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Dear enemy phenomenon in the leaf-cutting ant *Acromyrmex lobicornis*: behavioral and genetic evidence

Romina D. Dimarco,[a,b] Alejandro G. Farji-Brener,[a] and Andrea C. Premoli[a]
[a]Universidad Nacional del Comahue, Laboratorio Ecotono-Centro Regional Universitario Bariloche, Quintral 1250, 8400, Bariloche, Argentina and [b]Department of Ecology and Evolutionary Biology, University of Tennessee, 569 Dabney Hall, Knoxville, TN 37996, USA

The defense of territory through aggressive behavior is well known in animals. However, some territorial animal species respond less aggressively to intrusions by their neighbors than to intrusions by nonneighbors to minimize the costs of continuous fights, a phenomenon termed the dear enemy phenomenon (DEP). Although several studies show the existence of this phenomenon, little is known about the mechanism behind it. One possible explanation is the lower degree of genetic divergence between neighbors compared with nonneighbors. We tested the DEP hypothesis and whether genetic divergence among nests might drive the DEP in the leaf-cutting ant *Acromyrmex lobicornis* in Patagonia, Argentina, through behavioral and genetic studies. Individuals from nearby colonies interacted less aggressively than individuals from distant colonies. However, levels of genetic divergence between focal–close and between focal–nonneighbor nests attained similar values. Our results support the dear enemy hypothesis but suggest that the differential aggressiveness toward neighbors relative to nonneighbors is unrelated to genetic divergence among nests. Other possible causes of this behavior, such as habituation, are discussed. *Key words:* aggression, dear enemy phenomenon, isozymes, leaf-cutter ants. *[Behav Ecol]*

Many territorial animals behave less aggressively toward neighbors relative to nonneighbors or strangers (Wilson 1975; Heinze et al. 1996; Hernandez et al. 2002). This difference in the level of aggression has been called "the dear enemy phenomenon," hereafter DEP (Fisher 1954). The DEP posits that individuals of distant territories, that is, strangers, are potentially more dangerous than individuals from nearby territories because the former are more likely to be seeking new territory and, thus, pose a higher threat to resources (Temeles 1994). Moreover, the relatively peaceful coexistence between neighboring territorial species is considered adaptive because it avoids the costs of frequent fights (Jaeger 1981). Despite the fact that "dear enemy" behavior is common in social insects (e.g., Jutsum et al. 1979; Bennett 1989; Gordon 1989; Heinze et al. 1996; Kaib et al. 2002; Knaden and Wehner 2003; but see Sanada-Morimura et al. 2003; Boulay et al. 2007; Thomas et al. 2007; van Wilgenburg 2007; Thurin and Aron 2008), the mechanisms behind this phenomenon are poorly resolved.

Territorial behavior is common in ants, and species with relatively spatially fixed nests, such as leaf-cutter ants, commonly defend their territories against intrusion from con- and heterospecifics. Maintaining these territories should be advantageous for colony function (Hölldobler and Lumsden 1980). The fact that many ant species vary their level of aggressiveness according to neighbor status suggests some mechanism of intercolony recognition. It is well documented that colonies use low-volatility hydrocarbons localized in the exoskeleton for within-colony recognition (Bonavita-Cougourdan et al. 1987; Lahav et al. 1999; Wagner et al. 2000; Greene and Gordon 2007; Martin et al. 2008). Three sources are responsible for particular odors of the individuals within each colony: 1) the environment, including chemical compounds coming from sources such as food, construction materials of the nest, and microorganisms associated with the colonies (Breed et al. 1988; Heinze et al. 1996; Chen and Nonacs 2000; Buczkowski and Silverman 2006), 2) physical contacts among nestmates (Breed et al. 1992; Boulay et al. 2003), and 3) genetics, that is, heritable odors resulting from metabolites produced by the ants themselves, such as hydrocarbons, that constitute a group of cuticular heritable elements exhibiting high colony specificity (Crosland 1989).

