Insect. Soc. (2012) 59:241–250
DOI 10.1007/s00040-011-0211-9

Insectes Sociaux

RESEARCH ARTICLE

Colony spatial structure in polydomous ants: complimentary approaches reveal different patterns

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Received: 19 September 2011 / Revised: 14 November 2011 / Accepted: 21 November 2011 / Published online: 6 December 2011

Abstract Eusocial insects often live in colonies comprised of an extensive network of interconnected nests and estimating colony spatial structure and colony boundaries may be difficult, especially in cryptic, subterranean species. A combination of aggression assays and protein marking was used to estimate nest spatial distribution in field populations of the highly polydomous cornfield ant, *Lasius neoniger*. The estimates were first obtained via 1-on-1 aggression tests for workers collected from different nests within the research plots. The aggression tests were followed by mark-recapture field studies which utilized rabbit IgG protein. The ants were allowed to self-mark by feeding on sucrose solution spiked with the IgG protein. Colony spatial structure was detected by sampling ants from different nests and analyzing them for the presence of the marker using an ELISA test. Estimates based on aggression tests were substantially higher relative to those based on protein marking. The average colony size based on aggression tests was 2.0 ± 0.2 m^2 and was significantly higher than the 1.1 ± 0.4 m^2 estimate based on protein marking. The estimate based on protein marking was even lower, 0.2 ± 0.1 m^2, when a Fluon-coated ring restricted ant feeding to the focal nest and prevented ants from other nests from feeding on the protein-marked sucrose. No significant correlation was detected between internest aggression and internest distance. Likewise, no correlation was detected between distance from the focal nest and the percentage of workers testing positive for the protein marker. The results show that both approaches have their own limitations, but their simultaneous use allows for a more accurate assessment of colony spatial structure. The advantages and limitations of each technique are discussed.

Keywords Colony spatial structure · Cornfield ant · Immunomarking · *Lasius neoniger* · Protein marking · Trophallaxis

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Introduction

Social insects represent one of the pinnacles of organic evolution. The complexity of social insect colonies is manifested on many levels including division of labor, social communication, foraging behavior and specialization, and nesting architecture (Hölldobler and Wilson, 1990). In addition, social insects with flightless workers (i.e. ants and termites) often exhibit multinest colony structure known as polydomy which adds another level of complexity to their colonies. Ant colonies display enormous diversity in colony size and spatial organization and comprise a continuum ranging from colonies that occupy only one nest (monodomy) to colonies that occupy multiple nests (polydomy) (reviewed in Debout et al., 2007). The spatial complexity of ant colonies is most evident in large supercolonies that lack behavioral boundaries and are considered unicolonial (e.g. Passera, 1994; Holway et al., 1998; Giraud et al., 2000; Helanterä et al., 2009). While polydomy offers many ecological and evolutionary advantages (reviewed in Debout et al., 2007), the presence of spatially separate groups also creates multiple challenges for social insect colonies. These include the need to recognize individuals from distant nests (Vander Meer and Morel, 1998), possible queen–worker conflict over sex and resource allocation decisions (Snyder and Herbers, 1991; Backus, 1993; Herbers et al., 2001), increased predation as individuals move between the nests,

and the energetic cost of constructing additional nests (Debout et al., 2007). From a research perspective, polydomy makes it difficult to detect colony spatial structure. This difficulty is especially evident in large supercolonies which are typically assumed to operate as a single cooperative unit where food, workers, and brood, are freely exchanged among all nests. However, recent work demonstrates that large supercolonies are discontinuous with regard to resource flow and interactions among nests are spatially restricted (Buczkowski and Bennett, 2008; Heller et al., 2008).

The correct identification of colony boundaries is an essential prerequisite for empirical studies of social insect societies as numerous theories concerning social insect behavior and evolution are based on the concept of the colony (Debout et al., 2007). Yet, defining colony boundaries may be extremely difficult, largely due to the fact that ant colonies may be comprised of a large network of interconnected nests and many species are highly cryptic. For ants, there are various ways in which colony membership can be assessed. The most straightforward method is to simply observe worker movement along trails: two nests connected by a trail clearly belong to the same colony. However, not every nest is always connected to every other nest as colonies often form incomplete networks and observing worker movement along trails may be difficult for litter dwelling species or impossible for subterranean species. To aid in the identification of colony boundaries other techniques are often used in addition to direct observations. These include: nestmate recognition assays (i.e. aggression tests; reviewed in Breed, 2003; Roulston et al., 2003), genetic tests (e.g. Ross, 2001; Tsutsui and Case, 2001; Sundström et al., 2005), biochemical tests (i.e. analysis of cuticular hydrocarbon profiles; reviewed in Bradshaw and Howse, 1984; Howard and Blomquist, 2005; Hölldobler and Wilson, 2009), mark-and-recapture studies (e.g. Katzerke et al., 2006), and various tracking techniques such as dyes (reviewed in Hagler and Jackson, 2001) or radiolabels (Buffin et al., 2009). However, researchers rarely use multiple techniques simultaneously to verify the results of a single approach and the current study is the first attempt to examine the predictive power of various approaches.

