

# Susceptibility of Seven Termite Species (Isoptera) to the Entomopathogenic Fungus *Metarhizium anisopliae*

by

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## ABSTRACT

Seven termite species (Isoptera) from five families were tested for disease susceptibility against the entomopathogenic fungus *Metarhizium anisopliae* using a standard protocol: *Mastotermes darwiniensis* (Mastotermitidae), *Hodotermopsis sjoestedti* (Termopsidae), *Hodotermes mossambicus* (Hodotermitidae), *Kalotermes flavicollis* (Kalotermitidae), *Reticulitermes flavipes* and *Prorhinotermes canalifrons* (Rhinotermitidae), and *Nasutitermes voeltzkowi* (Termitidae). Our results showed a large diversity in disease susceptibility against *M. anisopliae* among the different species tested and we suggest that the evolution of disease resistance mechanisms in Isoptera may be influenced by the selective pressure of the nesting ecology of each species.

**Keywords:** termite, *Metarhizium anisopliae*, disease resistance, pathogen, nesting ecology.

## INTRODUCTION

Termites (Isoptera) consist of more than 2,600 described species, from seven families (Abe *et al.* 2000) and are a sister taxa to wood roaches (Inward *et al.* 2007a; Klass *et al.* 2008). Although all Isoptera are eusocial and share common traits, termites have spread and adapted to a large range of habitats resulting in high diversity of morphology, physiology, behavior, nesting ecology, and associated microbial community (Abe *et al.* 2000).

The interaction between termites and their potential pathogens has received particular attention in two distinct fields of research. The first is the use of a pathogenic agent for biological control against various pest species, mainly in

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the Rhinotermitidae (Grace 1997; Culliney & Grace 2000). Several laboratory assays have shown that termites are susceptible to some pathogenic fungi, nematodes, or bacteria, but their field trials showed no or limited efficacy to control a termite colony (Rath 2000). The second is the use of termites as a model for understanding the evolution of disease resistance of eusocial insects in an environment that can favor microbial growth and increase the risk of epizootics (Rosengaus *et al.* 1998a). *Metarhizium anisopliae* has probably been the most studied microorganism in both fields of research (Rath 2000). *Metarhizium anisopliae* is a soil fungus commonly found worldwide in tropical, sub-tropical, temperate, and near-arctic regions of the globe (Bidochka & Small 2005). Although *M. anisopliae* has the capability to survive and persist in the soil (Milner *et al.* 2003; St. Leger 2008; Chouvenc *et al.* 2008a), it is considered to be a generalist pathogen, and has been tested against a wide range of insects such as locusts, moths, beetles (Shah & Pell 2003), flies (Davidson & Chandler 2005), cockroaches (Patchamuthu *et al.* 1999), termites (Milner 2000) and other arthropods including ticks (Arruda *et al.* 2005). Some strains are considered more specifically virulent than others (Fargues *et al.* 1976; Bridge *et al.* 1997; Milner *et al.* 1998a).

In regard to termites' susceptibility, due to their ecological success and diversity, theoretical questions have been formulated about how termites evolved to resist disease from a solitary life of the wood roach-like ancestor to eusociality in an environment with intense selective pressure by pathogens (Fefferman *et al.* 2007). Disease resistance mechanisms in social insects have recently received particular attention (Cremer *et al.* 2007). In Isoptera, these include mechanisms such as grooming behavior (Rosengaus *et al.* 1998a; Yanagawa & Shimizu 2007), alarm behavior (Rosengaus *et al.* 1999a; Myles 2002a), avoidance of cadavers (Milner *et al.* 1998a), necrophagy (Myles 2002b), burial of infected cadavers (Jones *et al.* 1996), volatile chemicals in the nest (Wright *et al.* 2000; Rosengaus *et al.* 2000, 2004), colony demography (Rosengaus & Traniello 2001), immune response (Rosengaus *et al.* 1999b, 2007; Lamberty *et al.* 2001; Thompson *et al.* 2003; Bulmer & Crozier 2004, Xu *et al.* 2009; Chouvenc *et al.* 2009a) antimicrobial compounds produced by termite's gut (Rosengaus *et al.* 1998b; Siderhurst *et al.* 2005; Chouvenc *et al.* 2008b, 2009b), interactions with microorganisms (Rosengaus *et al.* 2003), and nest architecture (Pie *et al.* 2004). These studies provided valuable information

about independent potential defense mechanisms; however, it is unknown how do all these factors interact with each other in natural conditions, and more importantly, how did they evolve within the Isopteran lineage.

A comparative analysis of termite disease susceptibility relative to phylogeny could provide critical information for understanding the evolution of disease resistance in this group. However, as suggested by Fefferman *et al.* (2007), such an analysis is rendered difficult by the diversity of the nesting and foraging ecology that accompanied social evolution, with differences in selective pressures during adaptive radiation from a solitary wood roach-like ancestor to eusocial termites. Previously, termite susceptibility against several strains of *M. anisopliae* was tested for a wide range of species in different studies (Kramm *et al.* 1982; Lai *et al.* 1982; Hänel & Watson 1983; Milner *et al.* 1998b; Delate *et al.* 1995; Zoberi 1995; Rath & Tidbury 1996; Rosengaus & Traniello 1997; Myles 2002b; Milner 2003; Neves & Alves 2004; Wright *et al.* 2005; Dong *et al.* 2007; Maketon *et al.* 2007). Due to the variability of the fungal strain used, experimental conditions, and pathogen inoculation protocols, it is impossible to compare the results of these different studies and to interpret the variability of disease susceptibility among termite species.

In the present study, we tested the susceptibility of seven termite species to a single strain of *M. anisopliae* under standardized conditions. Based on the results, we discuss the potential role of nesting ecology as a selective agent on the evolution of disease resistance mechanisms against soil fungal pathogens within the Isoptera.

## MATERIALS AND METHODS

### Termite species

All termites used in the experiments were collected from the laboratory colonies maintained at the Université de Bourgogne in Dijon, France, for several years. Termite species were chosen to obtain a representative spectrum of the inferred phylogeny (Fig. 1), the geographical origin and the nesting ecology (Table 1). The seven termite species used in our study (Fig. 2) were, *Mastotermes darwiniensis* Froggatt (Mastotermitidae), *Hodotermopsis sjoestedti* Holmgren (Termopsidae), *Hodotermes mossambicus* (Hagen) (Hodotermitidae), *Kalotermes flavicollis* (Fabricius) (Kalotermitidae), *Reticulitermes flavipes* (Kollar) (syn. *R. santonensis*, cf. Brugerolle & Bordereau 2006), and

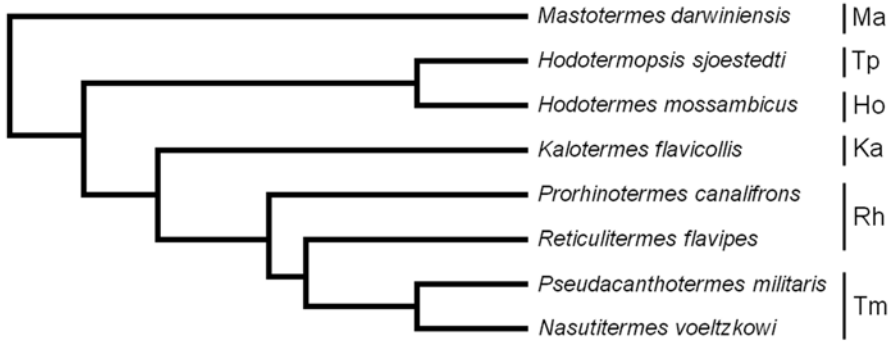


Fig.1. Simplified phylogeny of Isoptera inferred from simultaneous analysis of molecular and morphological data, modified from Inward *et al.* (2007b). Ma=Mastotermitidae, Tp=Termopsidae, Ho=Hodotermitidae, Ka=Kalotermitidae, Rh=Rhinotermitidae, Tm=Termitidae.

