

PAULO LUIZ DA SILVA

**BULA DA GLÂNDULA METAPLEURAL DE FORMIGAS CORTADEIRAS:
INFERÊNCIAS QUANTO À DEFESA ANTIMICROBIANA**

Dissertação apresentada à
Universidade Federal de Viçosa,
como parte das exigências do
Programa de Pós-Graduação em
Entomologia, para obtenção do
título de *Magister Scientiae*.

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RESUMO

SILVA, Paulo Luiz, M. Sc. Universidade Federal de Viçosa, setembro de 2008. **Bula da glândula metapleural de formigas cortadeiras: Inferências quanto à defesa antimicrobiana.** Orientador: Simon Luke Elliot. Coorientadores: Olinto Liparini Pereira, Robert Weingart Barreto e Terezinha Maria Castro Della Lúcia.

Formigas cortadeiras apresentam interações complexas. São cultivadoras de fungo o qual é alimentado por substratos vegetais. Tanto as formigas como o fungo do jardim correm o risco de serem parasitados por microrganismos. Para defenderem tanto o fungo quanto elas mesmas, as formigas utilizam defesas antimicrobianas, como substâncias secretadas através da glândula metapleural. Sendo que esta defesa está sujeita a pressão de seleção de acordo com o risco de ataque por parasitas, variações no investimento nela podem ser consequência deste risco. Ao mesmo tempo, pode haver indivíduos com diferentes valores para a colônia, dependendo em parte na área onde são encontradas, e podem ter maior ou menor investimento em defesas contra parasitas. Diante disso o trabalho propôs verificar a variação do volume do reservatório da glândula metapleural (bula) entre espécies, colônias e tarefas de formigas cortadeiras para verificar possíveis estratégias de defesas das operárias contra patógenos. Avaliou-se também a presença de uma bactéria mutualista, que é utilizada também como defesa contra parasitismo, e a possível relação entre esta e o volume do reservatório da glândula metapleural. Foram utilizadas um total de 11 colônias de quatro espécies de formigas cortadeiras, *Atta laevigata* (F. Smith, 1858), *Atta sexdens rubropilosa* Forel, 1908, *Acromyrmex subterraneus brunneus* (Forel, 1911) e *Acromyrmex disciger* (Mayr, 1887). Formigas foram coletadas de três áreas das colônias: as áreas de forrageamento, os jardins do fungo simbiote e o lixo. As informações tomadas foram: volume da bula da glândula metapleural, massa das formigas e uma estimativa do tamanho das colônias de bactéria mutualista. Análises de covariância foram utilizadas, com massa dos indivíduos como covariável, para determinar as contribuições de diversos fatores para explicar o tamanho desse órgão. Pelas análises obtidas, as duas espécies de *Atta* possuem bulas maiores em relação às duas espécies de *Acromyrmex*.

provavelmente refletindo não uma maior exposição a parasitas mas uma estratégia de maior investimento em defesas. As jardineiras e as forrageadoras das espécies *A. laevigata* e *Ac. subterraneus brunneus* tiveram bulas maiores em relação às lixeiras, isto mostrando uma menor qualidade (em termos de defesas contra parasitas) das lixeiras. Neste caso, suponhamos que estes indivíduos, sendo de menor qualidade, são alocados a tarefas mais perigosas e morrerão rapidamente. *Acromyrmex subterraneus brunneus* foi a única que apresentou colônias de bactéria mutualista, dispostas no tórax. Houve uma relação positiva do tamanho desta colônia com o volume da bula da glândula metapleurial. Essas variações nesses sistemas de defesas implicam que as formigas podem apresentar diferentes estratégias para defender o ninho contra patógenos e parasitas.

ABSTRACT

SILVA, Paulo Luiz, M. Sc. Universidade Federal de Viçosa, september, 2008. **Bulla of the metapleural glands of leafcutter ants: Inferences as the antimicrobial defences.** Adviser: Simon Luke Elliot. Co-Advisers: Olinto Liparini Pereira, Robert Weingart Barreto and Terezinha Maria Castro Della Lúcia.

