentatriacontane, might be critical to nestmate recognition in *L. humile*. Our results indicate that in California, the difference in the level of 15,19-Dimethylpentatriacontane between colonies raised on *B. germanica* and *S.*

longipalpa is 62% (2.80 vs. 7.30). In the Southeast, the difference is only 40% (4.60 vs. 7.68).

Discussion

Our results demonstrate profound geographic variation in Argentine ant response to certain environmentally-derived nestmate recognition cues. We show that Argentine ants belonging to the California supercolony are strongly affected by the imposition of prey-derived hydrocarbons and isolated colony fragments reared on distinct diets show high and injurious intraspecific aggression when reunited. In contrast, colonies of *L. humile* from the southeastern U.S. show little or no aggression when subjected to the same treatment.

Geographic variation in response to extrinsic environmental cues can result from proximate environmental effects, from underlying genetic factors, as well as the interaction between the two (Bradshaw 1965; Via and Lande 1985; West-Eberhard 1989). While natural selection has been traditionally considered to play a major role in producing phenotypic variants adapted to conditions present in a certain geographic region, evidence for the role of environmental factors inducing phenotypic plasticity is less common. Our results demonstrate that both environmental and genetic factors can influence regional differences in nestmate recognition behavior in *L. humile*. Evidence for the role of environmental factors comes from our examination of hydrocarbon profiles in *L. humile* from both ranges. Regional environmental differences may especially promote a differential response to prey-derived hydrocarbons. For example, regional differences in diet and nesting material diversity might cause qualitative and quantitative differences in cuticular hydrocarbon composition. Such differences might subsequently affect Argentine ants response to novel nestmate recognition cues, especially if new cues overlap with similar cues already present. Workers may not recognize newly acquired hydrocarbons as foreign, if such hydrocarbons

are initially present as part of the hydrocarbon profile. Such effects might be especially pronounced if there are threshold levels of hydrocarbons, above which increases in hydrocarbon levels do not cause a change in behavior. Our results indicate that relative to Argentine ants from California, ants from the southeastern U.S. had higher initial levels of hydrocarbons shared with *Supella*. Regional differences were especially evident with respect to 15,19-Dimethylpentatriacontane and 13- and 15- and 17- and 19-Methylheptatriacontane. Levels of the other two *Supella*-shared hydrocarbons were also higher, although not significantly different from those found in *L. humile* in California. Higher initial levels of *Supella*-shared hydrocarbons in colonies from the southeastern U. S. may, in part be responsible for the lack of aggression induction in ants from this region, as field-collected colonies already possessing relatively high levels of Supella-shared hydrocarbons may be less sensitive to additional increases in the levels of those hydrocarbons. Liang et al. (2001) suggested that certain *Supella*-derived hydrocarbons (especially 15,19-Dimethylpentatriacontane) might be critical to nestmate recognition in *L. humile* by altering hydrocarbon ratios within the range that is sensitive to such changes. In other ants, the proportion of certain hydrocarbons was also important for nestmate recognition (Bonavita-Cougourdan et al. 1993; Boulay et al. 2000). Our results show that Argentine ants in the Southeast not only have higher initial levels of *Supella*-shared hydrocarbons, but they also acquire lower amounts of those hydrocarbons, relative to ants from California. Liang et al. (2001) reported that *S. longipalpa* hydrocarbons altered nestmate recognition in the Argentine ant more profoundly than those from a number of other insects, including *B. germanica*. Therefore, differences in the levels of *Supella*-specific hydrocarbons might be especially important in explaining behavioral differences between regions. Our results

revealed that levels of three out of four *Supella*-shared hydrocarbons increased in California. At the same time, the levels of those hydrocarbons in the Southeast either decreased or increased only slightly. The level of 15, 19- and 17, 21- Dimethylheptatriacontane, however, increased in the Southeast and decreased in California. Interregional comparisons cannot be made by simply evaluating changes in *Supella*-specific hydrocarbons in ants raised on *S. longipalpa*. This is because field-collected colonies initially possess hydrocarbons in the range of those provided by *S. longipalpa* and our results show that colonies raised on *B. germanica* lose considerable amounts of Supella-specific hydrocarbons. Since our aggression tests are between colony fragments raised on both diets, we need to consider changes in *Supella*-specific hydrocarbons in colonies raised on *S. longipalpa* as well as *B. germanica*. The case of 5, 9- and 5, 11- Dimethylpentatriacontane (Table 2 and 3) provides a good example. Southeastern U.S. colonies raised on *S. longipalpa* experienced a decline in the level of this hydrocarbon (-1.07%, $P = 0.028$), while colonies in California exhibited an increase (2.11%, $P = 0.038$; difference between regions $P = 0.001$). Concurrently, colonies raised on *B. germanica* experienced a loss of *Supella*-specific hydrocarbons. In the Southeast the average loss was 2.68% ($P < 0.0001$), while in California it was 3.71% ($P < 0.0001$; difference between regions $P = 0.018$). As a result of these changes, the difference in the abundance of 5, 9- and 5, 11- Dimethylpentatriacontane in *L. humile* form California was relatively large (4.64% vs. 10.47%, $P < 0.0001$). In contrast, the levels of 5, 9- and 5, 11- Dimethylpentatriacontane in colonies from the southeastern U.S. were more similar, although still different (5.98 vs. 7.59%, $P = 0.0006$). If 5, 9- and 5, 11- Dimethylpentatriacontane is important for nestmate recognition, then regional differences in

the acquisition/loss of this hydrocarbon may explain differences in the level of aggression induction.

In addition to environmental factors, genetic factors may also promote differential responses to prey-derived hydrocarbons. Evidence for the role of genetic factors comes from our recent comparison of *L. humile* population genetic structure in California and the southeastern U.S. (Buczkowski et al. submitted). The southeastern *L. humile* population displayed elevated genotypic variability and high intercolony aggression relative to the California population. In the Southeast, aggression between neighboring colonies was high and we discovered 47 alleles across seven polymorphic microsatellite loci. In the California population, intercolony aggression was absent and only 23 alleles were found across the same seven loci. These results demonstrated that not all introduced populations within the U.S. are genetically and behaviorally uniform. These interregional differences in genetic diversity may further manifest themselves in differential responses to exogenous nestmate recognition cues. Specifically, regional differences in genetic diversity may affect the structure and operation of the nestmate recognition system, which in social insects is thought to consist of three distinct components: (1) expression, (2) perception, and (3) action (Sherman and Holmes 1985; Gamboa et al. 1986b; Waldman 1987; Reeve 1989). The expression component is the mechanism involved in cue production and acquisition. The perception component involves the development of the recognition template and the processing of that cue once perceived. Finally, the action component describes the process of cue-template matching, whereby the phenotype of an encountered individual is matched with a memory of a set of acceptable cues (the template) and an action is taken. In the Argentine ant, the expression component (cue ontogeny) has both genetic (Tsutsui et al. 2000; Giraud et

al. 2002; Suarez et al. 2002) and environmental (Chen and Nonacs 2000; Liang and Silverman 2000) origins. Our results (Table 1) show that field-collected colonies of Argentine ants show regional differences in the levels of certain *Supella*-specific hydrocarbons, although such differences are strictly quantitative, and not qualitative. It remains unknown however, whether such differences are due to genetic and/or environmental factors and whether colonies in either region show temporal variation in cuticular hydrocarbon patterns. In other ants, recognition cues are very dynamic and may change throughout the life of the colony (Vander Meer et al. 1989) and may exhibit seasonal variation (Ichinose 1991). Regional distinctions in genetic diversity may also cause differences in cue perception. The perception component of nestmate recognition involves the development of the recognition template and the processing of the perceived cues. The template is a collection of recognition cues that an individual considers acceptable (Waldman et al. 1988) and the cues are based on a referent. The referent can be anything that the individual contacts including: objects in the nesting environment, its diet, and most importantly, other individuals living in the same group (nest or colony). Argentine ant colonies are highly polygynous (Markin 1960, 1970) with each reproductive female possibly contributing unique recognition labels. As a result, workers learn the colony's collective odor cues, which results in a template that is uniformly accepted among workers (Breed and Bennett 1987). When the diversity of the referents that form the recognition system is wide, a broader template is formed and more labels are deemed acceptable. The breadth of the template ultimately depends on the genetic diversity among individuals that contribute to the collective colony odor. In California, the genetic diversity within colonies is relatively low comapred to colonies in the southeastern U.S. and as a result, the template may be relatively

narrow. The addition of *S. longipalpa* and/or *B. germanica* hydrocarbons may therefore trigger changes in perception and cause aggression between former nestmates raised on distinct prey diets. In contrast, in the southeastern U.S., where genetic diversity is relatively high, *L. humile* may have a wider template and it may therefore view foreign prey hydrocarbons as fitting within this template, resulting in no aggression between colony fragments.

In this study genetically different colonies were subjected to the same environmental conditions. Colonies from different regions exhibited differences in behavior when raised under identical conditions, thus suggesting a strong genetic basis for the differences seen between populations from different locations. Argentine ants from California and southeastern U.S. may therefore be separate ecotypes because they are genetically distinct and they differ in behavior and possibly other phenotypic traits. Alternatively, Argentine ants in California and the Southeast may simply be exhibiting phenotypic plasticity in response to environmental differences between the regions. Phenotypic plasticity in response to a wide variety of biotic and abiotic factors has been reported from several insect taxa (Gupta and Lewontin 1982; Nylin et al. 1996; Arnqvist and Johansson 1998; Greene 1989; Gotthard et al. 1999). These studies demonstrated that pronounced morphological and behavioral differences can be produced not only through selection, but also through phenotypic plasticity. The relative importance of phenotypic plasticity versus natural selection in producing regional behavioral differentiation in the Argentine remains untested.

Geographic variation observed in this study could prove useful for investigating evolutionary dynamics of invasive species. Invasive species evolve quickly in response to

new abiotic and biotic stresses and nestmate recognition behaviors are most likely under pressure from new selection factors. In California, loss of genetic diversity has promoted successful invasion and resulted in the formation of an extensive supercolony (Tsutsui et al. 2000). Our results indicate that in California the environmental component of nestmate recognition may play an important role in . Indeed, Chen and Nonacs (2000) reported aggression loss in California colonies raised under uniform laboratory conditions. In contrast, southeastern U.S. colonies seem to rely more heavily on endogenous cues. In the future, comparison of *L. humile* from both populations might offer insights into the ecological evolution of this species and possibly even initial stages of speciation between the two geographic regions.

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Refernces Cited

Arnqvist, G., and F. Johansson. 1998. Ontogenetic reaction norms of predator-induced defensive morphology in dragonfly larvae. Ecology. 79:1847–1858.

Bhatkar, A. D., and W. H. Whitcomb. 1970. Artificial diet for rearing various species of ants. Fl. Entomol. 53: 229–232.

Bonavita-Cougourdan, A., J. L. Clement, and C. Lange. 1987. Nestmate recognition: the role of cuticular hydrocarbons in the ant *Camponotus vagus*. Scop. J. Entomol.Sci. 22: 1–10.