Table 1. Ecological characteristics of the termite species

Species	T°C <sup>a</sup>	RH <sup>a</sup>	Life habitat <sup>b</sup>	Nesting type <sup>b</sup>	Origin <sup>c</sup>
<i>Mastotermes darwiniensis</i>	30°C	90%	Subterranean	Intermediate	Australia
<i>Hodotermopsis sjoestedti</i>	25°C	75%	Dampwood	One piece	Vietnam
<i>Hodotermes mossambicus</i>	28°C	40%	Subterranean sub-arid	Separate	South Africa
<i>Kaloterme flavicollis</i>	25°C	40%	Drywood	One piece	France
<i>Prorhinotermes canalifrons</i>	28°C	75%	Subterranean	Intermediate	Reunion Island
<i>Reticulitermes flavipes</i>	22°C	75%	Subterranean temperate	Intermediate	France
<i>Nasutitermes voeltzkowi</i>	28°C	75%	Arboreal	Separate	Mauritius island

<sup>a</sup>average temperature and relative humidity (RH) used in the laboratory. <sup>b</sup> according to Abe (1987), modified. <sup>c</sup> area where the original colony was collected.

*Prorhinotermes canalifrons* (Sjöstedt) (Rhinotermitidae), and *Nasutitermes voeltzkowi* (Wasmann) (Termitidae). Due to the difficulties of obtaining and maintaining termite colonies from such a diverse geographical repartition, we were only able to perform our experiments on a single colony for each species. Termites were collected from their respective colony and kept for 1h in groups of 20 individuals in a Petri dish with moistened filter paper at

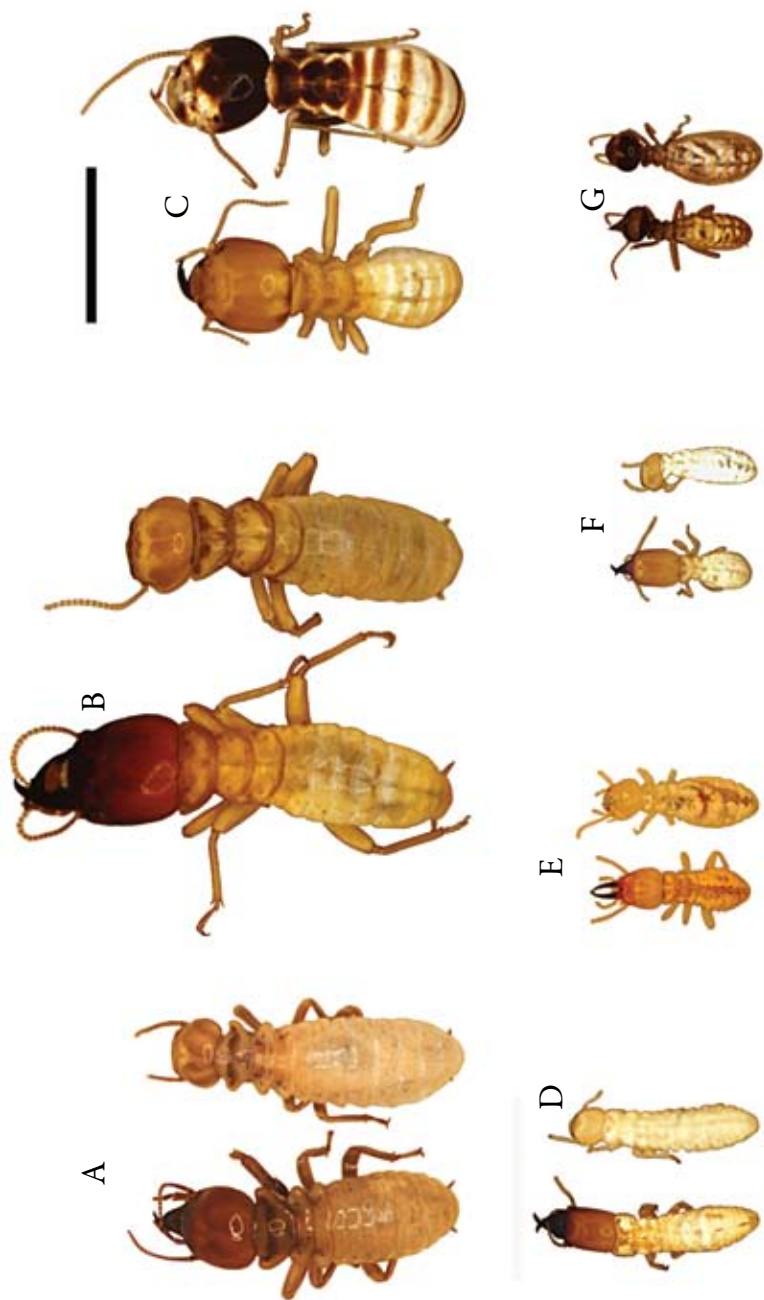


Fig. 2. The seven termite species tested for susceptibility against infection of *Metarhizium anisopliae*. (A) *Mastotermes darwiniensis*, (B) *Hodotermopsis sjoestedti*, (C) *Hodotermes mossambicus*, (D) *Kaloterms flavicollis*, (E) *Probinotermes canalifrons*, (F) *Reticulitermes canalifrons*, (G) *Nasutitermes voelzkowii*. Scale bar = 5mm.

25°C with 75% relative humidity (RH) before treatments. For each species, the caste ratio (workers/soldiers) was adjusted among the 20 individuals, according to the colony of origin so as to provide all the castes that may be involved in disease resistance. The caste ratios used were, *M. darwiniensis* (16 workers + 4 soldiers), *Hodotermopsis sjoestedti* (18 workers + 2 soldiers), *Hodotermes mossambicus* (10 large workers + 8 small workers + 2 soldiers), *K. flavicollis* (20 workers, no soldiers found), *R. flavipes* (19 workers + 1 soldier), *P. canalifrons* (18 workers + 2 soldiers) and *N. voeltzkowi* (16 workers + 4 soldiers). The average wet weight of each species was also measured.

### Preparation of conidial suspensions and susceptibility test

The *M. anisopliae* strain used in this study was American Type Culture Collection 90448. Conidia were spread for germination on 1/5 strength potato dextrose agar (1/5 PDA) and incubated at 27°C in the dark. After 48h, single-spore colonies were transferred to 1/5 PDA plates containing

