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ORIGINAL ARTICLE

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Disease susceptibility and the adaptive nature of colony demography in the dampwood termite *Zootermopsis angusticollis*

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Abstract The effect of demographic structure on disease resistance was studied in experimental colonies of the dampwood termite *Zootermopsis angusticollis* Hagen. Instar and group demography were found to be significant and independent predictors of susceptibility to infection after termites were exposed to 10^1–10^7 spores/ml suspension of the fungus *Metarhizium anisopliae* (or a spore-free medium) and subsequently maintained in isolation, or in homogeneous and heterogeneous instar groups. Nymphs, which were the oldest and largest individuals, had the highest survivorship. The youngest termites (instars III and IV) had approximately 3.6 and 2.0 times the hazard ratio of death of nymphs and were the most likely to succumb to disease. Termites in instar V had a hazard ratio of death 1.2-fold that of nymphs; termites in instar VI did not differ significantly from nymphs in susceptibility. Analysis of the survivorship patterns of each instar showed that (1) isolated termites exposed to fungal spores were significantly more susceptible to infection than similarly exposed individuals maintained in same-instar social groups, and (2) spore-exposed termites living in same-instar groups were significantly more susceptible to disease than similarly exposed individuals maintained in mixed-instar groups. Survivorship indices of spore-exposed termites in mixed-instar groups were the highest among all experimental treatments, approaching those of unexposed controls. Colony demography thus had a significant influence on individual survivorship and reduced mortality risk. *Z. angusticollis* colony demography results from the asynchronous development of termites which hatch during successive bouts of oviposition. We suggest that the demographic distribution of colony members produced by this overlap of generations reduces disease risk and is a previously unrecognized adaptive role of the caste distribution function.

Keywords Termite · Disease · Division of labor · Social behavior · Caste distribution function · Demography · Polymorphism · *Zootermopsis angusticollis* · *Metarhizium anisopliae*

Introduction

The influences of parasitism and disease will most likely be evident in social insect species with long-lived colonies that face chronic challenges from pathogens coexisting in the environments where they nest and feed. Disease-related selection pressures may influence the caste distribution function and patterns of polyethism as well as other significant aspects of the biology of social insects that enhance colony fitness (Wilson 1971; Hamilton 1987; Hamilton et al. 1990; Rosengaus and Traniello 1993a; Keller 1995; O'Donell 1997; Rosengaus et al. 1998a; Schmid-Hempel 1998; Baer and Schmid-Hempel 1999). Recent theories and comparative analyses suggest that key characteristics of sociality such as polymorphism and patterns of division of labor may be influenced by parasite and pathogen load. These components of social organization may have been favored by the genetic diversification generated by queen number and multiple mating, which adapted colonies to parasite prevalence and lower infection risk (Feener and Moss 1990; Schmid-Hempel and Schmid-Hempel 1993; Schmid-Hempel 1994; Keller 1995; Baer and Schmid-Hempel 1999; Cole and Wiernasz 1999; Schmid-Hempel and Crozier 1999; Villesen et al. 1999).

Previously, we found that dampwood termites, *Zootermopsis angusticollis* Hagen, exposed to the spores of the entomopathogenic fungus *Metarhizium anisopliae* and maintained in social groups have significantly higher survivorship than similarly exposed isolated nestmates (Rosengaus et al. 1998a). Sociality in *Z. angusticollis* benefits individuals through mutual grooming and the

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ability to communicate information about the presence of disease agents within the nest, and confers antibiotic protection from the cumulative use of exudes and glandular secretions in nest construction and maintenance (Rosengaus et al. 1998a, 1998b, 1999a, 2000a, 2000b). Group size is known to have a disease-reducing advantage (Rosengaus et al. 1998a), but does the demographic distribution of group members affect survivorship?

Current theories, which describe the influence of parasitism and disease on the evolution of polymorphism have mainly concerned ant species with discrete physical castes (Feener and Moss 1990; Keller 1995; Schmid-Hempel and Crozier 1999). Termites and ants differ in metamorphic development and caste differentiation and thus in the way in which polymorphism and the caste distribution function are generated. In contrast to the holometabolic ants and other social Hymenoptera, *Z. angusticollis* is hemimetabolic, as are all termites. Multiple bouts of oviposition by a primary queen are interspersed with periods of reproductive inactivity (Castle 1934; Weesner 1969; Greenberg et al. 1978; Thorne 1997), producing an overlap in age and size (instar) among groups of immature offspring which progress asynchronously through their developmental stages. Although colony-level demographic distributions have been analyzed functionally within the paradigm of caste theory and in light of the selection pressures of predation, competition, and resource distribution specified in its models, the influence of disease on the caste distribution function and division of labor in termites and other social insects has remained relatively unexplored (Schmid-Hempel 1998).

In termites, instar, size, and age are correlated, and various phylogenetic groups show patterns of division of labor involving polymorphism and polyethism (Crosland et al. 1998; Traniello and Leuthold 2000). *Z. angusticollis*, a lower termite, exhibits instar (size/age) polymorphism but appears to lack polyethism (Rosengaus and Traniello 1993b). In the present study, we examined the relationship of disease susceptibility and colony demography by manipulating the instar/size distribution of experimental groups of spore-exposed termites maintained in isolation and in homogeneous and heterogeneous instar groups. By comparing the survivorship of termites in these treatments, we found that susceptibility to a fungal disease is dependent upon instar. Moreover, termites in colonies with a heterogeneous demography had a significant advantage in resisting infection.

