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Intra- and interspecific agonism in *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) and effects of arena and group size in laboratory assays

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Summary

Displays of intraspecific and interspecific overt agonistic behavior between colonies of *Reticulitermes flavipes* (Kollar) and colonies of *Reticulitermes virginicus* (Banks) were observed in laboratory assays. All possible combinations of arena sizes (1.3, 3.5, 6.0, and 9.0-cm) and group sizes (1, 2, 5 and 10 workers per arena) were assessed for effects on interspecific and intraspecific agonism. Agonistic behavior was scored positive in arenas if half or less of the starting number of termites was alive after 24 h. Thirty-six percent of arenas with interspecific pairings were agonistic in all combinations tested while 4.5% of arenas with intraspecific combinations showed aggression (N = 544 and N = 288, respectively). Two interspecific pairings provided scores that were not statistically different from intraspecific combinations. These assays indicate that evidence of overt agonism between worker termites from these two Rhinotermitid species is not a reliable indicator to differentiate species. Encounters in the two larger arena sizes resulted in significantly more agonism than the two smaller arena sizes. Group size had little apparent effect on the frequency of positive agonistic interactions. Possible reasons for the variable overt agonism scores are discussed.

Introduction

Identification of species of subterranean termites in the genus *Reticulitermes* is difficult with available morphological keys for soldiers or alates (Haverty et al., 1996). The caste most commonly used to distinguish species is the alate (Weesner, 1965). Soldiers are also used to identify species because alates are found only during certain seasons and are rarely found in foraging groups (Scheffrahn and Su, 1994). However, soldiers of *Reticulitermes* species comprise 1 to 2% of an entire colony,

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and may not be found at an infestation site (Haverty and Howard, 1981). Workers are the most commonly found termites at infestation sites, but there are no morphologic keys for distinguishing between species using this caste. Because worker termites are always present at infestation sites, it would be worthwhile to pursue means of identifying termite species using this caste. Several studies have indicated intra- and interspecific encounters between different termite groups can result in aggressive, or agonistic behavior (Binder, 1988; Thorne and Haverty, 1991; Su and Scheffrahn, 1988; Jones, 1990). Agonistic behavior is defined by Kind (1973) as all behaviors displayed by individuals during contests to include both defensive and offensive behaviors of both attacker and defender. Agonism may occur when certain castes attempt to acquire nest and food resources, protect those resources, or compete for mates (Thorne and Haverty, 1991). Although soldiers are specially equipped to exhibit aggression, the worker caste members are also capable of demonstrating agonism (Andrews, 1911; Binder, 1988; Delaplane, 1991; Haverty and Thorne, 1989; Thorne, 1982; Jones, 1990).

We were interested in investigating the use of overt agonistic behavior in bioassays to identify or separate *Reticulitermes* species using the worker caste. Our hypothesis was that worker termites would only battle when placed with workers from another species. Recently, we have found evidence that the morphologic keys that we use to identify termites at our sites throughout Georgia are at times inadequate (Haverty et al., 1996).

Preliminary to examining the expression of overt agonistic behavior in laboratory assays, was the question of the effects of arena size and number of termites per arena. Agonism assays have typically placed termites from their nests into highly artificial conditions where the effects on social behavior could be questioned (Thorne and Haverty, 1991). Our goal was not only to investigate agonism among *Reticulitermes flavipes* (Kollar) and *Reticulitermes virginicus* Banks colonies in Georgia using laboratory methods, but also learn more about the methods themselves and their effects on the expression of agonism in *Reticulitermes* spp. If laboratory assays are going to be used to draw conclusions based on expression of agonism in termites, it would be useful to know if the frequency of agonism is not an artifact of the methods. This paper reports results from laboratory tests of our hypothesis and the effects of arena size and different number of workers per arena in expressing agonism using worker caste members.

Materials and methods

Termites

Four colonies each of *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) were collected from logs at the University of Georgia's Westbrook Farm, Spalding County, Georgia. Using techniques described by La Fage et al. (1983), termites were removed from logs brought into the laboratory. When present, alates were used to identify colonies to species (Weesner, 1965). When alates were not found, soldiers were used to identify colonies to species (Scheffrahn and Su, 1994). Each colony was maintained in separate clear plastic boxes with lids. Boxes were lined with damp pine (*Pinus* spp.) slats measuring 1-mm × 2-cm × 10-cm and moistened Whatman #1 filter paper disks. Colonies were maintained in an unlit environmental chamber at 24 °C from two weeks to seven months before inclusion bioassay. Individuals were transferred from these boxes to 9.0-cm petri pates lined with moistened filter paper at the start of this assay.

