Rosengaus RB, Moustakas JE, Calleri DV, Traniello JFA. 2003. Nesting ecology and cuticular microbial loads in dampwood (*Zootermopsis angusticollis*) and drywood termites (*Incisitermes minor, I. schwarzi, Cryptotermes cavifrons*). 6pp. *Journal of Insect Science*, 3:31, Available online: insectscience.org/3.31

Journal of Insect Science

insectscience.org

Nesting ecology and cuticular microbial loads in dampwood (*Zootermopsis angusticollis*) and drywood termites (*Incisitermes minor, I. schwarzi, Cryptotermes cavifrons*)

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Received 1 April 2003, Accepted 18 August 2003, Published 15 September 2003

Abstract

Termites form one-piece nests in wood that can vary in their moisture content and degree of decomposition, and thus microbial richness. To estimate the microbial load of nests and the potential risk they pose for colony members, we quantified the number of microbes in the nest and on the cuticle of the dampwood termite, *Zootermopsis angusticollis*, and three drywood termites, *Incisitermes minor, I. schwarzi*, and *Cryptotermes cavifrons*. The number of colony forming units (CFUs) cultured from nest material samples and washes of the cuticle of larvae and nymphs were determined. CFUs recorded from nest material was low (fewer than 60 CFUs/g) in the drywood termites and comparatively high in the dampwood species, as more than 800 bacterial and fungal CFUs/g were cultured from the nest material of *Z. angusticollis*. Similarly, cuticular microbial loads were negligible in the drywood termites sampled, ranging from 0.5 to fewer than 16 CFUs/cm^2, whereas approximately 200 CFUs/cm^2 were cultured from *Z. angusticollis*. The nesting and feeding habits of these basal species likely influence colony microbial load and the degree of pathogen exposure, which in turn could favor adaptations to resist disease that vary with termite nesting biology.

Keywords: Isoptera, ecology, pathogen load, social evolution

Abbreviation:
CFU colony forming unit

Introduction

The role of pathogens in the evolutionary ecology of termites has recently been the focus of a series of research efforts that describe and analyze the behavioral, biochemical and physiological mechanisms of disease resistance in the Isoptera. For example, *Zootermopsis angusticollis* nymphs and primary reproductives significantly increase the rate of allogrooming after exposure to pathogens (Rosengaus *et al.*, 1998, 2000) and use vibratory displays that convey information about the presence of lethal fungal spore concentrations to nestmates, which respond by absconding (Rosengaus 1999a). Additionally, the cephalic gland secretions of *Nasutitermes* soldiers and the sternal gland seceretions of *Z. angusticollis*, as well as its fecal pellets have fungistatic properties that reduce microbial growth within nest chambers (Rosengaus *et al.*, 1998, 2000). Termites also produce antibiotic peptides and show cellular and humoral immune responses that may be socially regulated (Rosengaus *et al.*, 1999b; Lamberty *et al.*, 2000; Traniello *et al.*, 2002).

It is likely that these individual and social mechanisms of disease resistance evolved in response to pathogen loads in nest environments, yet little is known about the ecology of disease risk in the Isoptera. We hypothesize that microbial loads are related to termite nest type and abiotic factors such as the moisture content of nest material. The nesting and feeding habits of the Isoptera are varied but have been categorized in general as one-piece, intermediate- and separate-nest types (Abe, 1987). In one-piece nest type species, which include the phylogenetically basal dampwood and drywood termites, moist, decayed wood, or dry wood, respectively, serve both as nest and food source throughout the colony's inception and maturation. Although the degree of wood decomposition and moisture content may be dramatically different

among species, one-piece nest termites do not forage outside of their nest. The intermediate nest-type species do not feed exclusively on the wood in which they nest, and also forage in the soil or on the ground and exploit other cellulose resources. In the separate nest-type species, the nest is distinct from the food source. If the nesting and feeding ecology of termites is correlated with microbial load and disease risk, then the heterogeneity of the pathogen environment could have been responsible for the evolution of adaptive mechanisms of infection resistance. Although some qualitative and quantitative data exist on the microbial communities inhabiting termite nests (Hendee, 1933; Meiklejhon, 1965; Sands, 1969; Keya *et al.*, 1982; Cruse, 1998; http://people.bu.edu/rrosenga/table1.htm), the relationship between nesting biology and microbial load is poorly understood. Here we report on the relative variation in nest and cuticular microbial loads associated with the nesting habits of representative species of North American dampwood and drywood termites.

