

Research article

## Differential resistance and the importance of antibiotic production in *Acromyrmex echinator* leaf-cutting ant castes towards the entomopathogenic fungus *Aspergillus nomius*

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**Abstract.** Paired exocrine metapleural glands are present in almost all ants and produce compounds with antibiotic properties towards a variety of pathogenic fungi and bacteria. In *Acromyrmex* leaf-cutting ants, small workers have relatively large metapleural glands compared to large workers, and thus harbour approximately half the number of gland cells of large workers, despite being only one-fifteenth their body mass. Here we present results showing that when the two worker castes of *A. echinator* are treated with spores of the pathogenic fungus *Aspergillus nomius* in doses that correspond to the difference in metapleural gland cell numbers they do not differ in survival. However, we also show, for the first time, that small workers survive significantly longer than large workers when both are challenged with a dose of spores that corresponds to their difference in body mass. Furthermore, the time until *Aspergillus nomius* hyphae and spores appear on the cadavers of workers dead from infection, is significantly increased in the small worker caste. In addition to supporting previous findings that the metapleural glands have an important defence function, the results of this study indicate that the relatively large glands in small workers makes this caste particularly well adapted to preventing pathogenic microorganisms from entering the colony.

**Keywords:** *Acromyrmex*, *Aspergillus*, caste, entomopathogenic fungi, metapleural glands.

### Introduction

Antibiotic production, in addition to innate immune defences, is crucial to many organisms when coping with microbial parasites and pathogens (Hajek and St. Leger, 1994; Schmid-Hempel, 1998; Vilcinskas and Götz, 1999). The rapid spread of infectious diseases is expected to be especially problematic in social insects, due to the large number of closely related individuals living together (Hamilton, 1987; Schmid-Hempel, 1994, 1998; Baer and Schmid-Hempel, 1999; Pie et al., 2004; Boomsma et al., 2005). Effective defence mechanisms are thus expected to be present. Among the social insects, defences of this kind are particularly needed in the leaf-cutting ants, since they have to protect both themselves and their mutualistic basidiomycetous fungus (Lepiotaceae, Leucocoprineae) (Chapela et al., 1994), which serves as the only food source for the ant brood and the main food source for adult ants (Möller, 1893; Quinlan and Cherrett, 1979; Hölldobler and Wilson, 1990; Silva et al., 2003).

Several generalist entomopathogenic fungi have been isolated in, or close to, leaf-cutting ant colonies, including *Metarhizium anisopliae* (Humber, 1992, Hughes et al., 2004b), *Beauveria bassiana* (Hughes et al., 2004b), *Aspergillus flavus* (Schmid-Hempel, 1998; Hughes and Boomsma, 2003; Hughes et al., 2004b), *Aspergillus tamarii* (H. Fernández-Marín, pers. comm.), and *Aspergillus nomius* (this study). *Metarhizium* and *Beauveria* are obligate entomopathogenic fungi, whereas *Aspergillus* is a facultative pathogen of many plants and animals (St. Leger et al., 2000; Boucias and Pendland, 1998). Entomopathogenic fungi are characterised by their ability to replicate internally after entering the insect, either by penetrating the cuticle using a

combination of physical forces and the secretion of enzymes, or in some cases by entering the hemocoel through the insect gut after ingestion (Hajek and St. Leger, 1994; Boucias and Pendland, 1998; Vilcinskas and Götz, 1999). After entering the hemocoel, the fungus normally kills the host within days, due to tissue penetration and nutrient depletion (Boucias and Pendland, 1998). These fungal pathogens thus have a semelparous life-history and are obligate killers, producing transmission stages only after host death. Furthermore, they are known to produce secondary metabolites that debilitate specific cells of the insect's innate immune defence. Appropriate defence mechanisms that prevent spore germination and early hyphal growth are therefore essential (Hajek and St. Leger, 1994; Boucias and Pendland, 1998; Vilcinskas and Götz, 1999).

