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## DIFFERENCES IN CELLULAR ENCAPSULATION OF SIX TERMITE (ISOPTERA) SPECIES AGAINST INFECTION BY THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE*

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### ABSTRACT

Termites (Isoptera) are eusocial insects, which live in an environment that can favor the spread of pathogens. To reduce the chance of an epizootic within a colony, termites have evolved many defense mechanisms. Most studies have focused on the social aspect of disease resistance, while the individual capacity of a termite to survive an infection remains poorly documented. We previously showed that when the eastern subterranean termite, *Reticulitermes flavipes* (Kollar), was exposed to the entomopathogenic fungus, *Metarhizium anisopliae* (Metch.) Sorokin, cellular encapsulation of the penetrating fungus was one of the last lines of defense for individual termites to prevent internal mycosis. The current study used histological preparations to (i) compare cellular encapsulation of *M. anisopliae* among 6 termite species from 5 families that evolved in habitats with different pathogenic pressures, and (ii) examine the effect of cellular encapsulation on the survival of termites exposed to *M. anisopliae*. Our results showed that all termite species were able to use hemocytes to encapsulate *M. anisopliae* when this fungus penetrated through the insect cuticle, but that the physiological cost to successfully encapsulate *M. anisopliae* varied greatly among termite species. We suggest that termite species, which evolved in a habitat with high pathogenic pressure, are adapted with more efficient immune reactions than those that evolved in a habitat with low pathogenic pressure.

Key Words: disease resistance, cellular encapsulation, hemocytes

### RESUMEN

Las termitas (Isoptera) son insectos eusociales, que viven en un ambiente que puede favorecer la diseminación de agentes patógenos. Para reducir la posibilidad de una epizootia en una colonia, las termitas han desarrollado varios mecanismos de defensa. La mayoría de los estudios se han enfocado en el aspecto social de la resistencia a enfermedades, mientras que la capacidad individual de una termita para sobrevivir a una infección sigue a un nivel pobremente documentado. Mostramos anteriormente que cuando la termita subterránea del este, *Reticulitermes flavipes* (Kollar), fue expuesto al hongo entomopatógeno, *Metarhizium anisopliae* (Metch.) Sorokin, la encapsulación celular del hongo que penetró fue una de las últimas líneas de defensa para las termitas individuales para evitar micosis interna. El estudio actual utilizó las preparaciones histológicas a (i) comparar la encapsulación celular de *M. anisopliae* entre 6 especies de termitas de 5 familias que se desarrollaron en hábitats con diferentes presiones de patógenos, y (ii) examinar el efecto de la encapsulación celular en la supervivencia de las termitas expuestas a *M. anisopliae*. Nuestros resultados mostraron que todas las especies de termitas pudieron utilizar los hemocitos para encapsular *M. anisopliae*, cuando este hongo penetró a través de la cutícula de los insectos, pero que el costo fisiológico para encapsular *M. anisopliae* exitosamente varió ampliamente entre las especies de termitas. Se sugiere que las especies de termitas, que se desarrolla en un hábitat con la presión alta de patogenicidad, se adaptan con reacciones inmunes más eficientes que las que se desarrolla en un hábitat con la presión de baja patogenicidad.