Bonavita-Cougourdan, A., J. L. Clement, and C. Lange. 1989. The role of cuticular hydrocarbons in recognition of larvae by workers of the ant *Camponotus vagus*: changes in the chemical signature in response to social environment (Hymenoptera: Formicidae). Sociobiology. 16: 49–74.

Bonavita-Cougourdan, A., J. L. Clement, and C. Lange. 1993. Functional discrimination (foragers and brood-tenders) in the ant *Camponotus vagus* Scop.: polymorphism of cuticular hydrocarbon patterns. J. Chem. Ecol. 19: 1461–1477.

Boulay R., A. Hefetz, V. Soroker, and A. Lenoir. 2000. *Camponotus fellah* colony integration: worker individuality necessitates frequent hydrocarbon exchanges. Anim.Behav. 59: 1127–1133.

Bradshaw, A. D. 1965. Evolutionary significance of phenotypic plasticity. Adv. Genet. 13: 115–155.

Breed, M D. 1983. Nestmate recognition in honey bees. Animal Behavior. 31: 86–91.

Cammaerts-Tricot. 1975. Ontogenesis of the defense reactions in the workers of *Myrmica rubra*. Anim. Behav. 23: 124–130.

Breed, M. D. and B. Bennett. 1987. Kin recognition in highly eusocial insects. pp. 243–285. *In* D. J. C. Fletcher and C. D. Michener [Eds.] *Kin recognition in animals*. J. Wiley & Sons Ltd, Chichester.

Buczkowski, G., E. L. Vargo, and Silverman, J. 2003. The diminutive supercolony: the Argentine ants of the southeastern U.S. Evolution. (submitted).

Cammaerts-Tricot. 1975. Ontogenesis of the defense reactions in the workers of *Myrmica rubra*. Anim. Behav. 23: 124–130.

Chen, J. S. C. and P. Nonacs. 2000. Nestmate recognition and intraspecific aggression based on environmental cues in Argentine ants (Hymenoptera : Formicidae). Ann. Entomol. Soc.of Am. 93: 1333–1337.

Crosland, M.W. J. 1989. Kin recognition in the ant *Rhytidoponera confusa*. I. Environmental odour. Anim. Behav. 37: 912–919.

Crosland, M. W. J. 1990. Variation in ant aggression and kin discrimication ability within and between colonines. J. Ins. Behav. 3: 359–379.

Fénéron, R. 1996. A new ethological test to study nestmate recognition in adult ants. Ins. Soc. 43:131–135.

Crozier, R. H. and M. W. Dix. 1979. Analysis of two genetic models for the innate components of colony odor in social Hymenoptera. Behav. Ecol. Sociobiol. 4: 217–224.

Gamboa, G. J., H. K. Reeve, and I. D. Ferguson, and T. L. Wacker. 1986a. Nestmate recognition in social wasps: the origin and acquisition of recognition odours. Anim. Behav. 34: 685–695.

Gamboa, G. J., H. K. Reeve, and D.W. Pfennig. 1986b. The evolution and ontogeny of nestmate recognition in social wasps. Ann. Rev. Entomol. 31: 431– 454.

Gamboa, G. J., H. K. Reeve, and W. G. Holmes. 1988. Conceptual issues and methodology in kin recognition research, a critical discussion. Ethology. 88: 109–127.

Gamboa, G. J., R.L. Foster, J. A. Scope, and A. M. Bitterman. 1991a. Effects of stage colony cycle context and intercolony distance on conspecific tolerance by paper wasps, *Polists fuscatus*. Behav. Ecol. Sociobiol. 29: 87–94.

Gamboa, G. J., Reeve, H. K. and W. G. Holmes. 1991b. Conceptual issues and methodology in kin recognition research, a critical discussion. Ethology. 88: 109–127.

Gordon, D. M. 1989. Ants distinguish neighbors from strangers. Oecologia. 81: 198–200.

Gotthard, K., S. Nylin, and C. Wiklund. 1999. Seasonal plasticity in two satyrine butterflies: state-dependent decision making in relation to daylength. Oikos. 84:453–462.

Greene, E. 1989. A diet-induced developmental polymorphism in a caterpillar. Science. 243: 643–646.

Gupta, A. P., and R. C. Lewontin. 1982. A study of reaction norms in natural populations of *Drosophila pseudoobscura*. Evolution. 36: 934–948.

Heinze, J., S. Foitzik, A. Hippert, and B. Hölldobler. 1996. Apparent dear-enemy phenomenon and environment-based recognition cues in the ant *Leptothorax nylanderi*. Ethology. 102: 510–522.

Hepper, P.G. 1991. Kin recognition: functions and mechanisms. Biol. Rev. 61: 63–93.

Hölldobler, B. 1976. Recruitment behavior, home range orientation and territoriality in harvester ants, *Pogonomyrmex*. Behav. Ecol. Sociobiol. 1: 3–44.

Hölldobler, B., and C. J. Lumsden. 1980. Territorial strategies in ants. Science. 210: 732–739.

Hölldobler, B., and C. D. Michener. 1980. Mechanisms of identification and discrimination in social Hymenoptera, pp. 35–58. *In* H. Markl [Ed.], Evolution of Social Behavior: Hypotheses and Empirical Tests. Weinheim: Verlag Chemie GmbH.

Holway, D. A., A. V. Suarez, and T. J. Case. 1998. Loss of intraspecific aggression in the success of a widespread invasive social insect. Science. 282: 949–952.

Howard, R. W. 1993. Cuticular hydrocarbons and chemical communication, pp.179–226. *In* Stanley-Samuelson, D. and D. Nelson [Eds.], Insect Lipids: Chemistry, Biochemistry, and Biology. University of Nebraska Press, Lincoln, Nebraska.

Hubbard, M. D. 1974. Influence of nest material and colony odor on digging in the ant *Solenopsis invicta* (Hymenoptera: Formicidae). J. Georgia Entomol. Soc. 9: 127–132.

Ichinose, K. 1991. Seasonal variation in nestmate recognition in *Paratrechina flavipes* (Smith) worker ants (Hymenoptera: Formicidae). Anim. Behav. 41: 1–6.

Jaffe, K. 1986. Nestmate recognition and territorial marking in *Solenopsis geminata* and in some Attini, pp. 211–222. *In* Lofgren C. S. and R. K. Vander Meer [Eds.], Fire ants and leaf-cutting ants: biology and management. Westview Press, Boulder, Colorado.

Jaisson, P. 1985. Social behaviour. pp. 673–694. *In* G. A. Kerkut and L. I. Gilbert [Eds.] Comprehensive Insect Physiology, Biochemistry, and Pharmacology. Vol. 15. Pergamon Press, Oxford.

Jutsum, A. R., T. S. Saunders, and J. M. Cherrett. 1979. Intraspecific aggression in the leaf-cutting ant *Acromyrmex octospinosus*. Anim. Behav. 27: 839–844.

Lacy R. C., and P. W. Sherman. 1983. Kin recognition by phenotype matching. Am. Nat. 121: 489–512.

Lahav, S., Soroker, V., Hefetz A., and R. K. Vander Meer. 1999. Direct behavioral evidence for hydrocarbons as ant recognition discriminators. Naturwissenschaften. 86: 246–249.

Le Moli, F., A. Mori , and D. A. Grasso. 1992. Nestmate and conspecific non-nestmate recognition in *Formica cunnicularia* Latr.: the effect of diet difference, pp. 161–165. *In* J. Billen [ed], Biology and Evolution of Social Insects. Leuven University Press, Leuven, Belgium.

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

Markin, G. P. 1968. Nest relationship of the Argentine ant, *Iridomyrmex humilis* (Hymenoptera: Formicidae). J. Kansas Entomol. Soc. 41: 511–516.

Markin, G. P. 1970. The seasonal life cycle of the Argentine ant, *Iridomyrmex humilis* (Hymenoptera: Formicidae) in southern California. Ann. Entomol. Soc. Am. 63: 1238–1242.

Morel, L., R. K. Vander Meer, and B. K. Lavine. 1988. Ontogeny of nestmate recognition cues in the red carpenter ant (*Camponotus floridanus*) – behavioral and chemical evidence for the role of age and social experience. Behav. Ecol. Sociobiol. 22: 175–183.

Nowbahari, M. and A. Lenoir. 1989. Age related changes in aggression in ant *Cataglyphis cursor* (Hymenoptera, Formicidae): Influence on intercolonial relationships. Behav. Proc. 18: 173–181.

Nylin, S., K. Gotthard, and C. Wiklund. 1996. Reaction norms for age and size at maturity in *Lasiommata* butterflies: prediction and tests. Evolution. 50:1351–1358.

Obin, M. S. 1986. Nestmate recognition cues in laboratory and field colonies of *Solenopsis invicta* Buren (Hymenoptera: Formicidae): Effect of environment and the role of cuticular hydrocarbons. J. Chem. Ecol. 12: 1965–1975.

Obin, M. S. and R. K. Vander Meer. 1988. Sources of nestmate recognition cues in the imported fire ant *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Anim. Behav. 36: 1361–1370.

Pfenning, D. W., and H. K. Reeve. 1980. Neighbor recognition and context-dependent aggression in a solitary wasp, *Sphecius speciosus* (Hymenoptera: Sphecidae). Ethology. 80: 1–18.

Pirk, C. W., P. Newmann, R. F. A. Moritz, and P. Pamilo. 2001. Intranest relatedness and nestmate recognition in the meadow ant *Formica pratensis* (R.). Behav. Ecol. Sociobiol. 49: 366–374.

Provost, E., G. Riviere, M. Roux, E. D. Morgan, and A. G. Bagneres. 1993. Change in the chemical signature of the ant, *Leptothorax lichtensteini* Bondroit with time. Insect Biochem. Molec. Biol. 23: 945–957.

Reeve, H. K. 1989. The evolution of conspecific acceptance thresholds. Am.Nat. 133: 407–435.

Roulston, T. H., G. Buczkowski, and J. Silverman. 2003. Nestmate discrimination in ants: effect of bioassay on aggressive behavior. Ins. Soc. 50: 151–159.

SAS Institute. 2002. SAS/STAT guide for personal computers, version 8.1. SAS Institute, Cary, NC.

Sherman, P. W. and W. G. Holmes. 1985. Kin recognition: issues and evidence. Fortschr. Zool., 31: 437–460.

Starks, P. T., D. J. Fischer, R. E. Watson, G. L. Melikian, and S. D. Nath. 1998. Context-dependent nestmate discrimination in the paper wasp, *Polistes dominulus*: a critical test of the optimal acceptance threshold model. Anim. Behav. 56: 449–458.

Suarez, A. V., D. A. Holway, D. Liand, N. D. Tsutsui, and T. J. Case. 2002. Spatio-temporal patterns of intraspecific aggression in the invasive Argentine ant. Anim. Behav. 64: 697–708.

Stuart, R. J. 1987. Transient nest mate recognition cues contribute to a multicolonial population structure in the ant, *Leptothorax curvispinosus*. Behav. Ecol. Sociobiol. 21: 229–235.