Methods

Collection and maintenance of termite colonies

Five termite colonies (ranging in size from approximately 500 to 1,500 individuals) were collected from Redwood Park and Roberts Park (East Bay Regional Park District) in Oakland, California. Termites were transferred to covered opaque plastic tubs (50×30×20 cm) lined with moist paper towel and were fed wood from their original nest supplemented with other decayed wood. The colonies were reared at 25°C and 65% relative humidity (RH) and were sprayed periodically with water to maintain high humidity. Termites of instars I and II were not used in our studies because they were scarce and too fragile to handle without causing injury and/or mortality. Instars III, IV, V, and VI were distinguished by their head width, and nymphs by the presence of wing buds (Yin 1972). Although the length of a stadium is variable and dependent on the conditions of both the individual and the entire colony, the duration of each stadium generally increases with development. This pattern is independent of sex (Yin 1972). Stadia duration are from 3 to 4 weeks for young termites to 9 to 10 weeks or longer for mature pseudergates (Yin 1972). Based on Heath (1903) and Yin (1972), we estimated the age of termites in various instars as follows: instar I (1–28 days); instar II (29–63 days); instar III (64–105 days); instar IV (106–154 days); Instar V (155–210 days); instar VI (211–480 days); nymphs (481–780 days). Individuals in instar VI and nymphs used in our experiments were thus more variable in age than younger or intermediate-age termites. Because termites are hemimetabolous, age is correlated with size and physical caste (Crosland et al. 1998), and the terms instar, age, and size are used interchangeably.

Preparation of spore solutions

M. anisopliae was used as a model pathogen (Rosengaus and Traniello 1997; Rosengaus et al. 1998a, 1999a, 1999b, 2000b). Although *M. anisopliae* has not been isolated from *Z. angusticollis* colonies, it is known to occur in colonies of several termites, including species that are sympatric with *Z. angusticollis* (Sands 1969; Ko et al. 1982; Zoberi 1995; Milner et al. 1998). Furthermore, *M. anisopliae* has been isolated from soils in forests inhabited by those sympatric species (Bhatt 1970; Keller and Bidochka 1998).

Spores were harvested from cadavers of termites that had been infected with the fungus *M. anisopliae*. The original source of the spores was American Type Culture Collection, batch 93-09, media 325, ATCC 90448. A detailed description of the preparation of Tween 80 fungal spore stock suspensions is provided by Rosengaus and Traniello (1997) and Rosengaus et al. (1998a). To ensure spore viability, 100 ml of a stock spore solution and seven serial dilutions, each differing by one order of magnitude, were freshly prepared. Spores in samples of the fourth dilution were counted with a hemocytometer to estimate its spore concentration. The concentration of the stock spore-solution was approximately 2×10^8 spores/ml. The final spore solutions used in our experiments contained 2×10^7, 2×10^4, and 2×10^1 spores/ml. Conidia viability was determined by plating 50 µl of the 2×10^4 spores/ml solution on a thin layer of potato dextrose agar (PDA), incubated at 25°C and 65% RH for 18 h. The average percent germination (±SD) recorded at ×400 was 91.2±1.5 (*n*=10 fields of vision). Control termites were exposed to a sporeless 0.1% Tween 80 solution.

Measuring the effect of demographic distribution on disease susceptibility

To determine the influence of an individual's instar and the demographic structure of a group on disease susceptibility, we manipulated group demography and the number of individuals in experimental subcolonies to produce the following three series of treatments using termites in instars III, IV, V, and VI, and nymphs: (1) isolated individuals; (2) homogeneous instar (same-instar) groups; and (3) heterogeneous instar (mixed-instar) groups. The latter social groups were composed of two individuals of each instar. These treatments allowed us to assess the susceptibility of termites in each instar to disease in the absence of social interactions, the role that instar and age/size-related social interactions play in infection control, and the significance of homogeneous and heterogeneous group demography on disease resistance. These assessments could not be answered through field experimentation given the cryptic nature of *Z. angusticollis*. Instead, we established "pseudomutant" colonies that allowed us to study the interaction between colony composition and disease susceptibility while controlling factors that influence disease susceptibility and survival, including pathogen load, temperature and humidity, and the amount and quality of food (Milner et al. 1997; Rosengaus and Traniello 1997). To minimize colony effects on disease susceptibility (Rosengaus et al. 1998a), all termite subgroups originated from the same stock colony. Termites were assumed to be at least siblings. To minimize the effect of spore count variation on survivorship, termites in all replicate treatments were exposed to spores originating from the same solution of conidia. To ensure the homogeneity of each spore suspension, flasks containing the suspensions were thoroughly mixed with a vortex immediately prior to exposing termites to spores.

Mortality risk of isolated termites

Isolating spore-exposed termites of different instars allowed us to determine instar-related physiological mechanisms of disease susceptibility while controlling for the effects of social interaction and colony organization on infection resistance (Rosengaus et al. 1998a). The susceptibility of isolated termites to fungal infection was studied by allowing ten termites of the same instar to walk over filter paper discs (90×10 mm; Whatman No. 5, fine porosity, particle retention >2.5 µm) moistened with 1 ml of either a 2×10^7, 2×10^4, or 2×10^1 spores/ml suspension, or a control solution (spore-free Tween 80) for 1 h (three replicates/instar per spore concentration; n=587 individuals). After exposure, each termite was transferred to a sterile plastic petri dish (60×10 mm) lined with a filter paper disc (Whatman No. 1) moistened with 300 µl of sterile water. These termites were then maintained in isolation. The petri dishes were stacked inside covered plastic boxes lined with moist paper towel and kept in an environmental chamber (25°C and 65% RH). Survivorship was recorded daily for 15 days. Infection by *M. anisopliae* was confirmed after surface sterilization, when termite corpses produced the spores that are diagnostic of this species of fungus (Rosengaus and Traniello 1997).

