Agonistic Behavior Between Individual Worker Termites from Three Cuticular Hydrocarbon Phenotypes of *Reticulitermes* (Isoptera: Rhinotermitidae) from Northern California

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ABSTRACT Bioassays examining aggression in termites have typically been performed by pairing groups of workers from different colonies. We examined whether similar results would be observed using bioassays with individual workers. We report the results of pairings of individual workers of *Reticulitermes* spp. of three different hydrocarbon phenotypes (CA-A, CA-B, and CA-C). Resulting agonistic behavior between individuals can be used to infer relatedness of workers from different foraging groups because identification of species of *Reticulitermes* is often difficult. Pairings consisted of two termites from the same colony or from different colonies with the same or different phenotypes. We recorded avoidance behavior and immediate aggression during 5-min observations and mortality at 24 h. Intercolonial/intraphenotype pairings paralleled group bioassays with low levels of immediate aggression; however, individual pairings had lower levels of mortality at 24 h. Bioassays using individual *Reticulitermes* spp. workers in interphenotype pairings yielded results comparable with previously reported group bioassays and could be used in lieu of group bioassays. However, if immediate aggression is lacking, it would be advisable to use group bioassays or increase the number of replications to differentiate intracolonial and intercolonial pairings. Survival rates were the same for all phenotypes when paired with either of the other two phenotypes. There were, however, significant differences in the number of avoidance responses; pairings with CA-C had the highest number of avoidance responses followed by pairings with CA-B and CA-A. There were significant differences in immediate aggression; CA-C was the most aggressive, followed by CA-B and CA-A. Different levels of aggression among phenotypes provide additional evidence that these phenotypes represent distinct taxa or species.

KEY WORDS aggression, competition, fighting, kin recognition, subterranean termites

STUDIES OF AGONISM BETWEEN termites increase our understanding of taxonomy, foraging biology, recognition cues, and communication abilities among termites. Agonism includes behaviors such as fighting, fleeing, or submitting that occur between termites from two different colonies of the same or different species (Haverty and Thorne 1989). Responses can vary depending on the individuals, colonies, and species involved. Variables such as genetic-relatedness, colony size, health and density, nutrient availability, and other environmental constraints may influence the expression of agonism (Su and Haverty 1991, Thorne and Haverty 1991, Grace 1996, Shelton and Grace 1996, 1997). Assessments of agonistic behavior between individuals (Nel 1968) or groups (Binder 1988, Jones 1990, Getty et al. 2000a, b) have been used to determine foraging territories and colony affiliation of termites (reviewed in Thorne and Haverty1991).

Ecological, behavioral, and genetic studies of *Reticulitermes*, a genus of economically and ecologically important subterranean termites commonly found in North America, are hindered by the woeful state of the taxonomy of this genus. To estimate colony size and extent of foraging territories (Haverty et al. 2000), we need to know whether adjacent foraging groups are of different species (obviously not the same colony) or the same species (may or may not be from the same colony). If the foraging groups are the same species, then mark-release-recapture (Haverty et al. 2000 and references within) or agonistic behavior (Haverty 1999a) bioassays need to be performed to be certain of colony membership. Large areas have the potential for numerous colonies to be present with contiguous or overlapping foraging territories. Because the number of suitable dyes available is limited (Su et al. 1991), agonistic behavior bioassays can be a useful method

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for delineating colony foraging territories and affiliation of foraging groups.

Keys to the species of North American *Reticulitermes* are based on morphology of alates and soldiers (Banks and Snyder 1920, Snyder 1954, Weesner 1965). Alates are found only seasonally and rarely in foraging groups. Soldiers are not common (Haverty 1977), but are collected with most foraging groups. For *Reticulitermes* spp. from the Pacific Coastal states, Weesner (1965) did not even attempt a key to the soldiers. The most recent key to the species of North American subterranean termites (Nutting 1990) did include a key to soldiers of *Reticulitermes* spp.; however, this key is based on the original descriptions of Banks and Snyder (1920) and subsequent synonymies by Snyder (1949).

Much of the taxonomic and biogeographical information on *Reticulitermes* spp. was developed in the first half of the last century (Banks and Snyder 1920, Light 1934, Pickens 1934a, b, Banks 1946, Miller 1949, Snyder 1954). No one has thoroughly reexamined the biogeographical or taxonomic treatments of *Reticulitermes* spp. from North America, even though there is agreement that the taxonomy of *Reticulitermes* spp. is desperately in need of revision (Weesner 1970, Nutting 1990, Scheffrahn and Su 1994).