Material and methods

Microbial load of termite nest material

Microbial loads were estimated from the number of colony forming units (CFUs; Smith, 1980) cultured from the nest material and cuticular washes of nymphs and workers of the dampwood termite *Zootermopsis angusticollis* Hagen (Family Termopsidae, 3 colonies sampled), and the drywood termites *Cryptotermes cavifrons* (2 colonies), *Incisitermes minor* (5 colonies) *and Incisitermes schwarzi* (5 colonies). All species are members of the Family Kalotermitidae (Kambhampati and Eggleton, 2000). All colonies were collected from natural field sites except four of the five colonies of *I. minor*, which were nesting in construction beams. Ten one-gram samples of wood adjacent to nest galleries were collected from each colony of each species. Each sample was placed in a sterile 1.5 ml Eppendorf tube with 100 µl of a 0.1% Tween 80 suspension medium, vortexed and then centrifuged at 300Xg at 4° C for 20 minutes. Twenty µl aliquots of supernatant were plated on potato dextrose agar N = 3 replicates/sample, total of 30 plates/colony, pH = 6. Twenty µl of a 0.1% Tween 80 solution (N = 3 replicates) served as controls. Potato dextrose agar favors the cultivation, isolation and enumeration of yeasts and moulds (Beever and Bollard, 1970; Cruise, 1998; BBL Becton, Dickinson and Company, www.bd.com) and thus was a suitable growth medium. Plates were inverted and incubated at 37˚C for 5 days and the number of CFUs at least 1mm in diameter was recorded for each plate.

Cuticular microbial loads

Cuticular microbial loads of termites were estimated from ten individuals/colony/species. Termites (sixth instar to nymph) were weighed individually, placed in sterile Eppendorf tubes with 550µl of a 0.1% Tween 80 solution and centrifuged at 300 x g at 4˚C for 20 minutes. Centrifugation of termites did not result in the rupture of the cuticle and the few samples contaminated with gut fluid were removed from the analysis and replaced with other nestmates. Thus, no obvious contamination of the sample took place and cuticular washes represent microbial loads carried on the exterior surface of the insect. Following centrifugation, 20µl aliquots of the supernatant were seeded on potato dextrose agar media (N = 3 replicates) on 100 mm diameter Petri dishes. CFUs of at least 1mm in diameter were counted following five days of incubation at 37˚C. To control for differences in body size and surface area among species, we estimated the cuticular area (in cm^2) using Meeh's formula:

Surface area = $kW^{2/3}$

where W is the mass (in grams) and k is the species-specific constant, which for termites is 12 (Sponsler and Appeal, 1990). Cuticular CFUs were thus standardized according to surface area (cm^2) across species. Samples contaminated with termite feces were not plated.

Statistical analyses

CFUs counts were not normally distributed; therefore, differences in the number of CFUs were analyzed with a Kruskal-Wallis one-way ANOVA or a Mann-Whitney U test. When multiple pairwise comparisons were performed, the significance value was adjusted using the Bonferroni correction (Rice, 1989), which set the threshold of significance at $p < 0.005$. Because one of the five *I. minor* colonies sampled was collected in the field while the other four nested in construction beams, the field colony was excluded from the main analysis. However, comparisons between the field and construction beam colonies were performed as well.

Results

Microbial loads of nest material and cuticule

The nest CFUs of either bacterial or fungal origin as well as the combined total number of bacterial and fungal CFUs isolated from nest material was significantly different among the dampwood and drywood species examined (Kruskal-Wallis One-Way ANOVA, $X^2 = 112.1$, df = 3, $P < 0.0001$; Table 1, Fig 1a). The highest loads were found in nests of *Z. angusticollis*: the combined number of bacterial and fungal CFUs was 824 ± 412 CFUs/g (average ± S.D., Figure 1a). In sharp contrast, nests of the drywood species had CFUs ranging from 0.4 ± 0.8 in *I. minor* to a maximum of 57.8 ± 37.4 CFUs/g in *I. schwarzi.* CFUs cultured from nest material differed significantly among the drywood termite species ($X^2 = 69.8$, df = 2, $P < 0.0001$). Pairwise comparisons between drywood species were significantly different (Mann-Whitney U Test, at $P = 0.005$) with the exception of *C. cavifrons* and *I. minor* ($U = 334$, $Z = -2.0$, $P = 0.04$). The average microbial load isolated from *I. minor* nests collected from construction beams (0.4 ± 0.8 CFUs/g) was significantly lower than the microbial load of the single colony collected in the field (4.1 ± 3.6 CFUs/g; $U = 85.0$, $Z = -5.6$, $P < 0.001$, Mann-Whitney U Test). Despite these differences between natural and lumber nests, the drywood species still had significantly lower microbial loads than *Z. angusticollis*.