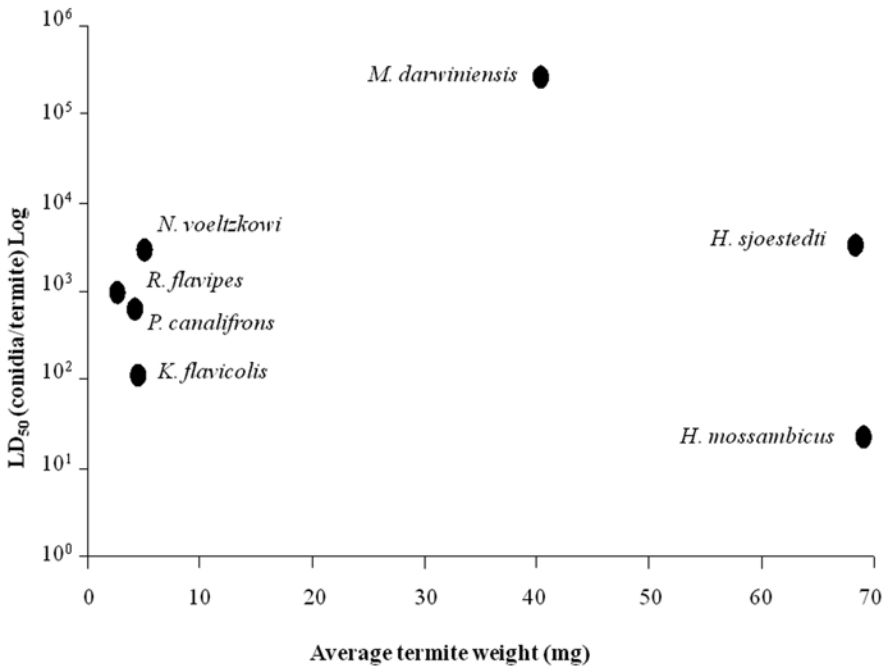


Fig. 3. Relationship between the average termite weight of the individual termites and their susceptibility to *M. anisopliae* exposure. No significant correlation was found.

one *R. flavipes* worker, previously killed and surface-sterilized for 1 h in a vial saturated with chloroform vapors. Inoculated plates were then incubated at 27°C for 14 d in the dark. Fresh conidia were harvested from these plates with a 0.1% Tween 80 solution, and a stock suspension of 10<sup>8</sup> conidia ml<sup>-1</sup> was prepared. Conidia stock suspensions were stored at 4°C before serial dilutions for the susceptibility test.

Seven conidia concentrations in 0.1% Tween 80 aqueous solution were tested for each termite species: 0 (control), 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup>, and 10<sup>8</sup> conidia/ml. Termites were chilled to 4°C for 20 min and individually treated on the dorsum with a 1µl droplet of solution to inoculate 0, 1, 10, 100, 1,000, 10,000, and 100,000 conidia per termite, respectively. Termites were then returned to 4°C for 20 min in order to prevent immediate grooming. Groups of 20 treated termites that received the same treatment were placed in a Petri dish provisioned with moistened filter paper. All Petri dishes containing *Hodotermes mossambicus* received fragments of oven-dried hay, while other species were provided filter paper as a food source. In addition to the seven treatments, groups of 20 termites that didn't receive any treatment (no

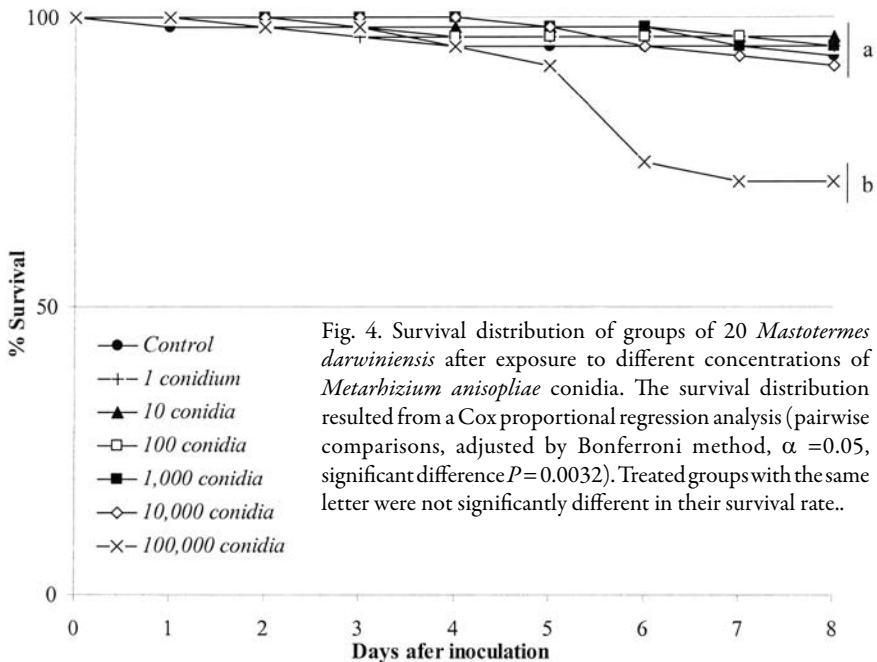


Fig. 4. Survival distribution of groups of 20 *Mastotermes darwiniensis* after exposure to different concentrations of *Metarbizium anisopliae* conidia. The survival distribution resulted from a Cox proportional regression analysis (pairwise comparisons, adjusted by Bonferroni method,  $\alpha = 0.05$ , significant difference  $P = 0.0032$ ). Treated groups with the same letter were not significantly different in their survival rate..

Tween80 solution, no cold exposure = naive termites) were prepared for each species and provisioned as above. Petri dishes were stored at 25°C with 75% RH for 8 d (all experiments were performed in these unique temperature and relative humidity conditions to avoid a different germination and growth rate of the fungus). Mortality was recorded and dead termites were also removed daily, for a total period of 8 days. Each of eight treatments (seven treatments + naive) consisted of three replicates of 20 termites each.

### **Statistical analysis**

For each species, the effective lethal time was achieved for all concentrations on the 8<sup>th</sup> d, mortality was corrected using Abbott's formula, and the LD<sub>50</sub> and their 95% fiducial limits (FL) were determined by Probit analysis (SAS Institute, 2002). The LD<sub>50</sub> is the median lethal dose required to kill 50% of the individuals in the tested population and it was considered that there were no significant differences of mortality when the 95% FL overlapped. To confirm the difference of mortality between species, a Cox proportional-hazard regression analysis (using the program R-Project for statistical computing, version 2.4; <http://cran.r-project.org/>) was performed to calculate the differential death rate of each species. Pairwise comparison of the death rates were adjusted by the Bonferroni Method ( $\alpha = 0.05$ ). In addition, for each species, a Cox proportional-hazard regression was performed to estimate the effect of the cold treatment and the Tween 80 solution treatment, and also to determine the effect of each conidial concentration exposure on the termite survival. Through the analysis the Wald statistic was generated and the resulting hazard function defines the instantaneous rate of death at a particular time, while controlling for the effects of other variables on survival.

## **RESULTS**

### **Naive termites and control termites survival**

Independent of the termite species tested, there were no significant differences in survival rates between the naive termites and the control termites (Wald statistic = 1.12,  $df = 1$ ,  $P = 0.29$ ) showing that the treatment with the Tween 80 solution combined with the time spent at 4°C had no significant effect on the termite survival for all species tested. Mortality observed in the termites treated with a conidia-free 0.1% Tween 80 solution (control) was used



Table 2. Median lethal dose for seven termite species exposed to *Metarhizium anisopliae* conidia

Species	<i>n</i> <sup>a</sup>	LD <sub>50</sub> <sup>b</sup>	(95% FL) <sup>b</sup>	Slope ± SE	χ <sup>2</sup>
<i>Mastotermes darwiniensis</i>	360	256,032	(126,353–1,106,424)	1.87 ± 0.41	20.60
<i>Hodotermopsis sjoestedti</i>	360	3,306	(2,079–5,351)	1.65 ± 0.17	90.88
<i>Hodotermes mossambicus</i>	360	22	(14–34)	1.91 ± 0.21	83.5
<i>Kaloterme flavicollis</i>	360	107	(77–150)	3.19 ± 0.43	55.07
<i>Probinotermes canalifrons</i>	360	616	(352–1,090)	2.25 ± 0.24	81.91
<i>Reticulitermes flavipes</i>	360	959	(645–1,427)	1.17 ± 0.11	104.57
<i>Nasutitermes voeltzkowi</i>	360	2,907	(1,601–5,303)	1.18 ± 0.12	94.63

<sup>a</sup> n = number of termites treated with the six different concentrations of conidia. The mortality was corrected using the termites treated with a control solution of Tween80. <sup>b</sup> LD<sub>50</sub> and 95% FL are expressed in number of conidia per termite

in the Abbott's formula to correct for control mortality in Probit analysis.