Termite taxonomy is a complex issue and has been the subject of lengthy controversies (Clément et al. 2001). Termites are notoriously "lacking in ornamentation and furnish few if any of those satisfyingly definite differences . . . which facilitate specific diagnoses. . . . Furthermore, termite species are extremely plastic and exhibit a wide range of (morphological) variation. Finally, the specific characters which are available express themselves almost entirely in the form of differences in range of size . . ." (Light 1927). The lack of a modern examination of the genus is likely responsible for the difficulty we have had identifying collections of *Reticulitermes* spp. Determination of species of *Reticulitermes* based on morphology (or geographic origin) of the sample is difficult and often equivocal (Haverty et al. 1996, 1999a, b, c, 2000, Haverty and Nelson 1997).

To make progress in our studies of *Reticulitermes* spp. and circumvent the difficulty with keys, our group has used characterization of cuticular hydrocarbons to delineate taxa. There is a growing body of evidence suggesting that termites have species-specific mixtures of cuticular hydrocarbons (Page et al. 2002 and references cited within). An additional, previously undescribed taxon, *Zootermopsis nevadensis nuttingi* Haverty and Thorne, was discovered and substantiated through studies of cuticular hydrocarbons, morphology, agonistic behavior, genetics, and biogeography (Haverty et al. 1988, Haverty and Thorne 1989, Thorne and Haverty 1989, Broughton and Kistner 1991, Korman et al. 1991, Thorne et al. 1995). In detailed studies using biochemical, biometric, chemical, and genetic techniques, Clément and coworkers distinguished criteria that elevated *R. santonensis* Feytaud to the rank of species, identified sibling species in the *R. lucifugus* (Rossi) complex, described a new species of *Reticulitermes*, and reported on the biosystematics of all known species of *Reticulitermes* in Europe (Clément et al. 2001 and references cited within). A key component of these studies was the characterization of the cuticular hydrocarbons and agonistic bioassays. Similar studies have been done with Asian species of *Reticulitermes* (Takematsu and Yamaoka 1999) and are in progress with North American species.

Repeatable chemical phenotype groups and biological information, such as soldier defense secretions, body weight, and dramatic interphenotype agonism, support the conclusion that cuticular hydrocarbon phenotypes represent distinct taxa (Haverty et al. 1999a, b, Nelson et al. 2001). All that remains is taxonomic description and naming of each taxon (M.I.H. and L. J. Nelson, unpublished data) and corroboration of taxa by morphological and genetic analyses. Agonistic bioassays will lend support to these taxonomic divisions of *Reticulitermes*. Until taxonomic descriptions are published we must rely on the assignment of cuticular hydrocarbon phenotype labels to *Reticulitermes* spp. in California (Haverty and Nelson 1997; M.I.H. and L. J. Nelson, unpublished data).

Haverty et al. (1999a) studied agonism between groups of workers of *Reticulitermes* spp. from northern California with the same or different cuticular hydrocarbon phenotypes. Pairing groups of workers from different cuticular hydrocarbon phenotypes usually resulted in immediate aggression and nearly always resulted in >50% mortality. In contrast, encounters between groups of workers of the same cuticular hydrocarbon phenotype from different colonies seldom resulted in immediate aggression, yet often resulted in >50% mortality.

The agonism bioassay, coupled with characterization of the cuticular hydrocarbons and mark-release-recapture studies, has enhanced our understanding of the dynamics of foraging territories and distributions of *Reticulitermes* spp. colonies in northern California (Haverty et al. 1999b, 2000, Getty et al. 2000a, b). This bioassay, however, requires 20 workers for each agonistic bout. Furthermore, it is nearly impossible to observe individual interactions. The need for hundreds of workers for replicated comparisons of only a few foraging groups led us to investigate agonism at the individual level. Do bioassays using single workers show similar results to previously reported bioassays using groups of workers? Do individual workers show variation in aggression?

We designed an agonism bioassay that forces workers to make recognition decisions and examines the interactions between individual worker termites. We observed and quantified interactions between individual workers from the same colony, from different colonies of the same cuticular hydrocarbon phenotype, and from colonies with different cuticular hydrocarbon phenotypes. Our primary goal was to determine whether levels of intercolonial and interphenotype agonism (or resulting mortality) are different to help us decide whether this one-on-one bioassay can be used in lieu of group assays that re-

quire many more individuals. Secondarily, we hoped to resolve with our bioassay whether aggressive activities are the result of one individual or a group effect. Finally, we wanted to determine whether certain phenotypes, believed to be different species (Haverty and Nelson 1997), are more aggressive than others.