Termites (Isoptera) represent more than 2,600 species from 7 families that have spread and adapted to a large range of habitats, resulting in high diversity in morphology, physiology, behavior, nesting ecology, and associated microbial community (Abe et al. 2000; Rosengaus et al. 2011). The relationship between termites and entomopathogens has received particular attention due to the potential use of such pathogens as biological control of termite pest species (Grace 1997; Culliney & Grace 2000); but no successful field applications have been reported (Grace 2003). To explain this lack of success, it was shown that termites have the ability to prevent the occurrence of epizootics within their nest (Chouvenc et al. 2008) due to the interaction of various disease resistance mechanisms (Cremer et al. 2007; Wilson-Rich et al. 2009; Chouvenc & Su 2010; Rosengaus et al. 2011). During the Isoptera radiation, termites evolved from the solitary lifestyle of the wood roach-like ancestor toward eusociality; and it was hypothesized that termites evolved such disease resistance mechanisms due to increased pressure from pathogens (Fefferman et al. 2007). However, various termite species showed different susceptibilities when exposed to a single strain of the entomopathogenic fungus *Metarhizium anisopliae* (Metch.) Sorokin (Chouvenc et al. 2009a), suggesting that each species has evolved differential resistance to this particular pathogen. In Chouvenc et al. (2009a) we hypothesized that the differences in susceptibility resulted from the type of habitat in which each termite species had evolved; and specific physiological changes may have occurred during the radiation of the isopteran lineage (Rosengaus et al. 2003, 2011; Calleri et al. 2010). In the eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Rhinotermitidae), we demonstrated that this termite species possesses a strong antifungal activity within its gut (Chouvenc et al. 2009b) and also possesses an efficient cellular encapsulation mechanism against the penetration of the fungal hyphae through the cuticle (Chouvenc et al. 2009c). We hypothesized that 1 of these 2 physiological parameters could be involved in the variability of susceptibility observed in the different species (Chouvenc et al. 2009a). However, the antifungal gut activity appears to be highly conserved in all termite species we tested (Chouvenc et al. 2010), which suggests that gut physiology is a critical factor in all termites against entomopathogenic fungi, and that the differential mortality among the termite species tested in Chouvenc et al. (2009a) was probably caused by other physiological factors rather than the gut antifungal activity.

Among the various disease resistance mechanisms described in termites (reviewed in Chouvenc & Su 2010; Rosengaus et al. 2011), we demonstrated the importance of cellular encapsula-

tion in *R. flavipes* as part of its defense against *M. anisopliae* at the level of the individual (Chouvenc et al. 2009c). Cellular encapsulation prevents the fungus from penetrating into the host and releasing toxins into the hemocoel, once it has successfully bypassed any other pre-penetration mechanisms (St. Leger 1991; Golkar et al. 1993). Although cellular encapsulation appears to be beneficial for the survival of an individual termite, the intensity of the nodule formation may have a physiological cost for the host due to the allocation of limited resources to the immune reaction (Chouvenc et al. 2009c). In *R. flavipes*, we showed that a healthy individual exposed to *M. anisopliae* had few nodules (1 to 4) formed at potential points of penetration of the fungus and, in moribund specimens, we observed up to 11 nodules (Chouvenc et al. 2009c). There are 2 possible proximal causes of death of the termite: 1) the multiple points of fungal infection increased the chance for 1 infective hypha to release the toxin early enough to bypass cellular encapsulation to kill the host and, 2) the formation of multiple nodules consumed all the available resources involved for nodule formation (i.e. hemocytes, phenols, etc.) and the physiological exhaustion of the host allowed additional hyphae to invade the hemocoel.

Although the first possibility may be the case for a highly virulent agent infecting the termite and leading to immunosuppression (Wang & St. Leger 2006), the second may better explain events with more moderately pathogenic fungal strains, where a high conidia dose is necessary to cause mortality in most termite species. Indeed this was the case in our experiment with *M. anisopliae* (Chouvenc et al. 2009a). Therefore, the chance for a given termite to survive multiple infections of *M. anisopliae* may partially depend on its ability to successfully encapsulate each infection at a low physiological cost. The cost of a cellular immune reaction can be estimated and quantified by the relative volume of hemocytes required by the host to encapsulate a penetrating hyphal body. If the formation of a single nodule requires a large quantity of resources, then the termite may only encapsulate a very limited number of penetrating hyphae at the time. By determining the cost of the immune reaction in all termite species in which the susceptibility to *M. anisopliae* is known (Chouvenc et al. 2009a), it should be possible to estimate the efficiency of the cellular encapsulation for each species.