Survivorship of spore-exposed termites in homogeneous-instar groups

The susceptibility to *M. anisopliae* infection of termites in same-instar groups was determined, as described above, by allowing ten termites of the same instar to walk freely over filter paper discs moistened with 1 ml of a spore suspension of either the 2×10^7, 2×10^4, or 2×10^1 spores/ml or a control solution for 1 h (seven replicates/ instar per spore concentration; n=1,555 individuals). Following exposure, termites were transferred as a group to a new petri dish (90×10 mm) lined with a filter paper disc (Whatman No. 1) moistened with 1 ml of sterile water. The dishes were then stacked inside a covered plastic box lined with moist paper towel and maintained at 25°C and 65% RH. Survivorship was determined from daily censuses for 15 days. Death and cannibalism were recorded during each census. Dead termites were removed daily and following surface sterilization were plated on PDA to confirm the cause of mortality, as described above. Although the size of our experimental colonies was small relative to that of mature colonies in the field, groups of ten individuals approximate the size of colonies during the incipient stages of colony development (Castle 1934; Nutting 1969; Rosengaus and Traniello 1993a). Moreover, this group size allowed us to compare survivorship data with results of previous studies on groups of similar size (Rosengaus et al. 1998a).

Survivorship of spore-exposed termites in heterogeneous-instar groups

The susceptibility to *M. anisopliae* infection of termites in mixed-instar groups was assessed by exposing two individuals in instars III–VI, and nymphs (a total of ten individuals) to either a 2×10^7, 2×10^4, or 2×10^1 spores/ml or control solution following the protocol described above. Four replicates/spore concentration were established (n=860 individuals). Death and cannibalism were recorded during the 15-day census and the cause of mortality was again determined by plating cadavers.

Statistical analysis

Several survival parameters were measured to analyze the relationships among risk of mortality due to infection, instar, and group demography. These parameters included the time course of survival (the survival distribution), percent survivorship at day 15, median survival time (LT_{50}), the spore dosage necessary to produce 50% mortality (LC_{50}), and the hazard ratio of death. All these estimators are complementary and should be evaluated together to provide a more complete understanding of the pathogenicity of *M. anisopliae* (Rosengaus et al. 2000a).

A Cox proportional regression analysis of 3,002 individuals was performed by including in the model the variables instar, spore concentration, colony of origin, and social treatment. The resulting hazard functions obtained characterize the instantaneous rate of death at a particular time, given that the individual survived up to that point, while controlling for the effect on survival of other variables (SPSS 1990; Smith et al. 1994). The hazard functions thus provide a relative measure of the degree of susceptibility of termites exposed to spores in different treatments while simultaneously controlling for the effect of instar, spore concentration, and colony of origin. Similarly, the hazard ratios of death analyzed as a function of instar allow comparisons among instars while controlling for the effects of all other variables included in the model. In this analysis, nymphs had the highest survivorship. Therefore, they were chosen as the reference group to which the survivorship of all other instars was compared.

The median survival time (LT_{50}) and the statistical comparison of the time course of mortality within and between treatments were analyzed with the Breslow statistic (Kaplan-Meier survival test; SPSS). If multiple pairwise comparisons were made, the α-value of significance was adjusted accordingly (Rice 1989). To estimate LC_{50}, survival data were analyzed with a log-transformed Probit analysis (SPSS), which allowed us to determine the strength of the relationship between spore concentration and mortality.

Cannibalism rates

Rates of cannibalism were recorded to measure the instar-related social response to diseased termites. Thus, rates of cannibalism were analyzed as a function of instar and spore concentration with a binomial logistic regression. The odds ratio of each variable in the model was compared with the Wald statistic after controlling for the effect of all other variables. Because death by cannibalism removed an individual from the risk of death by fungal infection, cannibalized individuals could not be considered censored data. Competing risk analysis was applied to obtain specific survival functions and the likelihood of death due to either fungal infection (–2 log likelihood=12,935.8, df=13) or cannibalism (–2 log likelihood=3,333.3, df=13) separately and in combination (–2 log likelihood=16,564.0, df=13; Allison 1995; Fig. 1a–c). Although the –2 log likelihood coefficients and hazard ratios differed significantly across the three models (likelihood ratio χ^2=294.9, df=13, P<0.0001), we believe that, given the disproportionate association between cannibalism and spore-exposure (Fig. 2), the most realistic and biologically meaningful model to understand the effects of *M. anisopliae* on termite survival is one in which mortality was coded as having resulted from both fungal infection and cannibalism. Furthermore, the hazard ratios of death and survival distributions followed very similar patterns when death was coded as due to both cannibalism and death by infection (Fig. 1a) and when death was coded as due to infection alone (i.e., when cannibalized individuals were coded as censored data; Fig. 1b). The first regression model showed that termites nesting in same- and mixed-instar groups were 67% and 85% less susceptible, respectively, than isolated termites (Fig. 1a). In the second model, termites nesting in