Leaf-cutting ants have several behavioural and chemical mechanisms to protect themselves and their mutualistic fungus against pathogenic microbes. Examples are: active cleaning (Wilson, 1980; Currie and Stuart, 2001), waste management (Hölldobler and Wilson, 1990; Bot et al., 2001a; Hart and Ratnieks, 2001), and the production of antibiotics by mutualistic bacteria (Currie, 2001; Currie et al. 1999, 2003, 2006). Also important are the paired exocrine metapleural glands that are present in all but a few ant species (Hölldobler and Engel-Siegel, 1984). These glands produce a secretion containing more than 20 different compounds in *Acromyrmex octospinosus* (do Nascimento et al., 1996; Ortius-Lechner et al., 2000), and these are known to have bactericidal and fungicidal properties (Maschwitz, 1974; Beattie et al., 1985, 1986; Ortius-Lechner et al., 2000; Bot et al., 2002; Poulsen et al., 2002a). The spread of gland secretion over the ant cuticle has previously been thought to be passive, but recent findings suggest that ants actively groom and apply the antibiotic secretion, especially in events of fungal infection (Bot et al., 2001b; Fernández-Marín et al., 2003, 2004, 2006), making it of hygienic importance for both the individual and the colony (Beattie et al., 1985, 1986; Schildknecht and Koob, 1970, 1971; Maschwitz et al., 1970; do Nascimento et al., 1996; Bot et al., 2002; Poulsen et al., 2002b).

Angus et al. (1993) examined the relationship between metapleural gland cell number and ant size and found a strong positive association between the two. However, in the genus *Acromyrmex* small workers have relatively large metapleural glands compared to large workers, so that a small worker has approximately half the number of gland cells of a major worker, despite being only one-fifteenth the body mass of the larger caste (Bot and Boomsma, 1996; Bot et al., 2001b; Poulsen et al., 2002a; Souza et al., 2006). Since the size of individual gland cells is not expected to be different between worker castes (cf. Angus et al., 1993 and references therein), possessing half the number of gland cells means that small workers are expected to produce half the amount of secretion and, hence, should be relatively better defended than large workers when exposed to pathogens. In the present study we challenged small and large workers of *A. echinator* with various spore doses of the pathogenic fungus *Aspergillus nomius* and examined their mortality. The dose applied to

the large worker caste was a standard dose, whereas the doses applied to small workers were reduced to correspond to either the ratio of metapleural gland cell number or the ratio of live body mass between the two castes, based on previously published estimates (Bot et al., 2001b; Poulsen et al. 2002a).

## Materials and methods

### Materials used

Ants from two *A. echinator* source colonies (Ae256 and Ae259) were used, which were collected in Gamboa, Panama, in May 2004 and kept in artificial nest boxes under normal Panamanian (non-airconditioned) indoor conditions. *Aspergillus* spores were obtained from a dead major worker of another *A. echinator* colony (Ae257), also excavated in Gamboa in May 2004. *Aspergillus* was isolated on Potato Dextrose Agar (PDA, 40 g/l; Becton, Dickinson and Co., MD 21151, USA), with 10 g/l agar (Becton, Dickinson and Co., MD 21151, USA) added as a solidifying agent, and 12.5 mg/l streptomycin sulphate and 12.5 mg/l penicillin-G (both from Fisher Scientific, NJ 07410, USA) added to inhibit bacterial growth. The culture was identified to the species level (*Aspergillus nomius*, synonymous to *A. zhaoqingensis*) by the Centraalbureau voor Schimmelcultures (www.cbs.knaw.nl). An initial *A. nomius* spore suspension was obtained by suspending a ca. 1 cm<sup>2</sup> piece of PDA covered with *A. nomius* spores in 10 ml sterile water (concentration  $C_{\max}$ :  $1.5 \times 10^6$  spores/ml).