After correcting for differences in surface area of the host species exoskeleton, bacterial and fungal microbial loads as well as the combined total loads on the cuticle were found to vary significantly among species ($X^2 = 216.4$, df = 3, $P < 0.0001$, Kruskal-Wallis One-Way ANOVA; Table 1 and Figure 1b). *Z. angusticollis* had on average (± S.D) 190.4 ± 108.7 CFUs/cm^2, but the maximum cuticular loads isolated from drywood termites were less than 4.0 CFUs/cm^2 (Figure 1b). Cuticular microbial loads also differed significantly among the drywood species ($X^2 = 62.1$, df = 2, $P <$

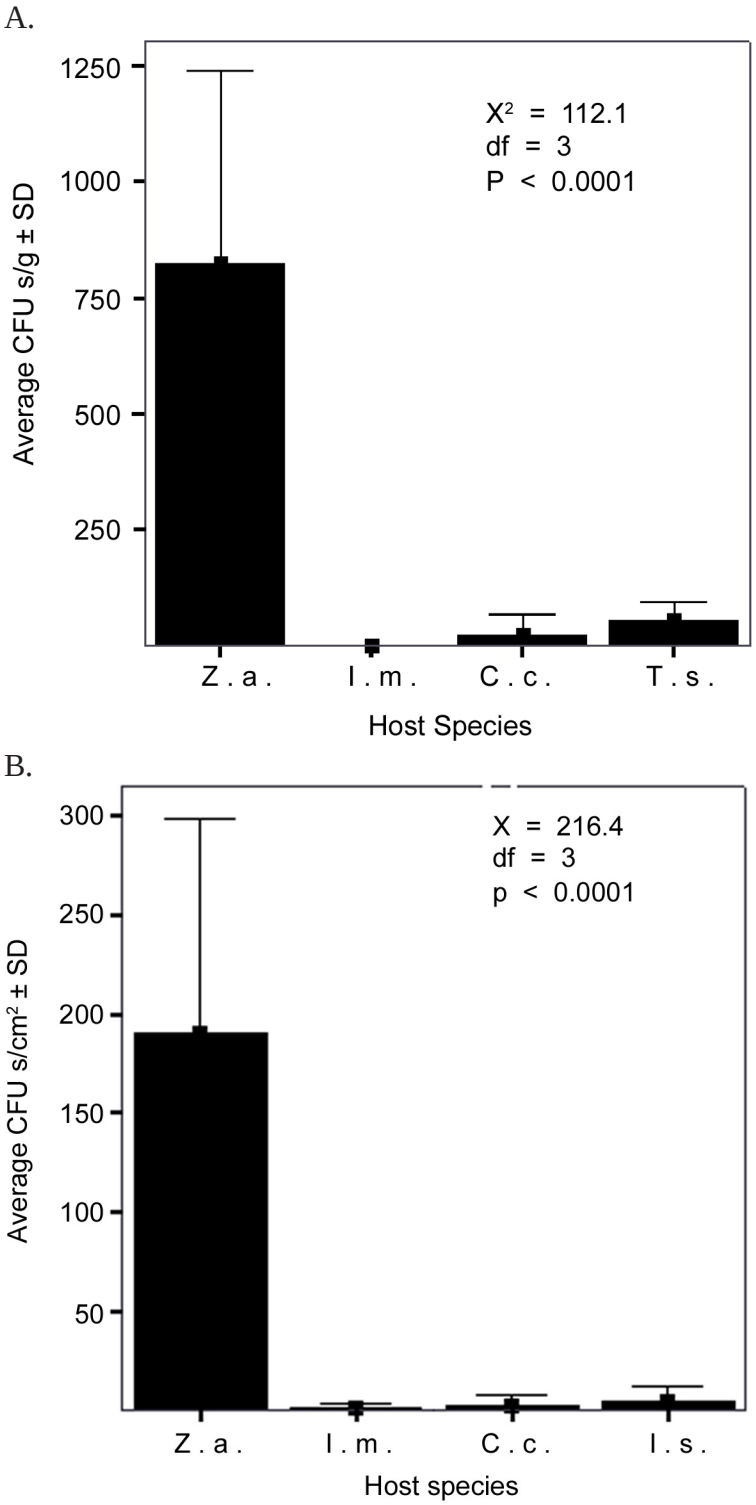

Figure 1. Average bacterial and fungal CFUs ± S.D. isolated from nests (a) and from the cuticule (b) of the dampwood termite *Z. angusticollis* (*Z.a.*) and the drywood species *I. minor (I.m.), C. cavifrons (C.c.)* and *I. schwarzi (I.s.).*

0.0001, Kruskal-Wallis One-Way ANOVA). Pairwise comparisons of cuticular CFUs between any two drywood species were significantly different (Mann-Whitney U Test, $P = 0.005$) with the exception of *C. cavifrons* and *I. schwarzi* ($U = 1428$, $Z = -2.0$, $P = 0.04$). The average cuticular microbial loads/cm^2 of *I. minor* termites collected in the field were not significantly different from termites

Table 1. Average nest and cuticular CFUs ± S.D. of bacterial and fungal origin.

	Z. angusticollis	*I. minor*	*C. cavifrons*	*I. shwarzi*	*P*
Nest CFUs/gram					
Bacterial	773.0±468.0	0.1±0.40	8.5±28.0	57.8±37.4	***
Fungal	50.9±107.0	0.3±0.8	14.7±38.8	0	***
Cuticular CFUs/cm^2					
Bacterial	190.2±108.7	0.4±2.1	0	3.8±6.6	***
Fungal	0.1±0.3	0.1±0.6	2.5± 4.8	0	***

*** denotes significance at $P < 0.0001$ by Kruskal-Wallis Test

collected from construction beams ($U = 2074$, $Z = -1.2$, $P = 0.2$).

Intercolony variability in nest and cuticular CFUs