The dispersion of sexual individuals from a focal nest is relatively centrifugal (i.e., from the nest that is a fixed point to the outside), so colonies that are closer might be more closely related to one another. Many studies suggest that an important genetic component exists in the recognition between individuals of the same species (Seppä 1992; Banschbach and Herbes 1996; Tsutsui et al. 2000; Pirk et al. 2001). When odor labels have a genetic base, the precision of the recognition is based on the levels of polymorphism and allelic frequencies at those loci (Tsutsui et al. 2003). Ant colonies often respond more aggressively toward less related individuals (i.e., more genetically divergent) because they do not share the same odors (Ydenberg et al. 1988). For example, in *Linephitema humile*, the level of aggression between ants is a function of genetic relatedness (Thomas et al. 2006). Therefore, genetic factors could play an important role to explain the less aggressive behavior between closely related colonies and could be an important factor in the DEP (Jutsum et al. 1979; Stuart 1987; Beye et al. 1998).

Another alternative explanation for the variation in aggressive behavior between ants by neighbor status is the phenomenon of habituation. The habituation phenomenon describes

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Address correspondence to R.D. Dimarco. E-mail: rdimarco@utk.edu.

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a lack of response toward a stimulus that it is experienced repetitively, such as a persistent odor (Orivel et al. 1997; Owen and Perrill 1998; Langen et al. 2000). Foraging ants could learn to recognize the cuticular compounds of the neighboring colonies independent of the genetic divergence between them.

The leaf-cutter ant *Acromyrmex lobicornis* is a good system to study the DEP because, like other leaf-cutting ant species, colonies exhibit intraspecific aggression in order to protect their territories (Hernandez et al. 2002; Ballari et al. 2007). In this study, we tested for the presence of the DEP in *A. lobicornis* and whether the degree of genetic divergence predicts the level of aggressiveness between colonies. We also consider the habituation hypothesis as an alternative explanation of the DEP. If the habituation hypothesis is correct, we expected that differential aggressiveness by neighbor status to be related simply to being neighbors or not and unrelated to the distance between the colonies and to the genetic divergence among colonies.

MATERIALS AND METHODS

Study system

Acromyrmex lobicornis is the leaf-cutter ant with large distribution in Argentina, extending to lat 44°S (Farji-Brener and Ruggiero 1994). In northwest Patagonia, *A. lobicornis* is the only species of leaf-cutter ants, and it persists at relatively high density.

Study site

This study was conducted in the Province of Neuquén, Argentina, in the east boundary of the Nahuel Huapi National Park.

Methods

We carried out individual behavioral assays whereby we placed ants from colonies of varying distances from a focal nest and observed their interactions. For the assays, 17 focal nests were randomly chosen, each of which were used to stage interactions with ants of its own colony as control, ants of a close neighbor nest, and ants of a nonneighbor nest. The combination of 3 experimental units, that is, focal, close neighbor, and nonneighbor nests, are hereafter referred as trios. Therefore, a total of 51 nests and 17 trios were utilized in this study. Distances between focal nests were measured. The minimum distance between focal nests was of 73 m, and the maximum distance was of 23 km. The close neighbor was the closest nest to the focal nest whose maximum distance to the focal nest did not exceed 50 m. Focal nests and their respective close neighbor always had overlapping foraging territories. The nonneighbor nest was located between 90 and 200 m away from the focal nest, and we took care to ensure that their foraging ranges did not overlap. We chose these distances because of the spatial distribution of nests in the study area as well as the extent of the area over which they forage.

Individual behavior assays were conducted along the foraging trail of each focal nest where the greatest foraging activity was detected. Foraging activity was measured by counting the number of ants that walked along 15 cm of the trail during a 20-s period. The activity was measured 3 times to obtain an average value.

We haphazardly collected 5 foraging ants of the focal nest (for the control treatment assay), 5 foraging ants of the close neighbor nest, and 5 foraging ants of the nonneighbor nest. The collection of the ants was made with tweezers cleaned with ethanol to minimize the mixing of odors between the colonies. We kept the collected ants of each nest in individual plastic containers inside a cooler at 5 °C to reduce their activity prior to the behavioral assays. Each ant was marked with acrylic paint on its thorax for identification purposes. This procedure does not produce alterations in behavior and is commonly used to mark ants (Roulston et al. 2003; Ballari et al. 2007).