### Ant-*Aspergillus nomius* bioassay

The experiment involved five treatments, each consisting of a group of 20 workers and being repeated with both colonies. In the first two treatments, large workers were treated with 0.5 µl of either the initial  $C_{\max}$  spore suspension (corresponding to ca. 750 spores) of *A. nomius* ( $L_{\text{standard dose}}$ ), or sterile water to control for any handling effect ( $L_{\text{control}}$ ). In the other three treatments, small workers were treated with either 1/15 of the *A. nomius* dose applied to large workers ( $S_{\text{mass dose}}$ ; corresponding to the approximate difference in worker body mass, i.e. ca. 50 spores were applied in 0.25 µl of a diluted spore suspension), half the dose of  $C_{\max}$  ( $S_{\text{cell dose}}$ ; corresponding to the approximate difference in metapleural gland cell number, i.e. 375 spores were applied in 0.25 µl of a diluted spore suspension), or 0.25 µl of sterile water ( $S_{\text{control}}$ ). In all cases, spores were applied by placing the droplet of suspension or sterile water on the thorax of the ant using a pipette. In order to prevent spore clumping, the suspension was mixed vigorously in the pipette prior to individual applications.

Following treatment, each ant was placed in a petri dish (diameter 4 cm) with a cotton plug soaked in saturated sugar water (ca. 2 g sucrose per litre sterile water). Mortality was recorded daily until all ants were dead, or until day 20 of the experiment, since no additional mortality from *Aspergillus* was expected after this period (cf. Hughes and Boomsma, 2004a,b; Hughes et al., 2004a). Dead ants were collected daily and checked under the microscope for the presence of hyphae and/or spores on the cuticle. After that, they were surface sterilised in sodium hypochlorite for three minutes followed by three washes of one minute in sterile water (Bidochka et al., 1998). This ensured that any subsequent fungal growth on the cadavers arose from fungi that had infected the ant while it was still alive and was not saprophytic growth after the ants had died. Following surface sterilisation, the ants were left on moist filter paper in closed petri dishes, and checked daily for hyphal growth and spore production using a binocular microscope.

### Statistical analyses

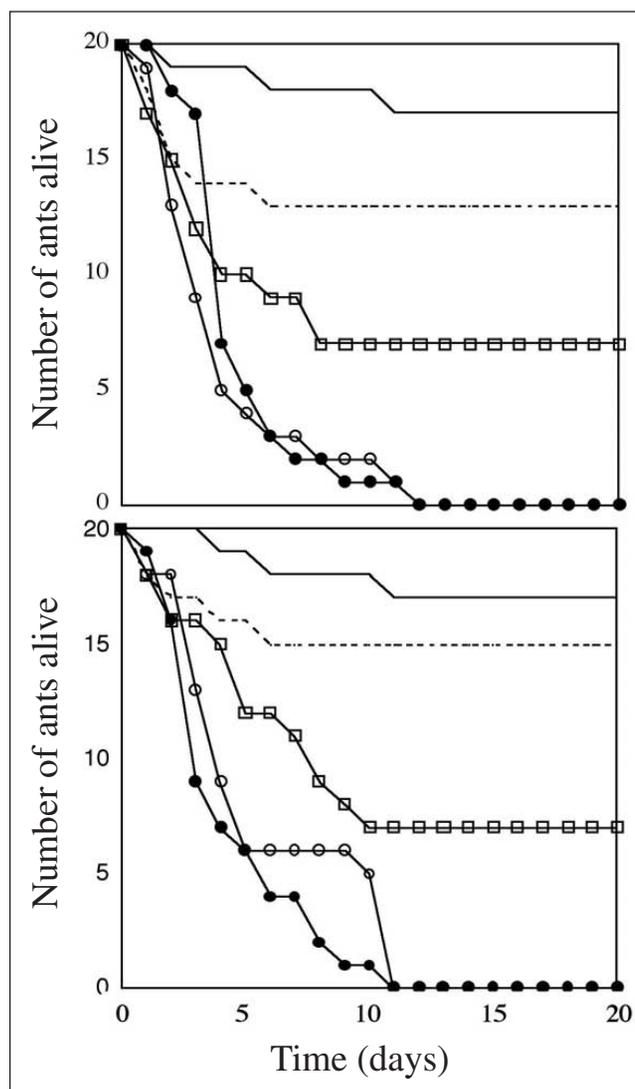
Since small workers were treated with two doses of *A. nomius* spores, one corresponding to body size and one corresponding to gland size, while large workers were treated only with one dose corresponding to both body size and gland size, three Cox's proportional hazard model tests were performed to test for differences in mortality between treatment groups over time (Volf, 1989). The first test only included small workers and had treatment ( $S_{\text{control}}$ ,  $S_{\text{cell dose}}$ ,  $S_{\text{mass dose}}$ ) and colony (Ae256 and Ae259) as factors. The second test was equivalent to this but performed for large workers only. Again, treatment ( $L_{\text{control}}$ ,  $L_{\text{standard dose}}$ ) and colony (Ae256 and Ae259) were included as factors. The third test excluded controls and was, hence, applied to test whether small workers treated with a dose corresponding to size survive significantly better than either small workers treated with a dose corresponding to cell number or large workers treated with the standard dose; again, treatment groups and colony of origin (Ae256 and Ae259) were included as factors.