Leafcutter ants live in a world of complex interactions, the most notable being their cultivation of a mutualistic fungus on vegetable substrates. These ants, and their mutualistic fungus gardens, are at risk from attack by parasitic microorganisms. To defend themselves and their mutualist, they have antimicrobial defences, such as substances secreted from their metapleural glands. As this defence is subject to selective pressures according to the risk of parasite attack, variations in the amount of defence may reflect variations in the risk. Meanwhile, there may be variation in the value of individual workers to the colony, dependent in part upon the area in which they work, and investment in their defences may also reflect this. In this study, the volume of the externally visible part (bulla) of the reservoir of the metapleural gland of workers was compared between species, colonies and tasks, to determine variations in defence strategies against pathogens. Additionally, colony sizes of a bacterial mutualist on the workers' thoraxes, were estimated, looking for a possible relationship with metapleural gland bulla size. A total of eleven laboratory colonies were used, of four species of leafcutter ants: *Atta laevigata* (F. Smith, 1858), *Atta sexdens rubropilosa* Forel, 1908, *Acromyrmex subterraneus brunneus* (Forel, 1911) and *Acromyrmex disciger* (Mayr, 1887). Workers were collected from three areas of the colonies: foraging areas, symbiotic fungus gardens and the refuse deposits. Parameters measured were: the volume of the bulla of the metapleural gland, the weight of ants and an estimate of the colony sizes of the symbiotic bacteria. Analyses of covariance were conducted using individual mass as a covariate, to determine the contributions of a range of parameters in explaining variation in the size of this organ. The two species of *Atta* were found to have larger bulla when compared to the two species of *Acromyrmex*., probably reflecting not a greater degree of exposure to parasites but a strategy of greater investment in defences. Gardeners and foragers of the

species *A. laevigata* and *Ac. subterraneus brunneus* had larger bulla than did waste workers, revealing a lower quality (in terms of parasite defence) of the waste workers. In this case, we expect that these individuals, being of lower quality, are allocated to more dangerous tasks, to die more rapidly. *Acromyrmex subterraneus brunneus* was the only species with visible colonies of the bacterial mutualist on the thorax. There was a positive relation between the size of this colony and the volume of the bulla of the metapleural gland. These variations in degrees of defence imply that the ants have different strategies to defend their colonies against pathogens and parasites.

INTRODUCTION

Leafcutter ant colonies are typically numerous and are characterized by complex social systems. The plant substrate they cut serves as food for the fungal symbiont upon which they depend and which they cultivate in subterranean chambers. The architecture of the nests varies according to the ant species. Thus, mature colonies of *Atta* species tend to have deep nests with a number of chambers, while *Acromyrmex* colonies have nests closer to the soil surface and with fewer chambers (Della Lucia 1993; Anjos *et al.* 1998). Concerning internal organisation of the colony, ants have division of labour, being differentiated according to their activities; thus, an individual may be a fungus gardener, a forager, a generalist, an excavator, a waste worker or a nursemaid (Wilson 1980; Camargo *et al.* 2007). To maintain this social system in the face of microbial parasites, ants have antimicrobial defences, notably secretions from the metapleural gland (Nascimento *et al.* 1996; Ortius-Lechner *et al.* 2000; Bot *et al.* 2001; Diehl & Junkeira 2001; Bot *et al.* 2002; Fernandez-Marin *et al.* 2006; Poulsen *et al.* 2006; Hughes *et al.* 2008) and a mutualist actinomycete bacterium which is adhered to the body of workers and which also produces antimicrobial substances (Currie *et al.* 1999; Schultz 1999; Poulsen *et al.* 2002a; Currie *et al.* 2003a; Poulsen *et al.* 2003; 2005; Kost *et al.* 2007). Apart from such direct antimicrobial defences, the organization of labour by social insects can be used to protect the colony from parasites and pathogens (Schmid-Hempel 1998; Pie *et al.* 2004).