Ants were permitted to acclimate to ambient temperature conditions for 10 min prior to each essay. We placed one marked ant 25 cm from the principal entrance of the focal nest, along the trail with greatest activity, and proceeded to record the behavior of ants of the focal nest toward the "intruder" ant for a period of 3 min. We assigned a semiquantitative value of aggression from 0–5 based on Suarez et al. (1999). The levels of aggression were 0: individuals of the focal nest ignore the intruder ant, 1: antennation, 2: touches with different parts of the body, 3: intruder ant is carried by the abdomen away from the nest and left near the trail without damage, 4: discontinued aggression with biting merely in the antennas, and 5: prolonged aggression between individuals, often consisting of both ants locking their mandibles onto a body part of the other and leg pulling. We carried out 15 individual behavior assays per trio with 5 replicates per pair, including 5 control assays to measure the interaction between ants of the same focal nest. The order in which we put each ant on the trail of the focal nest was at random and double blind. After 3 min, we placed the marked ant into a labeled Eppendorf tube. When maximum levels of aggression were registered (4, 5), we also collected an ant of the focal nest that was involved in the interaction. The tubes were kept at 5 °C until they were taken to the laboratory where they were stored at −80 °C until protein extraction for genetic analyses by means of isozymes.

Genetic study

Genetic relationships among *A. lobicornis* individuals of behavioral assays from focal, close neighbor, and nonneighbor nests were determined using horizontal electrophoresis on starch gels. A total of 372 individuals were genotyped, 201 individuals from focal nests, 86 individuals from close neighbor nests, and 85 individuals from nonneighbor nests. Enzyme extracts were prepared by grinding the head and abdomen of each individual in 0.25 ml of distilled water. Homogenates were absorbed onto Whatman No. 3 paper wicks that were loaded into 12% starch gels (Starch Art Corporation, Smithville, TX). Four enzymes coding for 4 putative isozyme loci were resolved using the Tris-ethylenediaminetetraacetic acid–boric acid–HCl buffer system (pH 6.7) modified from Shaw and Prasad (1970). These were MDH (malate dehydrogenase), PERc (cathodic peroxidase), PGI (phosphoglucoisomerase), and IDH (isocitric dehydrogenase) that were run at a constant current of 10 mA for about 18 h.

Statistics

We determined differences in the total frequencies in each level of aggression per treatment (control, close neighbor, and nonneighbors), using the total results of the fights per treatment independent of if there were replications of the same trio using a chi-square test. This method determines whether the proportion of different levels of aggression was independent of the distance between colonies. We independently compared the average proportion of each level of aggression displayed in each of the 3 treatments (focal–focal, focal–close neighbor, and focal–nonneighbor nests) using analysis of variance (ANOVA). In this case, each trio was considered as an experimental unit and the 5 repetitions as replicates. When the differences between treatments were significant, post hoc tests were performed with the Tukey–Kramer HSD method ($\alpha = 0.05$). We also performed a multiple regression including distance from the focal nest, genetic divergence (F_{st}), and the interaction between distance and genetic divergence (distance from focal nest $\times$ F_{st}) as predictors of the aggression. All the behavior analyses were performed with the program Statistica 7.0 (StatSoft Inc 2004).

To determine the level of genetic divergence between pairs of nests, we calculated F_{st} indices (Wright 1965). We obtained 34 values of F_{st} (2 values per trio), one for each focal nest–close neighbor pair and one for each focal nest–nonneighbor pair. Then, we calculated the average F_{st} value of the focal nest–close neighbor pair–and the focal nest–nonneighbor pair, respectively. To compare the average values, we performed a Wilcoxon nonparametric test due to the lack of normality in the data. We also calculated other 3 genetic distance indices (Rogers genetic distance index modified by Wright 1978, Nei's [1972] genetic distance index, and Nei's [1972] minimum genetic distance index). All the genetic analyses were performed using Biosys program 1.7 (Swofford and Selander 1989). Levels of isozyme variation of 17 focal nests of *A. lobicornis* were analyzed using POPGENE v.1.31 (Yeh et al. 1999). These levels were the total number of alleles (A_T), number of unique alleles (A_U), number of alleles in low frequency (A_K), mean number of alleles per locus (N_A), percentage of polymorphic loci by using no criteria ($P\%$), and observed (H_O) and expected (H_E) heterozygosity (See Supplementary Data).