Additionally, for both colonies we estimated the relationship between treatments ( $S_{\text{mass dose}}$ ,  $S_{\text{cell dose}}$ ,  $L_{\text{standard dose}}$ ) and the number of days from death to the external appearance of hyphae, the number of days from death to sporulation, and the number of days from the external appearance of hyphae to sporulation. ANOVA was performed using the software package JMP, with colony-of-origin as the main factor and with treatment group being a categorical factor; thus assuming that there is no fixed association between the doses applied and the responses observed across groups.

### Results

When the two castes of workers were challenged with a dose of *A. nomius* spores that corresponded to their 50% difference in metapleural gland cell number, they did not differ in survival (Fig. 1). However, when small workers were challenged with a dose of spores that corresponded to their 1:15 difference in body mass, they survived longer than large workers (Fig. 1). The Cox's proportional hazard model test for small workers only showed a strong effect of treatment ( $\chi^2 = 156.4$ ;  $df = 2$ ;  $P < 0.0001$ ), no effect of colony ( $\chi^2 = 1.632$ ;  $df = 1$ ;  $P = 0.2014$ ), but a significant interaction between colony and treatment ( $\chi^2 = 6.534$ ;  $df = 2$ ;  $P = 0.0381$ ). A similar pattern was found for large workers, where the treatment effect was highly significant ( $\chi^2 = 257.9$ ;  $df = 1$ ;  $P < 0.0001$ ), but in this test neither colony ( $\chi^2 = 0.041$ ;  $df = 1$ ;  $P = 0.8395$ ) nor the interaction between colony and treatment ( $\chi^2 = 0.00130$ ;  $df = 1$ ;  $P = 0.9713$ ) were significant. The third Cox's proportional hazard model, testing whether small workers treated with a dose corresponding to size survive better than either small workers treated with a dose corresponding to cell number or large workers treated with the standard dose, produced a highly significant treatment effect ( $\chi^2 = 209.1$ ;  $df = 2$ ;  $P < 0.0001$ ), no effect of colony of origin ( $\chi^2 = 0.632$ ;  $df = 1$ ;  $P = 0.4267$ ), and no significant effect of the interaction between colony and treatment ( $\chi^2 = 5.136$ ;  $df = 2$ ;  $P = 0.0767$ ).