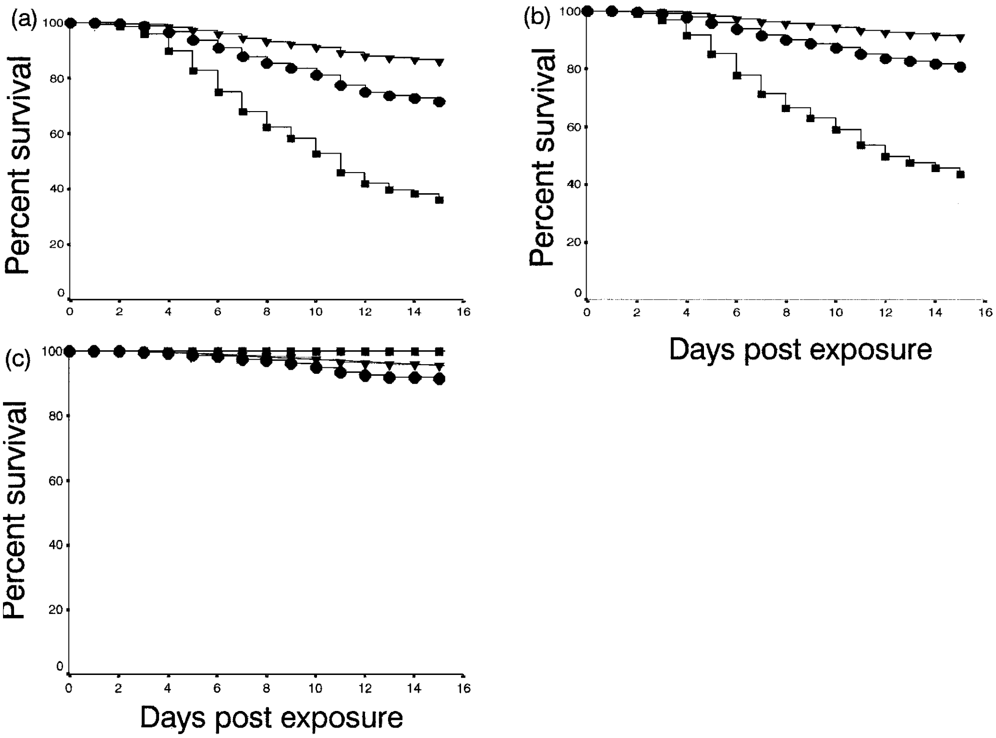


Fig. 1 Survival distributions of termites as a function of isolation and social treatment. In the regression models, mortality was coded as having resulted from both cannibalism and infection (**a**), infection only (**b**), and cannibalism only (**c**). Survival distributions as a function of termite instar also showed similar patterns and hazard rations between models (**a**) and (**b**), but not (**c**) (data not shown) (*squares* isolation treatment, *circles* same-instar groups, *triangles* mixed-instar groups)

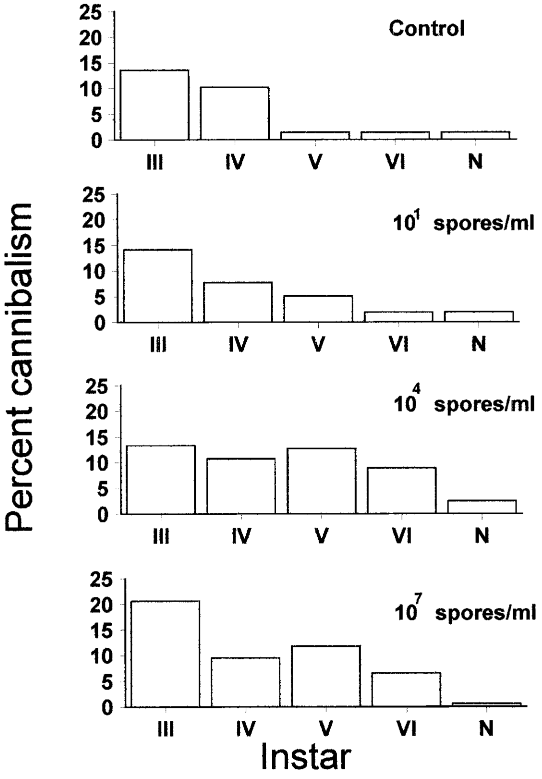


Fig. 2 Cannibalism rates as a function of termite age within each spore concentration. *N* denotes nymphs while *Control* denotes sporeless Tween 80 suspension

same- and mixed-instar groups were 74% and 89% less susceptible, respectively, than isolated termites. In contrast to these models, the regression model in which mortality was considered as a result of cannibalism alone showed that termites in same- and mixed-instar groups had, respectively, 9.8 and 9.1 times the hazard ratio of death compared to termites maintained in isolation (Fig. 1c). Moreover, the survival distributions of the first two models were strikingly different from those in which termites were considered dead only if they were cannibalized (compare Fig. 1c with Fig. 1a, b). All subsequent survival analyses were therefore based on the model that included both cannibalism and infection as causes of mortality.

Results

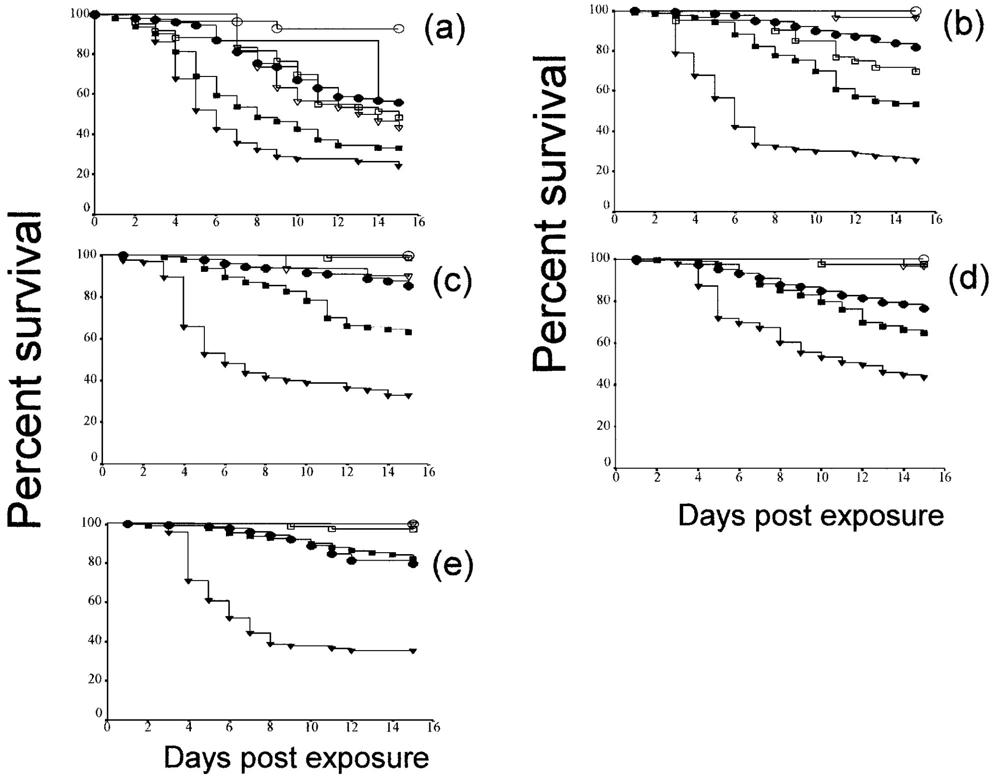
Factors influencing termite survivorship

A Cox proportional regression model showed that spore concentration, instar, colony of origin, and group demography were all significant and independent predictors of termite survival. The effect of each of these variables is discussed, in turn, below.