Materials and Methods

Termites. *Reticulitermes* spp. were collected from permanent monitoring stations or pieces of wood in soil contact at the USDA Forest Service, Pacific Southwest Research Station's Institute of Forest Genetics (IFG) near Placerville, CA. The permanent monitoring stations were established at IFG for studies of the foraging ecology of *Reticulitermes* spp. (Haverty et al. 1999b, 2000). The construction and installation of these monitoring stations were described, in detail, by Lewis et al. (1998). Monitoring stations consisted of two 30-cm sections of 10-cm-diameter ABS pipe adjoined with an ABS coupling, buried in the soil to a depth of ≈30 cm with ≈30 cm of the monitoring station above the soil, and capped with a screw-in lid. The inner volume of the in-ground portion was filled with soil to about the same level as the soil outside the monitoring station. The monitoring substrate was a bundle of 11–13 pieces of 30 by 3 by 1-cm, aged ponderosa pine, thoroughly soaked in water for 24 h, which was placed into the monitoring station so that the ends of all pieces of wood were in contact with the soil. The monitoring station was then sealed.

At this site, the *Reticulitermes* spp. have three distinct cuticular hydrocarbon phenotypes (Haverty et al. 1999b, 2000a). These phenotypes are characterized by extracting the hydrocarbons from the cuticle and then identifying and quantifying each cuticular hydrocarbon using a gas chromatograph/mass spectrometer. In northern California, these phenotypes have been designated as CA-A, CA-B, and CA-C (Haverty and Nelson 1997). The termites collected were brought to the laboratory and maintained for up to 9 mo in containers provided with sand, water, and wood from old monitoring station bundles (see above). Two hundred workers from each culture were used to determine cuticular hydrocarbon phenotypes using the methods of Haverty and Nelson (1997). Voucher specimens for each hydrocarbon phenotype were previously deposited in the Essig Museum, University of California, Berkeley (Haverty et al, 1999a).

Termites were collected from monitoring stations or pieces of wood on the ground that were >25 m from one another to increase the likelihood that the termites were from different colonies (Haverty et al. 2000). Five colonies each of phenotype CA-A and CA-B and four colonies of phenotype CA-C were used. For the purposes of this paper we defined termites collected from the same monitoring station or piece of wood on the ground as belonging to the same colony. Termites collected from each monitoring station over the 3-yr span of ecological studies at IFG (Haverty et al. 1999b, 2000) were only of one phenotype; more than one phenotype was never collected from a given monitoring station at any collection period. Rarely, one phenotype was displaced by another and was discovered by characterization of the cuticular hydrocarbons or by measuring the head capsules of soldiers in the foraging group (Haverty et al. 1999b). For this study, the cuticular hydrocarbon phenotype for each culture we used was verified before bioassays were conducted.

Agonism Bioassay. We paired individual workers from different colonies (or the same colony as a control) to determine whether or not they would show aggressive behavior. Rings (1.6 cm OD by 1.3 cm ID by 0.4 cm high) made from clean vinyl tubing were placed inside petri dishes (50 by 9 mm) containing a piece of moistened, absorbent filter pad (Gelman Sciences, Ann Arbor, MI). For identification, one termite was marked on the dorsal surface of the abdomen with green enamel paint (Testors Corp., Rockford, IL) using a 200-μl universal fit pipet tip (VWR Scientific Products, West Chester, PA). Paint was allowed to dry for 5 min before the termites were placed in the arena. The petri dishes were set up with a filter pad, water, ring, and one termite (either marked or unmarked). The lids were replaced until the dishes were opened again to add the second termite. Soft forceps were used to place the termites into the ring. After the second termite was added and the petri dish lid was replaced, a piece of red acetate paper was placed over the petri dish to filter out light during the experiment. The seal between the petri dish lid and the filter pad in the petri dish bottom did not allow escape of either termite.

The behavior of each termite was observed for 5 min immediately after the second termite was introduced. The behaviors observed were similar to those expressed by *Zootermopsis* spp. pseudergates (Haverty and Thorne 1989) and *Reticulitermes* spp. workers (Haverty et al. 1999a) as follows: (1) no apparent reaction, (2) antennation and grooming of one another, (3) clumping or thigmotaxis, (4) tapping of head or body on the substrate, (5) running in circles, (6) obvious avoidance of each other, or (7) attempted biting or actual bite. The total number of avoidance responses or repels (behaviors 5 and 6) and directional lunges (behavior 7) was recorded; passive behaviors (1–4) were not quantified. A repelling event was recorded whenever one of the two termites was repelled by, or attempted to "escape" or retreat from, the other. A lunging event was recorded when one of the termites lunged and bit, or attempted to bite, the other; careful attention was paid to see whether the painted or unpainted termite was lunging. Antennation, grooming, and shaking occurred, but were not the response variables of interest. After the 5-min observation period, the petri dishes with termites were set aside and mortality recorded at 24 h.