Stuart, R. J., and J. M. Herbers. 2000. Nest mate recognition in ants with complex colonies: within- and between-population variation. Behav. Ecol. 11: 676–685.

Suarez, A. V., D. A. Holway, D. Liang, N. D. Tsutsui, and T. J. Case. 2002. Spatiotemporal patterns of intraspecific aggression in the invasive Argentine ant. Anim. Behav. 64: 697–708.

Suarez, A. V., N. D. Tsutsui, D. A. Holway, and T. J. Case. 1999. Behavioral and genetic differentiation between native and introduced populations of the Argentine ant. Biol. Inv. 1: 1–11.

Thomas, M. L., Parry, L. J., Allen R. A., and M. A. Elgar. 1999. Geographic affinity, cuticular hydrocarbons and colony recognition in the Australian meat ant *Iridomyrmex purpureus*. Naturwissenschaften. 86:87–92.

Tsutsui, N. D., A. V. Suarez, D. A. Holway, and T. J. Case. 2000. Reduced genetic variation and the success of an invasive species. Proc. Natl. Acad. Sci. 97: 5948–5953.

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

Tsutsui, N. D., A. V. Suarez, and R. K. Grosberg. 2003. Genetic diversity, asymmetrical aggression, and recognition in a widespread invasive species. Proc. the Natl. Acad. Sci. 100: 1078–1083.

Vander Meer, R. K., and L. Morel. 1998. Nestmate recognition in ants. In: Pheromone Communication in Social Insects (Ed. by R. K. Vander Meer, M. D. Breed, K. E. Espiele, and M. L. Winston), pp. 79–103. Boulder, Colorado: Westview Press.

Vander Meer, R. K., D. Saliwanchik, and B. Lavine. 1989. Temporal changes in colony cuticular hydrocarbon patterns of *Solenopsis invicta*: implications for nestmate recognition. J. Chem. Ecol. 15: 2115–2125.

Venkataraman, A., and R. Gadagkar. 1992. Kin recognition in a semi-natural context: behavior towards foreign conspecifics in the social wasp *Ropalidia marginata* (Lep.) (Hymenoptera: Vespidae). Insect Society. 39: 285–299.

Via, S., and R. Lande. 1985. Genotype environment interaction and the evolution of phenotypic plasticity. Evolution. 39: 505–522.

Waldman, B. 1987. Mechanisms of kin recognition. J. Theor. Biol. 128: 159–185.

West-Eberhard, M. J. 1989. Phenotypic plasticity and the origin of diversity. Ann. Rev. Ecol. Syst. 20: 249–278.

Table. 1. Initial levels of hydrocarbons shared with prey in *L. humile* from California and southeastern U.S.

Cuticular hydrocarbon [a]	Region		Difference [b]	*P* [c]
	California	southeastern U.S.		
BG1	0.00 ± 0.00	0.32 ± 0.18	-0.32	0.168
BG2	0.26 ± 0.26	0.00 ± 0.00	0.26	0.220
BG1+BG2	0.26 ± 0.00	0.32 ± 0.00	-0.06	0.837
SL1	4.17 ± 0.14	6.78 ± 0.22	-2.61	< 0.0001
SL2	4.41 ± 0.41	6.10 ± 0.32	-1.69	0.005
SL3	6.61 ± 0.58	7.06 ± 0.26	-0.45	0.497
SL4	8.36 ± 0.27	8.66 ± 0.24	-0.30	0.414
SL1+ SL2+ SL3+ SL4	23.55 ± 1.32	28.60 ± 0.87	-5.05	0.016

[a] Hydrocarbon designations: *B. germanica* specific: BG1=11- and 13- and 15-Methylnonacosane; BG2=3-Methylnonacosane; and *S. longipalpa* specific: SL1=15, 19-Dimethylpentatriacontane; SL2=13- and 15- and 17- and 19-Methylheptatriacontane; SL3=15,19- and 17,21-Dimethylheptatriacontane; SL4=5,9- and 5,11-Dimethylpentatriacontane. Initial levels of cuticular hydrocarbons in field-collected colonies (day 0) expressed as percent of the total hydrocarbons. Values reported are means across colonies ± SE.

[b] Difference in hydrocarbon levels between regions (California – southeastern U.S.)

[c] Based on results of Student's *t*-test (SAS Institute 2002).

Table. 2. Initial cuticular hydrocarbon levels in California and southeastern U.S. colonies and changes in hydrocarbon levels after rearing on *B. germanica* and *S. longipalpa* prey.

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

[a] Hydrocarbon designations: *B. germanica* specific: BG1=11- and 13- and 15-Methylnonacosane; BG2=3-Methylnonacosane; and *S. longipalpa* specific: SL1=15, 19-Dimethylpentatriacontane; SL2=13- and 15- and 17- and 19-Methylheptatriacontane; SL3=15,19- and 17,21-Dimethylheptatriacontane; SL4=5,9- and 5,11-Dimethylpentatriacontane.

[b] Initial levels of cuticular hydrocarbons in field-collected colonies (day 0) expressed as percent of the total hydrocarbons. Values reported are means across colonies ± SE.

[c] Levels of hydrocarbons (day 52) after rearing on *B. germanica* or *S. longipalpa*.

[d] The change in hydrocarbon levels after rearing on cockroach prey (day 52 – day 0).

[e] Based on results of ANOVA (SAS Institute 2002).

Table. 3. Comparision of changes in cuticular hydrocarbon levels in *L. humile* colonies raised on *B. germanica* and *S. longipalpa* prey.

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

[a] Hydrocarbon designations: *B. germanica* specific: BG1=11- and 13- and 15-Methylnonacosane; BG2=3-Methylnonacosane; and *S. longipalpa* specific: SL1=15, 19-Dimethylpentatriacontane; SL2=13- and 15- and 17- and 19-Methylheptatriacontane; SL3=15,19- and 17,21-Dimethylheptatriacontane; SL4=5,9- and 5,11-Dimethylpentatriacontane.

[b] Net change in hydrocarbon levels after rearing on cockroach prey (day 52 – day 0; averaged over colonies).

[c] Based on results of ANOVA (SAS Institute 2002).

Table. 4. Final levels of prey-specific hydrocarbons in *L. humile* colonies raised on *B. germanica* and *S. longipalpa*.

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

[a] Hydrocarbon designations: *B. germanica* specific: BG1=11- and 13- and 15-Methylnonacosane; BG2=3-Methylnonacosane; and *S. longipalpa* specific: SL1=15, 19-Dimethylpentatriacontane; SL2=13- and 15- and 17- and 19-Methylheptatriacontane; SL3=15,19- and 17,21-Dimethylheptatriacontane; SL4=5,9- and 5,11-Dimethylpentatriacontane.

[b] Based on results of ANOVA (SAS Institute 2002).

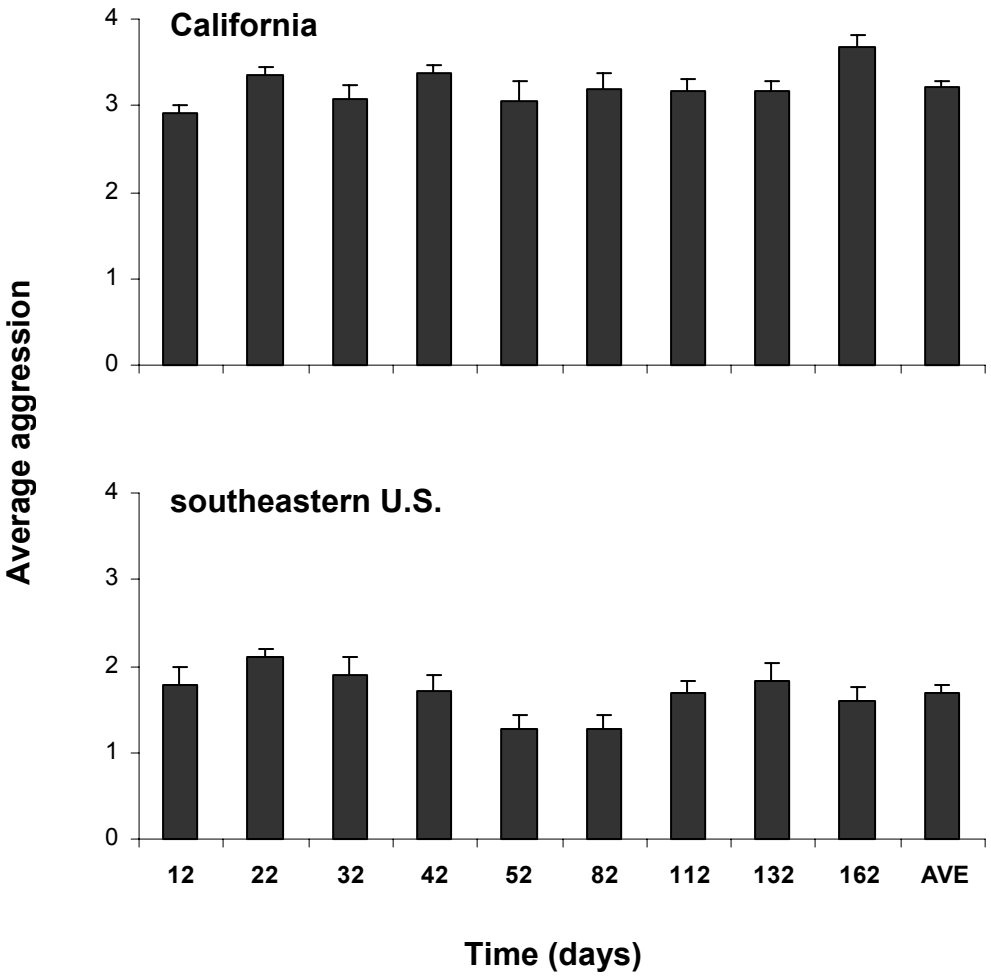

Fig. 1. Average aggression levels in southeastern U.S. (*n*=11) and California (*n*=7) colonies. Means for colonies ± SE are reported.

