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Research article

Nestmate discrimination in ants: effect of bioassay on aggressive behavior

T. H. Roulston *, G. Buczkowski and J. Silverman

Department of Entomology, North Carolina State University, Raleigh, North Carolina 27695-7613, USA
** Current address: University of Virginia, Blandy Experimental Farm, 400 Blandy Farm Lane, Boyce, Virginia 22620, USA, e-mail: thr8z@virginia.edu*

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Summary. 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.

Key words: Aggression assay, Formicidae, nestmate recognition, Argentine ants, *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; 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.

Methods

Ants (study species)

We used five colonies of Argentine ants (*Linepithema humile*) collected from four locations in the southeastern USA (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 USA exhibit strong intercolonial aggression (see below).

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 (Fénéron, 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 ant 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)

Colonies were maintained in soil-free, fluon-coated trays that ranged in size from 24 × 38 cm to 30 × 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 "Effect 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

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

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 "intruder" workers were allowed to walk onto a brush and were then introduced into rearing trays containing "resident" 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 "Effect 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, USA) 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 × 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 "fight" 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).

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.

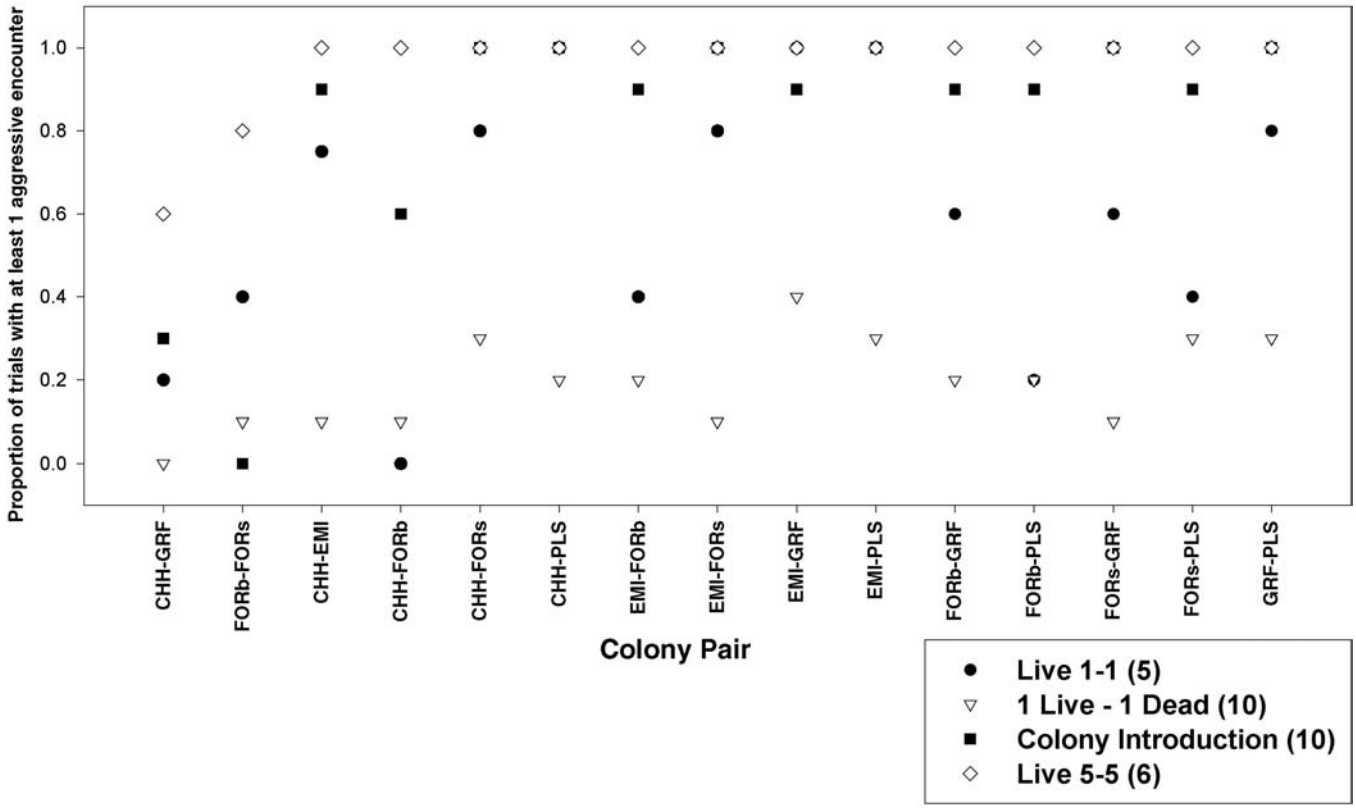

Figure 1. Proportion of trials with at least one aggressive encounter observed for each of four aggression assays. Number of trials in parentheses of legend. Pairs sorted by increasing aggression level