Z. angusticollis showed significant intercolony differences in cuticular microbes (CFUs/cm^2; $X^2 = 23.0$, df = 2, $P < 0.0001$; Kruskal-Wallis One-Way ANOVA) but no intercolonial differences were found in the total CFUs/cm^2 of *I. minor* ($X^2 = 3.0$, df = 3, $P = 0.4$), *I. schwarzi* ($X^2 = 9.4$, df = 6, $P = 0.1$) and *C. cavifrons* ($X^2 = 0.3$, df = 1, $P = 0.6$).

Discussion

Termite nesting ecology and microbial loads

The quantitative analysis of microbial loads allows the estimation of the relative microbe pressures that termites encounter within their nests. Colony forming units, however, do not necessarily reflect an accurate measure of pathogen loads, which requires the identification of all microorganisms and their role as infectious agents, a task beyond the scope of the present research. Although new genetic techniques can reveal microbial diversity with a higher resolution than culture-dependent methods or morphological identification, these techniques are also prone to sample biases (Hughes, et al., 2001). Because of the extreme diversity of microorganisms, particularly in decomposed wood samples and /or soil, an exhaustive inventory of microbial richness may not be possible (Hughes, et al., 2001). Yet, by estimating the abundance of nest microbes while controlling for the amount of wood sampled and differences in cuticular surface area among termites, we can infer the relative pathogenic pressures under which species with different nesting habits live. Our approach then, appears to be consistent with that proposed by Hughes et al. (2001) in their determination of microbial diversity. Our results show that the microbial loads in the nest material of the study species appeared to be positively correlated with the degree of wood decomposition and level of humidity within the nest. Other factors such as wood species, cellulose and lignin content, the presence of secondary plant compounds and antagonistic effects among the microorganisms themselves, could also affect colonization by both termites and microbes either directly through their impact on nutritive value or by influencing decay rate (e.g. Grace *et al.*, 1996; Ohtani *et al.*,