There is a rich literature on ant antimicrobial defences (Nascimento *et al.* 1996; Currie *et al.* 1999; Schultz 1999; Ortius-Lechner *et al.* 2000; Bot *et al.* 2001; Diehl & Junkeira 2001; Bot *et al.* 2002; Poulsen *et al.* 2002a; Currie *et al.* 2003a; Little *et al.* 2003; Poulsen *et al.* 2003; Baer *et al.* 2005; Poulsen *et al.* 2005; Fernandez-Marin *et al.* 2006; Poulsen *et al.* 2006; Cremer *et al.* 2007; Kost *et al.* 2007; Hughes *et al.* 2008) and these generally assume that there is a selective pressure imposed by pathogens on ants. However, the only studies which have demonstrated pathogen attack on ant colonies are those conducted by Evans and Samson (1982), who observed, over two years, consistent mortality of the ant *Cephalotes* sp. caused by the fungus *Cordyceps unilateralis* on the trunk of a single tree in Belém/PA. These observations, together with a

similar study conducted in Ghana (Evans 1974) are the only attempts to follow epizootics (in these cases it would probably be more appropriate to use the term enzootic) of a pathogen in a population of ants. These studies, although providing quantitative data on the number of ants found killed by fungi, did not quantify the mortality as a proportion of the total host populations. We therefore have little information on the pressure imposed by these pathogens. Subsequent to these studies, Hölldobler & Wilson (1990) commented that, in spite of the importance of ants to ecosystems and the diversity of fungi which attack them (Samson *et al.* 1988; Evans 2003), this interaction has never been quantified. Despite these considerations, however, we still find ants employing elaborate defence strategies against fungal pathogens and against other potentially antagonistic micro-organisms.

Members of the leafcutter ant (Attini) genera *Acromyrmex* and *Atta* have both economic and ecological importance (Samways 1983; Jones *et al.* 1997; Folgarait 1998) due to the damage caused as agricultural and forest pests (Della Lucia 1993; Anjos *et al.* 1998) and due to their role in seed dispersal (Peternelli *et al.* 2003). These insects are considered the principal primary consumers in terrestrial neotropical ecosystems (Beattie & Hughes 2002). In a review of twelve studies, Cherrett (1986) calculated that between 12 and 17% of the production of leaves in neotropical forests are cut by *Atta* spp. (and the inclusion of *Acromyrmex* spp. would increase this estimate). Meanwhile, in leafcutter ant exclusion experiments conducted in the Amazon Forest, Vasconcelos and Cherrett (1997) demonstrated a profound effect of ant activity on the establishment of tree seedlings which would, no doubt, affect the species composition of the forest.

Nests of *Atta* can reach 3.5 to 8 million individuals. Meanwhile, *Acromyrmex* colony growth in the laboratory can reach 1,000 individuals in a year (Della Lucia 1993; Anjos *et al.* 1998). Nests of *Atta* spp. constructed in the soil are deep and may have up to a hundred chambers bearing fungus garden, while those of *Acromyrmex* spp., which construct nests a few centimetres from the soil surface, may contain one to a few fungus garden chambers (Della Lucia 1993; Anjos *et al.* 1998). These characteristics may well affect exposure to disease and disease transmission and, therefore, defences employed by the ants. In a comparable system, termite colonies, a model of disease

transmission demonstrated, for example, that division of the nest into chambers could limit disease progress (Pie *et al.* 2004). We might therefore expect *Acromyrmex* spp., with their simpler nest architectures, to be more vulnerable to disease than *Atta* spp.

Division of labour can potentially contribute to keeping the nest free of pathogens. Ant colonies divide labour by social organization in castes and tasks (Wilson 1980). Thus, worker ants can be found nursing immatures, maintaining the fungus garden and caring for the queen, in addition to generalist workers and foragers (Wilson 1980; Camargo *et al.* 2007). This system is characteristic of social insects and could be employed to limit infections of the colony by pathogens (Schmid-Hempel 1998). Apart from division of labour at this level, individual tasks can be partitioned. Thus, foragers can divide tasks such as cutting and carrying leaves to the colony, between a series of individual workers (Hart & Ratnieks 2001; Hart *et al.* 2002); similar behaviour can be seen among workers that transport waste to the colony's refuse dump (Lacerda *et al.* 2006). The refuse dump is a region where workers could become contaminated with pathogenic fungi or other potentially harmful micro-organisms (Lacerda *et al.* 2006). From here, workers could potentially carry pathogens to other areas of the colony. However, task partitioning has the potential to limit this as an individual which has had contact with waste could be prevented from entering other, potentially vulnerable areas of the colony. In a similar fashion, a colony could have age-dependent polyethism, by which means workers could begin adult life conducting tasks within the colony and only leave, to come into contact with potential sources of pathogen inocula (e.g. waste or foraging trails) once they are older (Schmid-Hempel 1998). According to this model, ants which are exposed to disease risk outside the colony (Evans 1974; Evans & Samson 1982; Hughes *et al.* 2004) would not return to the more important areas of the nest, so avoiding bringing pathogens to these areas (Schmid-Hempel 1998). These behavioural mechanisms, if employed by leafcutter ants, would assist the colony in maintaining its complex social structure, but to date has been little investigated empirically.