Spore concentration

After controlling for the effect of all other variables, spore concentration was the most important predictor of termite survival (Wald statistic=685.8, *df*=3, $P<0.0001$). Termites exposed to a 10^1, 10^4, and 10^7 spores/ml had 2.1 (Wald statistic=33.1, *df*=1, $P<0.0001$), 5.5 (Wald statistic=191.2, *df*=1, $P<0.0001$) and 14.0 (Wald statistic=454.3, *df*=1, $P<0.0001$) times the hazard ratio of death of the controls, respectively.

Fig. 3 Survival distributions for control (*open symbols*) and spore-exposed (*filled symbols*) termites nesting either in isolation (*triangles*), single-instar groups (*squares*), and mixed-instar groups (*circles*) of instars III (**a**), IV (**b**), V (**c**), VI (**d**), and nymphs (**e**)



Instar

Instar was a significant predictor of survival (Wald statistic=260.4, *df*=4, *P*<0.0001). After controlling for the effect of all other variables, termites in instars III and IV were the most susceptible to infection, having 3.6 (Wald statistic=170.6, *df*=1, *P*<0.0001) and 1.9 (Wald statistic=42.0, *df*=1, *P*<0.0001) times the hazard ratio of death of nymphs, respectively. Termites in instar V had 1.2 times the hazard ratio of nymphs (Wald statistic=4.1, *df*=1, *P*<0.05), whereas termites in instar VI did not differ significantly from nymphs (Wald statistic=0.3, *df*=1, *P*>0.05). The higher susceptibility of younger-instar larvae was also evident when cannibalized individuals were censored (i.e., not considered dead). The hazard ratios of death for each instar relative to nymphs (3.0 for instar III, 1.7 for instar IV, 1.0 for instar V, and 0.9 for instar VI) were very similar to those generated when cannibalism was not censored (see Methods).

Colony of origin

Susceptibility of termites to *M. anisopliae* infection differed significantly according to colony of origin (Wald statistic=113.8, *df*=4, *P*<0.0001). After controlling for the effect of all other variables, colonies had hazard ratios ranging from 1.2 to 4.5 times that of the reference colony, which had the lowest susceptibility. All pairwise comparisons among colonies were significantly different (*P*<0.005 due to multiple comparisons, Cox proportional regression model, SPSS) with the exception of one colony (*P*=0.01).

Group demography

The demographic composition of subcolonies was a significant and independent predictor of termite survival (Wald statistic=228.9, *df*=2, *P*<0.0001). Relative to mixed-instar groups, which were the least susceptible to infection, same-instar groups and isolated termites had 2.2 (Wald statistic=96.3, *df*=1, *P*<0.0001) and 6.7 (Wald statistic=198.9, *df*=1, *P*<0.0001) times the hazard ratio of death, respectively.

Effects of isolation and social treatment on instar-related disease susceptibility

Disease susceptibility of isolated termites

Because termites maintained in isolation could not be cannibalized or interact socially, differences in their survival depended only on fungal pathology and the physiological response to infection of individuals in each instar. Third-instar larvae were particularly susceptible to fungal infection; roughly 60% of their susceptibility was explained by their isolation (see open triangles in Fig. 3a, Table 1). Despite the lower survival distributions of isolated termites in instars III and IV relative to other instars, significant differences in survivorship were only

Table 1 Survivorship parameters for the developmental stages of *Zootermopsis angusticollis* as a function of spore exposure, isolation, and demographic organization. Median (±SE) survival time (LT$_{50}$) (in days), percent survival 15 days post-exposure, LC$_{50}$ values [95% confidence interval (CI)], and significance of overall comparison of survival distributions across instars within each treatment are presented. > indicates that the LT$_{50}$ extended beyond the census period. LC$_{50}$ values and 95% confidence intervals were obtained by Probit analysis. The models built for each spore-exposed treatment fitted the data adequately (all Pearson goodness-of-fit tests were not significant at $P \geq 0.5$ and, therefore, no heterogeneity factors were used in the calculation of the 95% CI). The regression lines for each instar within a treatment were parallel with the exception of the isolation treatment ($\chi^2=147.6$, $df=4$, $P<0.0001$). The LC$_{50}$ and 95% CI for the controls were not calculated separately; instead, the survivorship data of controls was included in the Probit analysis of spore-exposed termites