1997; Madamanchi and Kuæ 1999; Muranaka *et a.l*, 1999). *Z. angusticollis* build one-piece nests in partially buried decayed and moist wood (Castle, 1934; Light and Illg, 1945), conditions that would seem to be highly favorable for the growth and development of a variety of microorganisms (Hendee, 1934; Arshad et al., 1982; Ignoffo, 1992). Because *Z. angusticollis* reside in and feed on their nests, termites of this species likely contact a dense population of diverse microbes, which are carried on the cuticle and may be ingested. Hendee (1934) reported on the frequency with which fungi were isolated from colonies of *K. minor* (= *I. minor*), the subterranean *Reticulitermes hesperus* and *Z. angusticollis*. Our quantitative analysis corroborates her qualitative results: colonies of *I. minor* had "much less luxuriant fungous growth" (Hendee, 1934, p. 107) than *Z. angusticollis*. Our research extends these earlier descriptions by quantifying both bacterial and fungal microbial loads within nests as well as on the cuticle.

Heterogeneity among colonies of *Z. angusticollis* in moisture, decay, wood species and chemistry may also explain the significant intercolony variability in CFUs we described. These factors, together with biogeographic variation in the diversity and abundance of microbe populations, could influence the structure of the microbial communities of termite nests. Given that the sampled *Z. angusticollis* colonies were collected at the same locality and within less than 100 meters from each other, we suggest that the microbial pressures in the dampwood termite may be specific for each colony. Kalotermitid species, like Termopsids, nest within their food source, but in contrast to these dampwood species, *I. minor, I. schwarzi,* and *C. cavifrons* inhabit relatively dry and solid wood that does not favor the growth of bacteria and fungi (Hendee, 1933, 1934; Ignoffo, 1992). We found no significant intercolony differences in CFUs isolated from nests of the drywood species, suggesting that the relative low moisture and the low degree of wood decay of the nests of these species are associated with reduced microbial activity. Moreover, nest and cuticular microbial loads for one colony of *Neotermes jouteli* (Family Kalotermitidae) were consistent with those of the other drywood species we sampled: approximately 43.9 ± 34.4 CFUs/g and 15.3 ± 23.6 CFUs/cm^2 were cultured from the nest and cuticular washes, respectively.

Microbial loads have been also estimated from the Australian *Mastotermes darwiniensis, Coptotermes lacteus* and *Nasutitermes exitiosus* (Cruse, 1998). *M. darwiniensis*, for example, has approximately 400 x10^3 CFUs/g of nest material and 1200 CFUs/cm^2 on the cuticle (Cruse, 1998). *C. lacteus* and *N. exitiosus* build mounds that are relatively dry and have low levels of organic material in their outer layers (Cruse, 1998). Approximately 100-190 x 10^3 CFUs/g and 74 CFUs/cm^2 were cultured from nest material and cuticular washes in *C. lacteus*, respectively, and 100-200 x10^3 CFUs/g and 200 CFUs/cm^2 in *N. exitiosus*. Cruse (1998) argued that the lower microbial loads of *C. lacteus* and *N. exitiosus* relative to *M. darwiniensis* reflect more efficient biochemical mechanisms to control microbes by these two species. Indeed, *N. exitiosus* may use defensive secretions to reduce intranidal and cuticular microbial loads because *Nasutitermes* soldiers produce terpenoid defensive compounds in the cephalic gland, of which the major constituent (α-pinene) significantly reduces the germination of fungal spores through direct contact and volatility (Rosengaus *et al.,* 2000). The volatile antifungal properties may result in an intranidal "fumigation" effect (Chen *et al.,* 1998). Frontal gland secretions of *N. exitiosus* could similarly control microbes within their nests.

We have hypothesized that the nesting and feeding ecology of termites is correlated with microbial load and the risk of infection. Alternatively, interspecific variation in nest and cuticular microbial loads could be accounted for by species-specific differences in biochemical defenses, as suggested by Cruse (1998): species with more efficacious biochemical protection should have lower nest and cuticular microbial loads. Although glandular secretions and exudates are undoubtedly important for the control of infectious agents (Batra and Batra, 1966; 1973; Sannasi and Sundara Rajulu, 1967; Olagbemiro *et al.,* 1988; Rosengaus *et al.,* 1998, 2000), we believe that the discrepancy between nest and cuticular loads may provide a better measure of the impact of termite antibiotics on nest microbes. While the microclimatic conditions and other physical attributes of the nest may support high microbial activity, effective antimicrobial secretions should minimize cuticular loads despite the high encounter rate with microbes. More detailed comparative studies are required to test these hypotheses.