The cornfield ant, *Lasius neoniger* (Emery) is the dominant open habitat species in the northeastern United States (Wilson, 1955). Despite its prominence and major ecological (Wang et al., 1995; Lopez and Potter, 2000) and economic (Vittum et al., 1999; Maier and Potter, 2005) importance, little is known about its colony spatial structure. Delimiting colony boundaries in *L. neoniger* is especially difficult for a number of reasons. First, colonies are highly polydomous and occur in dense populations (Traniello and Levings, 1986). Second, colonies are highly cryptic as nests are subterranean and connected via underground trails making direct observation of nest connectivity via worker movement impossible. Third, the ants mainly forage hypogaeically collecting honeydew from root Hemipterans and relatively little worker activity is observed above the ground. Finally, the spatial arrangement of nests may change seasonally and territorial expansion is based largely on seasonal food abundance (Traniello and Levings, 1986). The combination of these factors makes it difficult to delineate colony boundaries in *L. neoniger* and other ant species with similar nesting habits.

Here I use a combination two complimentary approaches, aggression assays and protein marking, to determine colony spatial structure in *Lasius neoniger*. Despite the fact that nestmate recognition system in *L. neoniger* is well-developed and workers defend both the nesting area and the foraging area (Levings and Traniello, 1981), recognition assays alone are not sufficient for estimating colony boundaries. This is because recognition assays sometimes produce erroneous results and lack of aggression does not always indicate same colony membership. The observed lack of aggression (which would tend to overestimate colony size) may be due to the lack of context among interacting individuals (Roulston et al., 2003; Buczkowski and Silverman, 2005), dear-enemy phenomenon (Heinze et al., 1996; Dimarco et al., 2010), or simply observer error and/or bias (Gamboa et al., 1991). To obtain a more accurate estimate of colony size and spatial structure, additional tools are needed. Marking insects is an effective tool especially when used as a mark-capture technique, whereby individuals are marked in the field, allowed to disperse, and are later collected and analyzed for the presence of the marker. To track the movement of the ants I used protein marking and double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA; Buczkowski and Bennett, 2006). Protein marking (a.k.a. immunomarking) has proven highly effective to study various aspects of ecology in a range of social insects including ants (Buczkowski and Bennett, 2006; Buczkowski and Bennett, 2007), honeybees (DeGrandi-Hoffman and Hagler, 2000), and termites (Buczkowski et al., 2007; Hagler et al., 2009) in field and laboratory settings. The main objective of this study was to evaluate the predictive ability of each approach to reveal colony spatial structure and intercolonial nest relationships in the highly cryptic and polydomous *L. neoniger*. Both approaches have their own limitations, but their simultaneous use may allow for a more accurate assessment of colony boundaries.

Methods

Biological model

The cornfield ant, *Lasius neoniger* is one of the most conspicuous and abundant mound-building ant species in North

America (Wilson, 1955). It thrives in open, sunny areas consisting of well-drained soils and low vegetative cover such as lawns, golf courses, and cultivated fields. Colonies are easily recognized by a crater-like ring of excavated soil around the nest entrance. Nests are shallow, interconnected chambers concentrated in the upper 30 cm of soil with occasional deeper vertical galleries (Wang et al., 1995). Colonies are monogynous (Wilson, 1955) Helanterä and occur in dense populations (Traniello and Levings, 1986). Workers generally tend subterranean Hemiptera for honeydew (Traniello, 1983), but are also known to be successful predators of pest insects in turf (Lopez and Potter, 2000).