We performed a simple regression between the proportion of the level of aggression 5 (higher level of aggression registered) in the treatment focal nest–nonneighbor nest in relation to the geographical distance between the focal nests and their nonneighbor nests. Level 5 was the most common behavior found in the treatment focal nest–nonneighbor nest. This analysis was conducted to obtain evidence for the habituation hypothesis. We expected that the aggressive behavior will not increase with distance and that the aggressive behavior observed in this study is related simply to being neighbors and not related to the distance between ants' colonies.

To investigate the correlation between the degree of genetic distance and the geographic distance and also to test if the scale used in this study was correct, we run a Mantel (1967) test, using the program GENALEX 6 (Peakall and Smouse 2006). The 2 distance metrics used to perform this test were the genetic distance (index of genetic distance of Rogers [Wright 1978]) and the geographic distance (in kilometers) between the 17 focal nests analyzed in this study (the focal nests were geographically separated up to distances of 23 km). Distances were estimated as the linear distance between the sampling pairs. The significance of this test was assessed using 999 permutations using the same program.

RESULTS

Aggressive behavior between ants

The levels of aggression between individuals of *A. lobicornis* increased with the distance between colonies. Ants within the same colony (i.e., focal nests) showed null to low levels of aggressive behaviors; ants from the focal and close neighbor nests presented intermediate levels of aggression; and ants from the focal and nonneighbor nests showed the highest levels of aggression. These results were revealed by both chi-square test using frequencies (Figure 1) and ANOVA analyses (Figure 2). The lowest level of aggression, that is, ignorance of intruder ant = 0, was most frequently observed in the control treatment but absent in the other treatments. The intermediate levels of aggression (2 and 3) were the most frequent in the interactions between focal and close neighbor nest. Together, these 2 levels constituted 61% of the interactions in this treatment. Finally, focal vs nonneighbor nest treatment yielded maximum levels of aggression (4 and 5), whereas the lower levels of aggression (0, 1, and 2) were almost absent and accounted by less than 10% of the interactions ($\chi^2 = 251.27$, degrees of freedom = 10, Figure 1).

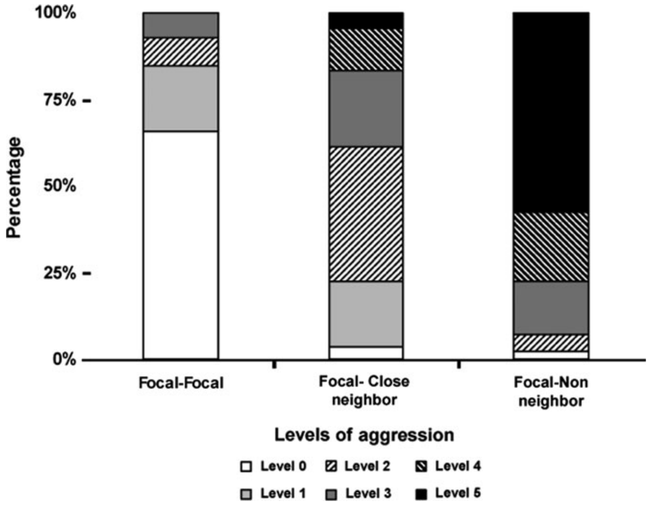

Figure 1
Percentage of the ascendant levels of aggression (0–5) observed in the different interactions between colonies of *Acromyrmex lobicornis*. Level 5 (prolonged aggression between individuals and often consisted of both ants locking their mandibles onto a body part of the other and leg pulling) is the highest level of aggression and is represented with the black color. Level 0 (individuals of the focal nest ignore the intruder ant) is the lowest level and is represented with the white color.