The first objective of this study was to describe cellular encapsulation in 5 termite species and compare these observations with what was previously observed in *R. flavipes* (Chouvenc et al. 2009c). The second objective was to estimate the relative physiological cost of cellular encapsulation in each termite species. The third and final objective was to check if the susceptibility to *M.*

*anisopliae* was dependent on the resources allocated for the cellular encapsulation. Therefore, in comparison with our observations made in *R. flavipes*, we estimated the relative cost of nodule formation in the 5 other termite species.

#### MATERIAL AND METHODS

Termites from 5 families that were used for the susceptibility test in Chouvenc et al. (2009a) were fixed for histological preparation, as previously described (Chouvenc et al. 2009c). Due to the differences in mortality among termite species observed at 6 d after exposure to *M. anisopliae*, the availability for specimens was unequal among termite species. As a result, the observation of cellular encapsulation formation was limited for some species. Among all specimens prepared for histological analysis, there were 25 for *Hodotermopsis sjoestedti* Holmgren (Termopsidae) including 4 soldiers, 8 for *Hodotermes mossambicus* (Hagen) (Hodotermitidae) including 3 soldiers, 13 for *Kaloterms flavicollis* (Fabricius) (Kalotermitidae), 10 for *Prorhinotermes canalifrons* (Sjöestedt) (Rhinotermitidae), and 2 for *Nasutitermes voeltzkowi* (Wasmann) (Termitidae). In addition, histological preparations of 20 randomly selected specimens of *R. flavipes* used in Chouvenc et al. (2009c) were re-used in current experiment for comparison with the data from the other termite species. For each specimen, all histological sections were observed for cellular encapsulation and each nodule was measured at its largest diameter. The relative physiological cost of cellular encapsulation in all 6 species was estimated by calculating the ratio of the average nodule size by the average weight of the species (in  $\mu\text{m} / \text{mg}$ ). The size of cellular encapsulation and the relative physiological cost of each species were subjected to analysis of variance (ANOVA). A Tukey HSD test (post hoc,  $\alpha = 0.05$ ) was performed to compare the average size of encapsulation and the relative physiological cost among the 6 termite species. Regression analyses were performed to test for correlations between the size of the encapsulations and 2 other factors: the average wet weight for each of the termites and the susceptibility to *M. anisopliae* ( $\text{LD}_{50}$  values obtained from Chouvenc et al., 2009a). Finally, a survival trade-off value was generated by calculating the ratio of the  $\text{LD}_{50}$  by the relative physiological cost. Survival trade-off estimates termite survival efficiency resulting from the physiological investment for cellular encapsulation, with higher values indicating relatively higher efficiency.

#### RESULTS AND DISCUSSION

##### Nodule Formation in 6 Termite Species

Termites inoculated with *M. anisopliae* revealed differences in the process of cellular encapsulation among the 6 tested species (Fig. 1). Size of encapsulation, relative physiological cost, and survival trade-off are summarized in Table 1. The sclerotization was easily visible in *Hodotermopsis sjoestedti* and *K. flavicollis* (Supplementary Fig. 1 online at InfoLink1) while a microscope was needed to observe the nodules in 4 other species. The formation of nodules in the termites differed in size and intensity and detailed descriptions of the cellular encapsulation for each of the 6 species are available in the supplementary material (Supplementary Figs. 2-7 online at InfoLink1).

*Reticulitermes flavipes*

##### *Reticulitermes flavipes*

The full description of the cellular encapsulation in *R. flavipes* was provided in Chouvenc et al. (2009c), from which 49 different encapsulations of various sizes were located among 20 randomly chosen specimens. Most of them were found in areas where the cuticle folds, or at arthrodial membrane locations, especially at the leg-thorax junctions. The average size of the nodules was  $78.51 \pm 43.51 \mu\text{m}$  and the relative physiological cost was estimated at  $28.77 \pm 15.94 \mu\text{m} / \text{mg}$ .