Study sites and research plots

Suitable research sites were located by visual inspection of likely nesting areas. All research sites were in the vicinity of West Lafayette, Indiana, USA and included four areas: Squirrel Park (SP), Horticulture Park (HP), Lindberg Village (LV), and Ackerman Hills Golf Course (AH). All sites were low-maintenance lawns or naturalized golf course roughs and were similar in the degree of anthropogenic influence. All sites: received periodic mowing to approximately 5–8 cm in height, received annual N-fertilizer and selective herbicides, had similar soil texture best classified as silty clay loam, were covered by a mixture of cool-season perennial and annual grasses, had no evidence of other ants nesting in the plots, and were medium to high use areas. Four 4 m × 4 m research plots were selected at each of the four sites. To select the plots, the ground was inspected for the presence of soil craters (piles of excavated soil) and the largest and most active crater constituted the center of each plot. The corners of each plot were marked with wooden stakes driven into the ground and replicate plots within a study site were separated by at least 15 m. The number of soil craters visible on the surface of each plot was counted and the craters were categorized as either active or inactive. Active craters were those that were being actively maintained by the ants, had visible ant traffic, and a clear, unobstructed opening. Inactive craters were no longer maintained, had a weathered appearance, no ant activity, and a blocked opening. It is unclear whether each crater is an entrance to a single nest, multiple nests, or whether multiple craters lead to a single nest. Therefore, the number of craters is not necessarily indicative of the number of subterranean nests. Herein, craters will be referred to as nests. Due to extremely high nest density within the plots it was impractical to utilize all nests. Ten active nests were selected for further consideration: the focal nest in the center of the plot (labeled nest 1) and 9 additional nests surrounding the focal nest. A combination of distance from the focal nest and preliminary aggression tests toward the focal nest were used to select the surrounding nests. The goal was to achieve a combination of aggressive/non-aggressive nests at various distances from the focal nest. For each plot 3 categories of nests were selected: 3 nests non-aggressive toward the focal nest and in proximity to the focal nest (nests 2–4), 3 nests non-aggressive toward the focal nest and further away from the focal nest (nests 5–7), and 3 nests on the periphery of the plot and aggressive toward the focal nest (nests 8–10). The preliminary aggression tests utilized a 1–4 aggression scale (see "Nestmate recognition assays" below for details) and nest pairings exhibiting non-injurious aggression (score of 2 or below) were considered non-aggressive whereas nest pairings exhibiting injurious aggression (score of 3 and above) were considered aggressive. The distance between the focal nest and each of the surrounding nests was recorded in centimeters. All 10 nests were marked with numbered flags for future identification. Two approaches, described in more detail below, were used to examine the spatial distribution of nests within *L. neoniger* colonies: nestmate recognition assays and protein marking.

Nestmate recognition assays

The spatial pattern of nest distribution and colony range were first examined via nestmate recognition assays. The level of aggression between ants from different nests was tested by using worker dyad interactions within a neutral arena (Buczkowski and Bennett, 2008). Within each plot, aggression was examined in pairwise tests between ants from the focal nest (nest 1) and every other nest (nests 2–10). Three replications were performed for each pairing for a total of 27 aggression tests per plot. Workers were selected at random as they exited nest openings, were allowed to walk onto a toothpick, and were placed sequentially into a glass vial (2-dram). The top half of the vial was coated with Fluon™ to prevent the ants from escaping and to restrict the ants to a small area to maximize the chance of encounters. Ant interactions were scored on a 1–4 scale [Suarez et al., 2002; 1 = ignore, 2 = avoid, 3 = aggression (lunging, brief bouts of biting and/or pulling), 4 = fighting (prolonged aggression, also abdomen curling to deposit defensive compounds)]. In each replicate the ants were allowed up to 25 encounters, each instance of direct physical contact between the ants was regarded as an encounter. For each replicate, the maximum score of 25 encounters was used in data analysis (Roulston et al., 2003). In all assays, individual ants were not tested in more than one trial. For each plot, the location of all ten nests was mapped with a tape measure and coordinates were determined to the nearest cm. The nests were plotted onto a grid and a polygon encompassing all nests that were non-aggressive was drawn. Subsequently, the area of the polygon was calculated as an estimate of the colony's size.