Results obtained using the trio as experimental units (focal–focal, focal–close neighbor, and focal–nonneighbor nest) were consistent with those obtained from the analysis of frequencies. The levels of aggression 0, 2, and 5 were the most frequent levels in each interaction. The absence of aggression was, on average, greater in the control than in the other interactions ($F_{2,48} = 97.1$, $P < 0.001$; post hoc Tukey test). The frequency of an intermediate level of aggression (2) was, on average, higher in the focal nest versus close neighbor treatment compared with the other interactions ($F_{2,48} = 35.7$, $P < 0.001$; post hoc Tukey test). The highest level of aggression (5) occurred most frequently in interactions between individuals of the focal nest vs nonneighbor nest ($F_{2,48} = 84.2$, $P < 0.001$; post hoc Tukey test). Finally, in the levels of aggression 1, 3, and 4, there were not higher frequencies on a single treatment. In the level of aggression 1, the control and the focal nest versus close neighbor interactions were more similar than the focal nest versus nonneighbor nest interaction ($F_{2,48} = 8.6$, $P < 0.001$; post hoc Tukey test). In the levels of aggression 3 and 4, the focal nest versus close neighbor and the focal nest versus nonneighbor nest interactions were more similar than the control treatment ($F_{2,48} = 3.9$, $P = 0.027$; $F_{2,48} = 11.4$, $P < 0.001$; post hoc Tukey test) (Figure 2).

Results from the multiple regression showed that distance from the focal nest was the only significant predictor of aggression, whereas the genetic divergence (F_{st}) and the interaction term were not significant (whole model: $F_{3,30} = 8.96$,