We designed our experiment to emphasize intercolonial pairings consisting of two termites from different colonies but of the same cuticular hydrocarbon phenotype. Haverty et al. (1999a) reported that responses of intercolonial pairing of groups usually re-

sult in low levels of immediate aggression and high levels of delayed aggression (i.e., high mortality at 24 h). Based on previous studies (Haverty et al. 1999a, Getty et al. 2000a), we expected intracolonial pairings (two termites from the same colony) to be benign and interphenotype pairings (two termites of different cuticular hydrocarbon phenotypes) to result in immediate aggression and high mortality.

Intracolonial pairings were replicated four times for each colony (see *Appendix 1*). Each possible intercolonial (intraphenotype) pairing was replicated 10 times; there were 10 replicates of a worker from colony A-1 versus a worker from colony A-2, 10 replicates of a worker from colony A-1 versus a worker from colony A-3, etc. In each of these sets of 10 replicates, the worker from the first colony was marked with paint in five replicates, and the worker from the second colony was marked in the other five replicates (see *Appendix 1*). Each possible interphenotype pairing was replicated four times; there were four replicates of a worker from colony A-1 versus a worker from colony B-1, four replicates of a worker from colony A-1 versus a worker from colony B-2, etc. In each of these sets of four replicates, the worker from the first colony was marked with paint in two replicates, and the worker from the second colony was marked in the other two replicates (see *Appendix 1*). Occasional shortages of workers in phenotype CA-C colonies affected sample sizes. All bioassays were conducted at ambient laboratory temperatures (≈20–23°C); no attempt was made to regulate temperature.

Statistical Analyses. The responses recorded for each pairing and used in statistical analyses were (1) total number of avoidance responses (repels) per 5 min, (2) the number of lunges per 5 min for each of the painted or unpainted workers and the total for each replication, and (3) the mortality of the painted or unpainted workers at 24 h and the total for each replication. Statistical significance of (1) the means for the total responses (repels, lunges, and 24-h mortality) among phenotypes or combinations of phenotypes within each type of pairing (intracolonial, intercolonial, interphenotype); (2) the grand means for the total responses for all intracolonial, intercolonial, and interphenotype pairings; and (3) the mean number of lunges by painted individuals toward unpainted individuals and vice versa in each type of pairing was determined at the 5% level by a Student's *t*-test using the Bonferroni adjustment (Miller 1981, Christensen 1987). Statistical significance of the mean number of dead painted or unpainted termites per replicate at 24 h for each phenotype within each type of pairing was determined at the 5% level by McNemar's test for paired binomial data (Zar 1999).

Hierarchy in agonism among phenotypes in interphenotype pairings was determined by summing (1) the total number of avoidance responses (repels), (2) the total number of lunges, and (3) 24-h survival for individual workers of each phenotype (painted and unpainted) when paired with either of the other phenotypes. For example, we combined the results of CA-A versus CA-B and CA-C, etc. Statistical significance of the means for the number of repels, the number of lunges, and 24-h survival was determined at the 5% level by a Student's *t*-test using the Bonferroni adjustment (Miller 1981, Christensen 1987).

Results

Intracolonial Agonism. As an evaluation of the bioassay (control), we paired two workers from the same colony (intracolonial pairings) and painted one of the individuals to test the effect of paint. There were no significant differences in the mean number of repelling events, lunges, or dead termites at 24 h among different phenotypes (Table 1). Termites that died in these controls were completely intact and showed no signs of damage. Painting the abdomen did not stimulate aggression in workers of any cuticular hydrocarbon phenotype (Table 1). Neither the number of lunges nor the number dead at 24 h was significantly greater for painted or unpainted individuals for any phenotype. From these results we conclude that the conditions of our bioassay did not promote aggressive behavior of either individual of a pair.

Intercolonial Agonism. The mean number of repels for cuticular hydrocarbon phenotypes CA-A and CA-C were not significantly different from one another, while CA-B had significantly more repels (Table 1). The mean number of lunges by cuticular hydrocarbon phenotype CA-A was not significantly different from CA-B or CA-C; however, CA-B and CA-C were significantly different from each other. There were no significant differences in the total number of dead termites at 24 h (Table 1).