##### *Hodotermopsis sjoestedti*

Before *M. anisopliae* could penetrate the procuticle, it was observed that the cuticle of *Hodotermopsis sjoestedti* had the ability to trigger the prophenoloxdase cascade within the cuticle itself, without the involvement of hemocytes from the hemocoel. This resulted in a localized melanization of the cuticle, but it was only observed a couple of times. As previously reported in *R. flavipes*, this did not prevent the fungus from penetrating deeper into the cuticle, which suggests that this early sclerotization is not a major mechanism in a termite's defense against fungal infection.

*Hodotermopsis sjoestedti* was the largest termite tested in our study. The cuticle at some point was the thickest among all the species studied and ranged from  $4 \mu\text{m}$  (at the spiracle and the sclerites) to  $40 \mu\text{m}$  (at the articulation points or muscle insertions point). This particularity, when compared with *R. flavipes*, implies that the penetration peg of *M. anisopliae* had to go through a longer distance before reaching the hemocoel, which had 2 direct consequences. First, *M. anisopliae* had enough space to grow laterally in the cuticle, assimilate the nutrients obtained from the cuticle degradation, and spread between the exocuticle and the endocuticle without being in direct contact with the hemocytic activity of the hemocoel. Second, the thickness of the cuticle provided additional time for the hemocytic reaction to take place and localize of the fungal spread.

Therefore, the large cuticle thickness in *Hodotermopsis sjoestedti* provided a different re-