Among the means available to ants to combat disease, they possess a pair of 'metapleural' glands that produce secretions with antimicrobial properties (Nascimento *et al.* 1996; Poulsen *et al.* 2006). These glands are localized within

the thorax and are connected to a reservoir ('bulla') which is visible as a bulge in the cuticle (Fig. 1). The the externally visible component of the bulla is proportional in size to its internal volume (Bot *et al.* 2001). In workers of *Atta sexdens rubropilosa*, *A. cephalotes* and *Acromyrmex octospinosus* (Reich, 1793), substances extracted from the metapleural glands are capable of controlling the growth of bacterial colonies and of the fungal mycoparasite *Trichoderma viride* (Nascimento *et al.* 1996). Recently, Rodrigues *et al.* (2005) demonstrated that the removal of worker ants from nests of *Atta sexdens rubropilosa* resulted in the growth of 11 fungal species in the fungal garden, among these the mycoparasites *Escovopsis weberi* and *Trichoderma* sp., demonstrating the importance of ant tending in maintenance of the health of the fungus garden. Meanwhile, in ants infected with *Aspergillus tamaritii*, a positive correlation between metapleural gland size and individual ant survival has been demonstrated (Poulsen *et al.* 2006). In this instance, survival is probably assisted by ant grooming, through which means metapleural secretions are passed over the body (Fernandez-Marin *et al.* 2006). More recently, Hughes *et al.* (2008) found variaion in the size of the bulla of the metapleural gland among attine species (*Atta*, *Acromyrmex*, *Trachymyrmex* e *Sericomyrmex*), and based their study upon na assumption that workers with smaller bulla (and thus metapleural gland) would be less resistant to disease in comparison with species ith larger bulla. They found colonies of *Atta* and *Acromyrmex* to have larger bulla in comparison to the more basal species and proposed a correspondingly elevated resistance to disease. Besides these variations between species, it is possible that the volumes of the bulla vary according to the location where the worker is to be found within the colony and between colonies, potentially representing corresponding variation in investment in defence against disease.

Similarly, ants could present variation in the degree to which they employ the mutualistic actinomycete bacterium *Streptomyces* (Kost *et al.* 2007). In *Acromyrmex octospinosus* this bacterium is to be found adhering to the cuticle of workers. Meanwhile, a similar actinomycete, *Pseudonocardia*, is associated in a similar fashion with other species of *Acromyrmex* as well as *Trachymyrmex* and *Apterostigma* (Cafaro & Currie 2005). A mutualism has been demonstrated between these bacteria and their host leafcutter ants (Currie *et al.* 1999; Currie

et al. 2003a), based upon experimental observations of a suppression of growth of the fungal mycoparasite *E. weberi* which is specific to the leafcutter symbiont fungus *Leucoagaricus* (Currie *et al.* 2003b; Kost *et al.* 2007). In *Acromyrmex octospinosus*, the acinomyceete is to be found at its highest concentrations on the tegument of workers which are found in the fungus garden (compared with foragers). Similarly, in the fungus garden, workers at the top of the garden have larger bulla of the metapleural gland but less actinomyceete (Poulsen *et al.* 2002a).

Faced with these studies, we chose to investigate leafcutter species with different habits. Thus, we investigated two species of *Acromyrmex* and two of *Atta*. The former of these genera constructs comparatively smaller and more superficial nests, with fewer chambers, fewer individuals and less structured societies, we initially postulated a greater exposure to disease risk and a correspondingly elevated investment in defence. We measured, as a correlate of this investment, the size of the bulla of the metapleural gland (Hughes *et al.* 2008). We further postulated that variation would be seen in this parameter and also in the amount of actinomyceete symbiont observable (in the species in which it was to be found), according to task being conducted (caring for the garden, foraging or working on the waste).