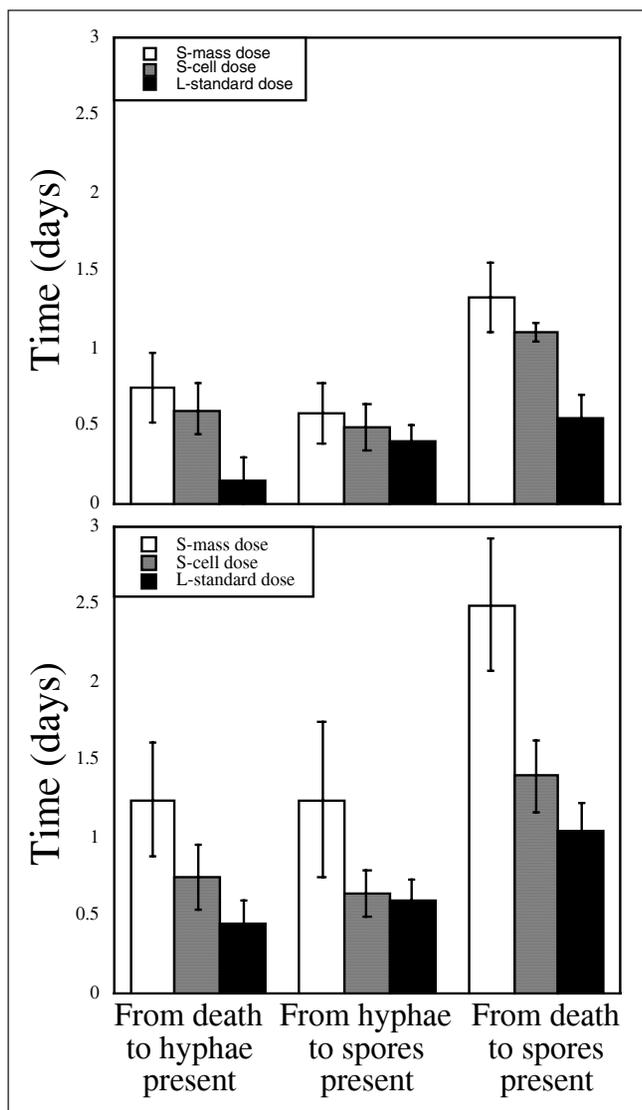
The duration from death to hyphal growth and spore production varied between the treatment groups (Fig. 2). The ANOVA showed that the number of days from death to the appearance of hyphae did not differ significantly between colonies, the effect of treatment was marginally non-significant, and there was no significant interaction between



**Figure 1.** The cumulative mortality in control and treatment groups for colony *A. echinator* 256 (top) and for colony *A. echinator* 259 (bottom) after exposure to *A. nomius*. Control groups are shown as dashed lines for small workers and solid lines for large workers; symbols for treatment groups are:  $\circ = S_{\text{cell dose}}$ ,  $\square = S_{\text{mass dose}}$ , and  $\bullet = L_{\text{standard dose}}$ . The number of ants with subsequent *A. nomius* sporulation from their cadaver out of the total number of dead ants in each group was: 2/9 in  $S_{\text{control}}$ , 18/20 in  $S_{\text{cell dose}}$ , 13/13 in  $S_{\text{mass dose}}$ , 1/3 in  $L_{\text{control}}$ , and 20/20 in  $L_{\text{standard dose}}$  for *A. echinator* 256; and 1/5 in  $S_{\text{control}}$ , 20/20 in  $S_{\text{cell dose}}$ , 7/13 in  $S_{\text{mass dose}}$ , 0/3 in  $L_{\text{control}}$ , and 20/20 in  $L_{\text{standard dose}}$  for *A. echinator* 259.

colony and treatment (Table 1). Only colony-of-origin appeared to have a significant effect on the number of days between the appearance of hyphae and spores (Table 1). However, the number of days from death to sporulation differed significantly between both colonies and treatments, with there being no significant interaction between the factors. Interestingly, in colony Ae259 sporulation following hyphae emergence on the cadavers occurred in only 61.5% of the cases in  $S_{\text{mass dose}}$ , whereas the other two treatment

groups ( $S_{\text{cell dose}}$  and  $L_{\text{standard dose}}$ ) had sporulation frequencies of 100% of the infected workers. This difference between groups was not present in Ae256, where the three treatment



**Figure 2.** The number of days from death until *A. nomius* hyphae (left) and spores (right) were observed on the cadavers, and the time from hyphae were observed until spores were present (middle) for the three treatment groups and the two colonies *A. echinator* 256 (top) and *A. echinator* 259 (bottom).

groups had sporulation frequencies of 92.3% ( $S_{\text{mass dose}}$ ), 90.0% ( $S_{\text{cell dose}}$ ), and 100.0% ( $L_{\text{standard dose}}$ ), respectively. Note that a few (four out of twenty) workers in the control groups also exhibited *Aspergillus* sp. sporulation from cadavers while the experiment was running (Fig. 1); however, this most likely reflects the latent presence of *Aspergillus* present on the ants (cf. Hughes and Boomsma, 2004b).