Protein marking

In addition to nestmate recognition assays, protein marking was used as a comparative approach to examine nest dispersion in *L. neoniger*. The goal was to compare the predictive ability of these two different approaches for revealing colony size and spatial organization. Protein marking was utilized as a mark-recapture technique whereby the workers were provided with a food source containing the marker and were later sampled. Two types of tests were performed. In the first series of tests, only the focal nest received the food containing the protein marker and ants from all other nests were prevented from having direct access to the food. Thus, the only way for the marker to show up in nests other than the baited focal nest was for the ants to distribute the maker below the ground, thus revealing the intricacies of the tunnel system, nest connectivity, and the overall colony spatial structure. A plastic, Fluon^{TM}-coated ring (9 cm diameter × 5 cm high) was placed around the focal nest and the bottom 1–2 cm of the ring was pressed into the soil to prevent the ants from escaping under the ring. The ring was coated with Fluon^{TM} on both sides and prevented protein-marked ants from migrating to other nests directly (i.e. above the ground) and also prevented ants from unprovisioned nests from directly accessing the food. A vial containing 2 mL of 30% sucrose solution containing technical grade rabbit immunoglobin (IgG) protein (Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 0.5 mg IgG/mL sucrose was placed inside the ring, next to the nest opening. This concentration was selected based on the results of previous studies that revealed that the increases in optical density were minimal above 0.5 mg/mL (Buczkowski and Bennett, 2006). The ants were allowed to feed on the sucrose solution for 3 h. To estimate the amount of protein marker present within the different nests we randomly sampled 10 workers from all 10 nests within each plot 24 h after feeding. The 10 nests were the same as used in the aggression assays. This was repeated for all plots within each research site and across all research sites. All individuals were frozen in individual tubes at −20°C and later analyzed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA, see below) using previously described methodology (Buczkowski and Bennett, 2006; 2007). In a second series of tests, the dispersal of the protein marker was investigated across the same colonies while allowing open access to the food. The test was repeated exactly as above, except that no ring was provided thus allowing ants from multiple nests (or colonies) to feed simultaneously. The test was performed 7 days after the first test to assure that the marker was completely purged from the colonies (i.e. digested, excreted, or diluted beyond detection). The period of 7 days was selected based on the results from previous field tests (Buczkowski and Bennett, 2006) and was additionally confirmed by performing ELISA analysis on workers randomly selected from several test plots. Once again, the location of all ten nests was mapped within each plot using a tape measure and coordinates were determined to the nearest centimeters. The nests were plotted onto a grid and a polygon encompassing all nests that contained at least one worker testing positive for the protein marker was drawn. Subsequently, the area of the polygon was calculated as an estimate of the colony's size.

The ELISA procedure

Sandwich ELISA was performed on individual ant samples using previously described techniques (Hagler, 1997; Buczkowski and Bennett, 2007). Briefly, ant samples were individually homogenized in 150 µL phosphate buffered saline (pH = 7.4) and assayed for the presence of the rabbit immunoglobin protein. Each well of a 96-well microplate was coated with 100 µL of anti-rabbit IgG (developed in goat) (Sigma Chemical Co., St. Louis, MO) diluted 1:500 in distilled water and incubated for 2 h at 4°C. After incubation, the primary antibody was discarded and 310 µL of 1% non-fat dry milk (Bio-Rad Laboratories, Hercules, CA) in distilled water was added to each well to block any remaining non-specific binding sites. After 30 min incubation at 26°C the milk was discarded. Ant samples were vortexed, added to each well, and incubated for 1 h at 26°C. The samples were then discarded and each well was washed 3 times with PBS Tween 20 (0.05 %) and 2 times with PBS. Anti-rabbit IgG conjugated to horseradish peroxidase (50 µL) diluted 1:1,000 in 1% non-fat milk was added to each well and incubated at 26°C for 1 h. All wells were washed again as above and 50 µL of TMB HRP substrate (BioFX Laboratories, Owings Mills, MD) was added to each well and incubated for 30 min. Samples were analyzed on a Beckman Coulter AD 340 Absorbance Detector set at 620 nm. Six negative controls (ants never exposed to food containing rabbit IgG) and 6 blanks (PBS buffer only) were run on each plate.

Statistical analysis

The mean (±SE) optical density value and the percentage of samples scoring positive for rabbit immunoglobin protein were determined for all nests and plots. The samples were scored positive for the presence of the protein marker if the ELISA optical density value exceeded the mean negative control value by three standard deviations (Sutula et al., 1986; Buczkowski and Bennett, 2006). The percentage of samples testing positive for the protein was tabulated by first calculating the percent of individuals testing positive within a replicate and then averaging across the four replicates. An

ANOVA test was used to test for differences in total nest number, percent active nests, and nest density among the sites. This was accomplished by using the PROC GLM procedure in SAS 9.2 (SAS, 2008), followed by post hoc Tukey's HSD tests to separate the means by site. An ANOVA test was also used to test whether the estimates of colony size varied among the different tests. The testing assay was set as a fixed factor (categorical variable under the *class* statement) and colony size was set as the response variable. This was accomplished by using the PROC UNIVARIATE procedure on square root transformed data followed with a Bonferroni correction. The correlation between internest aggression and internest distance and between distance from the focal nest and the percentage of workers testing positive for the protein marker was examined using the PROC GLIMMIX procedure. The observational error for all tests was assumed to follow a normal distribution and the normality distribution of residuals was tested using the UNIVARIATE procedure. The level of significance for all tests was set at $\alpha = 0.05$.