Treatment		Instar					P[a]
		III	IV	V	VI	Nymph	
Control							
Isolation ▽	LT$_{50}$	13.0±3.4[c]	>15.0[b]	>15.0[b]	>15.0[b]	>15.0[e]	***
	Percent survival	43	97	90	97	100	
Same-instar □	LT$_{50}$	15.0±2.6[c]	>15.0[c]	>15.0[b]	>15.0[b]	>15.0[b]	***
	Percent survival	45%	68	99	97	96	
Mixed-instar[d] ○	LT$_{50}$	>15.0[b]	>15.0[b]	>15.0[b]	>15.0[b]	>15.0[b]	NS
	Percent survival	93	100	96	96	100	
Spore-exposed							
Isolation ▼	LT$_{50}$	6.0±0.4[c]	6.0±0.4[c]	6.0±0.7[c]	12.0±2.6[c]	7.0±0.6[c]	**
	Percent survival	24	26	33	43	36	
	LC$_{50}$	3.5×10^{1}	5.3×10^{1}	1.7×10^{2}	2.1×10^{3}	6.0×10^{2}	
	(95% CI)	(1.3×10^{1}–1.0×10^{2})	(1.8×10^{1}–1.6×10^{2})	(5.6×10^{1}–5.6×10^{2})	(6.8×10^{2}–6.1×10^{3})	(1.8×10^{2}–1.9×10^{3})	
Same-instar ■	LT$_{50}$	8.0±0.8[c]	>15.0[c]	>15.0±0.6[c]	>15.0±0.6[c]	>15.0[b]	***
	Percent survival	33	50	61	58	76	
	LC$_{50}$	2.0×10^{1}	1.0×10^{4}	6.2×10^{5}	1.8×10^{5}	2.9×10^{8}	
	(95% CI)	(1.4×10^{0}–1.9×10^{2})	(1.2×10^{3}–9.5×10^{4})	(6.8×10^{4}–7.0×10^{6})	(2.2×10^{4}–1.7×10^{6})	(2.2×10^{7}–7.3×10^{9})	
Mixed-instar ●	LT$_{50}$	>15.0[c]	>15.0[c]	>15.0[b]	>15.0[c]	>15.0[c]	***
	Percent survival	48	71	78	69	62	
	LC$_{50}$	3.0×10^{3}	1.2×10^{9}	2.3×10^{11}	4.8×10^{8}	1.2×10^{7}	
	(95% CI)	(2.1×10^{1}–3.0×10^{5})	(7.0×10^{6}–8.1×10^{12})	(5.0×10^{8}–1.7×10^{16})	(3.2×10^{6}–1.8×10^{12})	(1.2×10^{5}–9.5×10^{9})	

[a] ** and *** denote significance in the overall comparison of survival distributions among instars within a treatments at $P<0.005$ and $P\leq0.0005$, respectively, by the Breslow statistic, $df=4$, Kaplan-Meier survival test, SPSS (1990). *NS* indicates no significant differences.

[b] and [c] indicate significant pairwise comparisons within each instar between each social treatment and its corresponding control mixed-instar group ([d] the reference treatment). These pairwise comparisons should be viewed in conjunction with Fig. 3a–e

[e] Pairwise comparison not possible because of the 100% survivorship in this group

observed between instars III and VI ($P<0.0001$, Breslow statistic=21.7) and instars IV and VI ($P=0.001$, Breslow statistic=9.6; Fig. 4a). No significant pairwise differences were found in the survival distributions of isolated instars V, VI, or nymphs ($P>0.005$, Breslow statistic, Kaplan Meier survival test, SPSS; Fig. 4a). We confirmed that *M. anisopliae* was the cause of death of isolated termites in 89.7–100% of the cases ($n=318$ deaths across concentrations). There was no apparent instar-related bias in the confirmation rates.

Spore-exposed termites of all instars maintained in groups, whether in demographically homogeneous or heterogeneous subnests, consistently had higher survivorship than spore-exposed isolated termites of the same instar (Fig. 3a–e, Table 1). Social contact thus lowered the susceptibility to infection of all termites, independent of instar.

Disease susceptibility in demographically homogeneous groups

The susceptibility to infection of termites maintained in same-instar groups was dependent upon instar (Fig. 4b, Table 1). Third-instar larvae were again found to be significantly more susceptible to infection than termites in other instars: they exhibited the lowest survival distribution (Fig. 4b), percent survival on day 15 post-exposure and values of LT$_{50}$ and LC$_{50}$ (Table 1). Although instars IV, V, VI, and nymphs were equivalent in median survival, percent survivorship at the end of the census period and the survival distributions of each instar were significantly greater with increasing age/size, with the exception of instars V and VI (Fig. 4b, Table 1). These results suggest that the social interactions of termites in homogeneous groups composed of instars III and IV, in contrast to older instars, are insufficient to overcome the pathological effects of *M. anisopliae* infection. Death by

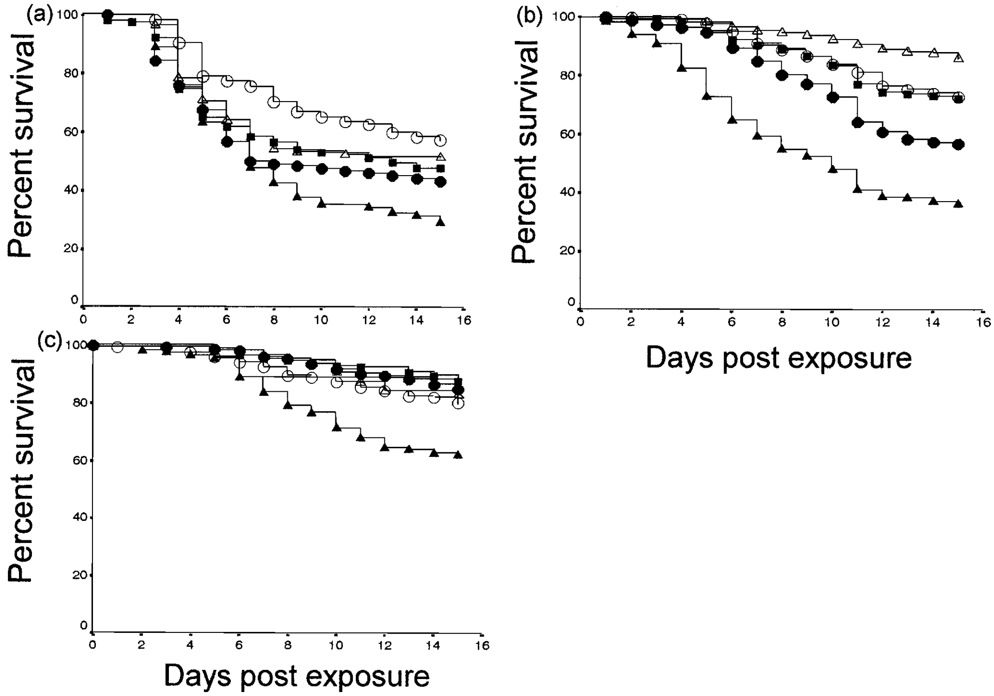


Fig. 4 Survival distributions for instars (*closed triangles* III, *closed circles* IV, *closed squares* V, *open circles* VI, *open triangles* nymph) as a function of social treatment: isolation (**a**), same-instar groups (**b**), and mixed-instar groups (**c**). The distributions are corrected for control mortality

M. anisopliae in demographically homogeneous groups was confirmed in 62.5–100% of cases (n=423 deaths across concentrations excluding cannibalized individuals), with no apparent instar bias.