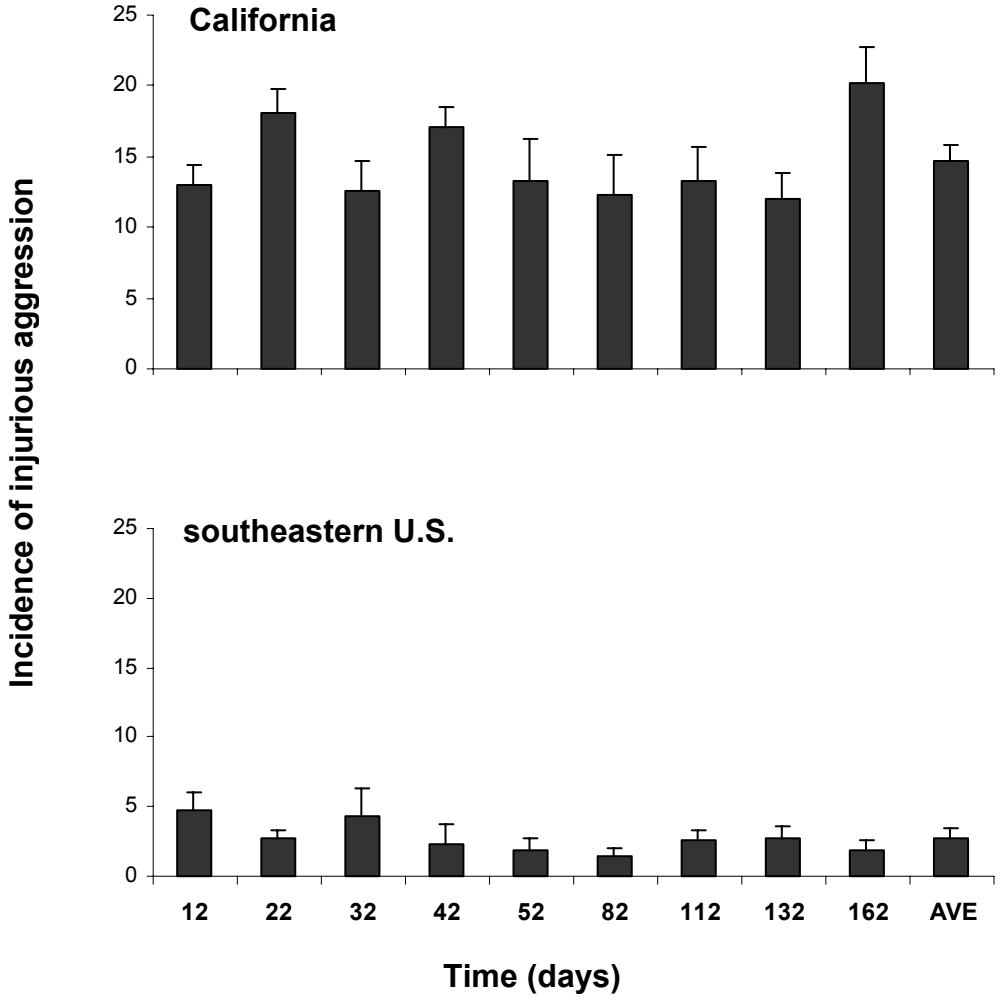

Fig. 2. Average level of injurious aggression (level 3 or higher) in southeastern U.S. (*n*=11) and California (*n*=7) colonies. Means for colonies ± SE are reported.

APPENDIX I

Nestmate Discrimination in Ants: Effect of Bioassay on Aggressive Behavior. [1]

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[1] Roulston, T. H., Buczkowski, G. and J. Silverman. 2003. Nestmate discrimination in ants: effect of bioassay on aggressive behavior. Insectes Sociaux, 50: 151–159.

Abstract. Aggression assays are commonly used to study nestmate recognition in social insects. Methods range from detailed behavioral observations on small numbers of insects to counts of individuals fighting in group interactions. These assays vary in the equipment used and the intensity and duration of observations. We used the Argentine ant, *Linepithema humile*, to compare four aggression bioassays for consistency between replicates, similarity between assays, and ability to predict whole colony interactions. The assays included were 1 live - 1 dead ant interactions, live 1-1 battles, live 5-5 battles, and 1 ant introduced to a foreign colony. We tested six ant colonies in all pairwise combinations using four different assays and two to three scoring methods per assay. We also conducted a colony merging experiment to see which assays were capable of predicting this ecologically important event. We found that scoring methods within assays yielded very similar results, giving us no reason to favor observationally intense procedures, such as continuous scanning, over less observationally intense systems, such as snapshot surveys. Assays differed greatly in their consistency between replicates. No two replicates of the 1 live - 1 dead assay were significantly correlated. The live 5-5 and the colony introduction assays were the most consistent across replicates. The mean scores of the live 1-1, live 5-5 and colony introduction assays were all significantly correlated with each other; only the live 5-5 assay was significantly correlated with the 1 live - 1 dead assay. Assays that utilized the greatest number of live ants were the most likely to reveal high levels of aggression. The aggression scores of all but the 1 live - 1 dead assay were positively correlated with the number of ants that died during whole colony encounters and negatively associated with colony merging. We conclude that all live ant assays tested are useful tools for analyzing aggressive interactions between colonies, but that the pairing of a live and dead ant produced

inconsistent results and generally lower levels of aggression. We found relatively low consistency between trials using the live 1-1 assay, but found that with sufficient replication its results were highly correlated with the assays using more interacting ants.

We suggest that isolated aggressive acts in assays do not necessarily predict whole colony interactions: some colonies that fought in bioassays merged when the entire colonies were allowed to interact.

Keywords: aggression assay, Formicidae, nestmate recognition, Argentine ant, *Linepithema humile*

Introduction

Social insects such as termites and many Hymenoptera exhibit agonistic behavior toward individuals that come from different nests (Hölldobler and Michener, 1980). Such exclusionary behavior is important because it allows colonies to stockpile resources during times of abundance and use these resources to feed nestmates, who are likely to be kin (Hamilton, 1972). It also allows colonies to protect their developing offspring (which are often abundant, helpless, and nutritious) against predation or enslavement. Colony threats may be either intraspecific (e.g., Pollock and Rissing, 1989; Breed et al., 1999) or interspecific (e.g., Sakagami, 1993; Mori et al., 2000).

During the past 25 years, nestmate recognition has received considerable attention from researchers working on ants, bees, and termites. The topics that have elicited the most interest are the mechanisms of nestmate discrimination and the relative influence of inheritance and environment on expression or detection of the nestmate phenotype (for reviews and discussion, see Hölldobler and Michener, 1980; Breed and Bennett, 1987; Jaisson, 1987; Waldman, 1987; Gamboa et al., 1991; Vander Meer and Morel, 1998; Lenoir, et al., 1999). In social insects, nestmate recognition likely depends on cuticular hydrocarbons (e.g., Obin, 1986; Bonavita-Cougourdan et al., 1987; Bonavita-Cougourdan et al., 1989, but see Vander Meer and Morel, 1998 for a criticism of commonly used methods) located on the exoskeleton. Cuticular hydrocarbon profiles are determined by genetic (e.g., Ross et al., 1987) and environmental factors such as diet (e.g., Jutsum et al., 1979; Liang and Silverman, 2000), nest material (e.g., Breed et al., 1988; Heinze et al., 1996) and physical contact with colony members (e.g., Breed et al., 1992).

Experiments designed to determine nestmate recognition require the induction of behavioral responses between interacting pairs or groups of organisms. Nearly all researchers use aggression bioassays in which colony members are shown to ignore or favor nestmates but display various amounts of aggressive behavior toward non-nestmates or toward nestmates that have been altered by experimental treatments. Aggression assays are irreplaceable tools to sort out the various hypotheses governing the mechanisms, heritability, and plasticity of nestmate recognition systems. These assays, however, are extremely varied (Table 1) and often there is no apparent relationship between the assay chosen, the question asked, and the species studied. Researchers seldom justify their choice of a particular assay, and rarely compare results from different assays, even when introducing a new one. The only apparent consistency is that individual researchers tend to use the same assay repeatedly.

Aggression assays vary in their duration, observational detail, analytical complexity, number and physical state of experimental subjects, and size and complexity of the experimental arena. They can be simple counts, such as the number of ants that died after 3 days spent in a box with ants from a different nest (Nowbahari and Lenoir, 1989) or counting the number of individuals of a given group permitted into a foreign nest (Greenberg, 1979; Breed et al., 1988; Mintzer, 1989). Assays may also be extremely detailed, such as scoring 20 pairwise encounters between an introduced ant and a host colony on a 1-9 scale based on specific behaviors thought to represent increasing aggression (Obin and Vander Meer, 1989), or labor intensive, such as tying four ants to different sections of a petri dish and recording aggression and spatial associations of an untethered ant walking among them (Fénéron, 1996). While such detailed observations can lend themselves to fine-scale analyses of

behavior, most researchers process the data from aggression assays into a single number (either an average, a maximum, or a summation over time) to represent the aggressiveness in an experimental trial. In such cases, it is unclear if these detailed analyses result in different representations of trials and different conclusions to study questions than less observationally intense trials.

It would be impractical and of questionable value to compare every aggression assay, including every duration and arena size that has been published. Instead, we have chosen to focus on several categorical differences among the designs and analytical approaches used in aggression bioassays and to examine their mathematical intercorrelations and possible differences in interpretation.

Materials and Methods

Ants (study species)

We used five colonies of Argentine ants (*Linepithema humile*) collected from four locations in the southeastern U.S.A. (Chapel Hill, North Carolina, CHH; Emerald Isle, North Carolina, EMI; Winston-Salem, North Carolina, FORb and FORs; Griffin, Georgia, GRF) and one colony from the western U.S.A. (Pleasanton, California, PLS). Experiments were carried out on all 15 pairwise combinations, as well as intracolony controls. Unlike the vast unicolonial structure of the Argentine ant population in California and elsewhere, in which workers from one nest can be readily transferred to another nest hundreds of kilometers away (Way et al., 1997; Suarez et al., 1999, but see Chen and Nonacs, 2000), some of the spatially isolated Argentine ant colonies in the southeastern U.S.A. exhibit strong intercolonial aggression (see below).

Colonies were maintained in soil-free, fluon-coated trays that ranged in size from 24 x 38 cm to 30 x 57 cm, depending on the size of the ant colony. Nests comprised plastic dishes filled with moist grooved plaster.

Assays

We performed a series of experiments to examine the effect of arena size, number of workers, and context (intruder or resident) on nestmate discrimination ability. The colony merging assay (see below) was carried out Apr 25 - May 28, 2001. All other assays were carried out from Mar 6-April 5th, 2001. In all experiments except the colony merging assay, the observer who recorded the data did not know the identity of the interacting colonies.

Individual ants were not tested in more than one trial. Unless stated otherwise, aggression scores were derived from the 0-4 scale of Suarez et al. (1999) [0 = ignore, 1 = touch, 2 = avoid, 3 = aggression (including lunging, and brief bouts of biting or pulling), and 4 = fighting (prolonged aggression, which also includes abdomen curling and apparent attempts to spray defensive compounds)]. For statistical analyses that use aggression as a binary character (i.e., aggressive or not aggressive), observations of 3 or 4 on this scale were considered aggressive.

Effect of arena size on aggression

To test the effect of arena size on aggression score, we carried out a series of one on one (live 1-1) assays using arenas of 1.3 cm, 3.2 cm, 5.7 cm, and 8.9 cm in diameter. Arenas were plastic rings or dishes with fluon-coated walls. For the three larger arenas, an open-ended, fluon-coated ring was placed in the center of the arena prior to each trial. One forager from each of two ant colonies was transferred by a brush to the arena, with one ant being placed inside and one outside the central tube. After 1 min. acclimatization, the central ring was removed so that the two ants could interact. For the smallest arena, which was too small to permit an inner chamber, the second ant was placed into the arena from a fluon-coated centrifuge tube 1 min. after being removed from its colony. An observer recorded data from 6 colonies simultaneously, recording the highest aggression observed during a 5-10 sec. scan each min. for 10 min. All colony pairings (15 intercolony and 6 intracolony) were replicated 3 times.