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 average score of all scan samples during a trial and the adjusted score weighted by time was 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

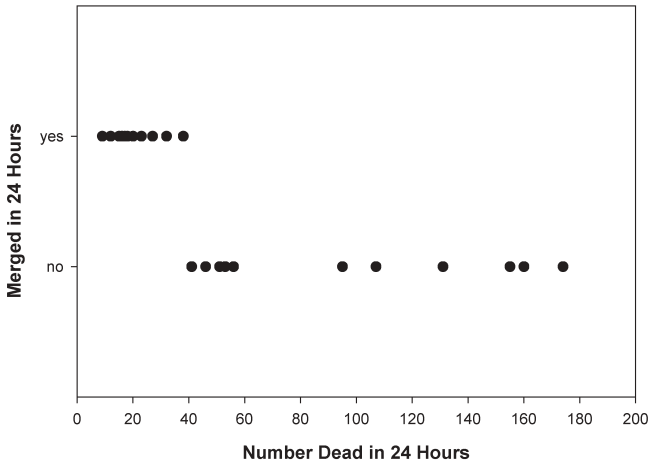

Figure 2. Relationship between number of ants killed in first 24 hours of contact and whether or not colonies merged within 24 hours

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.

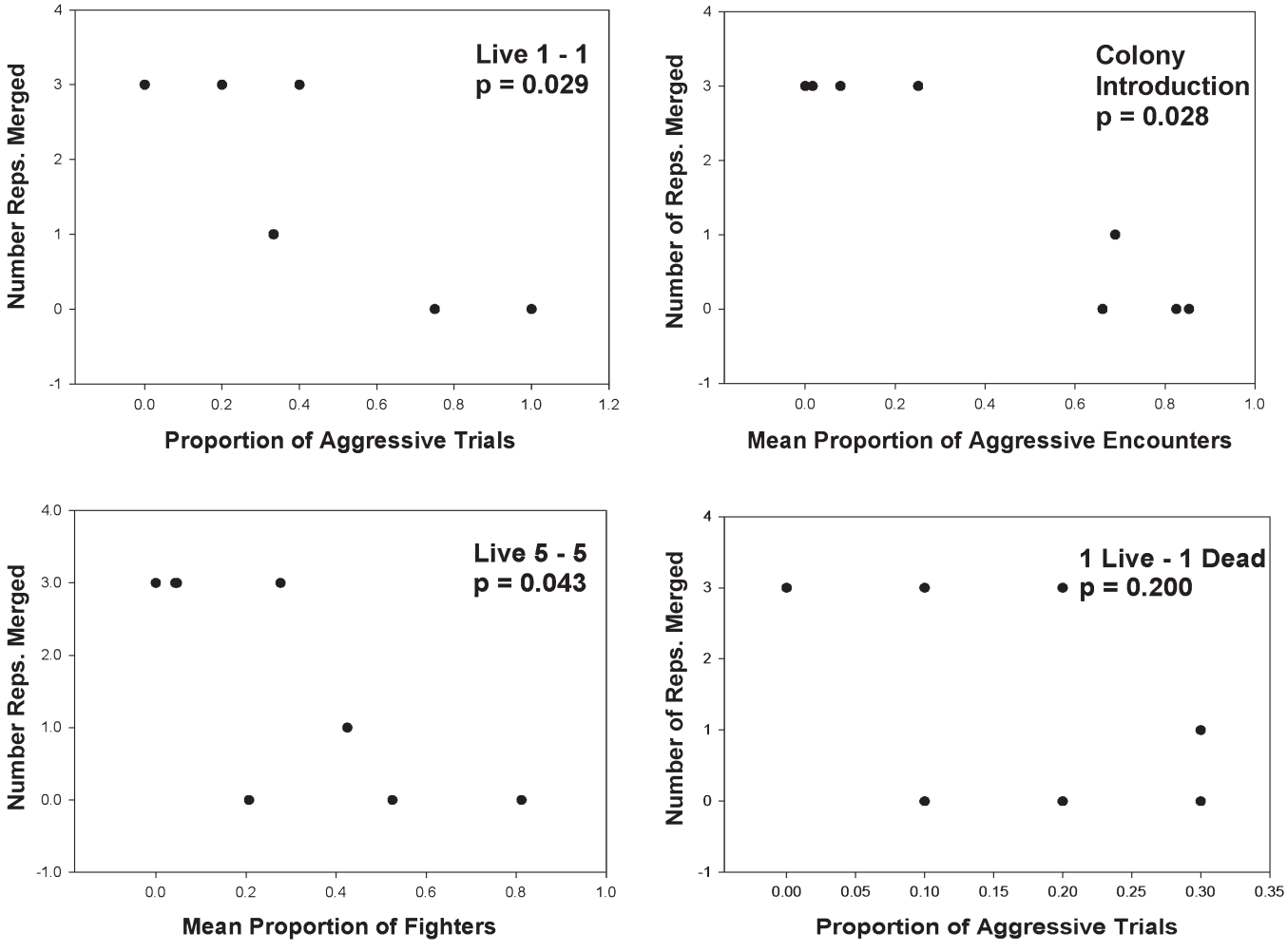