Bioassay and data analysis

Paired combinations of workers from the four colonies were placed into 1.3, 3.5, 6.0 or 9.0-cm diameter arenas. The three largest arena sizes consisted of polystyrene petri plates. The smallest arena was constructed with 1 × 2.5-cm (OD) tygon tubing attached with silicone to a glass microscope slide. Each arena was lined with a single Whatman #1 filter paper. Paper was moistened with a measured amount of distilled water, according to the size of the arena to attain an initial percentage moisture of approximately 52% (w/w). One, 2, 5, or 10 worker termites from two different colonies were placed into an arena sealed with a lid. Termites that were attached to filter paper were removed by grasping the filter paper with one hand and striking that hand with the other so that termites would become detached. Termites would then fall about 10-cm into clean, separate weigh boats. These weigh boats, that contained different colonies, were then tipped over test arenas so that individuals could fall into the arena from a height of 4-cm. This was performed in order to avoid handling with forceps that could cause injury. Each colony was added to test arenas less than 5 minutes from one another. Attention was taken in using only those workers that appeared uninjured during transfer to the arenas.

Each colony combination, arena size, and number of workers were replicated at least 4 times, using new arenas each time. From these tests we chose a measure of agonism we term overt agonism to avoid subjective descriptions of behaviors, facilitate a large number of replicates, and to avoid attempting to separate similar looking individuals into their respective colonies. After 24 h, overt agonistic behavior was scored positive in those arenas that contained half or less of the starting number of live termites. This scale was based on overt agonism that presumed an aggressive encounter would result in the death of at least half of the individuals involved. The scale used to score overt agonism was 0 or 1, with 1 representing agonism had occurred and 0 denoting no agonism had occurred. The data were analyzed using analysis of variance and protected least significant difference mean separation technique ($P < 0.05$). (SAS Institute, 1988).

Results

In all arenas scored positive for overt agonism, it was evident that the dead individuals died as a result of an aggressive encounter because appendages were injured or missing and many of the individuals decapitated. Overt agonistic behavior between *R. flavipes* and *R. virginicus* worker termites does not appear to be a reliable indicator for differentiating between these two species among Georgia populations. It is also apparent that these tests cannot be used to differentiate at the colony level.

Table 1. Proportion of replicates scored positive for agonistic behavior from inter- and intraspecific pairings by colony for all arena size and group size combinations

Pairings[a]	n[b]	Mean agonism score[c]	
Rf3, Rv4	64	0.609	A
Rf1, Rv2	80	0.525	A, B
Rv1, Rf1	64	0.438	B
Rv3, Rf3	64	0.422	B
Rv2, Rf2	64	0.297	C
Rv3, Rf4	64	0.219	C
Rv1, Rf2	80	0.188	C, D
Rv4, Rf4	64	0.094	D, E
Rv3, Rv4	64	0.078	D, E
Rv1, Rv2	80	0.034	E
Rf3, Rf4	64	0.031	E
Rf1, Rf2	80	0.013	E

[a] Rv followed by a number indicates the *R. virginicus* colony used. Rf followed by a number indicates the *R. flavipes* colony used.
[b] Number of colony pairings that includes all combinations of arena size and number of termites per arena.
[c] Means followed by the same letter are not significantly different using LSD with $P < 0.05$.

Table 2. Proportion of replicates scored positive for overt agonistic behavior from interspecific pairings by arena size

Arena size	Mean agonism score[a, b]	
9.0 cm	0.544	A
6.0 cm	0.463	A
3.5 cm	0.221	B
1.3 cm	0.169	B

[a] Means followed by the same letter are not significantly different using LSD with $P < 0.05$.
[b] $N = 136$ for each mean and includes all interspecific pairings and all numbers of termites per arena.

Tables 3. Proportion of replicates scored positive for overt agonistic behavior from interspecific pairings by number of individuals per arena

Termites per arena	Mean agonism score[a, b]	
4	0.426	A
2	0.346	A, B
20	0.316	B
10	0.309	B

[a] Means followed by the same letter are not significantly different using LSD with $P < 0.05$.
[b] $N = 136$ for each mean and includes all interspecific pairings and all arena sizes.

Data for each inter- and intraspecific pairing were combined because the analysis provided no interaction between arena size and number of termites per arena. None of the interspecific pairings displayed 100% overt agonistic behavior toward one another ($F = 27.72$; $df = 10$; $P < 0.0001$) (Table 1). In addition, none of the intraspecific combinations resulted in 100% lack of overt agonistic behavior; several of the intraspecific combinations had mean agonistic scores not significantly different from the interspecific combinations (Table 1).