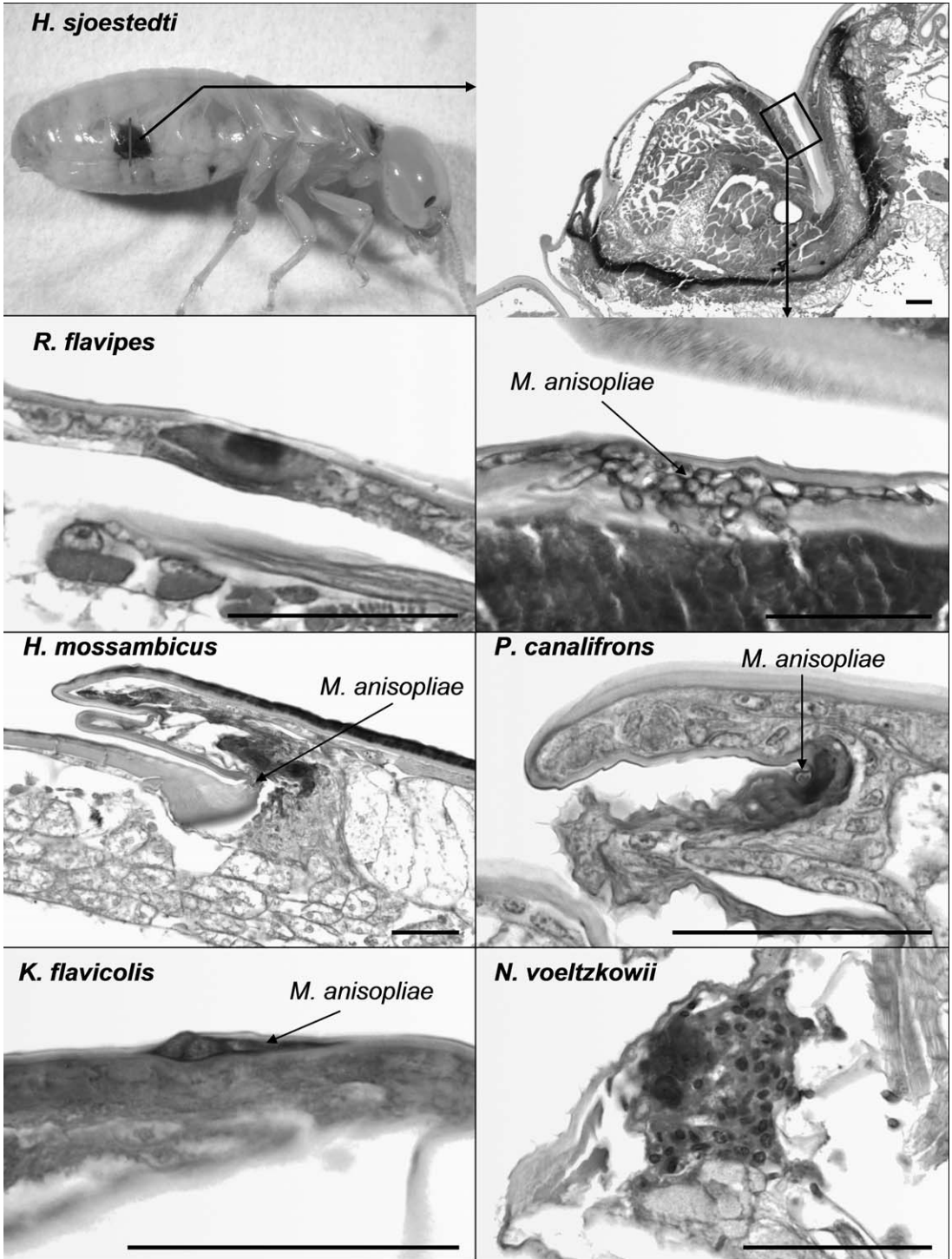


Fig. 1. Cellular encapsulation in 6 termite species against the infection of *Metarhizium anisopliae*. Histological observations. The horizontal bar = 50  $\mu$ m. *Hodotermopsis sjoestedti* actual size: 2.1 cm. The termite species are: *Hodotermopsis sjoestedti*, *Hodotermes mossambicus*, *Kaloterme flavicollis*, *Prorhinotermes canalifrons*, *Reticulitermes flavipes*, and *Nasutitermes voeltzkowii*. More elaborate descriptions of encapsulation of *M. anisopliae* for all of these termite species are available in the supplementary figures at <http://www.fcla.edu/FlaEnt/fe943.htm#InfoLink1>.

TABLE 1. DIFFERENTIAL CELLULAR ENCAPSULATION *METARHIZIUM ANISOPLIAE* IN 6 TERMITE SPECIES.

	Average Weight (mg)	LD <sub>50</sub> (Conidia / termite)	Cellular encapsulation size (µm) mean ± SD	Relative physiological cost (µm / mg) mean ± SD	Survival trade-off* Survival trade-off*	Number of encapsulations observed
<i>Hodotermopsis sjoestedti</i>	68.50	3,306	370.54 ± 303.40 a	5.40 ± 4.42 a	612.22	54
<i>Hodotermes mossambicus</i>	69.17	22	223.43 ± 127.62 b	3.23 ± 1.84 a	6.81	23
<i>Kaloterme flavicollis</i>	4.55	107	221.98 ± 177.04 b	48.73 ± 38.87 b	2.19	55
<i>Prorhinotermes canalicifrons</i>	4.25	616	77.59 ± 62.59 c	18.26 ± 14.72 c	33.73	34
<i>Reticulitermes flavipes</i>	2.73	962	78.51 ± 43.51 c	28.77 ± 15.94 c	33.43	49
<i>Nasutitermes voeltzkowi</i>	5.11	2,907	108.47 ± 108.47 bc	21.20 ± 8.49 c	137.00	6

\*The survival trade-off estimates represent how efficient the physiological investment for cellular encapsulation is in the survival in each species, with high values indicating relatively high efficiency. Identical letters in the same column indicate no significant difference (ANOVA, HSD post hoc, = 0.05). Average wet weight and LD<sub>50</sub> values are from Chouvenec et al. (2009a).

relationship between the cellular reaction and the infective hypha when compared with *R. flavipes* and the dynamic in this relationship resulted in massive aggregations of hemocytes and the formation of large melanized nodules. The average size of these nodules was 370.54 ± 303.40 µm and the relative physiological cost was estimated at 5.40 ± 4.42 µm / mg. The survival trade-off value for *Hodotermopsis sjoestedti* was the highest among all tested species, which suggests that besides having large nodules, the cellular encapsulation was efficient for survivorship at a relatively low physiological cost in this large species. In addition, we observed 1 case of fungal infection in a spiracle, which was the only case of fungal penetration through the respiratory tract observed through the entire study, which suggest that this way of penetration is unlikely in natural conditions.