Painting significantly affected lunges between nonnestmates (Table 1). Overall, workers with paint on their abdomen were more aggressive toward nonnestmates. This difference is strongly skewed by the results of CA-A and CA-B pairings. There were significantly more painted termites dead at 24 h in cuticular hydrocarbon phenotype CA-C; painting did not significantly affect mortality in cuticular hydrocarbon phenotypes CA-A and CA-B (Table 1).

The grand means for repels and mortality at 24 h in the intercolonial bouts were significantly greater than the intracolonial responses while significantly less than the interphenotype bouts (Table 1). However, the grand mean for number of lunges was not significantly different from those in the intracolonial bouts but significantly less than in the interphenotype bouts.

Interphenotype Agonism. When termites of different cuticular hydrocarbon phenotypes were placed together, there was usually immediate aggression and high mortality. Statistically significant differences in the mean number of repels, lunges, and dead termites at 24 h were observed between the different combinations of interphenotype pairings (Table 1). The greatest response resulted from pairing CA-B with CA-C.

Painting the termites prompted immediate aggression between different phenotypes; workers with paint on their abdomen were significantly more aggressive toward unpainted workers (Table 1). This

Table 1. Mean (SE) response from three types of pairings of individual workers of *Reticulitermes* spp. with different cuticular hydrocarbon phenotypes (CA-A, CA-B, and CA-C) from northern California

Type of pairing	*n*	Mean no. of repels per 5 min[a]	Mean no. of lunges			Mean no. dead at 24 h		
			P → U[b]	U → P[b]	Total[a]	Painted[c]	Unpainted[c]	Total[a]
Intracolonial								
CA-A	20	0.25 (0.12)a	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)a	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)a
CA-B	20	0.05 (0.05)a	0.05 (0.05)	0.10 (0.10)	0.15 (0.15)a	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)a
CA-C	14	0.00 (0.00)a	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)a	0.07 (0.07)	0.07 (0.07)	0.14 (0.10)a
Grand mean[d]	54	0.10 (0.04)γ	0.02 (0.02)	0.03 (0.03)	0.05 (0.05)β	0.02 (0.02)	0.02 (0.02)	0.05 (0.03)γ
Intercolonial								
CA-A	100	0.28 (0.08)b	0.24 (0.20)	0.00 (0.00)	0.24 (0.20)ab	0.10 (0.03)	0.06 (0.02)	0.16 (0.04)a
CA-B	100	1.08 (0.20)a	0.84 (0.36)	0.14 (0.09)	0.98 (0.40)a	0.13 (0.03)	0.14 (0.03)	0.27 (0.06)a
CA-C	45	0.44 (0.10)b	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)b	0.13 (0.05)	0.00 (0.00)	0.13 (0.05)a
Grand mean[d]	245	0.60 (0.08)β	0.36 (0.14)	0.05 (0.03)	0.40 (0.15)β	0.12 (0.02)	0.07 (0.01)	0.19 (0.03)β
Interphenotype								
CA-A versus CA-B	100	2.46 (0.30)c	3.99 (0.76)	4.63 (0.99)	8.62 (1.21)c	0.49 (0.05)	0.51 (0.05)	1.00 (0.07)c
CA-A versus CA-C	64	10.89 (0.93)b	11.41 (1.76)	10.00 (1.65)	21.41 (2.33)b	0.72 (0.06)	0.66 (0.06)	1.38 (0.07)b
CA-B versus CA-C	62	16.27 (1.27)a	20.50 (1.83)	9.74 (1.53)	30.24 (2.68)a	0.82 (0.05)	0.87 (0.04)	1.69 (0.06)a
Grand mean[d]	226	9.87 (0.54)α	11.97 (0.88)	8.12 (0.82)	20.09 (1.25)α	0.68 (0.03)	0.68 (0.03)	1.36 (0.04)α

[a] Means for the total responses (repels, lunges, and dead) of each phenotype within each type of pairing (intracolonial, intercolonial, and interphenotype) followed by the same letter (a, b, or c) are not significantly different at the 5% level by a Student's *t*-test using the Bonferroni adjustment.

[b] Mean number of lunges of the painted individual toward the unpainted individual (P→U) and vice versa (U→P). The grand means for intercolonial and interphenotype pairings for P→U and U→P are significantly different at the 5% level by a Student's *t*-test using the Bonferroni adjustment.