Results

Lasius neoniger occurs in dense populations and each population is comprised of distinct colonies with one or more nest entrances (Table 1). A total of 1,126 nests were detected in all study plots. The average number of nests per plot did not vary significantly among research plots (ANOVA, $df = 3$, $F = 1.72$, $P = 0.216$) and ranged from 79.5 ± 7.5 nests in Horticulture Park to 60.3 ± 8.1 nests in Lindberg Village, with an average of 70.4 ± 7.7 nests across all plots. Likewise, nest density (the number of nests per 1 m^2) averaged 4.4 ± 0.5 nests per m^2, ranged from 5.5 ± 0.5 nests in Horticulture Park to 3.8 ± 0.5 nests in Lindberg Village, and did not vary significantly among the plots (ANOVA, $df = 3$, $F = 1.69$, $P = 0.221$). Of the 1,126 nests mapped in this study, 287 (26%) were active. The percentage of active nests averaged $26.0 \pm 3.1\%$, ranged from 22% in Squirrel Park to 33% in Ackerman Hills, and did not vary significantly among research sites (ANOVA, $df = 3$, $F = 2.93$, $P = 0.077$), although minor differences did exist.

Estimates of colony size and boundaries varied widely depending on the technique used (Table 2; Fig. 1). In general, colony sizes revealed by aggression tests were significantly larger relative to those based on protein marking (ANOVA, $df = 2$, $F = 33.3$, $P < 0.0001$). The average colony size based on aggression tests was 2.0 ± 0.2 m^2, ranged from 1.7 ± 0.2 m^2 at Ackerman Hills to 2.7 ± 0.3 m^2 at Horticulture Park, and varied significantly among sites (ANOVA, $df = 3$, $F = 5.72$, $P = 0.011$). These values are in direct agreement with those reported by Traniello and Levings (1986) who used aggression tests to

Table 1 Nesting activity of *Lasius neoniger* at the four study areas

Study area	No. active nests	No. inactive nests	Total nests	Percent active nest	Nest density	Distance from focal nest
Squirrel Park	16.5 ± 2.1	62.3 ± 7.2	78.8 ± 6.1 a	21.5 ± 3.3 a	4.9 ± 4.4 a	133 ± 7 a
Horticulture Park	17.8 ± 2.8	61.8 ± 6.7	79.5 ± 7.5 a	22.5 ± 3.0 a	5.5 ± 0.5 a	127 ± 5 a
Lindberg Village	16.5 ± 3.6	43.8 ± 5.1	60.3 ± 8.1 a	26.9 ± 2.7 a	3.8 ± 0.5 a	128 ± 6 a
Ackerman Hills	21.0 ± 4.1	42.0 ± 5.6	63.0 ± 9.1 a	32.9 ± 3.3 a	3.9 ± 0.6 a	127 ± 4 a
Average all areas	17.9 ± 3.2	52.4 ± 6.2	70.4 ± 7.7	26.0 ± 3.1	4.4 ± 0.5	128 ± 3

Values reported for each area (mean ± SEM) are an average of four 16 m^2 research plots. Nest density is per square meter and is based on the total number of nests. Distance from focal nest is the distance in centimeters from the focal nest (center of plot) to every other nest averaged across all plots within a study site. Numbers within columns followed by the same letter are not different based on a Tukey test ($P = 0.05$)

Table 2 Colony size as revealed by aggression assays and protein marking

Study area	Aggression assay	Protein marking, ring present	Protein marking, ring absent
Squirrel Park	1.76 ± 0.10 a, a	0.21 ± 0.08 a, b	1.18 ± 0.43 a, a
Horticulture Park	2.74 ± 0.34 a, a	0.21 ± 0.06 a, c	0.83 ± 0.13 a, b
Lindberg Village	1.78 ± 0.05 a, a	0.18 ± 0.12 a, b	1.66 ± 0.73 a, a
Ackerman Hills	1.73 ± 0.20 a, a	0.23 ± 0.10 a, c	0.71 ± 0.29 a, b
Average all areas	2.00 ± 0.17	0.21 ± 0.09	1.09 ± 0.39

Values reported for each area (mean ± SEM) are in square meters and represent an average of four 16 m^2 research plots. Negative values represent decreases in colony size. First letter indicates within column comparisons, second within row comparisons and numbers followed by the same letter are not different based on a Tukey test ($P \leq 0.05$)