*Hodotermopsis sjoestedti* is a damp wood 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 led to an efficient immune response to survive constant exposure to such pathogens.

*Hodotermes mossambicus*

Cellular encapsulation in *Hodotermes mossambicus* was similar to that observed in *Hodotermopsis sjoestedti* in terms of size and intensity. The average size of the nodules was 223.43 ± 127.62 µm and the relative physiological cost was estimated at 3.23 ± 1.84 µm / mg. Although the relative physiological cost was low in comparison with other termite species, the resource investment in *Hodotermes mossambicus* was one of the least efficient because the survival trade-off value was among the lowest. As a matter of fact, most of the nodules failed to successfully encapsulate the penetrating hyphae which confirmed that the large encapsulations in *Hodotermes mossambicus* were not efficient in preventing the infective hyphae from reaching the hemocoel. When comparing the survival trade-off values, the cellular encapsulation in *Hodotermes mossambicus* was 90 times less efficient than that of *Hodotermopsis sjoestedti* and 15 times less efficient than that of *R. flavipes*.

*Hodotermes mossambicus* is a species with an underground nest and forages on ground surface in the sub-arid climate in the East and South African region. Because it has permanent contact with the soil, we expected a similar result to that obtained with *Hodotermopsis sjoestedti*. Instead, *Hodotermes mossambicus* showed acute mortality to the fungal exposure and a poor immune reaction. We suggest that the relatively dry habitat of this termite reduced the pressure from fungal

pathogens such as *M. anisopliae*, as the humidity requirement for fungal germination (Sun et al. 2003) may not be present in the termite nests of this particular species. It is therefore possible that the lack of successful encapsulation in this termite is a consequence of the environmental conditions this species evolved in, with a relatively low pathogenic pressure, which lead to the loss of an efficient immune reaction.

#### *Kaloterms flavicollis*

Although *K. flavicollis* is much smaller than *Hodotermes mossambicus*, the size of their cellular encapsulations were similar ( $221.98 \pm 177.04 \mu\text{m}$  for *K. flavicollis*) which resulted in an elevated relative physiological cost for *K. flavicollis* ( $48.73 \pm 38.87 \mu\text{m} / \text{mg}$ ), and was the highest relative physiological cost among all termite species tested. As observed for *Hodotermes mossambicus*, most of the nodule formations failed to stop the progression of the fungus into the hemocoel. Thus, even if the cost to produce such cellular encapsulations was high, the trade-off for survival was minimal. The cellular encapsulation in *K. flavicollis* was 278 times less efficient than that of *Hodotermopsis sjoestedti*, which suggests that it is poorly adapted to the infection by *M. anisopliae*.

*Kaloterms 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 provided very little selective pressure to maintain a costly immune mechanism (Rosengaus et al. 2003). It is therefore possible that *K. flavicollis* has lost some of its disease resistance mechanisms against soil pathogens during evolution.

#### *Prorhinotermes canalifrons*

The cellular encapsulation of *Prorhinotermes canalifrons* was very similar in shape, size and intensity to *R. flavipes*, which resulted in a similar relative physiological cost and survival trade-off values. Both species are subterranean termites and are commonly exposed to soil pathogens. Although their overall trade is smaller than *Hodotermopsis sjoestedti*, it was shown that for subterranean termites, the cellular encapsulation is one of the many components involved in the termite's disease resistance (Chouvenc & Su 2010). This suggests that most of the pre-penetration defense mechanisms are sufficiently efficient to lower the pathogenic pressure to maintain a relatively cheap immune reaction that can protect individual termites. Alternatively, due to the large number of individuals in subterranean termite colonies, it may be more beneficial for the colony to invest a minimal amount of resources for individual survivorship, and instead isolate the in-

fecting termites by necrophagy or burial (Myles 2002; Chouvenc & Su 2010). In comparison, *Hodotermopsis sjoestedti* individuals are much bigger, and the size of their colonies is smaller. This may be an alternative strategy where the individual investment is more noticeable in such species.