## Discussion

Parasite virulence depends on the density of parasites infecting a host (e.g. Milner and Prior, 1994; Ebert et al., 2000; Hughes et al., 2004a; Hughes and Boomsma, 2004b), which also applies in leaf-cutting ants, where defences to cope with pathogens include self-grooming, allo-grooming, antibiotic production, and both humoral and cellular immune system components (Kermarrec et al., 1986; Gillespie et al., 1999; Hughes et al., 2002; Poulsen et al., 2002b; Sumner et al., 2003; Baer et al., 2005). In this study we find that small workers survive longer than large workers when the dose is equivalent to body mass (so parasite density is controlled) (treatment groups  $S_{\text{mass dose}}$  and  $L_{\text{standard dose}}$ ; Fig. 2), implying that small workers are more resistant to *A. nomius* than large workers. Furthermore, there was no apparent difference when the dose applied was controlled for the number of metapleural gland cells (treatment groups  $S_{\text{cell dose}}$  versus  $L_{\text{standard dose}}$ ), suggesting that the different responses of *A. echinator* worker castes towards *Aspergillus* are likely to be due to either the substantially larger metapleural gland size in small workers (Bot and Boomsma, 1996; cf. Poulsen et al., 2002a) or a caste specific difference in grooming ability (Wilson, 1980; Hölldobler and Wilson, 1990). As we did not observe a significant difference in mortality within the same worker castes when exposed to spore concentrations that were proportional to metapleural gland cell number ( $S_{\text{cell dose}}$  and  $L_{\text{standard dose}}$  treatments), it is most likely that metapleural gland secretion is the primary factor to explain this result by effectively preventing *Aspergillus* spore germination.

The importance of ant defence against potentially virulent pathogens is substantiated by the fact that the observed mortality after exposure to *A. nomius* could not have been caused by instantaneous effects of toxins present in the spore suspension followed by superficial growth on the cadaver's cuticle, because: 1. The ants were surface sterilised following death, killing any spores or hyphae that might have been present on

Main factors	From death until hyphae visible			From hyphae visible until spores visible			From death until spores visible		
	F	df	P	F	df	P	F	df	P
Colony-of-origin	1.7514	1	0.1890	5.335	1	0.0231	11.62	1	0.001
Treatment	6.715	2	0.0019	2.495	2	0.0881	10.30	2	<0.0001
Colony x Treatment	0.4132	2	0.6627	1.005	2	0.3702	1.570	2	0.2136

**Table 1.** One-way ANOVA testing the effects of colony-of-origin and treatment (*A. nomius* exposure) on the number of days from ant death until hyphae were visible on the cadaver (left), from when hyphae were visible until spores became visible (middle) and from the time of ant death until spores were visible on the cadaver (right).

their cuticle, 2. The external appearance of *A. nomius* always began around the intersegmental membranes and leg joints, which is typical of fungus growth originating from inside the host (Boucias and Pendland, 1998), and 3. *Aspergillus* toxins are produced by the hyphae (Boucias and Pendland, 1998), and hyphae were not present on the cuticles of ant cadavers when ants were first observed dead. Thus, the observed mortalities appear to directly reflect individual ant ability to prevent *A. nomius* spore germination after initial exposure.