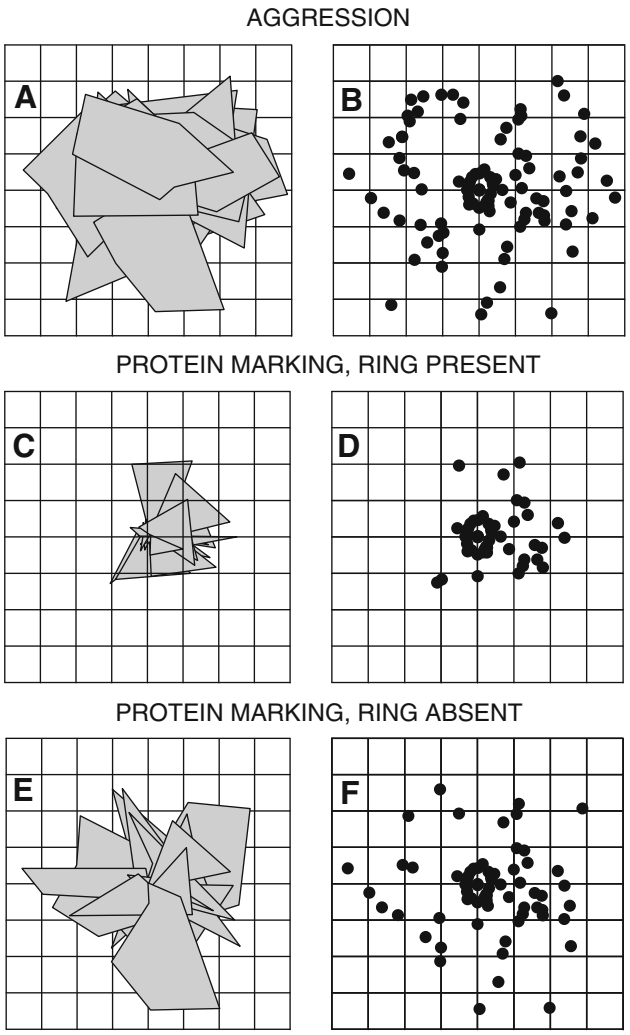

Fig. 1 Spatial distribution of *L. neoniger* nests in 16 m^2 plots as revealed by aggression tests (**a**–**b**) and protein marking (**c**–**f**). Each *chart* is an overlay of all plots from all study sites (total of 16 plots). The *polygons* are estimates of colony size formed by connecting nests that showed no mutual aggression or contained workers testing positive for the protein marker. The *dot graphs* are estimates of the colony size and represent nests that were non-aggressive toward the focal (*center*) nest or contained workers testing positive for the marker provided in the focal nest

estimate colony size in *L. neoniger*. Interestingly, the site with the highest nest density (Horticulture Park) was also the site where the colonies occupied the largest area. Estimates of colony size based on aggression tests were 89% higher that those based on protein marking with the ring present and 43% higher than those based on protein marking with the ring absent.

Protein marking with the ring present was the most restrictive test and produced the smallest estimates of colony size. The average colony size was 0.2 ± 0.1 m^2 and did not vary significantly among sites (ANOVA, $df = 3$, $F = 0.06$, $P = 0.981$). Estimates of colony size based on protein marking with the ring present were significantly

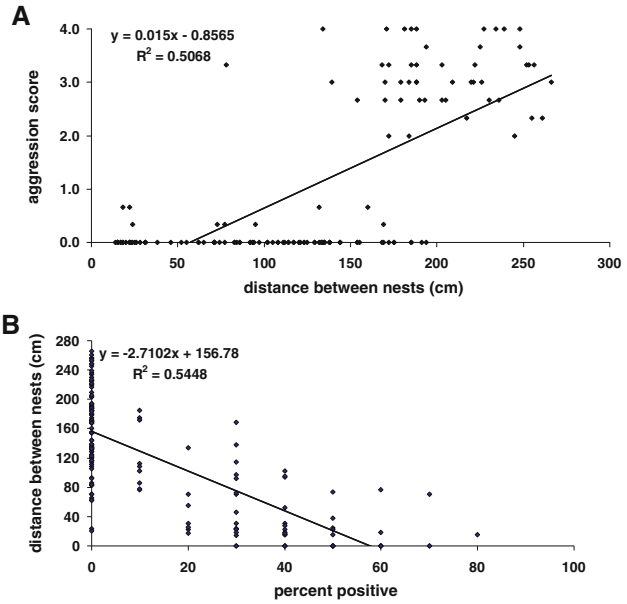

Fig. 2 The relationship between **a** internest aggression and internest distance ($P = 0.916$), **b** internest distance and the percentage of workers testing positive for the protein marker ($P = 0.732$)

lower relative to those based on aggression tests (ANOVA, $df = 1$, $F = 23.5$, $P = 0.012$). When the ring was absent, the average colony size was 1.1 ± 0.4 m^2 and did not vary significantly among sites (ANOVA, $df = 3$, $F = 0.89$, $P = 0.474$). Estimates based on protein marking with the ring absent were approximately 43% lower relative to those based on aggression tests (ANOVA, $df = 1$, $F = 45.1$, $P < 0.0001$) and 70% higher than those based on protein marking with the ring present (ANOVA, $df = 1$, $F = 45.1$, $P < 0.0001$).