### Effect of termite weight on survival

Because all the termite species had a different average biomass, multiple regression models analysis was performed to estimate the effect of average termite weight on survivorship (Fig. 3). However, no significant correlation was found ( $R^2 < 0.03$ ) suggesting that disease susceptibility was independent of termite weight. Thus, all the LD<sub>50</sub> values were given on an individual number basis and not biomass.

### Median lethal dosage for each termite species

The comparison of LD<sub>50</sub> values (Table 2) shows that *Hodotermes mossambicus* was the most susceptible (LD<sub>50</sub> = 22) to an infection of *M. anisopliae*, followed by *K. flavicollis* (LD<sub>50</sub> = 107). *Reticulitermes flavipes* and *P. canalifrons* had a moderate susceptibility, (LD<sub>50</sub> = 959 and LD<sub>50</sub> = 616 respectively), *N. voeltzkowi* and *Hodotermopsis sjoestedti* had a relatively low susceptibility to the fungal infection (LD<sub>50</sub> = 2,907 and LD<sub>50</sub> = 3,307, respectively) and finally, *M. darwiniensis* was virtually not affected by the exposure of high concentrations of *M. anisopliae* (LD<sub>50</sub> = 256,032).

### Effect of conidial concentrations for each species

The effect of *M. anisopliae* conidial concentration was analyzed separately for each species after confirming that the treatment with Tween 80 solution combined with the time spent at 4°C had no significant effect on the termite mortality of all species.

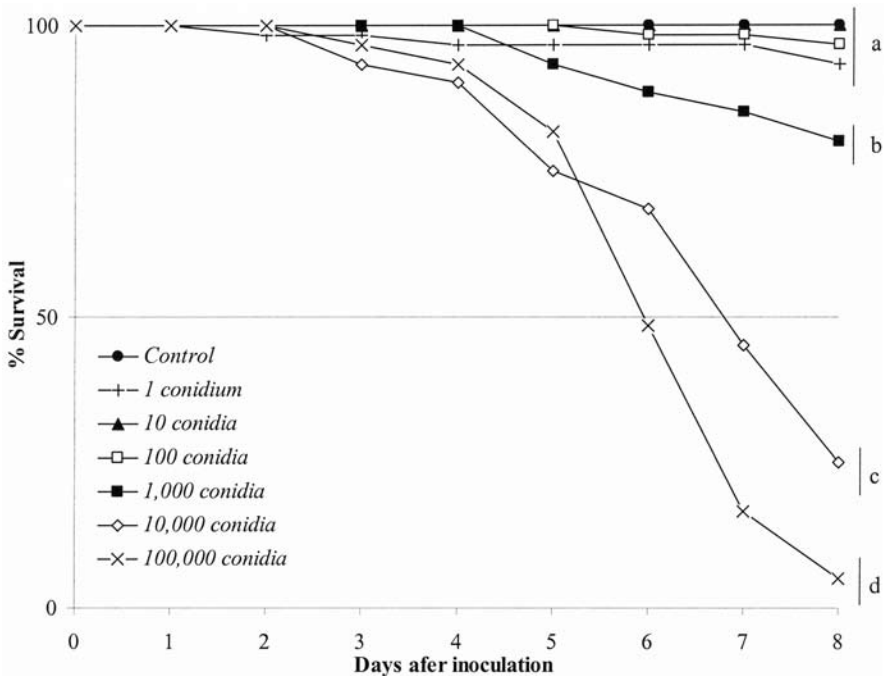


Fig. 5. Survival distribution of groups of 20 *Hodotermopsis sjoestedti* after exposure to different concentrations of *Metarhizium anisopliae* conidia. The survival distribution resulted from a Cox proportional regression analysis (pairwise comparisons, adjusted by Bonferroni method,  $\alpha = 0.05$ , significant difference  $P = 0.0032$ ). Treated groups with the same letter were not significantly different in their survival rate.

***Mastotermes darwiniensis*:** After controlling the effect of all other variables, the conidial concentration was a significant predictor of *M. darwiniensis* survival (Wald statistic = 32.5,  $df = 7$ ,  $P < 0.001$ , Fig. 4). However, pairwise comparisons among concentrations indicated that the survival of control termites was not significantly different from those of termites treated with 1, 10, 100, 1,000 and 10,000 conidia (multiple comparisons,  $P > 0.40$ ). Only termites treated with 100,000 conidia were significantly different in survival, with 6.2 times the hazard ratio for death when compared with the controls (Wald statistic = 8.46,  $df = 1$ ,  $P = 0.003$ ).

***Hodotermopsis sjoestedti*:** The conidial concentration of *M. anisopliae* was a significant predictor of *Hodotermopsis sjoestedti* survival (Wald statistic = 174,  $df = 7$ ,  $P < 0.001$ , Fig. 5). However, pairwise comparisons among concentrations indicated that control termites were not significantly different

in survival from those of termites treated with one conidium (Wald statistic = 0.93,  $df = 1$ ,  $P = 0.33$ ), 10 conidia (Wald statistic = 0.56,  $df = 1$ ,  $P = 0.57$ ) or 100 conidia (Wald statistic = 0.34,  $df = 1$ ,  $P = 0.49$ ). Meanwhile, termites exposed to 1,000, 10,000, and 100,000 conidia had 13.2 (Wald statistic = 6.16,  $df = 1$ ,  $P = 0.001$ ), 80.7 (Wald statistic = 18.8,  $df = 1$ ,  $P < 0.001$ ) and 167 (Wald statistic = 25.4,  $df = 1$ ,  $P < 0.001$ ) times the hazard ratio of death of the controls, respectively.

***Hodotermes mossambicus*:** The conidial concentration was a significant predictor of *Hodotermes mossambicus* survival (Wald statistic = 257,  $df = 7$ ,  $P < 0.001$ , Fig. 6). However, pairwise comparisons among concentrations indicated that the survival of control termites was not significantly different from those of termites treated with one conidium (Wald statistic = 1.6,  $df = 1$ ,  $P = 0.30$ ) or 10 conidia (Wald statistic = 6.94,  $df = 1$ ,  $P = 0.008$ ). Meanwhile, termites exposed to 100, 1,000, 10,000, and 100,000 conidia, had (Wald statistic = 6.94,  $df = 1$ ,  $P = 0.008$ ), 8.9 (Wald statistic = 38.4,  $df = 1$ ,  $P < 0.001$ ), 17 (Wald statistic = 59.1,  $df = 1$ ,  $P < 0.001$ ), 17.2 (Wald statistic = 59.5,  $df = 1$ ,  $P < 0.001$ ), and 26.6 (Wald statistic = 58,  $df = 1$ ,  $P < 0.001$ ) times the hazard ratio of death of the controls, respectively.

***Kaloterme flavicollis*:** The conidial concentration was a significant predictor of *K. flavicollis* survival (Wald statistic = 250,  $df = 7$ ,  $P < 0.001$ , Fig. 7). However, pairwise comparisons among concentrations indicated that survival of control termites did not differ significantly from those of termites treated with one conidium (Wald statistic = 0.21,  $df = 1$ ,  $P = 0.643$ ), or 10 conidia (Wald statistic = 0.68,  $df = 1$ ,  $P = 0.41$ ). Meanwhile, termites exposed to 100, 1,000, 10,000, and 100,000 conidia had 17.2 (Wald statistic = 15,  $df = 1$ ,  $P < 0.001$ ), 103 (Wald statistic = 39.6,  $df = 1$ ,  $P < 0.001$ ), 134 (Wald statistic = 42.5,  $df = 1$ ,  $P < 0.001$ ) and 147 (Wald statistic = 43.1,  $df = 1$ ,  $P < 0.001$ ) times the hazard ratio of death of the controls, respectively.