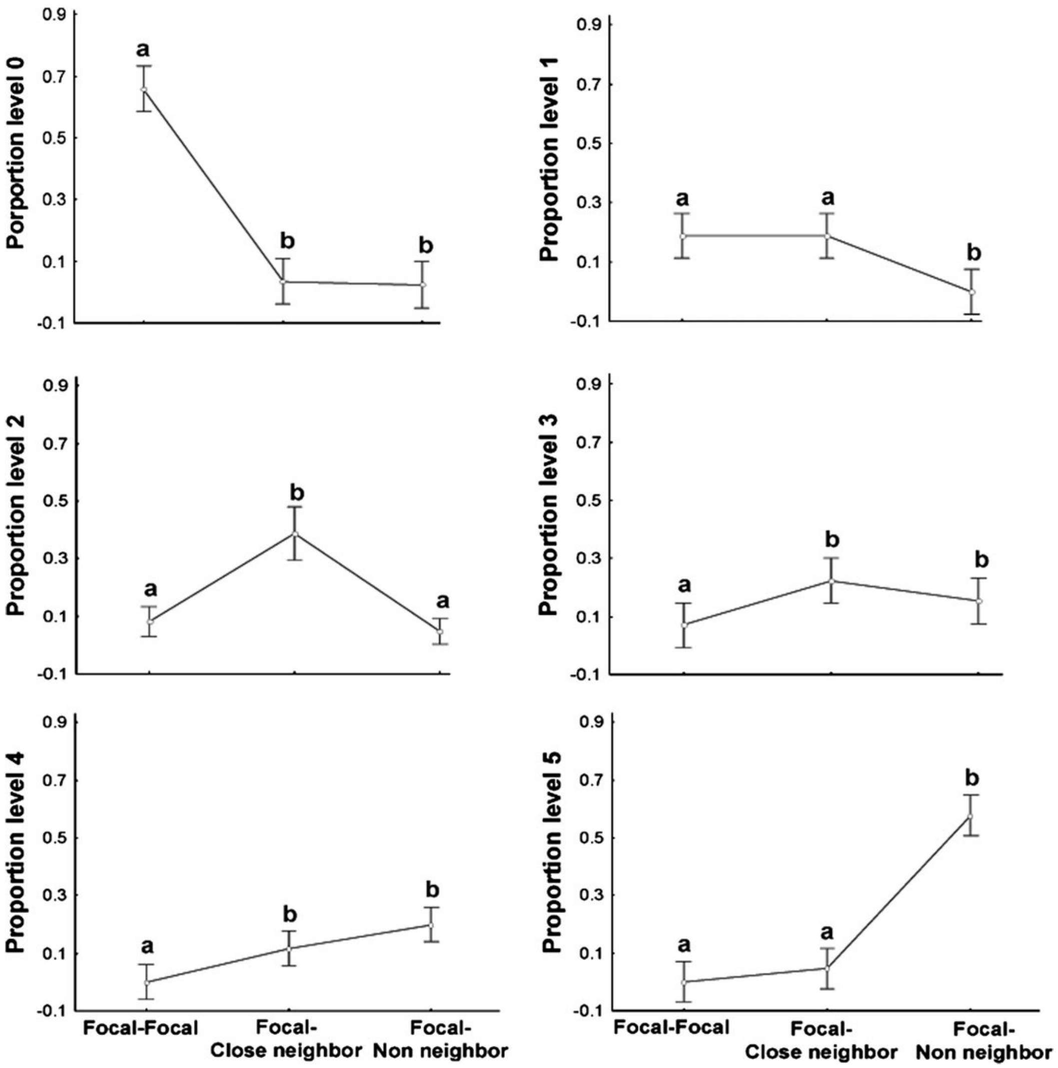

Figure 2
Average proportion of the 6 levels of aggression (0–5) in the 3 treatments: focal nest–focal nest, focal nest–close neighbor, and focal nest–nonneighbor with their standard errors. Different letters indicate statistical significant differences ($P < 0.05$). Level 5 is the highest level of aggressions, and level 0 represents absence of aggression.

$P = 0.0002$, $R^2 = 0.47$, probability of the t ratios for each term: F_{st} $P = 0.7$, distance $P < 0.0001$, interaction $P = 0.35$).

Genetic results

All 4 isozyme loci were polymorphic in the analyzed focal nests of *A. lobicornis*. Focal nests hold uniformly high genetic diversity for all calculated parameters (Appendix 1). Degree of genetic divergence between focal–close ($F_{st} = 0.055$, 95% confidence interval [CI] = 0.036–0.075) and focal–nonneighbor nests ($F_{st} = 0.046$, 95% CI = 0.032–0.059) attained greater than 0 values. These suggest moderate significant genetic divergence (Hartl and Clark 1997) between focal and either close or nonneighbor nests. Nevertheless, no significant differences were found between both F_{st} estimates as by comparison of CIs. In addition, we failed to detect a significant difference between paired by locus average values of F_{st} between focal and close neighbor and nonneighbor nests (Wilcoxon test $Z = 0.50$, $P = 0.62$, Figure 3). Similarly, other such tests using 3 genetic distance indices (Rogers [Wright 1978]; Nei's [1972] and minimum distance of Nei [1972]) failed to detect a difference.

Effect of geographical distance between focal nests and nonneighbor nests

We failed to detect a relationship between the variation of the most aggressive behavior (level of aggression 5) and the distance between focal and nonneighbor nests ($P = 0.46$, $R^2 = 0.0363$, $F_{1,15} = 0.57$, Figure 4) in the studied range (from ca. 80 to 200 m from the focal nest). This suggests that aggression does not increase with distance in nonneighbor nest.

Effect of geographic distance and genetic distance

After conducting a Mantel test, we found a negative trend between genetic distance and geographic distance. Although little of the variance in genetic distance of the studied nests was explained by geographic distance ($R^2 = 0.047$, $P = 0.05$, $n = 17$, Figure 5).