No significant correlation was detected between internest aggression and internest distance (Fig. 2; ANOVA, $df = 1$, $F = 0.01$, $P = 0.916$) even though aggression is generally expected to increase as the distance from the focal nests increases. Likewise, no correlation was detected between distance from the focal nest and the percentage of workers testing positive for the protein marker (Fig. 2; ANOVA, $df = 1$, $F = 0.15$, $P = 0.732$).

Discussion

The results reported here highlight the difficulties in estimating colony spatial structure in natural populations of polydomous ants. This is especially true for cryptic hypogaeic species where little above-ground foraging takes place. The two approaches used in this study, nestmate recognition assays (aggression tests) and protein marking, produced vastly different results, raising a question as to which approach is more accurate. Aggression tests are often

used to estimate colony boundaries in populations of ants (e.g. Breed, 2003; Roulston et al., 2003; van Wilgenburg and Elgar, 2007). Yet, aggression tests are not a perfect tool and have the propensity to overestimate colony boundaries. This is because aggression tests often fail to detect aggression. The observed lack of aggression may be due to the lack of context when individuals from mutually aggressive colonies are placed in artificial test settings (e.g. glass vials) and fail to display aggression when they interact in the absence of social and/or ecological cues (Roulston et al., 2003; Buczkowski and Silverman, 2005). Lack of observed aggression may also be due to the dear-enemy phenomenon whereby individuals respond less aggressively to neighbors relative to non-neighbors to minimize the cost of fights (Heinze et al., 1996; Dimarco et al., 2010). Finally, observer error and/or bias (Gamboa et al., 1991) may also result in lower estimates of aggression and thus higher estimates of colony's territory. In the current study, estimates of colony size based on aggression testing were approximately tenfold higher than protein marking with the ring present and twofold higher than protein marking with the ring absent.

It is not clear whether aggression testing overestimated colony size, but some evidence suggests that the estimates may indeed be accurate. First, previous studies demonstrate that *L. neoniger* has a well-developed nestmate recognition system whereby the workers defend both the food resources and the crater area and respond with high aggression to alien conspecifics (Levings and Traniello, 1981; Traniello and Levings, 1986). High intercolony aggression would have facilitated the likelihood of detecting aggression in glass vials, even if some of the context had been absent. Second, allowing the ants 25 encounters and using the maximum score of 25 encounters in data analysis would have increased the likelihood of detecting aggression (Roulston et al., 2003). Colony sizes observed in this study are well within the limits observed by other researchers who also determined colony membership by aggression assays (Traniello, 1983; Traniello and Levings, 1986). However, if aggression assays in general have a tendency to be biased then the similarity of the current results to previously published results says nothing about the accuracy of the aggression tests.

Estimates of colony size and spatial nest distribution produced by protein marking were significantly lower than those produced by aggression tests, especially when the ring was present. Protein marking with the ring present was designed to examine food distribution from the focal nests to satellite nests via the subterranean trail network that might be connecting the nest chambers. The advantage of this approach is that the ring prevents ants from neighboring competitive colonies from having access to the food and therefore guarantees that individuals testing positive for the marker are indeed from the same colony. Another advantage is that the presence of the ring has the potential to reveal the subterranean nest connectivity as food is shared via underground tunnels. Nest connectivity is otherwise impossible to detect simply by observing worker movement above the ground and requires more elaborate techniques such as total colony excavation following nest casting (e.g. Mikheyev and Tschinkel, 2004; Tschinkel, 2004; Hölldobler and Wilson, 2009). Despite the advantages, including the ring has the potential to exclude nests that belong to the colony, but may not be directly connected to the focal nests via underground tunnels.

Another disadvantage is that food distribution in the colony may be incomplete and workers in the more distant nests may receive less food relative to workers in the focal nest. Although no relationship was detected between distance from the focal nest (food source) and the percentage of workers testing positive for the protein marker, other studies show that food distribution generally decreases as distance from the food source increases (Vega and Rust, 2003; Buczkowski and Bennett, 2006; Buczkowski and Van-Weelden, 2010). Protein marking with the ring absent was less restrictive and allowed open access to the food by ants from multiple nests and/or colonies. The estimate of colony size produced by protein marking with the ring absent was on average 70% higher than the estimate produced by protein marking with the ring present. This demonstrates that excluding the ring is indeed a less restrictive test and one that may more accurately estimate the actual colony size. However, the potential disadvantage of excluding the ring is that colony size may be underestimated if ants from some nests do not find the food or choose not to feed. Indeed, previous studies in *L. neoniger* suggest that foragers show strong fidelity to a single crater and move rarely between subterranean chambers associated with craters. Traniello and Levings (1986) examined nest fidelity in field populations of *L. neoniger* by marking foragers from individual nest craters with different color paints and releasing them back into the nests. Results showed that 90–95% of the marked ants continued to forage on the crater where they were marked. This suggests that foragers have strong fidelity to a single nest and specialize in foraging within a particular area, possibly to reduce search time by having detailed information about their immediate nest environment. Another potential disadvantage of excluding the ring is that estimates of colony size may be erroneous if the defense of colony boundaries and/or food resources is weak and ants from different colonies gain access to the same food source. In the current study, no fighting was ever observed at the baits, suggesting that recruitment was by ants from a single colony.