Disease susceptibility in demographically heterogeneous groups

With the exception of instar III, all termites in mixed-instar groups had similar survivorship. Although LT_{50} values across all instars exceeded 15 days, the survival distribution of termites in instar III was significantly lower than those of instars IV, V, VI, and nymphs (Fig. 4c). No other paired comparison was significantly different (Fig. 4c). The percent survivorship at 15 days post-exposure for instars IV, V, VI, and nymphs ranged from 69 to 78%, which was higher than the survivorship of termites in instar III (48%) at the end of the census period (Table 1). The LC_{50} values increased dramatically from 3.0×10^3 spores/ml for termites in instar III to greater than 1×10^7 for termites in all other instars (Table 1).

Generally, termites in groups with mixed demographies had greater survivorship, as measured by all survival indices, relative to groups of termites of the same instar (Fig. 3a–c, Table 1). The exception was the insignificant difference in the survival distribution, and the similar LT_{50} values and percent survival 15 days post-exposure of instar VI larvae and nymphs in same- and mixed-instar groups (Fig. 3d, e and Table 1). This suggests that social contact among nestmates of different developmental stages benefits relatively young and intermediate-age individuals (instars III, IV, and V). The LC_{50} values of instars IV and V, which were higher relative to those of instars VI and nymphs in both homogeneous and heterogeneous groups, again indicates that termites in instars IV and V are at a developmental stage which appears to benefit most from nesting with older individuals. Death by *M. anisopliae* in demographically heterogeneous groups was confirmed in 42.9–100% of cases (n=182 deaths across concentrations excluding cannibalized individuals), with no apparent instar bias.

Cannibalism

Cannibalism generally occurred when termites were moribund but not yet dead. The frequency of cannibalism did not differ between treatments involving different demographic distributions (Wald statistic=4.9, df=1, P>0.01 due to multiple comparisons). Therefore, subsequent analyses of the effect of cannibalism were based on pooled data. Instar was the most important independent predictor of cannibalism (Wald statistic=73.4, df=4, P=0.003). After controlling for the effect of spore concentration, termites in instars III, IV, V, and VI had 11.0, 6.2, 5.1, and 3.0 times the probability of being cannibalized, respectively, of nymphs (all pairwise comparisons P<0.0001, df=1; Fig. 2). Spore concentration was also a significant and independent predictor of cannibalism, although its influence was less important than instar (Wald statistic=15.9, P=0.001, df=3). After controlling for the effect of instar, cannibalism in termites exposed to a 10^1 spores/ml suspension was not significantly greater than in controls (Wald statistic=0.3, df=1, P=0.6). In contrast, termites exposed to suspensions of 10^4 and 10^7 spores/ml had approximately twice the probability of being cannibalized, relative to control termites (Wald statistic=8.6 and 9.1, *df*=1, *P*≤0.003; Fig. 2).

Discussion

The adaptive nature of the caste distribution function of social insect colonies has traditionally been analyzed in reference to its role in division of labor and the optimization of ergonomic efficiency (Oster and Wilson 1978; Hölldobler and Wilson 1990; reviewed in Schmid-Hempel 1992; Traniello 1997) and polymorphism has almost invariably been linked to a process of task partitioning that has ecological significance (Hölldobler and Wilson 1990; but see Beshers and Traniello 1994, 1996). Environmental factors thought to favor adaptive colony demography include predation, the size-frequency distribution of food resources, and others related to foraging, competitive interactions at the community level, and size-dependent physiological requirements associated with overwintering (Calabi and Traniello 1989; reviewed by Schmid-Hempel 1992; Tschinkel 1993; Beshers and Traniello 1994; Passera et al. 1996; Traniello 1997; Traniello and Leuthold 2000). The etiology of social insect disease has only recently been incorporated into models of division of labor (Schmid-Hempel and Schmid-Hempel 1993; Schmid-Hempel 1998) and evaluated as a selective force in the evolution of caste polymorphism (Keller 1995). The nesting and feeding ecology of the dampwood termite *Z. angusticollis* favor the exposure of colony members to a great variety of microbes including fungi, bacteria, protozoa, viruses, spirochetes, and nematodes (Rosengaus et al. 1998b). We have quantified the cuticular microbial loads of *Z. angusticollis* nymphs as well as the microbial richness of the nest material itself. We cultured an average of 350.4±28.2 colony-forming units (CFUs)/20 µl, (of which 183.2±19.8 were fungal in origin) from cuticular washes of individual termites (R.B. Rosengaus, J.F.A. Traniello, J. Moustakas, unpublished data). In addition, we cultured 824.3±412.0 CFUs/20 µl from the nest material (Rosengaus et al., unpublished data). These results demonstrate that *Z. angusticollis* does indeed live in contact with an abundant microbial community.