Effect of group size on aggression score

To test the effect of group size on aggression score, we chose one colony pair of moderate aggression and one pair of low aggression for 10 replicate comparisons of 5-5 and 20-20 interactions. For each trial we transferred a sample of foragers (5 or 20) from each nest to a 9 cm diameter fluon-coated dish. The foragers from one nest were placed inside a central, fluon-coated, open-bottomed tube within the arena, while the foragers from the second nest were placed in the arena outside the tube. After 1 min. acclimatization, the central tube was removed and the ants interacted. An observer watched 5 arenas simultaneously and recorded the number of fights and the number of ants engaged in fights during scan surveys taken each min. for 10 min.

Live 1-1 in arena

After detecting no effect of arena size on aggression level, we carried out 5 replicate live 1-1 assays on all colony pairings using a 1.3 cm diam. circular arena (see AEffect of arena size on aggression@ above for methods). We chose the smallest arena in order to videotape multiple trials simultaneously with as large an ant image as possible for observing behavior. Videotaping then scoring afterward for both the live 1-1 and the 1 live - 1 dead assay (below) minimized the total time that elapsed from the beginning until the end of all assays. The live 1-1 assays were scored in two ways, during two viewings of the videotape. First, each pair was scored during 5-10 sec. scans taken each min. for 10 min. For comparison of scoring methods within the assay, these data were analyzed as the average score recorded during each trial (over 10 observation periods), and the maximum score recorded during each trial. Second, each pair was scored for the duration (sec.) of behavior at

each aggression level for the first 3 min. following initial contact. The resultant statistic was derived from the equation

$$\log_{10}[(3_{i=1\text{-}4} \text{ total sec. at aggression level } i * i)/180 \text{ sec.}]$$

following Lahav et al. (1998). For comparison among assays, we used the proportion of trials in which aggression was observed at least once for each colony pair.

1 live - 1 dead in arena

This assay was identical to the live 1-1 assay, except that the second ant had been frozen to death then warmed prior to introduction. As above, all trials were videotaped for later analysis. Colony pairings were replicated 10 times, 5 with the first colony as the live ant and 5 with the second colony as the live ant. Each trial was scored by a 5-10 sec. scan each min. for 10 min. For comparison of scores within the assay, these data were analyzed as the average score recorded during each trial (over 10 observation periods), and as the maximum score recorded during each trial. For comparison among assays, we used the proportion of trials in which high aggression was observed at least once for each colony pair.

Colony introductions

We used a colony introduction assay that measured mortality and the level of aggression during intercolony worker introductions. Individual Aintruder" workers were allowed to walk onto a brush and were then introduced into rearing trays containing Aresident@ ants. For each test, we allowed the intruder ant to go through up to 25 encounters

with resident ants. Each instance of direct physical contact between the intruder and any of the residents was regarded as an encounter. If the intruder ant was seized by a resident ant and engaged in a highly aggressive encounter (level 4 aggression) for more than 10 sec., then the trial was terminated at that encounter and a 1.2 cm fluon-coated ring was placed around the fighting ants. Mortality among the ants that fought was checked 1 h later. After each test the intruder was removed and discarded and the residents were allowed to calm down before being used again. There were 10 replicates per colony pair, 5 replicates with colony 1 as the resident and 5 replicates with colony 1 as the intruder. Data were analyzed as the maximum score per trial, the average encounter score per trial, the number of dead ants per trial, and the proportion of highly aggressive encounters per trail. For comparison among assays, we used the average proportion of aggressive encounters per trial.

Live 5-5 in arena

After detecting no difference on aggression score when using groups of five versus groups of 20 ants (see AEffect of group size on aggression score@ above) we chose to use groups of five individuals in order to reduce the loss of ants from experimental colonies during trials. We carried out 6 replicates of the live 5-5 assay for all inter- and intracolony pairs. We counted the number of simultaneous fights and the number of ants in fights during scan surveys taken each min. for 10 min. For comparison among assays, we used the average proportion of ants involved in fights at one time across all colony pairs.

Colony Merging Experiment

In order to determine if the aggression assays using few ants would predict the outcome of whole colony interactions, we carried out a colony merging experiment and recorded the aggressive interactions between workers, and subsequently, the degree of mixing between colonies. We were unable to use all of the colony pairs from the aggression assays in the colony merging experiment because some colonies contained too few workers for subsequent use. Instead, we chose pairs that represented low, intermediate, and high aggression based on the aggression assays. We used a total of 8 different colony pairs, 3 replicates per pair.

Each colony contained ~100-150 workers, ~20 brood, and a single queen. For each colony pair, all ants in one colony were marked on the abdomen with white acrylic paint (Apple Barrel Colors #20782, Plaid Enterprises Inc., Norcross Georgia, U.S.A.) using a 10/0 spotter brush. Colonies were placed in separate nesting containers (17 cm by 25 cm by 11 cm high) and were allowed to colonize artificial nests that consisted of foil-covered glass tubes half-filled with water and stopped with cotton. After a 48 h starvation period within their nesting containers, each pair of colonies was given simultaneous access to a common foraging arena (17 by 25 cm) through separate 30 cm long vinyl tubes. The foraging arena contained 25% sucrose solution in a 50 mm x 4 mm vial. After 12 h, the sucrose vial was removed. The following day, we recorded the number of dead ants (marked and unmarked) in each container and piece of tubing then placed a vial containing 7 dead flies (*Drosophila melanogaster*) in the foraging arena. After 30 min., we counted and removed the flies remaining in the foraging arena and inserted a vial of 25% sucrose. Workers in the foraging arena were counted every hour for the next 5 hours. Daily, from d 5 - d 9 of the experiment,

colony pairs were inspected for merging and worker mortality. Merging was defined as the presence of the two queens and all brood in the same nest and the intermingling of marked and unmarked workers without fighting. In no case did the queens share a nest while the workers remained segregated. Experiments were terminated on the day that colonies merged or after d 9 if colonies still hadn=t merged.

Statistical analysis

Except as noted, all analyses were carried out using MINITAB 13.1 (MINITAB Inc., State College, Pennsylvania, USA) or SAS 8.2 (SAS Institute Inc., Cary, North Carolina) statistical software. The influence of arena size on aggression score was tested by a repeated measures logistic regression model using the genmod procedure of SAS 8.2. Colony pair and arena size were included as factors in the model with aggression score as the dependent variable, which was recorded once a minute for 10 minutes during each trial. The influence of group size on proportion of ants fighting was tested using proc mixed in SAS 8.2 with colony pair and group size as factors in the model, observation per trial as a random variable, and the proportion of total ants fighting per trial (after arcsin transformation) as the dependent variable. Throughout this manuscript the term arcsin transformation indicates the arcsin of the square root of the proportion (Sokal and Rohlf, 1981).

To test for an effect of being the resident versus the intruder colony during colony introductions, we used a nested ANOVA model with the proportion of aggressive encounters per trial (transformed with the arcsin) as the dependent variable and colony pair and resident colony within colony pair as treatment factors. We compared the number of ants killed

during the colony introduction assay using a non-parametric test (Kruskal-Wallis) because the data were not normally distributed.

Scoring methods within assays (e.g., average recorded score per session, highest score per session) were compared using the Spearman=s rank correlation of the score of each colony pairing averaged across replications. Consistency of results across replications is given as the Spearman=s rank correlation of the average score per trial. The Pearson product moment correlation was used to compare the proportion of aggressive trials per colony pairing among the the 4 assays. Differences in sensitivity to detecting aggressiveness among the four assays were determined through a general linear model ANOVA with colony pair and assay as main effects, proportion of aggressive trials as the dependent variable, and mean separation by Tukey=s simultaneous tests. For this test, aggression was interpreted as a level 3 or 4 response for assays using the 0-4 aggression index (Suarez et al., 1999) and as at least one fight in the live 5-5 assay.

We determined if aggression scores from the various aggression assays could explain the results of the colony merging experiment in two ways: first, we performed simple linear regression using the average intercolony aggression score from each assay as the independent variable and the number of ants killed within 24 hours of colony interaction as the dependent variable. Next, we performed binary logistic regression with the average intercolony aggression score as the independent variable and colony merging (for statistical analysis, colonies were considered to have merged if they merged in all three trials) as the dependent variable. Because the data set was small and the independent variable was unreplicated (i.e., each aggression score represented a single colony pairing), the logistic regression equation could not be solved by the maximum likelihood algorithm for most assays. Instead, we

solved the equation with LogXact software (Cytel Software Corporation, Cambridge, Massachusetts), using Monte Carlo simulations to derive the parameters.

Results

Neither arena size ($p > 0.37$ for all comparisons) nor group number ($p = 0.72$) influenced aggression score in our preliminary assays. Mean correlation coefficients (r) for the 5-6 replicates within aggression assays ranged from -0.15 - 0.79. The 1 live - 1 dead assay produced the least consistent results, with correlation coefficients ranging from -0.15 - 0.18 for all pairwise comparisons within the 5 replicates. The other assays all produced results that were positively correlated between replicates, with the live 5-5 the most consistent ($r = 0.79$, all replicates signficantly correlated), followed by the colony introduction assay ($r = 0.61$, all replicates significantly correlated) and live 1-1 ($r = 0.34$, 3 of 10 pairs of replicates significantly correlated). Within assays, all alternate scoring methods produced highly correlated results (range 0.78-0.98). Between assays, the live 1-1, live 5-5 and colony introduction assays produced significantly correlated results (Table 2). Correlations between each of these assays and the 1 live - 1 dead assay were modest and it was only statistically significant for the live 5-5 assay.

Among assays, there were differences in the likelihood of detecting acts of aggression (scored as a 3 or 4, on the 0-4 scale, or as a Afight@ in the live 5-5 assay). All four assays differed in the proportion of trials per colony pair in which at least one aggressive encounter was recorded, with the 1 live - 1 dead assay the least likely to detect aggression and the live 5-5 the most likely to detect aggression (Fig. 1) ($F_{.05\,(2,59)} = 53.5$, $p < 0.001$, all means different, general linear model ANOVA).

Two assays comprised asymmetrical presentations of one colony to the other. When one live ant was presented to a second colony (colony introductions), the proportion of

aggressive encounters was independent of which colony acted as the resident colony and which colony contributed the intruder ($F_{.05\ (15,120)} = 1.37$, $p = 0.172$, nested ANOVA). The 1 live - 1 dead assay also comprised an asymmetrical presentation but the preponderance of very low scores in all trials precluded a valid statistical analysis for the nested effect.

All assays resulted in a numerical ordering of the 15 colony pairs from least to most aggressive, rather than a binomial outcome (e.g., aggressive or not aggressive), even though observations were inherently binomial (e.g., ants fighting versus not fighting) or categorical on an arbitrary numerical scale (e.g., 0 - 4). Despite the depiction of apparent intermediate levels of aggression, all rankings from the present assays derived mainly from the relative proportions of aggressive and non-aggressive encounters rather than observations of individuals displaying intermediate aggression.