Despite their exposure to a dose corresponding to metapleural gland cell number ( $S_{\text{cell dose}}$ ) and this dose resulting in similar mortality, small workers took significantly longer to show evidence of hyphal growth and spore production than large workers ( $L_{\text{standard dose}}$ ) (Fig. 2). The more rapid appearance of hyphae and spores on the cuticle of  $L_{\text{standard dose}}$  than on  $S_{\text{cell dose}}$  is curious, because if other components delayed the growth and sporulation of the pathogen, these should also have resulted in a difference in the survival curve, which we did not find (Fig. 1). One possible explanation for the similarities in survival between castes, but subsequent differences in hyphal growth and events of sporulation, could be that larger ants may provide better conditions for fungal growth than the smaller caste. Similarly, differences in the nutritional values of workers to *A. nomius* between colonies could explain the highly significant differences in the number of days from ant death to hyphae and spore appearance on the cuticle of ants originating from the two *A. echinator* colonies (Fig. 2). However, the use of only two colonies in this study makes it hard to quantify actual colony-level differences in mortality to pathogen exposure in general; nevertheless, the variation observed indicates that this is an important feature that needs to be taken into account when examining pathogen virulence.

Castes and/or colonies could differ in their susceptibility to the within-host effect of the fungus (starvation, toxin production, etc.), and thus in the threshold level of parasite growth that they can survive, e.g., if workers differ in physical condition at the time of exposure to spores, which, if true, should also have been apparent in the survival curves. They could also differ either quantitatively or qualitatively in their gland secretion, in grooming ability, or in their immune response (Bot and Boomsma, 1996; Hughes et al., 2002; Ortius-Lechner et al., 2004; Baer et al., 2005). However, if castes and colonies differ in grooming, immunity or metapleural gland secretion (Ortius-Lechner et al., unpubl. data), such differences would be expected to result in differences in survival and possibly in differences in time to hyphal and spore presence, but no differences in survival were found. Possible explanations for this may be: 1. that there were in fact differences in survival, but that these remained undetected in this study, e.g. due to a limited sample size of 20 workers per treatment group; 2. that the host-parasite interaction is more complex and the differences in defence affecting the time to sporulation would have resulted in differences in survival except that they were counterbalanced by something else, for example that a caste and/or colony was more susceptible to the toxins; and/or 3. that there were qualitative differences in the metapleural gland secretion that did not affect survival but did affect sporulation (for example if some compounds

inhibit hyphal growth but not spore production as shown by Bot et al., 2002).

The presence of physical worker castes with different tasks within the same colony, and specific behavioural and physiological adaptations to these tasks, is not uncommon in social insect societies (e.g. Hölldobler and Wilson 1990). The primary roles of small workers are brood care, fungus farming, and the preparation and incorporation of leaf-material into the garden (Weber, 1972; Wetterer, 1999). Small workers are particularly abundant in the top section of the fungus garden where leaf-material, potentially harbouring pathogenic microbes, is incorporated (Poulsen et al., 2002a). It is, therefore, likely the small workers that form the key barrier in preventing the entry of microorganisms into the fungus garden and, thus, protecting the vulnerable fungi, brood, and queen. Effective defence mechanisms against pathogens will therefore be particularly important to the small workers. The antibiotic-producing metapleural glands of the small worker caste are disproportionately large and these ants are also more effective at grooming than large workers (Bot and Boomsma, 1996; Hughes et al., 2002). Interestingly, Hughes et al. (2002) found that small workers were more resistant than large workers when treated with the same dose of *Metarhizium anisopliae*, whereas we find here that they are more resistant to *A. nomius* than large workers only when treated with a dose corresponding to their body size. This suggests that the difference in resistance between small and large workers is greater against *Metarhizium* than *Aspergillus*, in concordance with *Metarhizium* spores being more sensitive to the metapleural gland secretion than *Aspergillus* spores (Bot et al., 2002). Differences in gland potency against specific microbes may thus explain the observed differences in resistance to the two parasites (Hughes et al., 2002; this study), and hence confirm the importance of the metapleural gland defence function in addition to adding more evidence to the hypothesis that the small worker caste is very well equipped to prevent the introduction and subsequent spread of virulent pathogens.

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