Examining the interspecific pairings for effects of arena size did provide significant differences in mean overt agonistic behavior scores (Table 2). The two largest arena sizes provided higher mean overt agonistic behavior scores (Table 2). The two largest arena sizes provided higher mean overt agonistic behavior scores than the two smallest arena sizes ($F = 27.36$; $df = 3$; $P < 0.0001$). When examining the number of termites per arena in the interspecific pairings, no obvious biologically significant trend in the incidence of overt agonistic behavior was seen ($F = 2.38$; $df = 3$; $P < 0.690$) (Table 3).

Discussion

It is apparent from these tests that agonism bioassays are not a useful tool in distinguishing between *R. flavipes* and *R. virginicus.* Although a lack of agonism among populations of *R. flavipes* has been observed (Grace, 1996; Traniello and Thorne, 1994), variability in occurrence of agonism among other termite species has also been seen (Haverty and Thorne, 1989; Pearce et al., 1990; Thorne, 1982). Thorne and Haverty (1991) cite evidence that the occurrence of agonism is contingent on species, colonies, nests, castes, individuals, and climatic conditions. Altering their environment could have an influence on how they would typically behave. Termites placed in unfamiliar surroundings may not react to stimuli in the same way as they would in field conditions.

Results from the two variables tested were counterintuitive (Tables 2, 3). It was expected that as the arena size decreased and the number of individuals per arena increased the overt agonism scores would increase. Results from a study with *Heterotermes aureus* Snyder (Isoptera: Rhinotermitidae) by Binder (1988) showed that soldier and worker encounters decreased with an increase in arena size perhaps because escape was more frequent in the larger dishes. Displays of overt agonism from our inter- and intraspecific pairings were certainly inconsistent (Table 1). Our data suggest that agonism may not be an innate behavioral response. It may suggest that kin recognition, as evidenced by agonistic behavior in *Reticulitermes*, is governed by a series of behavioral and chemical cues (Su and Haverty, 1991).

Additional information is needed concerning which cues are being used and how they are being utilized. Kin recognition in an artificial environment may be better rated on a scale of behavioral responses instead of a measure of overt agonism. Clement (1981 and 1982) has already identified differences in greeting behaviors between *Reticulitermes* species when recognizing their nestmates. There has been evidence that there is a correlation between seasonality and aggression in that colonies become open, or more tolerant to outsiders during warmer seasons (Clement, 1986). Another possible effect that would cause equivocal occurrence of agonism, is the laboratory environment. There has been evidence that when termites are held in the laboratory their level of aggression decreases over time (Nel, 1968; Clement, 1986; Shelton and Grace, 1997). Our tests were conducted from 8/9 through 9/4. Colonies RF2, RV2 and RV4 were collected only 2 weeks prior to the assay, while colonies RF1 and RF3 were collected 1 month before inclusion bioassay. Colonies RF4 and RV1 were maintained in the laboratory for 3 months while colony RV3 had been collected 7 months before testing. However, collection time did not seem to affect colony aggression level in this assay. It is possible that season could have had an affect in that our tests were conducted when these societies would be expected to be open or more tolerant (Clement, 1986). However, agonism data collected from out field sites over two years did not indicate a seasonal fluctuation in expression of aggressive behavior (Forschler, unpubl. data). Pearce et al. (1990) observed *Microtermes* spp. colonies isolating themselves by constructing soil barriers rather than initiating battles. These and other behavioral responses need to be pursued to fulfill our understanding of termite kin recognition before further attempts are conducted at using agonism as a basis for termite species identification.

The variability in aggressive behavior we observed could also be explained if non-nestmate aggressive behavior was relegated to specific individuals within the termite worker caste rather than being a generic behavioral expression in worker termites. This may be an example of polyethism among worker caste members. Temporal polyethism has been found in *Reticulitermes fukienensis* Light among workers of different sizes (Crosland et al., 1996). The larger workers were found to scout sooner, tunnel farther, display alarm behavior and bury corpses more frequently than did the smaller workers. It is possible that the same is true for displays of agonism in which it is expressed differentially in workers of different sizes. Further studies in possible polyethism within the worker caste need to be pursued in order to answer which tasks are being divided and if agonism is included. Because tests for agonism usually consist of a low number of individuals placed into an arena, it is possible that the probability of a fighter existing in that group is low. As indicated in our tests for the interspecific combination RV4RF4, insufficient replicates could have produced a false negative conclusion. Therefore, it is important to replicate termite agonism tests a significant number of times, at least 30, as demonstrated by this experiment before concluding species or colony relatedness in *Reticulitermes* spp. due to the lack of overt agonism.

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