***Prorhinotermes canalifrons*:** The conidial concentration was a significant predictor of *P. canalifrons* survival (Wald statistic = 132,  $df = 7$ ,  $P < 0.001$ , Fig. 8). However, pairwise comparisons among concentrations indicated that the survival of control termites was not significantly different from those of termites treated with one conidium (Wald statistic = 0,  $df = 1$ ,  $P = 0.995$ ), 10 conidia (Wald statistic = 0.15,  $df = 1$ ,  $P = 0.70$ ) or 100 conidia (Wald statistic = 3.08,  $df = 1$ ,  $P = 0.07$ ). Meanwhile, termites exposed to 1,000, 10,000, and

100,000 conidia had 3.8 (Wald statistic = 17.4,  $df = 1$ ,  $P < 0.001$ ), 5.7 (Wald statistic = 27.9,  $df = 1$ ,  $P < 0.001$ ), and 9.4 (Wald statistic = 47.6,  $df = 1$ ,  $P < 0.001$ ) times the hazard ratio of death of the controls, respectively.

***Reticulitermes flavipes*:** The conidial concentration was a significant predictor of *R. flavipes* survival (Wald statistic = 219,  $df = 7$ ,  $P < 0.001$ , Fig. 9). However, pairwise comparisons among concentrations indicated that survival of control termites did not differ significantly from those of termites treated with one conidium (Wald statistic = 0,  $df = 1$ ,  $P = 0.983$ ), 10 conidia (Wald statistic = 1.39,  $df = 1$ ,  $P = 0.238$ ) or 100 conidia (Wald statistic = 1.38,  $df = 1$ ,  $P = 0.24$ ). Meanwhile, termites exposed to 1,000, 10,000, and 100,000 conidia had 10.5 (Wald statistic = 19.5,  $df = 1$ ,  $P < 0.001$ ), 32.7 (Wald statistic = 43.9,  $df = 1$ ,  $P < 0.001$ ) and 37.4 (Wald statistic = 46.8,  $df = 1$ ,  $P < 0.001$ ) times the hazard ratio of death of the controls, respectively. Moreover, termite

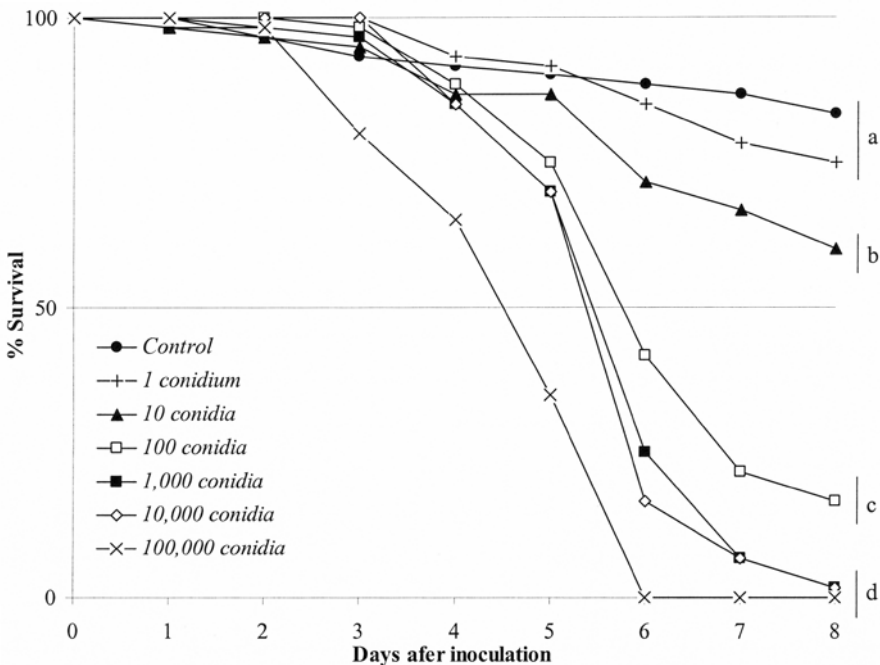


Fig. 6. Survival distribution of groups of 20 *Hodotermes mossambicus* after exposure to different concentrations of *Metarhizium anisopliae* conidia. The survival distribution resulted from a Cox proportional regression analysis (pairwise comparisons, adjusted by Bonferroni method,  $\alpha = 0.05$ , significant difference  $P = 0.0032$ ). Treated groups with the same letter were not significantly different in their survival rate.

treated with 10,000 and 100,000 conidia had similar survival (Wald statistic = 1.43,  $df = 1$ ,  $P < 0.23$ ).

***Nasutitermes voeltzkowi*:** The conidial concentration was a significant predictor of *N. voeltzkowi* survival (Wald statistic = 179,  $df = 7$ ,  $P < 0.001$ , Fig. 10). However, pairwise comparisons among concentrations indicated that survival of control termites did not differ significantly from those of termites treated with one conidium (Wald statistic = 0,  $df = 1$ ,  $P = 0.99$ ), or 10 conidia (Wald statistic = 0.32,  $df = 1$ ,  $P = 0.58$ ) Meanwhile, termites exposed to 100, 1,000, 10,000, and 100,000 conidia had 12.1 (Wald statistic = 11.3,  $df = 1$ ,  $p < 0.001$ ), 13.8 (Wald statistic = 12.6,  $df = 1$ ,  $P < 0.001$ ), 17.1 (Wald statistic = 14.9,  $df = 1$ ,  $P < 0.001$ ) and 146 (Wald statistic = 43.4,  $df = 1$ ,  $P < 0.001$ ) times the hazard ratio of death of the controls, respectively.

### Comparison of Survival among Seven Termite Species Exposed to

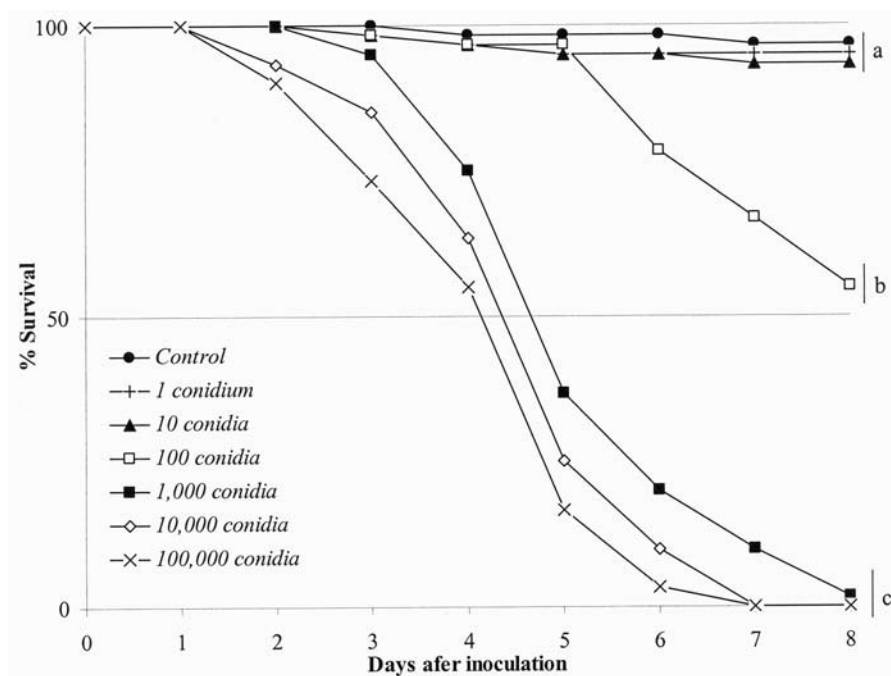


Fig. 7. Survival distribution of groups of 20 *Kalotermes flavicollis* after exposure to different concentrations of *Metarhizium anisopliae* conidia. The survival distribution resulted from a Cox proportional regression analysis (pairwise comparisons, adjusted by Bonferroni method,  $\alpha = 0.05$ , significant difference  $P = 0.0032$ ). Treated groups with the same letter were not significantly different in their survival rate.