Aggressiveness rankings for most colony pairs were similar across assays, with CHH-GRF and FORb-FORs always showing low aggression and GRF-PLS and CHH-PLS always showing high aggression. Some pairs, however, such as FORb-GRF, were notably inconsistent across assays. Although the 1 live - 1 dead assay was less consistent across replicates, it provided a ranking of colony pair aggressiveness similar to other assays when all replicates were considered simultaneously.

In the live 1-1 assay, pairs of ants tended to ignore each other or fight vigorously. Level 2 aggression (avoidance) was only recorded frequently in the colony introduction assay. Recording data continuously from trials and factoring in the time spent at each aggression level, rather than taking scan samples at regular intervals, did not influence our interpretation of the aggressiveness of a given trial. The correlation coefficient between the 0.91.

The colony introduction assay should be sensitive to aggression because each trial permitted up to 25 ants to interact with each intruder. Not all colony pairs that exhibited fighting, however, did so in every trial. Only two of 10 trials of CHH-GRF, resulted in fighting while 10 of 10 trials led to fighting in five other colony pairs. The mean number of encounters until fighting occurred (excluding trials in which fighting did not occur) varied among colony pairs ($F_{.05\,(13,101)} = 1.98$, $p = 0.041$, 1-way ANOVA), as did the number of ants killed during fights ($H = 24.7$, $df = 12$, $p = 0.016$, Kruskal-Wallis test).

The colony merging assay generally resulted in either a combination of high initial mortality, poor food retrieval, and no merging, or low initial mortality, efficient food retrieval, and merging. The number of dead ants after 24 hours was a strong predictor of merging (Fig. 2). Two colony pairings gave delayed or inconsistent results: one of the three replicates of FORs-PLS merged within 24 hours but the other two replicates never merged. All three replicates of CHH-EMI merged, one each after 24, 48, and 72 hours. The number dead due to initial fighting for these two colony pairs was intermediate. The one replicate of the FORs-PLS pairing that merged showed less mortality than the two replicates that did not merge.

Results of all but the 1 live - 1 dead assay were significantly correlated with both the number of dead ants after 24 hours of encounters and the frequency of colony merging (Figs. 3-4). Colony pairs identified as the most aggressive in all assays showed high mortality and no merging while those of low aggression showed low mortality and merging within 24 hours. The colony pairs that were intermediate in merging (delayed or inconsistent across replicates) had intermediate aggression scores in most assays.

Discussion

Aggression bioassays use many different scoring systems and experimental designs (Table 1). In the present work with Argentine ants, we found that scoring methods within all assays were correlated, but that some assays (ants using a live and a dead ant or only 2 live ants) were less consistent than others.

All assays were not equally likely to reveal aggressive acts during individual trials. The live 5 - 5 and the colony introduction assays were more likely than the live 1-1 and the 1 live - 1 dead assays to reveal highly aggressive encounters (Fig. 1). These differences could reflect either the number of ants involved in the assays or the ecological context that the assay most closely approximates. The assays that generated the highest aggression scores were the assays in which the most ants were involved. If individual ants vary in their chemical profiles and/or their tendency to attack non-nestmates (e.g., Cammaerts-Tricot, 1975; Nowbahari and Lenoir, 1989; Fénéron, 1996) then increasing the number of interacting ants increases the likelihood of a potentially aggressive pair interacting.

These assays mimic different ecological contexts in which ants may encounter non-nestmates. The nest introductions resemble a stray forager or raiding party approaching the nest of another colony, but 1 - 1 assays more closely resemble isolated encounters between foragers away from their nest. Because aggressive interactions are potentially lengthy and lethal to both participants (e.g., De Vita, 1979), workers should be more likely to initiate fights when they have the most to gain by winning (Starks et al., 1998). Therefore, workers may exhibit particularly high levels of aggression when competing for food (e.g., Fellers, 1987; Cerdá et al., 1998; Holway, 1999) or when defending their nest from foreign ants (e.g.,

Hölldobler, 1976), and assays that mimic these ecological contexts may induce more aggression than assays that mimic casual encounters between non-nestmates (Starks et al., 1998).

The 1 live - 1 dead assay yielded the most variable results and could easily underestimate intercolony hostility with inadequate replication. This assay also sometimes showed aggression when none was expected. It was the only assay in which data from controls (same colony pairings) weren=t easily discerned from non-controls. For 5 of the 6 controls, there was at least 1 trial in which an aggressive encounter was recorded. These may have been instances of actual aggression, or may have been attempts to carry the dead ant that were misinterpreted as aggression. Although we found the 1 live - 1 dead assay to be the least consistent between trials, the use of dead individuals is appropriate for research designs that require eliminating one individual=s behavior, but not its chemical profile, on the behavior of another individual (Gamboa et al., 1991).

Researchers may wish to use aggression assays to explain various aspects of the ecology of a species. We found that three of the four assays, despite their differences in promoting highly aggressive behavior, were mathematically capable of explaining which colony pairs merged (i.e., there was an average aggression score that served as a threshold value for merging in all assays). In no assay, however, could we predict where that threshold value would lie. One colony pair that fought in some replicate assays (CHH-FORb) and one colony pair that fought in most replicates (CHH-EMI), merged. The CHH-EMI pairs didn=t merge until 2-4 days had elapsed and over 25% of the workers had died during fights. This could indicate either that the most aggressive individuals or the individuals most easily recognized as foreign were killed, which allowed for colony merging (e.g., Crosland, 1990),

or that sufficient non-fatal interaction had occurred to homogenize the chemical profile of the two colonies (e.g., Breed et al., 1992). In either case, these results serve as a warning that observations of instantaneous aggression between isolated individuals in assays do not result in clear predictions concerning whole colony interactions over time. Colony merging is of potential ecological significance in this species because reduced intercolonial territorial behavior has been implicated as one of the most important factors contributing to the invasiveness of Argentine ants (Holway et al., 1998; Holway, 1999; Suarez et al., 1999).

Unexpectedly, we found a poorer correlation between trials within an assay (including no significant correlation between replicates for the 1 live - 1 dead assay) than between assays, indicating substantial heterogeneity in chemical cues, perceptive abilities, or aggressiveness of individual colony members. Our mean inter-replicate correlation coefficient (0.34) was much lower than that reported by Tsutsui et al. (2000) (0.81) using the same aggression assay and the same species of ant. Their trials included many colony pairs that never fought, which may account for their greater consistency across replicates. Mintzer (1989) noted variation similar to ours with a colony introduction assay using leaf-cutter ants. He noted 30-100% rejection of foreign ants at the nest entrance, depending on the colony pairing. For this reason, adequate replication, particularly with assays using few individuals per trial, is essential.

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References Cited

Allies, A.B., A.F.G. Bourke and N.R. Franks. 1986. Propaganda substances in the cuckoo ant *Leptothorax kutteri* and the slave-maker *Harpagoxenus sublaevis*. J. Chem. Ecol. 12: 1285–1293.

Bonavita-Cougourdan, A., J.L. Clement and C. Lange. 1987. Nestmate recognition: the role of cuticular hydrocarbons in the ant *Camponotus vagus* Scop. J. Entomol. Sci. 22: 1–10.

Bonavita-Cougourdan, A., J.L. Clement and C. Lange. 1989. The role of cuticular hydrocarbons in recognition of larvae by workers of the ant *Camponotus vagus*: changes in the chemical signature in response to social environment (Hymenoptera: Formicidae). Sociobiol. 16: 49–74.

Breed, M.D. and B. Bennett. 1987. Kin recognition in highly eusocial insects. In: *Kin Recognition in Animals* (D.J.C. Fletcher and C.D.Michener, Eds.), J. Wiley & Sons Ltd., Chichester, pp. 243–285.

Breed, M.D., T.P. McGlynn, E.M. Stocker and A.N. Klein. 1999. Thief workers and variation in nestmate recognition behavior in a ponerine ant, *Ectatomma rubidum*. Ins. Soc. 46: 327–331.

Breed, M.D., L.E. Snyder, T.L. Lynn and J.A. Morhart. 1992. Acquired chemical camouflage in a tropical ant. Anim. Behav. 44: 519–523.

Breed, M.D., K.R. Williams and J.H. Fewell. 1988. Comb wax mediates the acquisition of nest-mate recognition in honey bees. Proc. Natl. Acad. Sci. 85: 8766–8769.

Cammaerts-Tricot. M.C., 1975. Ontogenesis of the defense reactions in the workers of *Myrmica rubra*. Anim. Behav. 23: 124–130.

Cerdá, X., J. Retana and S. Cros. 1998. Prey size reverses the outcome of interference interactions of scavenger ants. Oikos 82: 99–110.

Chen, J.S.C. and P. Nonacs. 2000. Nestmate recognition and intraspecific aggression based on environmental cues in Argentine ants (Hymenoptera: Formicidae). Ann. Entomol. Soc. Am. 93: 1333–1337.

Cohen, J. 1988. Statistical Power Analysis for the Behavioral Sciences, 2nd ed. Lawrence Earlbaum Associates, Hillsdale, New Jersey, U.S.A.

Crosland, M. W. J. 1990. Variation in ant aggression and kin discrimination ability within and between colonies. J. Insect. Behav. 3: 359–379.

De Vita, J. 1979. Mechanisms of interference and foraging among colonies of the harvester ant *Pogonomyrmex californicus* in the Mojave Desert. Ecology 60: 729–737.

Fellers, J. H. 1987. Interference and exploitation in a guild of woodland ants. Ecology 68: 1466–1478.

Fénéron, R. 1996. A new ethological test to study nestmate recognition in adult ants. Ins. Soc. 43: 131–135.

Gamboa, G. J., H. K. Reeve and W.G. Holmes. 1991. Conceptual issues and methodology in kin recognition research, a critical discussion. Ethology 88: 109–127.

Greenberg, L. 1979. Genetic component of bee odor in kin recognition. Science 206: 1095–97.

Hamilton, W. D. 1972. Altruism and related phenomena, mainly in social insects. Ann. Rev. Ecol. Syst. 3: 193–232.

Hefetz, A., C. Errard, A. Chambris and A. Le Negrate. 1996. Postpharyngeal gland secretion as a modifier of aggressive behavior in the myrmicine ant *Manica rubida*. J. Insect Behav. 9:

709–717

Heinze, J., S. Foitzik, A. Hippert and B. Hölldobler. 1996. Apparent dear-enemy phenomenon and environment-based recognition cues in the ant *Leptothorax nylanderi*. Ethology 102: 510–522.

Hölldobler, B. 1976. Recruitment behavior, home range orientation and territoriality in harvester ants, *Pogonomyrmex*. Behav. Ecol. Sociobiol. 1: 3–44.

Hölldobler, B. and C. D. Michener. 1980. Mechanisms of identification and discrimination in social Hymenoptera. *In*: Evolution of Social Behaviour: Hypotheses and Empirical Tests. Dahlem Konferenzen 1980 (H. Markl, Ed.), Verlag Chemie GmbH, Weinheim, pp. 35–57.