DISCUSSION

We found support for the dear enemy hypothesis in the leaf-cutting ant *A. lobicornis*: Ants of this species exhibit less

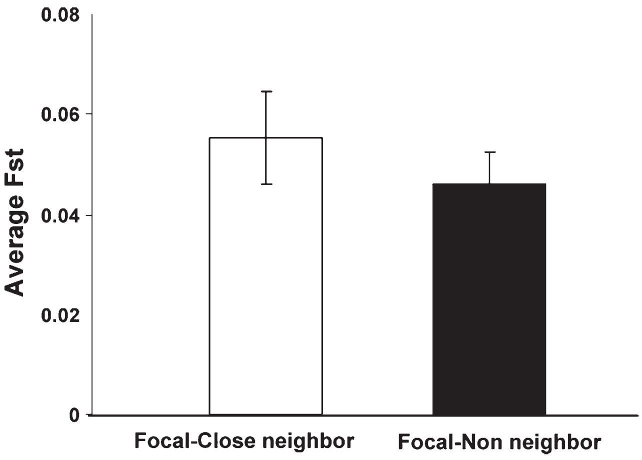

Figure 3
Average values of F_{st} with their standard errors. The white bar represented the average genetic divergence between the pair focal nest–close neighbor nest, and the black bar represented the average genetic divergence for the pair focal nest–nonneighbor nest (higher genetic divergence when $F_{st} = 1$, lower genetic divergence when $F_{st} = 0$).

aggressive behavior with individuals of neighboring colonies than toward individuals of nonneighbor colonies. However, we failed to detect evidence that genetic divergence among colonies explain this pattern, in spite of significant but moderate genetic divergence between focal and nearby colonies and between focal and nonneighbor ones as well.

The lack of a genetic underpinning to this pattern could be due to the low number of isozymes analyzed (4 loci) and the fact that these enzymes codified for basic physiology, not necessarily for the cuticular substances that give the odor involved in the mechanism of recognition to the colony. Basic enzymes, because of their importance, could have a low degree of variation because they are fixed in a conservative way by natural selection.

However, in other ant studies, the enzyme electrophoresis was successfully used to determine genetic relatedness and genetic divergence. For example, Mäki-Petäys et al. (2005) studied 2 wood ant species *Formica aquilonia* and *F. lugubris* using enzyme electrophoresis, and genetic differentiation was estimated by using F_{st}. Also, Eaton and Medel (1994) and Diehl et al. (2001) used the same techniques to analyze genetic relatedness in the ant species *Camponotus chilensis* and *A. heyeri* and *A. striatus*, respectively. Therefore, we consider our analyses, despite their limitations, an adequate measure of genetic divergence between colonies. Granted, there are other molecular methods (e.g., microsatellite and single nucleotide polymorphisms) that allow detection of genetic variation between 2 individuals to the level of base pairs; however, to date, there are no microsatellites developed for this ant species. Microsatellites have been developed for *A. echiniator* (Ortius-Lechner et al. 2000), but no information exists on their transferability to *A. lobicornis*. Future studies might use these molecular methods to better assess the genetic patterns in populations of *A. lobicornis*.

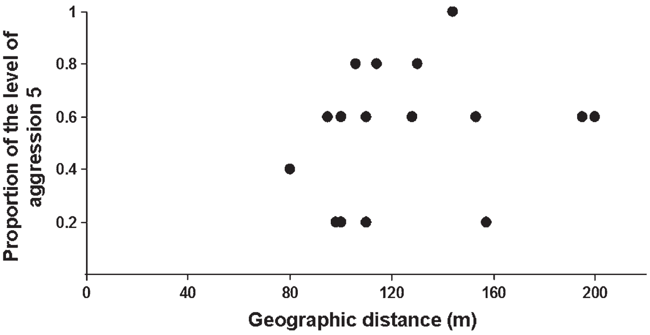

Figure 4
Proportion of the level of aggression 5 (higher aggressive behavior) found in the treatment focal nest–nonneighbor nest in relation to the geographical distance between the focal nests and their nonneighbor nests ($P = 0.46$, $R^2 = 0.0363$, $F_{1,15} = 0.57$).