### *Metarhizium*

The following analysis compares mortalities among the seven species of individuals that were exposed to relevant concentrations of *M. anisopliae* (Fig.11). A Cox regression analysis was performed to generate the relative ratio of death among the different species exposed to the fungus (pairwise comparisons, adjusted by Bonferroni method,  $\alpha = 0.05$ , significant difference  $P = 0.0032$ ). Only the individuals exposed to 100, 1,000, and 10,000 conidia were used to generate the values, due to the low variability of mortality among species at 0, 1, 10, and 100,000 conidia. The analysis was therefore performed independently of the conidial concentrations.

When exposed to *M. anisopliae*, *Hodotermes mossambicus* had 50.2 times the hazard ratio of death of *M. darwiniensis* (Wald statistic = 127,  $df = 1$ ,  $P < 0.001$ ), 6.6 times the hazard ratio of death of *Hodotermopsis sjoestedti*

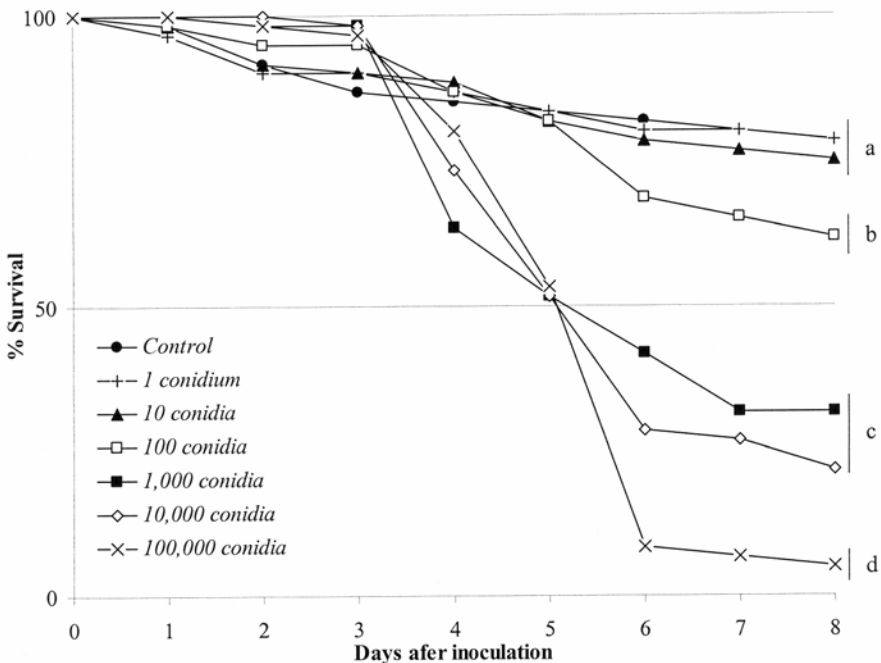


Fig. 8. Survival distribution of groups of 20 *Prorhinotermes canalifrons* after exposure to different concentrations of *Metarhizium anisopliae* conidia. The survival distribution resulted from a Cox proportional regression analysis (pairwise comparisons, adjusted by Bonferroni method,  $\alpha = 0.05$ , significant difference  $P = 0.0032$ ). Treated groups with the same letter were not significantly different in their survival rate.

(Wald statistic = 142,  $df = 1$ ,  $P < 0.001$ ), 1.9 times the hazard ratio of death of *P. canalifrons*, (Wald statistic = 26.1,  $df = 1$ ,  $P < 0.001$ ), 1.95 times the hazard ratio of death of *R. flavipes* (Wald statistic = 25.3,  $df = 1$ ,  $P < 0.001$ ), and 3.68 times the hazard ratio of death of *N. voeltzkowi* (Wald statistic = 72.2,  $df = 1$ ,  $P < 0.001$ ). *Hodotermes mossambicus* and *K. flavicollis* that were exposed to *M. anisopliae* did not have a significantly different hazard ratio of death (Wald statistic = 2.6,  $df = 1$ ,  $P = 0.107$ ).

When exposed to *M. anisopliae*, *K. flavicollis* had 32 times the hazard ratio of death of *M. darwiniensis* (Wald statistic = 101,  $df = 1$ ,  $P < 0.001$ ), 4.48 times the hazard ratio of death of *Hodotermopsis sjoestedti* (Wald statistic = 91.6,  $df = 1$ ,  $P < 0.001$ ), 1.56 times the hazard ratio of death of *P. canalifrons*, (Wald statistic = 12.1,  $df = 1$ ,  $P < 0.001$ ), 1.53 times the hazard ratio of death of *R. flavipes* (Wald statistic = 10.3,  $df = 1$ ,  $P = 0.001$ ), and 2.75 times the hazard

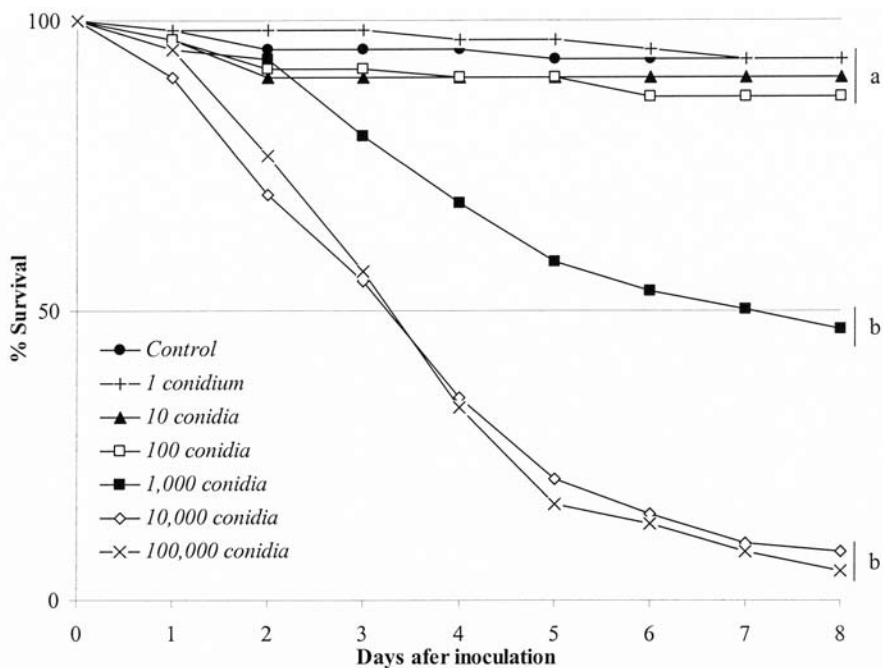


Fig. 9. Survival distribution of groups of 20 *Reticulitermes flavipes* after exposure to different concentrations of *Metarhizium anisopliae* conidia. The survival distribution resulted from a Cox proportional regression analysis (pairwise comparisons, adjusted by Bonferroni method,  $\alpha = 0.05$ , significant difference  $P = 0.0032$ ). Treated groups with the same letter were not significantly different in their survival rate.

ratio of death of *N. voeltzkowi* (Wald statistic = 15.46,  $df = 1$ ,  $P < 0.001$ ).