[c] The grand mean of the number of dead termites at 24 h for painted versus unpainted for the intercolonial pairings is significantly different at the 5% level by McNemar's test for paired binomial data.

[d] The grand means for all intracolonial, intercolonial, and interphenotype pairings for the total responses (repels, lunges, and dead) followed by the same Greek letter (α, β or γ) are not significantly different at the 5% level by a Student's *t*-test using the Bonferroni adjustment.

difference was strongly affected by results of the CA-B versus CA-C pairings; painted workers lunged at unpainted workers more than twice as often. Pairing CA-A versus CA-B and CA-A versus CA-C yielded similar results between painted and unpainted termites (Table 1). Fortunately, we compensated for this discrepancy by using an equal number of individuals from each colony that were either painted or unpainted (with exceptions resulting from an occasional shortage of workers). For example, if we paired one termite from Group A (painted) and one termite from Group B (unpainted) then we did the reciprocal in the second replicate (see *Appendix 1*).

Painting did not significantly affect mortality observed across all pairings of phenotypes, because the grand means of all interphenotype pairings (painted or unpainted) were identical (Table 1). The grand means for repels, lunges, and mortality at 24 h in the interphenotype bouts were significantly greater than either the intercolonial or intracolonial responses (Table 1). From these results, we conclude that most individual workers quickly recognized workers from different phenotypes and responded aggressively to them.

Hierarchy in Agonism among Phenotypes. Pairings with CA-C had significantly more repels than CA-A or CA-B, whereas the number of repels in pairings with CA-A and CA-B were not significantly different (Table 2). Matches involving CA-A had significantly fewer lunges per bout than CA-B or CA-C, but there were no significant differences in lunges between CA-B and CA-C (Table 2). Moreover, we did not see any significant differences in mean 24-h mortality among phenotypes (Table 2).

Table 2. Mean ± SE number of repels, lunges and 24-h survival from pairings of workers of *Reticulitermes* spp. with different hydrocarbon phenotypes (CA-A, CA-B, and CA-C) with either of the other two hydrocarbon phenotypes (interphenotypic pairings)

Phenotype[a]	*n*	Number of repels[b] (Mean ± SE)	Number of lunges[c] (Mean ± SE)	24-h survival[d] (Mean ± SE)
CA-A	164	6.69 ± 1.01a	3.51 ± 0.92a	0.69 ± 0.07a
CA-B	162	9.36 ± 1.31a	10.20 ± 1.90b	0.66 ± 0.07a
CA-C	126	13.63 ± 1.60b	16.68 ± 2.37b	0.70 ± 0.08a

Means within columns followed by different letters are significant at $\alpha = 0.05$.

[a] All interphenotypic pairings of an individual worker of CA-A, CA-B, or CA-C (painted and unpainted) with a worker of either of the other phenotypes. For example, CA-A represents one-on-one bioassays of CA-A versus CA-B and CA-A versus CA-C, etc.

[b] Number of repels when individual workers of CA-A, CA-B, or CA-C (painted and unpainted) were paired with a worker of either of the other phenotypes.

[c] Number of lunges by individual workers of CA-A, CA-B, or CA-C (painted and unpainted) when paired with a worker of either of the other phenotypes.

[d] Twenty-four-hour survival of individual workers of CA-A, CA-B, or CA-C (painted and unpainted) when paired with a worker of either of the other phenotypes.

Discussion

Our bioassay forced two workers to encounter one another within an arena. Termites in this situation could only fight or not fight; they could not flee or avoid the other worker. Avoidance may occur in natural settings (see review by Thorne and Haverty 1991) and has been reported for *Heterotermes aureus* (Snyder) in laboratory bioassays (Binder 1988). Although the surroundings were unnatural and may have affected behavior, we did observe attempted avoidance behavior (repels) between many individuals within the arenas.

We found that workers recognized and accepted colony members that had been painted and handled. We thought actions like these and other disturbances might provoke individuals. This was not the case as demonstrated by the lack of aggressive behavior and mortality observed in the intracolonial pairings, i.e., controls. Paint does affect aggression in non-nestmates and should be taken into account when designing an agonism bioassay. Both the intercolonial and interphenotype pairings showed significantly higher levels of immediate aggression (lunges) of the painted individuals toward the unpainted individuals. However, there were no statistically significant differences observed in mortality caused by painting in either the intercolonial or interphenotype pairings.