Our research demonstrates a relationship between instar, colony demography, and susceptibility to infection, and suggests a significant pathogen-related role for the caste distribution function. Instar-related differences in the composition of the larval cuticle (Charnley 1984; Wood and Grula 1984; St. Leger et al. 1988; Boucacia and Pendland 1991; Khan et al. 1993; Susilo et al. 1994) and/or the immunocompetence of the various developmental stages (Rheins and Karp 1985; Rosengaus et al. 1999b) and behavior (Rosengaus et al. 1998a) may render older instars more resistant to *M. anisopliae* infection than younger nestmates. This may explain why mixed-instar groups had a lower susceptibility to fungal disease. If the anatomical and/or physiological characteristics of older individuals retard the onset of disease symptoms, then more mature instars, including nymphs, may behaviorally and/or biochemically reduce disease risk for longer periods of time. This could lower infection risk in younger nestmates. Furthermore, although *Z. angusticollis* colonies seem to lack age-based division of labor (Rosengaus and Traniello 1993b), termites of different instars may alter their behavior in the presence of pathogens. Older larvae and nymphs might engage in significantly higher rates of allogrooming when exposed to a fungal pathogen, thus reducing the cuticular spore loads of younger nestmates (Oi and Pereira 1993; Rosengaus et al. 1998a, 2000a). Adaptive changes in foraging behavior in the presence of parasites have been reported for several ant genera (Feener 1988; Feener and Brown 1992; Orr 1992; Porter et al. 1995; but see Poulin 1995). A similar context-dependent behavioral response to pathogens in *Z. angusticollis* could explain the apparent asymmetry in the infection-reducing benefits gained by termites living in multi-instar groups. Termites in instars III, IV, and V in mixed-instar groups have greater survivorship relative to same-instar groups, but termites in instar VI and nymphs maintained in mixed-instar groups are as susceptible as individuals in instar VI and nymphs maintained in same-instar groups (Fig. 3a–e). Younger termites maintained in homogeneous groups may not allogroom as effectively as their older nestmates, perhaps elevating the disease risk of the entire group. Because isolated termites can behaviorally reduce their cuticular spore loads only through selfgrooming, our finding that susceptibility to fungal infection was the highest in solitary individuals is not surprising.

In addition to allogrooming, cannibalism appears to reduce the colony-level risk of disease transmission. Cannibalism is prevalent in termites and has been described as a means to supplement nitrogen (O'Brien and Slaytor 1982; Lenz 1994; Shellman-Reeve 1994, 1997; Curtis and Waller 1997) and as a social behavior which eliminates dead or injured individuals from a colony (Wilson 1971; Visscher 1983). Our results indicate a significant association between cannibalism, instar, and infection risk. Instar appears to be the principal determinant of cannibalism, but lethal pathogen loads also increase its prevalence, particularly for infected termites in instars III and IV. The disproportionate cannibalism of younger instars may be explained by an instar-related physiological susceptibility to *M. anisopliae* infection (see above). Cannibalism directed at younger instars may reduce the likelihood of disease transmission in a colony although its role could be restricted to lowering the incidence of fungal infection rather than infection in general. *M. anisopliae* does not invade a host through the gut (Kramm and West 1982). Therefore, the potential costs of spreading a fungal infection through cannibalism should be minimal because passage through the alimentary tract may inactivate fungal spores (Dillon and Charnley 1991). The significant association between cannibalism and exposure to lethal spore concentrations contrasts with results from other studies in which sporulating dead termites were isolated by burial rather than eliminated by cannibalism (Kramm et al. 1982; Logan et al. 1990; Zoberi and Grace 1990). Our results do not support the hypothesis that pathogen transmission is a selective force against cannibalism (Pfennig et al. 1998). In *Z. angusticollis*, cannibalism could accomplish the same disease-reducing function as the removal of dead workers by undertaker bees (Visscher 1983).

The evolution of the demographic distribution created by the overlap of queen(s) and workers has been described in terms of the genetic and ecological factors that might influence the benefits and costs of associating in stable groups composed of more than a single generation of adults (reviews in Bourke and Franks 1995; Crozier and Pamilo 1996). A survivorship and fitness advantage has been ascribed to this aspect of colony demography in haplodiploid species (Queller 1989; Gadagkar 1990). Our study demonstrates in the diploid termites that a heterogeneous demographic distribution of offspring can improve the realization of such fitness benefits through the reduction of disease susceptibility. *Z. angusticollis* and other dampwood termites nest in groups and must cope with direct and social exposure to disease agents (Hendee 1934; Sands 1969; Rosengaus et al. 1998a and references therein). Primary reproductives are susceptible to infections transmitted from mates (Rosengaus and Traniello 1993a; Rosengaus et al. 2000a); the presence of larvae hatched from the first clutch of eggs may assist parents in lowering disease risk if immatures remove fungal spores, bacteria, and other microbes during bouts of allogrooming. Previously, we showed that a relatively small increase in group size significantly reduces disease susceptibility in *Z. angusticollis* and that allogrooming appears to have a primary role in lowering individual susceptibility to infection (Rosengaus et al. 1998a). The multiple bouts of oviposition of a *Z. angusticollis* primary queen yields a multiage (multi-instar) demography resulting from the temporal overlap of immature offspring which hatch from consecutive broods (Heath 1903; Yin 1972; Thorne 1997). We have also shown that the caste composition of groups of *Nasutitermes*, a higher termite, is a significant and independent predictor of survival (Rosengaus et al. 2000b). Here we suggest that the generational overlap between the queen and her offspring as well as the overlap among the offspring of successive broods may increase colony fitness by increasing disease resistance.

Disease risk, which has been considered an inherent cost of group living (Freeland 1976, 1979; Hochberg 1991; Lipsitch et al. 1995; Myers and Rothman 1995), may have acted separately or in consort with other environmental factors to promote the evolution of polymorphism and social organization. In the lower termites, disease-related selection for polymorphism may have been independent of selection for colony-level efficiency in task performance. In this scenario, the microbial ecology of a termite nest may have favored the evolution of adaptive size/age heterogeneity. Alternatively, nutritional constraints on reproduction and hemimetabolic development may have generated a colony demography which indirectly had the effect of increasing colony-level disease resistance.

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