#### *Nasutitermes voeltzkowi*

The small number of available specimens of *N. voeltzkowi* for histological preparation limited our observations for cellular encapsulation, but the available data suggested that the nodule size was similar to that observed in *R. flavipes* and *P. canalifrons*, and that the relative physiological costs were also not significantly different from these 2 species. Due to the relatively low susceptibility of *N. voeltzkowi* to *M. anisopliae*, the survival trade-off value suggested that the cellular encapsulation in *N. voeltzkowi* was 4 times more efficient than the cellular encapsulation observed in *R. flavipes* and *P. canalifrons*. The relatively high survivorship of *Nasutitermes* sp. may partially be explained by the presence of a potent humoral immunity (Bulmer et al. 2009) and the fungistatic activity of some of the compounds produced by frontal gland of the soldiers (Rosengaus et al. 2000; Fuller 2007).

#### Relationships Between Cellular Encapsulation and Other Known Immunity Factors

Three variables obtained from empirical data of this study included 1) the average wet weight of the species, 2) the median lethal dosage ( $LD_{50}$ ) of each species when exposed to concentrations of *M. anisopliae*, and 3) the average size of the cellular encapsulation for all species. Chouvenc et al. (2009a) showed that the susceptibility was independent of the mass of the termite. With the cellular encapsulation size variable presented in this study, we examined if this factor was dependent on the weight or the  $LD_{50}$  of the termite species.

#### The Relative Physiological Cost

Termite body size can be an important factor for its relative vulnerability to a variety of harmful environmental factors (Nalepa 2011a). In our study, the relative physiological cost value was generated by dividing the average size of the cellular encapsulation by the termite's average weight. This ratio can be interpreted as the amount of resources allocated to immunity relative to the available resources of the termite. If this value was similar for all termite species, it would suggest that the size of encapsulation was mainly dependent on the weight of the termite. However, as shown previously, this value varied



significantly among species, and a linear regression analysis found a weak correlation ( $R^2 < 0.18$ ,  $F = 50.28$ ,  $df = 219$ ,  $P < 0.001$ ) between the termite weight and the size of cellular encapsulation (Supplementary Fig. 8 online at InfoLink1). Therefore, the size of cellular encapsulation in termites was not strongly dependent on their weight and the amount of resources allocated for nodule formation mainly depended on other factors.

#### Survival Trade-Off

The survival trade-off value was generated by dividing the relative susceptibility to *M. anisopliae* ( $LD_{50}$  value) by the relative physiological cost. The resulting ratio is considered an indicator of relative profitability of the cellular encapsulation. A high ratio suggests that the physiological investment by a termite for cellular encapsulation was highly profitable for survival and that the resource allocation was efficient.

If the trade off values were similar among the 6 species, it would suggest that the survivorship of a termite was dependent on the relative amount of resource allocated for encapsulation. However, as shown previously, this ratio strongly varied from species to species, and a linear regression analysis found a weak correlation ( $R^2 < 0.14$ ,  $F = 37.68$ ,  $df = 219$ ,  $P < 0.001$ ) between the termite susceptibility and the relative physiological cost for cellular encapsulation (Supplementary Fig. 9). Therefore, the susceptibility of a termite was not strongly dependent on the relative physiological cost for cellular encapsulation, and termite investment in cellular immunity had a different profitability in survivorship among all 6 tested species, with *K. flavicollis* cellular encapsulation being the least profitable and *Hodotermopsis sjoestedti* being the most profitable.