The colonies of *A. lobicornis* studied here had recently colonized the area, and they are at the current edge of the species range (Farji-Brener 1996, 2000). Thus, the values of genetic divergence and genetic distance reported here might be consequence of a relatively recent founder effect. In particular, in natural populations with stepping-stone model of dispersion, divergence occurs gradually, and therefore, more generations are probably needed in order to detect the divergence measure by F_{st} (Efremov 2004). Thus, the colonies studied may not be genetically divergent enough at neutral traits. This could be tested comparing our behavioral results with behavioral studies in areas where *A. lobicornis* has been established for a longer time (e.g., center and north of Argentina).

Another possible explanation for the lack of greater differentiation in genetic divergence with nest distance is that sexual individuals may reach distances greater than 200 m. Therefore, the scale selected between nests may not be large enough to capture higher genetic divergence between focal and nonneighbor colonies. However, even if the scale was not proper to detect genetic differences between colonies, it was adequate to study variation in aggression between neighbors and nonneighbors, strongly suggesting that factors other than genetic divergence contribute to the DEP.

There is evidence that the sexual individuals of *Atta* (the other genus of leaf-cutter ants) can fly up to 12 km (Cherrett 1968). If this is so in *A. lobicornis*, we could be evaluating colonies that are closely related in the maximum range used in this work (200 m). Nonetheless, the results of the Mantel test suggest that the scale used in this study is correct for evaluating the DEP and that the populations studied have a significant but relatively low degree of genetic divergence and that insufficient time has elapsed to produce genetic divergence among nests.

The phenomenon of habituation is an alternative explanation for the variation in aggressive behavior between ants and distance (Orivel et al. 1997; Owen and Perrill 1998; Langen et al. 2000). If this is the case, foraging individuals of *A. lobicornis* could learn to recognize the odors of the foraging ants of neighboring colonies independent of the levels of genetic divergence. If this hypothesis is correct, we might expect aggressiveness to respond to neighbor status, but not necessarily the distance between the colonies. Our data are consistent with the habituation hypothesis: We failed to detect a relationship between the incidence of the highest level of aggressive behavior (and most frequent) and the distance between nonneighboring nests (Figure 4). All colonies of *A. lobicornis* in our study area encounter similar vegetation; thus, it is unlikely that dietary variation alone could generate enough odor diversity to account for colonies' distinct chemical signatures. However, chemicals studies on the cuticular composition of foraging individuals are required in order to discard this explanation. Though our data are consistent with the habituation hypothesis, we caution that these data are an indirect assessment.

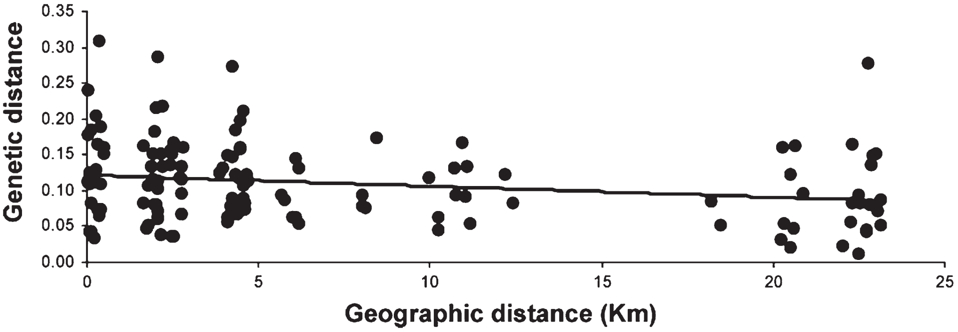

Figure 5
Mantel test between genetic distance (index of genetic distance of Rogers [Wright 1978]) and the geographic distance (in kilometers) of the 17 focal nests ($R^2 = 0.047$, $P = 0.05$).

In sum, we found evidence of the dear enemy hypothesis in the ant *A. lobicornis*, but variation in aggressive behavior was not associated with among-colony genetic divergence. Indirect evidence suggests that habituation might be the mechanism minimizing the aggression between neighboring colonies, but chemical assays are required to confirm this explanation. The habituation hypothesis also appears the most adaptive explanation, regardless of the genetic divergence between neighboring nests, as frequently aggressive interactions between neighbors would result in wasted energy.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/.

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