When exposed to *M. anisopliae*, *P. canalifrons* had 18.2 times the hazard ratio of death of *M. darwiniensis* (Wald statistic = 69.6,  $df = 1$ ,  $P < 0.001$ ), 2.62 times the hazard ratio of death of *Hodotermopsis sjoestedti* (Wald statistic = 35.2,  $df = 1$ ,  $P < 0.001$ ), and 1.7 times the hazard ratio of death of *N. voeltzkowi* (Wald statistic = 11.6,  $df = 1$ ,  $P < 0.001$ ). *Prorhinotermes canalifrons* and *R. flavipes* that were exposed to *M. anisopliae* did not have a significantly different hazard ratio of death (Wald statistic = 1,  $df = 1$ ,  $P = 0.93$ ).

When exposed to *M. anisopliae*, *R. flavipes* had 15 times the hazard ratio of death of *M. darwiniensis* (Wald statistic = 60.2,  $df = 1$ ,  $P < 0.001$ ), 2.28 times the hazard ratio of death of *Hodotermopsis sjoestedti* (Wald statistic = 24.7,  $df = 1$ ,  $P < 0.001$ ), and 1.66 times the hazard ratio of death of *N. voeltzkowi* (Wald statistic = 10.2,  $df = 1$ ,  $P < 0.001$ ).

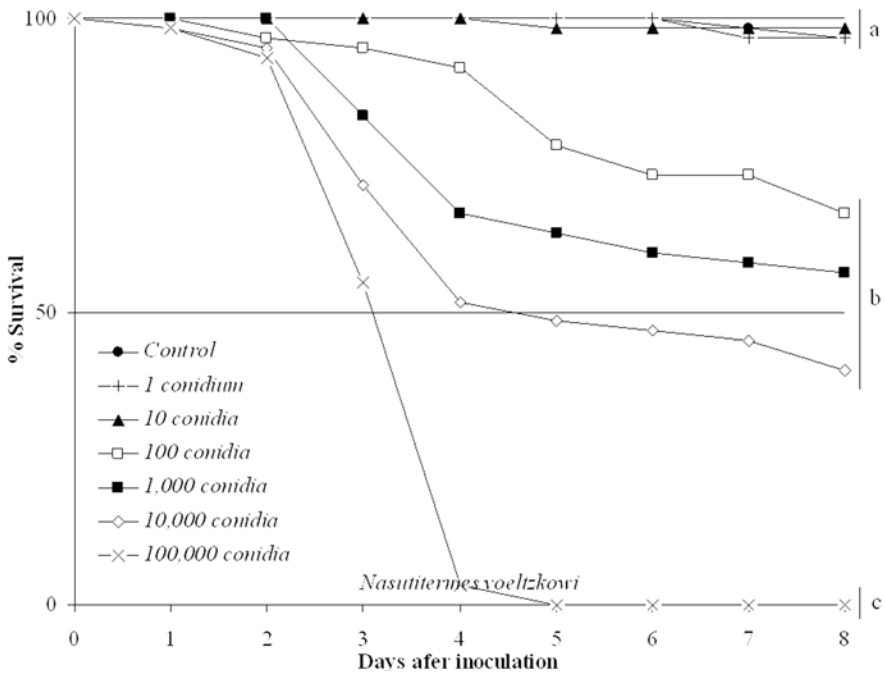


Fig. 10. Survival distribution of groups of 20 *Nasutitermes voeltzkowi* after exposure to different concentrations of *Metarhizium anisopliae* conidia. The survival distribution resulted from a Cox proportional regression analysis (pairwise comparisons, adjusted by Bonferroni method,  $\alpha = 0.05$ , significant difference  $P = 0.0032$ ). Treated groups with the same letter were not significantly different in their survival rate.



Finally, when exposed to *M. anisopliae*, *Hodotermopsis sjoestedti* had 7.1 times the hazard ratio of death of *M. darwiniensis* (Wald statistic = 60.2,  $df = 1$ ,  $P < 0.001$ ) and *N. voeltzkowi* had 9.4 times the hazard ratio of death of *M. darwiniensis* (Wald statistic = 39.9,  $df = 1$ ,  $P < 0.001$ ), but *Hodotermopsis sjoestedti* and *N. voeltzkowi* did not have a significantly different hazard ratio of death (Wald statistic = 3.11,  $df = 1$ ,  $P = 0.077$ ).

### DISCUSSION

Our study revealed some of the difficulties of standardizing comparative tests of termite susceptibility to fungal pathogens. A standard test is needed for comparison, but each species has a different habitat and may respond differently to the stress imposed by the experimental conditions. We successfully minimized the control mortality (<18%) in the seven tested species,

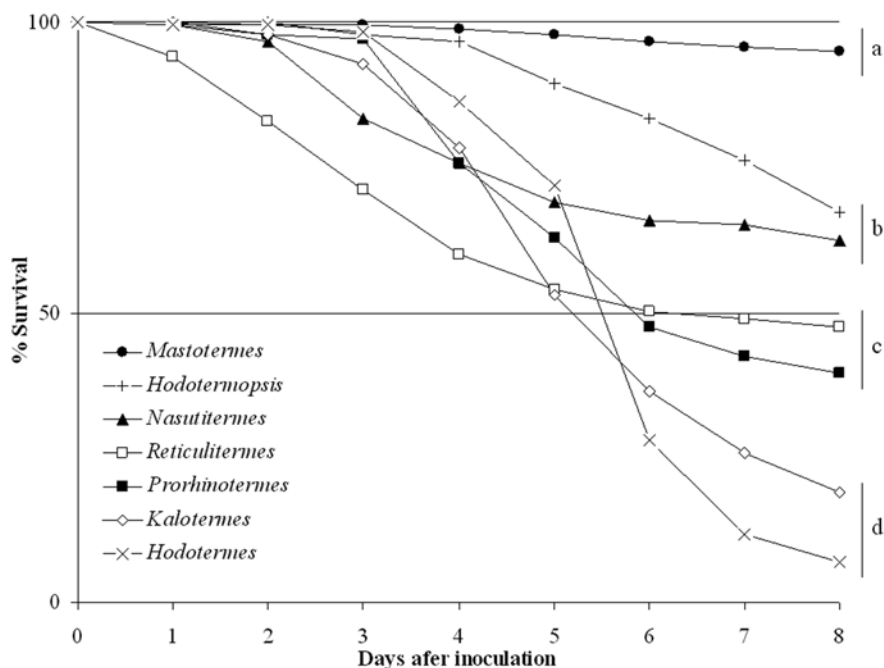


Fig. 11. Survival distribution of groups of 20 individuals from seven termite species after exposure to *Metarhizium anisopliae* (pooled data from individuals exposed to 100, 1,000 and 10,000 conidia). The survival distribution resulted from a Cox proportional regression analysis (pairwise comparisons, adjusted by Bonferroni method,  $\alpha = 0.05$ , significant difference  $P = 0.0032$ ). Species with the same letter were not significantly different in their survival rate.

allowing us to provide unique information about relative disease susceptibility for these species. However, due to the laboratory availability of a single termite colony of each species from diverse geographical origin, this study could not provide data about intra-specific colony variability. Rosengaus & Traniello (2001) showed the existence of variability in disease susceptibility among different colonies of *Zootermopsis angusticollis* (Hagen), Termopsidae. Although such variability in each of the species tested in the present study cannot be confirmed, the following interpretation was made under the assumption that the intra-species (inter-colonial) variability was lower than the observed interspecies variability.