Our 1-on-1 bioassay allowed us to corroborate previous results of a 10-on-10 bioassay (Haverty et al. 1999a), using termites of the same phenotypes from the same site, although not necessarily the same colonies. Our design in this study was balanced by pairing each colony with each of the other colonies the same number of times. As Haverty et al. (1999a) discussed, aggression between different cuticular hydrocarbon phenotypes (likely different species) is much greater than that between individuals or groups from different colonies of the same cuticular hydrocarbon phenotype.

Our 24-h mortality data and observations of immediate versus delayed aggression using individual workers are very similar to those using groups of 10 workers. Thus, individual workers can be used in lieu of groups for agonism bioassays. This eliminates the need for large numbers of workers. Results from interphenotype pairings of individual workers were similar to those of group bioassays, i.e., aggression or violence is nearly instantaneous and mortality usually exceeds 50%.

The results of intercolonial pairings are more difficult to interpret because the mean mortality was similar to that of intracolonial pairings. Demonstration of intercolonial relationships between foraging groups of unknown relatedness would require a large number of replications. For example, suppose we have two foraging groups, A and B, which were collected 5 m apart. For our bioassay, we would use five arenas and place one worker from group A and one worker from group B into each arena. If we noticed a great deal of avoidance behavior and/or lunging and at least one dead termite at 24 h in each arena, we could probably say, with confidence, that groups A and B were different phenotypes or species. If, however, we noticed very few avoidance or lunging events and all five arenas had two live termites after 24 h, what could we conclude based on these results? The results are inconclusive and may indicate the groups are from the same colony. To be certain, we would either have to greatly increase the number of replications, maybe as high as 30 (Polizzi and Forschler 1998) or conduct a power analysis designed to estimate the risk of obtaining a false negative result (Cohen 1988, O'Brien 1998).

Clearly, interactions between groups or individuals of the same phenotype or species provide the greatest diagnostic challenge. Individual pairings resulted in <15% 24-h mortality, whereas group interactions (Haverty et al. 1999a) resulted in 24–48% mortality (Table 3). There may be differences in threshold levels of aggression among individuals within a colony (Polizzi and Forschler 1998). For example, placing two nonaggressive individuals together may not result in immediate or delayed aggression because of avoidance or tolerance of the other termite. Placing 20 individuals together increases the likelihood of at least 1 aggressive individual being present. This individual may initiate an aggressive act that starts a progression

Table 3. Comparison of mortality at 24 h in individual and group interactions from three types of pairings of workers of *Reticulitermes* spp. with different hydrocarbon phenotypes (CA-A, CA-B, and CA-C)

	1-on-1 interactions		10-on-10 interactions[b]	
	n	Percent mortality at 24 h[a]	*n*	Percent mortality at 24 h[c]
Intracolonial				
CA-A	20	0.00		
CA-B	20	0.00		
CA-C	14	7.14[d]		
Mean	54	1.85	81	0.00
Intercolonial				
CA-A	100	8.00		
CA-B	100	13.50		
CA-C	45	6.67		
Mean	245	10.00	486	≥24.04
Interphenotype				
CA-A versus CA-B	100	50.00		
CA-A versus CA-C	64	68.75		
CA-B versus CA-C	62	84.68		
Mean	226	64.82	261	≥48.85

[a] Includes mortality of both members of the pair.

[b] From Table 1, Haverty et al. (1999a).

[c] Mortality at 24 h for group interactions was based only on the number of replicates in which high mortality was observed. High mortality was defined as 0–10 workers out of 20 alive after 24 h. Exact mortality levels were not reported. Thus, percent mortality is at least one-half (≤10 of the 20 individuals in the replicate) of the percentage of the bouts with high mortality. For intercolonial bouts, 225 (25 + 151 + 49) of the 468 bouts (48.08%) resulted in high mortality. Therefore, at least 24.04% of the workers died in the 10-on-10 intercolonial bioassays that resulted in high mortality. For the interphenotype bouts, 255 (71 + 45 + 139) of the 261 bouts (97.70%) resulted in high mortality. Therefore, at least 48.85% of the workers died in the 10-on-10 interphenotype bioassays that resulted in high mortality.

[d] Termites dead at 24 h were completely intact and showed no signs of aggressive behavior having taken place.

of aggressive behaviors, resulting in high mortality. However, this requires 10 times the number of individuals for each replicate of the bioassay. Matsuura (2001) reported that gut contents of *R. speratus* Kolbe contain chemical cues that are important in nestmate recognition; therefore, it is possible that during aggressive group encounters, the release of these chemicals may incite frenzy.