Microbes and termite sociality

The relatively high cuticular microbial loads of the basal *Z. angusticollis* provide insight into the pathogen-related selective pressures that may have affected social behavior in extant lower termite species. The risk of infection from microbes is likely exacerbated during the incipient stages of colony foundation, when colony size is small and demography is skewed toward younger instars (Rosengaus and Traniello, 2001). Larvae do not provide labor until the third instar and they rarely allogroom nestmates (Rosengaus and Traniello, 1993), a behavior that appears to be an extremely important social mechanism of infection control (Rosengaus *et al.,* 1998, 2000; Rosengaus and Traniello, 2001). Nesting in microbially rich environments with a small cohort of immature and behaviorally limited offspring may in part explain the high failure rates of incipient colonies (Shellman-Reeve, 1994; Rosengaus *et al.,* 2000 and references therein).

Preliminary data on the cuticular microbial loads of the subsocial woodroach *Cryptocercus punctulatus*, a species believed to be closely related to termites (Nalepa, 1984; Lo *et al.,* 2000), suggest that significant microbial selection pressures may have been also encountered by subsocial termite ancestors. *C. punctulatus* lives in small family units and nests in fallen decayed and moist wood on which it feeds (Seelinger and Seelinger, 1983; Nalepa, 1984). The average cuticular microbial load of *C. punctulatus* was 234.3 ± 137.5 CFUs/cm^2 (N = 30 roaches originating from two family units, three replicates/roach), a level not significantly different from that estimated for *Z. angusticollis* (U=1112.0, Z=-1.5, p = 0.1, Mann-Whitney U test). Indeed, cockroaches have one of the highest cuticular microbial loads of any arthropod, averaging 10^5 CFUs/mg (Le Guyader *et al.,* 1989) and hosting 42 and 85 different species of fungi and bacteria, respectively (Roth and Willis, 1967). In spite the controversy surrounding the phylogenetic relationship of *Cryptocercus* to termites (Grandcolas, 1999a,b; Maekawa and Kitade, 1999; Nalepa and Bandi, 1999, 2000; Kambhampati and Eggleton, 2000; Lo *et al.,* 2000; Eggleton, 2001), it is generally agreed that termites evolved from a subsocial roach-like insect that lived in logs and digested cellulose with the assistance of intestinal

symbionts (Noirot, 1970; Hamilton, 1978; Nalepa, 1994; Thorne, 1997; Lo *et al.*, 2000). The prototermite may have lived under significant microbial pressures that accompanied these insects during their transition from a subsocial to a eusocial existance. The quantification of microbial loads in *Cryptocercus* and *Z. angusticollis* suggests that the pathogen-related pressures experienced by prototermites may have been similar to those of extant basal species.

Although such inferences are limited, disease, in addition to other ecological factors, could have played a contributing role in the evolution of termite sociality (Rosengaus and Traniello, 1993; Thorne and Traniello, 2003). Eusociality in the Isoptera was most likely favored by several factors (reviewed in Thorne, 1997), including a shift in dependent care from parents to older offspring (Nalepa, 1984, 1994). This hypothesis is particularly interesting in light of the interplay between disease susceptibility, group size and demography of termite colonies (Rosengaus *et al.*, 1998; Rosengaus and Traniello, 2001). Detailed studies on the methods of disease resistance are required to understand the significance of pathogens in termite social biology.

Variation in nest and cuticular microbial loads of termites may favor the evolution of interspecific differences in mechanisms of infection control. Comparative studies on the disease-related aspects of the socioecology, as well as the immunology, of these species could afford the opportunity to examine the influence of nesting ecology on the evolution of individual and social modes of disease resistance.

Acknowledgements

We thank Dr. Christine Nalepa for providing us with family units of the woodroach *C. punctulatus*, Drs. Rudi Scheffrahn, Vernard Lewis and Micheal Crosland for providing *C. cavifrons* and *I. minor* colonies. Dr. Drion Boucias suggested an important reference. We also thank the administrators of the Redwood East Bay Regional Park District for allowing us to collect *Zootermopsis* colonies and the suggestions of two anonymous reviewers. This research was supported by NSF grant IBN-0116857 to Traniello and Rosengaus.

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