Figure 3. Number of trials (out of 3) that colony pairs merged plotted against the mean aggression score of each colony pair. Analyzed statistically using binary logistic regression. All proportions transformed using the arcsin

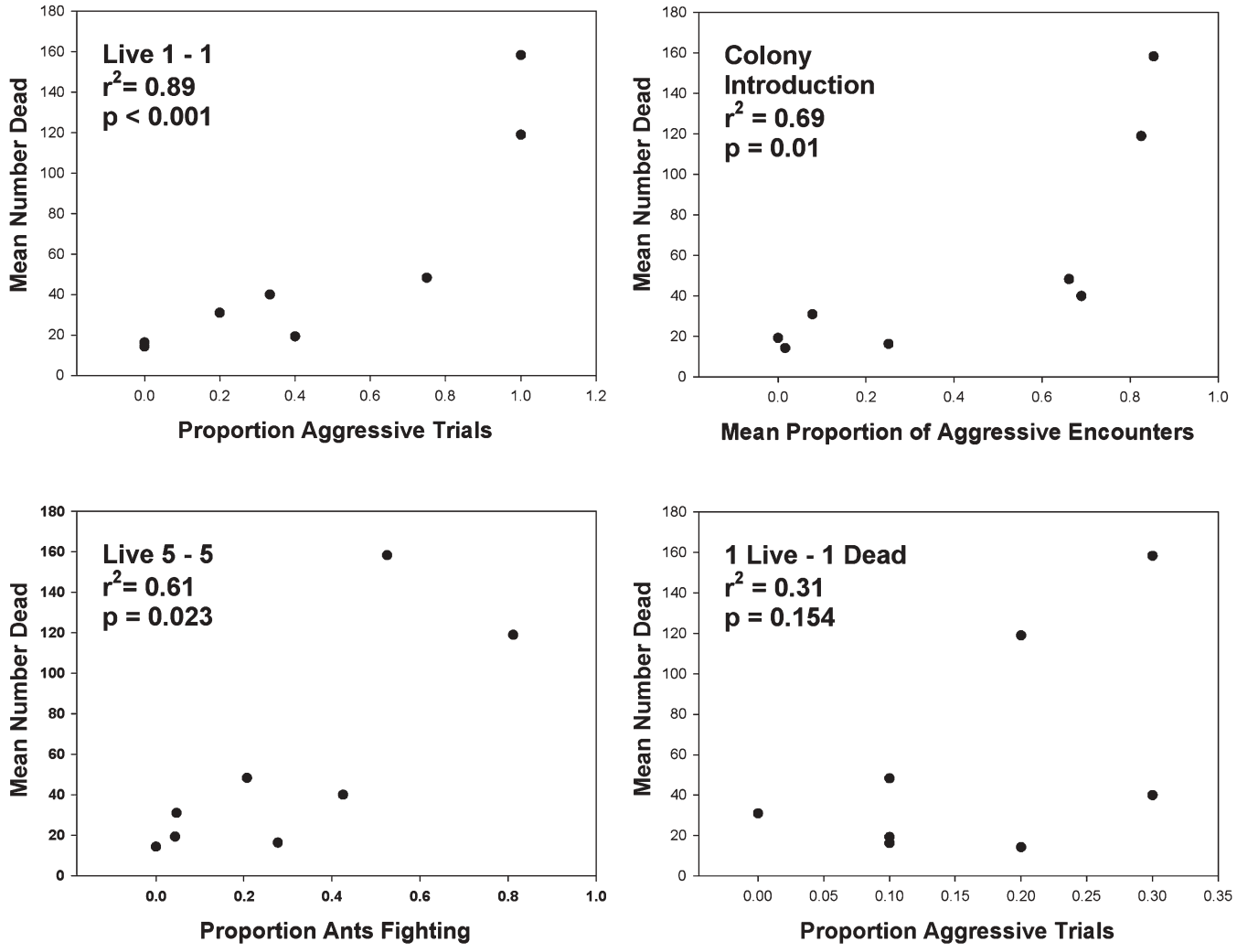

Figure 4. Mean number of ants killed during the first 24 h after colony interaction plotted against the mean aggression score of each colony pair

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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