No significant correlation was detected between internest aggression and internest distance. According to theoretical predictions, aggression is expected to increase as distance

between nests increases because dispersal in natural populations is usually localized and the relatedness between two individuals taken from different groups typically decreases as the distance between the groups increases (Lehmann and Rousset, 2010). However, in this study, the great majority of nests in the research plots belonged to the same colony (centered on the focal nest) and most of the nests showed no aggression regardless of the distance from the focal nest. Figure 2a demonstrates that aggression in the test plots was either low (score of 0) or high (score of 4) and very few values were recorded in the middle of the 0–4 aggression scale. This emphasizes the fact that nestmate recognition is *L. neoniger* is well-developed and the workers aggressively attack alien conspecifics (Levings and Traniello, 1981; Traniello and Levings, 1986). It appears that the switch from no aggression to high aggression occurred at the distance of approximately 175 cm from the focal nest, although the significance of 175 cm is unknown. Another possible reason for the lack of significant association between aggression and distance is the fact that territoriality in *L. neoniger* has both a spatial and a temporal component (Traniello and Levings, 1986). Furthermore, it is important to remember that the relationship between distance and aggression is based on a subset of nests that were chosen based on aggression and distance. A different relationship might have been observed if the nest pairings were chosen randomly. Likewise, no correlation was detected between distance from the focal nest and the percentage of workers testing positive for the protein marker (Fig. 2; ANOVA, $df = 1, F = 0.15, P = 0.732$). Generally, the percentage of workers testing positive is expected to decrease with increasing distance from the source of the protein marker (focal nest) as has been demonstrated in other protein marking studies in ants (Buczkowski and Bennett, 2006; Buczkowski and VanWeelden, 2010). Lack of detectable relationship in this study may be due to numerous experimental factors such as marker dilution via trophallaxis or specific sampling methodology (3 h feeding time, 24 h to sample collection, 10 workers per nest). Similar results were observed by Chapuisat et al. (2005) who found that neither worker aggression nor trophallaxis rates were significantly associated with geographic distance between nests in a unicolonial population of *Formica paralugubris*.

In summary, aggression testing and protein marking are two complimentary approaches that may be used to detect colony spatial structure in field populations of polydomous ants. Both techniques appear suitable, but each has limitations that need to be considered when interpreting the results of experimental studies. It appears that well-replicated, properly conducted, and properly analyzed aggression tests (Roulston et al., 2003) followed by careful observations of worker movement may be a more accurate method of detecting colony spatial structure in ants, especially cryptic subterranean species. While protein marking has been successfully used to track colony structure in other cryptic insects such as subterranean termites (e.g. Buczkowski et al., 2007; Hagler et al., 2009; Baker et al., 2010) it may be more suitable for detecting colony structure in highly active epigaeic species where above-ground trails facilitate the detection and tracking of movement patterns. Indeed, previous laboratory and field studies show that protein marking is an excellent tool to investigate worker movement and colony spatial structure in epigaeic ants such as *Tapinoma sessile* which have well-established and highly active above-ground trails that facilitate the distribution and detection of workers carrying protein-marked sugar water (Buczkowski and Bennett, 2006, 2007). Another important aspect to consider is that the results of the different tests will depend on the specific methods used to collect the data. For example, the results of aggression assays may be affected by the specific assay used to detect aggression or the way the results are analyzed (Roulston et al., 2003). Similarly, the results of protein marking assays may be affected by numerous factors such as marker concentration, the number of workers sampled for ELISA, and the sampling intervals (Sutula et al., 1986; Buczkowski and Bennett, 2006; Hagler et al., 2009; Buczkowski and VanWeelden, 2010). Therefore, the conclusion that aggression tests produce higher estimates of colony size relative to protein marking is not a general one, and may depend on the specific methods. Simultaneous use of multiple experimental and analytical approaches including aggression testing, protein marking and a variety of other tools including genetic and biochemical tests will allow for the most accurate assessment of colony spatial structure.

Acknowledgments I thank X. Chen for advice on data analysis, M. VanWeelden, C. Bacon, and M. Nour for technical assistance, the staff of Birck Boilermaker Golf Complex for allowing access to research sites, and D. Richmond, I. Kaplan and two anonymous reviewers for comments on the manuscript. This study was supported in part by the Industrial Affiliates Program at Purdue University.

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