Of the seven species tested, *M. darwiniensis* was the most tolerant to exposure to *M. anisopliae*. *Mastotermes darwiniensis* is often considered the termite species with conserved ancestral traits that are common to wood cockroaches, *Cryptocercus* sp. (Klass *et al.* 2008). Although no data are available on *Cryptocercus* susceptibility to *M. anisopliae*, some experiments were conducted on Blattodea, and the LD<sub>50</sub> observed for *Blattella germanica* L. by Patchamuthu *et al.* (1999) and Quesada-Morega *et al.* (2004) was in the same order of magnitude as that of *M. darwiniensis* in this study. The reason for the low susceptibility of *M. darwiniensis* is unknown, but we hypothesize that it could be a form of immune competence inherited from a cockroach-like ancestor. As a matter of fact, a *M. darwiniensis* transferrin involved in immune response to infection closely resembles a cockroach transferrin (Thompson *et al.* 2003). Another factor that could be an important mechanism in disease resistance of *M. darwiniensis* involves glandular secretions. Although the chemistry of the salivary glands is poorly known in *M. darwiniensis*, some preliminary studies (Moore 1968; Prestwitch 1979a) showed the presence of quinones in soldier salivary secretions. Further studies are needed to characterize these chemicals and quantify their antifungal property in order to confirm their role in *M. darwiniensis* resistance to fungal infection.

Although the disease resistance observed in *Hodotermopsis sjoestedti* was not as high as that in *M. darwiniensis*, our results showed that its resistance to *M. anisopliae* infection was higher than all other termite species tested (except for *N. voeltzkowi*). This resistance might be partially attributed to the nesting ecology of this species because *Hodotermopsis sjoestedti* is a dampwood termite that lives in a moist environment in contact with the soil. Such an

environment is favorable for the natural occurrence of *M. anisopliae*, and the long evolutionary history of exposure of *Hodotermopsis sjoestedti* to the fungus may have promoted tolerance of this termite species. An adaptive immune reaction may have been conserved from an ancestral trait, or may have evolved against the fungus, i.e. an immune reaction such as the one observed in another termopsid, *Z. angusticollis* (Rosengaus *et al.* 1999b, 2007). Moreover, Rosengaus *et al.* (2004) showed that the sternal gland secretion of *Z. angusticollis* had fungistatic activity. Such secretions have yet to be reported in *Hodotermopsis sjoestedti*.

Disease susceptibility of *N. voeltzkowi* was similar to that of *Hodotermopsis sjoestedti*, and was also highly resistant to fungal infection. However, the life habitat and nesting ecology of *N. voeltzkowi* are drastically different from those of the Termopsidae. *Nasutitermes voeltzkowi* builds arboreal nests with above-ground galleries and the potential exposure to *M. anisopliae* is probably limited. Contrary to *Hodotermopsis sjoestedti*, the high survivorship observed in *N. voeltzkowi* can not be explained by an evolutionary adaptation to a constant exposure to the fungus. However, multiple terpenoid compounds that have been described from the frontal secretion of the soldiers in the genus *Nasutitermes* (Prestwich 1979b) may contribute to its resistance. These terpenoids (originally described as components of the glue secreted by the soldiers for defense against potential enemies) may have attained a secondary antimicrobial function, because some of these chemicals have been shown to have antifungal activity (Rosengaus *et al.* 2000). However, it is not known if soldiers of *Nasutitermes sp.* actually “spray” these chemicals within the nest structure. Although the frontal gland secretion in *Nasutitermes* could be a major factor for their high survivorship in our experiment, other factors such as antifungal peptides (Bulmer & Crozier 2004) may be involved and *Nasutitermes* could be an interesting model for the evolution of alternative defense mechanisms against pathogens in termites.

In the Rhinotermitidae, *R. flavipes* and *P. canalifrons* are subterranean termites that are in constant contact with soil and may be regularly exposed to *M. anisopliae*. As we suggested for *Hodotermopsis sjoestedti*, Rhinotermitidae may also have evolved adaptive defense mechanisms against soil fungi. For this reason, the susceptibility observed in the Rhinotermitidae was expected to be similar to that observed in *Hodotermopsis sjoestedti*, yet, *R. flavipes* and *P.*

*canalifrons* showed moderate susceptibility to *M. anisopliae* when compared to the species discussed above. As suggested by Chouvenc *et al.* 2008a, subterranean termites may not be at full capacity to inhibit the pathogen when in small groups and in a Petri dish. The LD<sub>50</sub> observed in our study may be underestimated under these standardized conditions. Although the social interactions and the nest architecture are important in all termite species, their roles might be particularly critical in the Rhinotermitidae.

*Kalotermes flavicollis* is a drywood termite that infests a single piece of wood. Contact with soil fungi is unlikely and we suggest that the relative *Metarhizium*-free habitat in which this termite evolved had very little selective pressure to maintain a costly immune mechanism. It is therefore possible that *K. flavicollis*, and potentially other drywood termites species, have lost most of their disease resistance against soil pathogens during the evolutionary process.

*Hodotermes mossambicus* is a species with an underground nest and forages on the surface of the ground in the East and South African region. Because it has permanent contact with the soil, we expected a similar result to that obtained with the Rhinotermitidae. Instead, *Hodotermes mossambicus* showed acute mortality to the fungal exposure. Interestingly, Langewald *et al.* (2003) reported that *Hodotermes mossambicus* was not susceptible to a majority of strains of *M. anisopliae* originating from their native area. The strain we used in our experiment may have an acute effect on *Hodotermes mossambicus*, in the hypothesis that this termite's defense is not adapted to strains it never evolved with. Also, old workers of *Hodotermes mossambicus* have very little grooming activity (C. Bordereau, *pers. com.*), which is usually performed by younger instars (not melanized yet). In our experiment, we mainly used melanized workers (80% of the individuals), so in these conditions, a small number of individuals actually performed grooming activity. Grooming is one of the major mechanism that help removing pathogens from the surface of the termite cuticle (Yanagawa & Shimizu 2007), The high mortality of *Hodotermes mossambicus* in our experiment may simply be due to an inaccurate representation of the caste ratio of the colony, with few young instars that may have reduced the overall conidial load from their nestmates' cuticle surface, by performing an efficient allogrooming.

In conclusion, our results suggest that disease resistance is not phylogenetically consistent, although work with more than one colony for each species would be necessary to confirm our findings. Some relatively closely related species differed considerably in their disease susceptibility (i.e. *Hodotermopsis sjoestedti* and *Hodotermes mossambicus*), while distant species were similar in their disease susceptibility. We suggest that the resistance to *M. anisopliae* infection is an ancestral trait, inherited from a cockroach-like ancestor, which was conserved in some species, or lost in others during evolution due to differential selective pressure. However, it appears that some independent species have evolved unique defense mechanisms in addition to the ancestral mechanisms (i.e. *Nasutitermes* and *Mastotermes*, using putative chemical secretions). Contrarily, some species appear to have partially lost some of their disease resistance mechanisms (i.e., *Hodotermes* and *Kalotermes*). This could be explained by the minimal selective pressure over evolutionary time due to a habitat that was relatively *Metarhizium*-free. In addition, individual biomass did not appear to be a factor contributing to termite survival. Thus, body size in termite evolution was probably not pathogen-driven. Finally, because the nesting ecology appears to be important in termite survival against pathogens (Rosengaus *et al.* 2003; Pie *et al.* 2004; Chouvenc *et al.* 2008a), further studies on the evolution of disease resistance mechanisms in relation to their habitats are warranted.

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