Holway, D. A. 1999. Competitive mechanisms underlying the displacement of native ants by the invasive Argentine ant. Ecology 80: 238–251.

Holway, D. A., A. V. Suarez and T. J. Case. 1998. Loss of intraspecific aggression in the success of a widespread invasive social insect. Science 282: 949–952.

Ichinose, K. 1991. Seasonal variation in nestmate recognition in *Paratrechina flavipes* (Smith) worker ants (Hymenoptera: Formicidae). Anim. Behav. 41: 1–6.

Jaisson, P. 1987. The construction of fellowship between nestmates in social Hymenoptera. *In*: From Individual to Collective Behavior in Social Insects: Les Treilles Workshop (J. M. Pasteels and J. L. Deneubourg Eds.), Birkhauser, Basel, pp. 313–331.

Jutsum, A. R., T. S. Saunders and J. M. Cherrett. 1979. Intraspecific aggression in the leaf-cutting ant *Acromyrmex octospinosus*. Anim. Behav. 27: 839–844.

Lahav, S., V. Soroker, R. K. Vander Meer and A. Hefetz. 1998. Nestmate recognition in the ant *Cataglyphis niger*: do queens matter? Behav. Ecol. Sociobiol. 43: 203–212.

Liang, D, and J. Silverman. 2000. "You are what you eat": Diet modifies cuticular

hydrocarbons and nestmate recognition in the Argentine ant, *Linepithema humile*. Naturwissenschaften 87: 412–416.

Lenoir A., D. Fresneau, C. Errard and A. Hefetz. 1999. The individuality and the colonial identity in ants: the emergence of the social representation concept. *In*: Information Processing in Social Insects (C. Detrain, J. L. Deneubourg and J. M. Pasteels, Eds), Basel, Birkhäuser Verlag, pp 219–237.

Mintzer, A. 1982. Nestmate recognition and incompatibility between colonies of the acacia-ant *Pseudomyrmex ferruginea* [sic]. Behav. Ecol. Sociobiol. 10: 165–168.

Mintzer, A. 1989. Incompatibility between colonies of acacia ants: Genetic models and experimental results. In: *The Genetics of Social Evolution* (M.D. Breed and R.E. Page Jr.,Eds), Westview Press, Boulder, pp. 163–182.

Morel, L., R. K. Vander Meer and B. K. Lavine. 1988. Ontogeny of nestmate recognition cues in the red carpenter ant (*Camponotus floridanus*) - behavioral and chemical evidence for the role of age and social experience. Behav. Ecol. Sociobiol. 22: 175–183.

Mori, A., D. A. Grasso and F. LeMoli. 2000. Raiding and foraging behavior of the blood-red ant, *Formica sanguinea* Latr. (Hymenoptera, Formicidae). J. Insect Behav. 13: 421–438.

Nowbahari, M. and A. Lenoir. 1989. Age related changes in aggression in ant *Cataglyphis cursor* (Hymenoptera, Formicidae): Influence on intercolonial relationships. Behav. Processes 18: 173–181.

Obin, M. S. 1986. Nestmate recognition cues in laboratory and field colonies of *Solenopsis invicta* Buren (Hymenoptera: Formicidae): Effect of environment and the role of cuticular hydrocarbons. J. Chem. Ecol. 12: 1965–1975.

Obin, M. S., L. Morel and R. K. Vander Meer. 1993. Unexpected, well-developed nestmate

recognition in laboratory colonies of polygyne imported fire ants (Hymenoptera: Formicidae). J. Insect Behav. 6: 579–589.

Obin, M. S., and R. K. Vander Meer. 1989. Mechanism of template-label matching in fire ant, *Solenopsis invicta* Buren, nestmate recognition. Anim. Behav. 38: 430–435.

Pollock, G. B., and S. W. Rissing. 1989. Intraspecific brood raiding, territoriality, and slavery in ants. Am. Nat. 133: 61–70.

Provost, E., 1989. Social environmental factors influencing mutual recognition of individual in the ant *Leptothorax lichtensteini* Bondr. (Hymenoptera: Formicidae*).* Behav. Processes 18: 35–39.

Ross, K. G., R. K. Vander Meer, D. J. C. Fletcher and E. L. Vargo. 1987. Biochemical phenotypic and genetic studies of two introduced fire ants and their hybrid (Hymenoptera: Formicidae). Evolution 41: 280–293.

Sakagami, S. F. 1993. Ethology of the robber bee *Lestrimelitta limao* (Hymenoptera: Apidae). Sociobiology 21: 237–277.

Silverman, J., and D. Liang. 2001. Colony disassociation following diet partitioning in a unicolonial ant. Naturwissenschaften 88: 73–77.

Sokal, R., and F. Rohlf. 1981. *Biometry*. W. H. Freeman and Company, New York.

Starks, P. T., D. J. Fischer, R. E. Watson, G. L. Melikian and S. D. Nath. 1998. Context-dependent nestmate discrimination in the paper wasp, *Polistes dominulus*: a critical test of the optimal acceptance threshold model. Anim. Behav. 56: 449–458.

Stuart, R.J. 1987. Individual workers produce colony-specific nestmate recognition cues in the ant, *Leptothorax curvispinosus*. Anim. Behav. 35: 1062–69.

Stuart, R. J., and J. M. Herbers. 2000. Nest mate recognition in ants with complex colonies:

within- and between-population variation. Beh. Ecol. 11: 676–685

Suarez, A. V., N. D. Tsutsui, D. A. Holway and T. J. Case. 1999. Behavioral and genetic differentiation between native and introduced populations of the Argentine ant. Biol. Invasions 1: 1–11.

Tsutsui, N. D., A. V. Suarez, D. A. Holway and T. J. Case. 2000. Reduced genetic variation and the success of an invasive species. Proc. Natl. Acad. Sci. 97: 5948–5953.

Vander Meer, R. K., and L. Morel, 1998. Nestmate recognition in ants. *In*: Pheromone Communication in Social Insects: Ants, Wasps, Bees, and Termites (R.K. Vander Meer, M. D. Breed, K. E. Espelie and M. L. Winston (Eds), Westview Press, Boulder, pp. 79–103.

Wagner, D., M. Tissot, W. Cuevas and D. M. Gordon. 2000. Harvester ants utilize cuticular hydrocarbons in nestmate recognition. J. Chem. Ecol. 26: 2245–2257.

Waldman, B. 1987. Mechanisms of kin recognition. J. Theor. Biol. 128: 159-185.

Wallis, D.I. 1962. Aggressive behaviour in the ant, *Formica fusca.* Anim. Behav. 10: 267–274.

Way, M. J., M. E. Cammell, M. R. Paiva, and C. A. Collingwood. 1997. Distribution and dynamics of the Argentine ant *Linepithema* (*Iridomyrmex*) *humile* (Mayr) in relation to vegetation, soil conditions, topography and native competitor ants in Portugal. Ins. Soc. 44: 415–433.

Whitehouse, M. E. A., and K. Jaffe. 1995. Nestmate recognition in the leaf-cutting ant *Atta laevigata*. Ins. Soc. 42: 157–166.

Table 1. Examples of published aggression assays with scoring methods and representative references.

Participants from colonies		Place of encounter	Scoring method to quantify aggressiveness
Colony 1	Colony 2		
1 live ant	1 live ant	neutral arena	Integer scale (Tsutsui et al. 2000), time summation (Lahav et al. 1998), count aggressive interactions (Heinze et al. 1996)
1 live ant	whole colony	in colony 2	Integer scale (Obin et al. 1993), accept/reject (Mintzer 1982), proportion of aggressive to non-aggressive interactions (Wallis 1962), attacked/not attacked (Stuart 1987)
1 live ant	3-5 live ants	neutral arena	Integer scale (Ichinose 1991), time summation (Hefetz et al. 1996), bitten or not (Allies et al. 1986)
whole colony	whole colony	colonies connected	Merged/not merged (Provost 1989), number of ants moving between colonies (Silverman and Liang 2001)
20 live ants	20 live ants	neutral arena	Count ants fighting (Chen and Nonacs 2000)
1 tethered ant	whole colony	in colony 2	Integer scale (Stuart and Herbers 2000)
1 free ant	4 tethered ants	neutral arena	Count aggressive acts and determine spatial orientation (Feneron 1996)
1 chilled ant	whole colony	in colony 2	Accept/reject (Breed et al. 1992)
1 pinned live	1 pinned live	in colony 2	Count number of times bitten in 2 min (Whitehouse and Jaffe 1995)
1 dead ant	whole colony	in colony 2	Number of ants aggressive toward dead and at 1 min. intervals (Morel et al. 1988)
1 dead ant	1 live ant	neutral arena	Time spent by live ant biting dead ant (Crosland 1990)
cuticular wash	whole colony	foraging area	Count number of ants aggressive toward extract on glass block (Wagner et al. 2000)

Table 2. Correlations among aggression assays.

Assay	Assay	*r*	*p*
colony introduction[1]	live 5-5[2]	0.91	<0.001
colony introduction[1]	live 1-1[3]	0.77	<0.001
colony introduction[1]	1 live - 1 dead[3]	0.39	0.076
live 5-5[2]	live 1-1[3]	0.70	<0.001
live 5-5[2]	1 live - 1 dead[3]	0.43	0.049
live 1-1[3]	1 live - 1 dead[3]	0.48	0.058

[1] Mean proportion of aggressive encounters per trial

[2] Mean proportion of ants fighting

[3] Proportion of trials revealing aggression

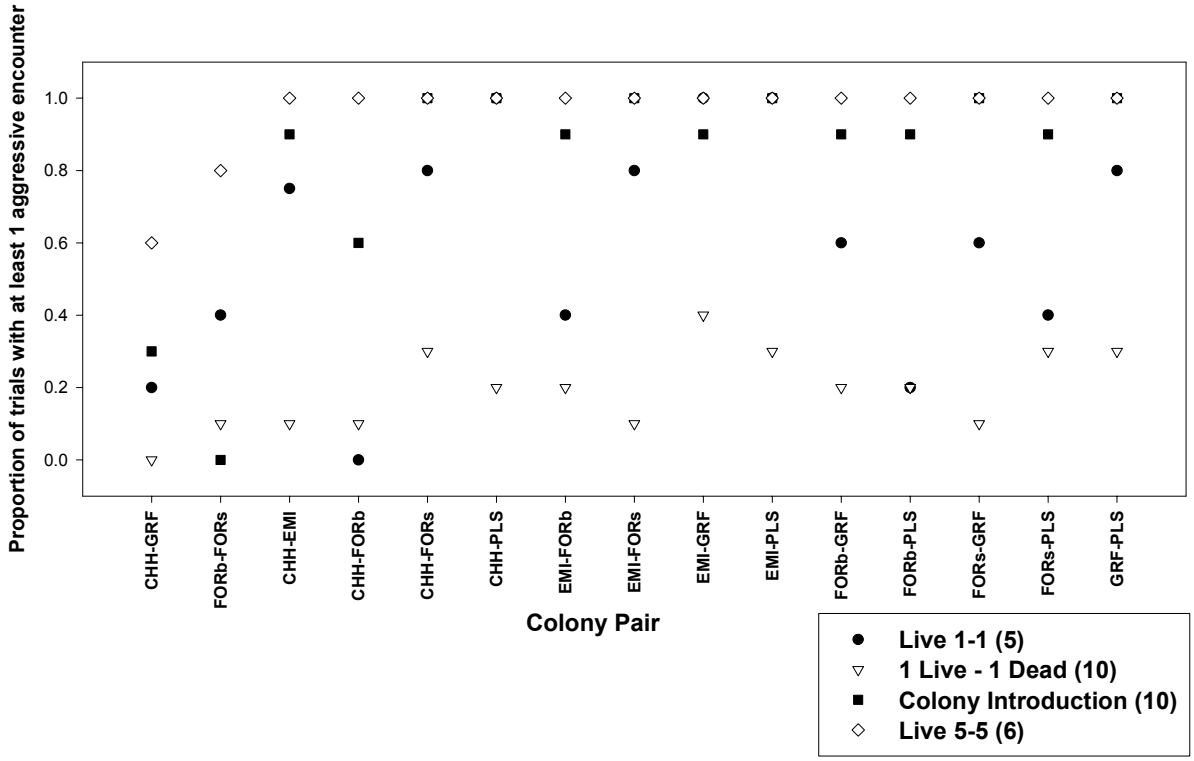

Fig. 1. Proportion of trials with at least one aggressive encounter observed for each of four aggression assays. Number of trials in parentheses of legend. Pairs sorted by increasing aggression level.