Results from our studies of agonism among cuticular hydrocarbon phenotypes revealed the following hierarchy in levels of aggression: CA-C > CA-B > CA-A. We saw significantly higher levels of avoidance behavior when CA-C was paired with either CA-A or CA-B and significantly more immediate aggressive behavior by CA-C when paired with CA-A. However, mean mortality at 24 h does not seem to be dependent on phenotype; once one termite was mortally wounded, the other generally survived until 24 h passed. These results imply that CA-C is the aggressor and CA-A and CA-B are exhibiting avoidance or defense by fighting back in these pairings. This may be because of the following ecological explanation.

During studies of the cuticular hydrocarbons of *Reticulitermes* spp. in California, Haverty and his colleagues have made hundreds of collections. Of the cuticular hydrocarbon phenotypes used in this study of agonism, CA-A is the most commonly encountered in the northern half of the state. CA-B seems to be restricted to the foothills of the Sierra Nevada; CA-C has thus far only been collected at the Institute of Forest Genetics near Placerville, CA. At the 4-ha collection site for the termites used in our study, cuticular hydrocarbon phenotypes CA-A, CA-B, and CA-C occur sympatrically. Of the 68 monitoring stations used by *Reticulitermes* spp., 53 were occupied by CA-A, 10 by CA-B, and 5 by CA-C (Haverty et al. 2000). From limited experimental evidence (Haverty et al. 1999a, 2000) and empirical observations (by MIH), CA-A colonies often occupy more than one monitoring station; all CA-B and CA-C colonies collected occupy only one monitoring station. This would indicate that the foraging territories and total populations of CA-A are much larger than either CA-B or CA-C. Workers of CA-A are larger than CA-B and CA-C workers (Haverty et al. 1999b). Likewise, soldiers of CA-A are larger than those in CA-B and CA-C (Haverty and Nelson 1997). Therefore, it follows that for these smaller, less abundant termites to survive or compete for resources as they become available, they must be more aggressive.

Our results suggest that group agonistic behavior may be prompted by only one or a few aggressive individuals within a group. They likely trigger the release of chemical and/or behavioral cues that change behavior resulting in high mortality. This is based on the low 24-h mortality seen in intercolonial pairings of individual workers where we would have expected much higher mortality if most of the individuals were aggressive. We were able to corroborate that the high levels of aggression between different cuticular hydrocarbon phenotypes in group bioassays is also higher in one-on-one bioassays. In contrast, the results of intercolonial pairings suggests there are subtle cues detected by antennation or grooming involved in recognition between colonies of the same phenotypes, lending further evidence to the suggestion that different cuticular hydrocarbon phenotypes represent different species. We determined that there are differences in aggression levels among cuticular hydrocarbon phenotypes. The next logical step would be to investigate differences among colonies within the same taxon. Our bioassay proved useful in comparing and contrasting individual versus group bioassays. However, we had the advantage of knowing the phenotypes of the termites before we began the study. Without this prior knowledge this bioassay may need to be used in conjunction with group bioassays.

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Appendix 1. The number of pairings (or replicates) of one worker versus another worker for all possible combinations of five colonies each of *Reticulitermes* spp. phenotypes CA-A and CA-B and four colonies of phenotype CA-C: in each pairing one of the workers was painted and the other unpainted

Colony	Painted worker													
	Phenotype CA-A					Phenotype CA-B					Phenotype CA-C			
	A-1	A-2	A-3	A-4	A-5	B-1	B-2	B-3	B-4	B-5	C-1	C-2	C-3	C-4
A-1	4	5	5	5	5	2	2	2	2	2	2	2	2	2
A-2	5	4	5	5	5	2	2	2	2	2	2	2	2	2
A-3	5	5	4	5	5	2	2	2	2	2	2	2	2	2
A-4	5	5	5	4	5	2	2	2	2	2	2	2	2	2
A-5	5	5	5	5	4	2	2	2	2	2	2	2	2	2
B-1	2	2	2	2	2	4	5	5	5	5	2	2	2	2
B-2	2	2	2	2	2	5	4	5	5	5	2	2	2	2
B-3	2	2	2	2	2	5	5	4	5	5	2	2	2	2
B-4	2	2	2	2	2	5	5	5	4	5	2	2	2	2
B-5	2	2	2	2	2	5	5	5	5	4	2	2	2	2
C-1	2	2	2	2	2	2	2	2	2	2	4	5	5	5
C-2	2	2	2	2	2	2	2	2	2	2	5	4	5	5
C-3	2	2	2	2	2	2	2	2	2	2	5	5	4	5
C-4	2	2	2	2	2	2	2	2	2	2	5	5	5	4