#### CONCLUSIONS

All studied termite species presented a cellular encapsulation reaction to the infection of the entomopathogenic fungus *M. anisopliae*. Most of the observations about the occurrence of such an immune reaction were similar to that previously observed in *R. flavipes* (Chouvenc et al. 2009c), *i.e.* most points of infection were found in areas where the cuticle folds and at arthroal membrane locations, which usually are areas out of reach for allogrooming by nest mates. However, the profitability of the resource investment for nodule formation varied depending on the species. Among those species, *Hodotermopsis sjoestedti* was the most efficient at cellular encapsulation, followed in decreasing order by *N. voeltzkowi*, *R. flavipes*, *P. canalifrons*, *Hodotermes mossambicus*, and finally *K. flavicollis*. The immune reaction of *Hodotermopsis sjoestedti* appeared to be well adapted to a habitat rich with soil patho-

gens (damp wood) while *Hodotermes mossambicus* (semi-arid soil) and *K. flavicollis* (dry wood), species from habitats having a potentially relatively low pathogenic load (Rosengaus et al. 2003), showed a poorly adapted immune response. If poorly adapted to the fungal pathogen, a termite cellular encapsulation would not successfully prevent the fungal progression, as suggested by Avulova & Rosengaus (2011). This can be the case for *K. flavicollis* and *Hodotermes mossambicus* in which we observed cases of fungal growth through the nodule itself. However, for all other termite species tested herein, it appears that the hyphal body was not able to grow through the cellular nodule, which indicated an efficient cellular defense. These termite species would only be infected by *M. anisopliae* when exposed to high concentrations of conidia (Chouvenc et al. 2009a), supporting the hypothesis that the termites ran out of hemocytes to successfully encapsulate the too many points of infection, as previously described in Chouvenc et al. (2009c).

One limit of our study was the restricted access to a large sample size of individuals from various species and that all individuals from each species originated from a single laboratory colony. It is likely that the termite physiological responses to the exposure of an entomopathogen may vary within a species, depending of the colony of origin (Rosengaus & Traniello 2001). Reichheld (2010) suggested that the immune responses may also vary among the different castes within a colony, but the limited amount of samples obtained could not allow us to confirm it. The access to laboratory colonies of a wide range of species for standard tests remains a problematic limitation when dealing with live termites. Other than Chouvenc et al. (2009a), no other study was able to perform a standard bioassay on more than 3 species, preventing the accumulation of standardized results across species in the literature. Therefore, the current study presented a unique opportunity to provide insight in the evolution of termite disease resistance against pathogens.

Disease resistance in termites results from a complex interaction of defense mechanisms and grooming, antifungal gut activity and individual immunity were suggested to be the key elements of the termites' survivorship when exposed to pathogens (Chouvenc & Su 2010; Rosengaus et al. 2011). However, it was previously observed that all termite species have the ability to perform grooming on their nest mates (Yanagawa & Shimizu, 2007; Chouvenc et al. 2009a), which appears to be an ancestral trait (Nalepa 2011b), and the results of the current study corroborate that *M. anisopliae* was mainly able to penetrate the termite's cuticle in areas that are out of reach for grooming. In addition, the gut antifungal activity appears highly conserved in all termite species (Chouvenc et al. 2010). Our results suggest that

the variability in the efficiency of cellular encapsulation among the tested species is one of the factors involved in this difference in susceptibility to the entomopathogenic fungus. However, the immune system in insects has many interconnected pathways capable of eliminating invaders and this is particularly true in social insects such as termites with their myriad behavioral and molecular mechanisms involved in resistance to diseases (Rosengaus et al. 1998, 2007, 2011; Traniello et al. 2002; Chouvenc & Su 2010). It will be necessary to identify the various genes and their relative expression during the immune response and their diversity within the Isoptera as previously suggested by Bulmer et al. (2009) to elucidate how disease resistance may have evolved in termites.

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