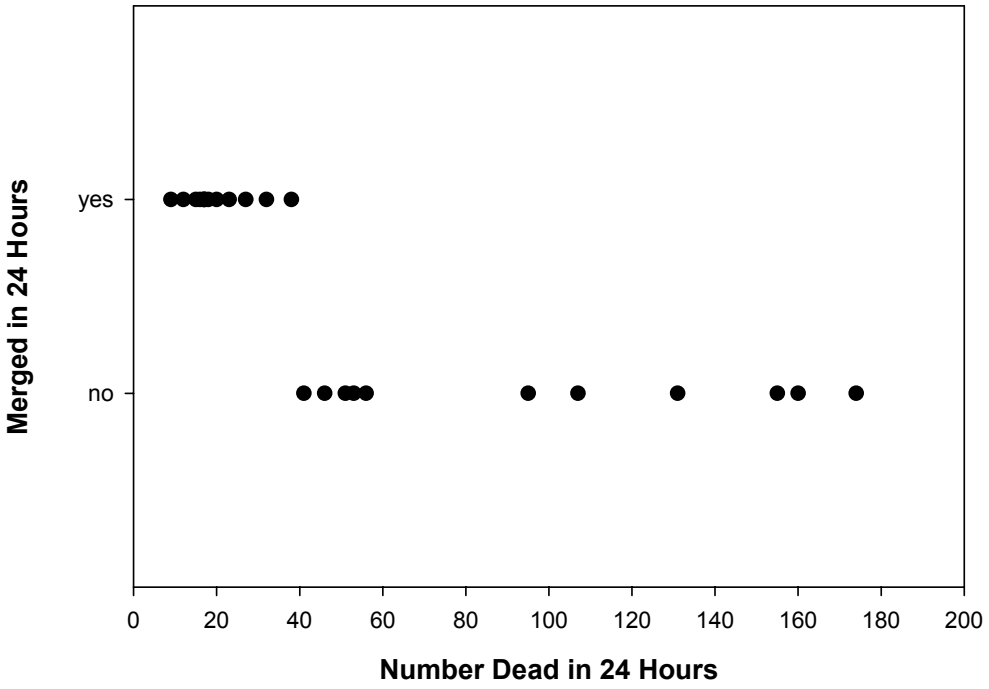

Fig. 2. Relationship between number of ants killed in first 24 hours of contact and whether or not colonies merged within 24 hours.

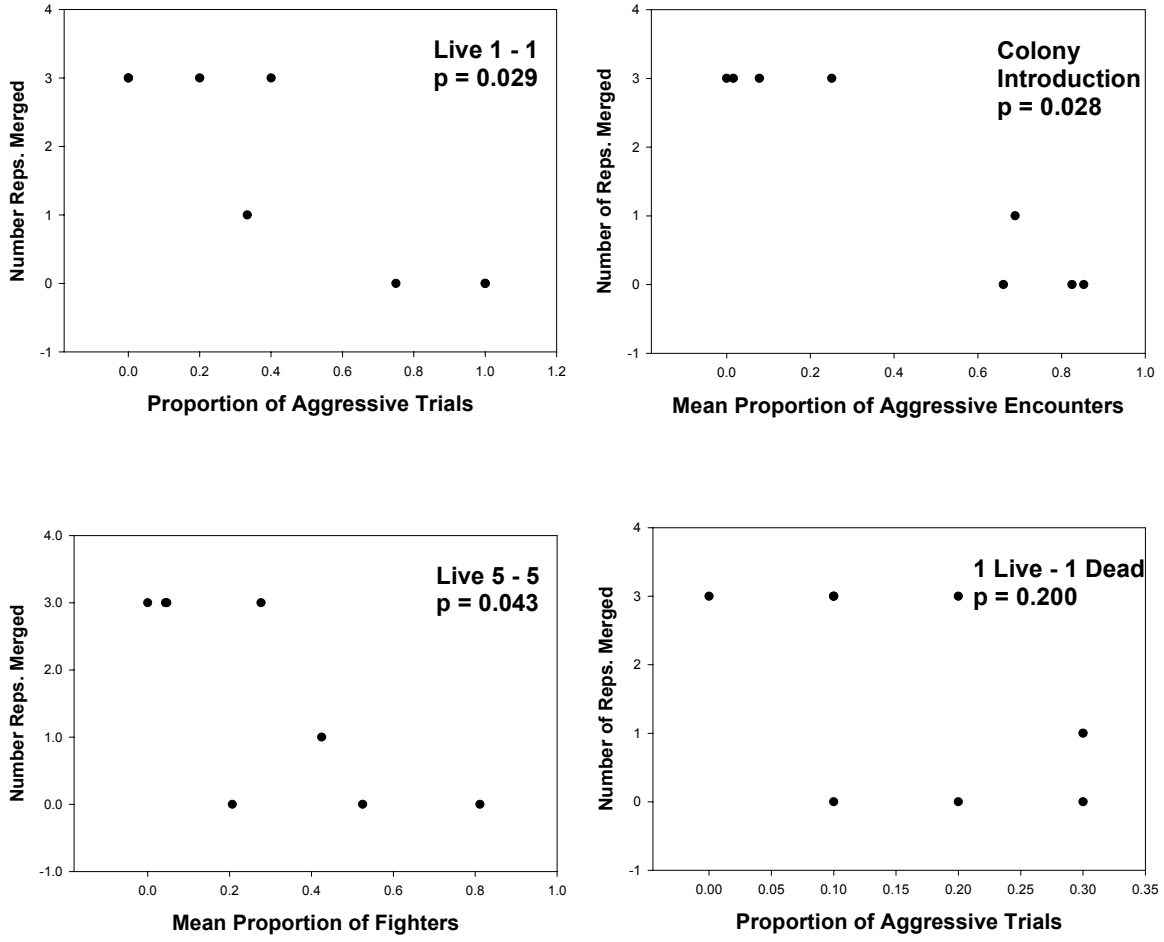

Fig. 3. Number of trials (out of 3) that colony pairs merged plotted against the mean aggression score of each colony pair. Analyzed statistically using binary logistic regression. All proportions transformed using the arcsin.

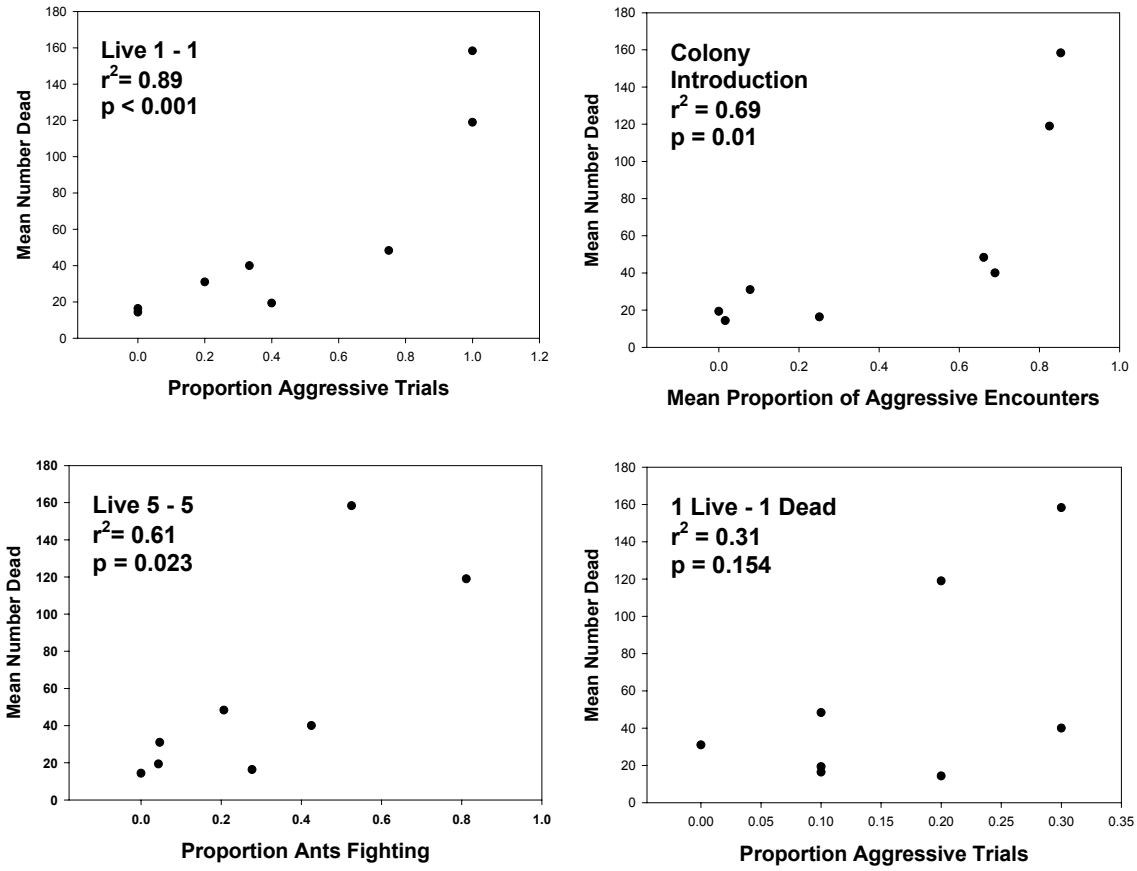

Fig. 4. Mean number of ants killed during the first 24 h after colony interaction plotted against the mean aggression score of each colony pair.