MATERIALS AND METHODS

Species and colonies of study leafcutter ants

The colonies of leafcutter ants used in this study were collected (i.e. excavated) in the vicinity of Viçosa, state of Minas Gerais (20° 45'05,76''S, 42°52'51,80''W), Brazil, and were taken to the laboratory. They were subsequently maintained for over a year in a climate-controlled room with temperature of $25 \pm 2.5^{\circ}\text{C}$ and relative humidity of $75 \pm 10\%$ (Della Lucia 1993) in the Insectary of the Animal Biology Department of the Federal University of Viçosa. They were daily provided leaves of *Ligustrum* sp. (Oleaceae, 'privet') and *Acalypha wilkesiana* (Euphorbiaceae). For the present study, three colonies of each of *A. laevigata*, *A. sexdens rubropilosa* and *Ac. subterraneus brunneus* were used, in addition to two colonies of *Ac. disciger* (a third colony of this species was not available).

To obtain rough estimates of colony sizes, three 50ml samples of the fungal symbiont were taken from three of the colonies. These were refrigerated (to 3°C) for two hours and the now-immobilized ants were counted. The volumes of the symbiont fungus growth in the colonies was determined with a rule. The products of these two numbers gave an estimate of the numbers of individuals in the fungus gardens, of use for comparative estimates of colony size (Pereira & Della Lucia 1998). Estimates obtained were 375,500 workers for *Atta* colonies (one *A. sexdens rubropilosa* colony was used as all six colonies were very similar), 104,600 workers for *Ac. subterraneus brunneus* and 108,300 for *Ac. disciger*.

Sampling of ants

Ants were sampled from the foraging and waste areas as well as the fungus garden of each colony. For the purposes of this study, these workers are henceforth termed foragers, waste workers and gardeners respectively. The foragers and waste workers were collected with forceps as they crossed a line visualized with a 30cm rule (i.e the rule was held above the substrate); this was to avoid human bias due to worker size. To collect gardeners, 300ml samples of

fungus garden were transferred to trays. These samples were gently broken to free the ants which were collected using a rule as described above. All collected ants were placed individually into 1.5ml lidded Eppendorf tubes and refrigerated to 3°C for a maximum of two hours. They were then removed from their tubes and weighed individually using a precision balance, then returned to the tubes whereupon 70% ethanol was added.

With the above procedure, an excess of ant workers was collected. Based on the weights obtained, three or four mass categories were calculated for each species, e.g. 0.10 to 5.15mg, 5.16 to 10.20mg, 10.21 to 15.25mg and 15.26 to 20.30mg for *A. laevigata*. Equal numbers of individuals from each weight category were subsequently used to render between-location comparisons comparable (i.e. a number of ants were discarded so as to have samples approximately equal by weight).

Further, independent sampling was conducted, following the procedure described above, in order to obtain weight distributions for workers from the different sample areas for the four species.

Estimates of the volume of ant bulla

Measurements of three dimensions of the external surfaces of the bulla were taken with a graduated ocular in a stereomicroscope at a magnification of 40x (Figure 1). Despite partial distortion into an ovoid, these structures approximate spheres so estimated volumes were calculated as: $\text{Volume} = \frac{4}{3} \times \pi \times \text{width}/2 \times \text{length}/2 \times \text{height}$. Note that this incorporates an assumption that half of the structure is hidden within the insect, as measured height is taken as one of the three radii.

(a)



Figure 1. Measurements of the bulla (reservoir) of the metapleural gland of leafcutter ants. (a) Arrow indicates location of bulla on *Atta sexdens rubropilosa*; superior (b) and lateral (c) views showing measurements of width (b: dotted line), height (c: solid line) and length (c: dashed line).

(b)



(c)



Estimation of colony sizes of the symbiont actinomycete bacterium in *Ac. subterraneus brunneus*

The symbiotic actinomycete which is to be found on the cuticle of leafcutter ants was visible only in the case of *Ac. subterraneus brunneus*. Estimates of the sizes of these colonies were taken from individuals whose bulla had been measured (above), using a stereomicroscope as detailed above. The extent of the colonies was measured on the propleura of the thorax, using only one dimension (anterior-posterior length; Figure 2).

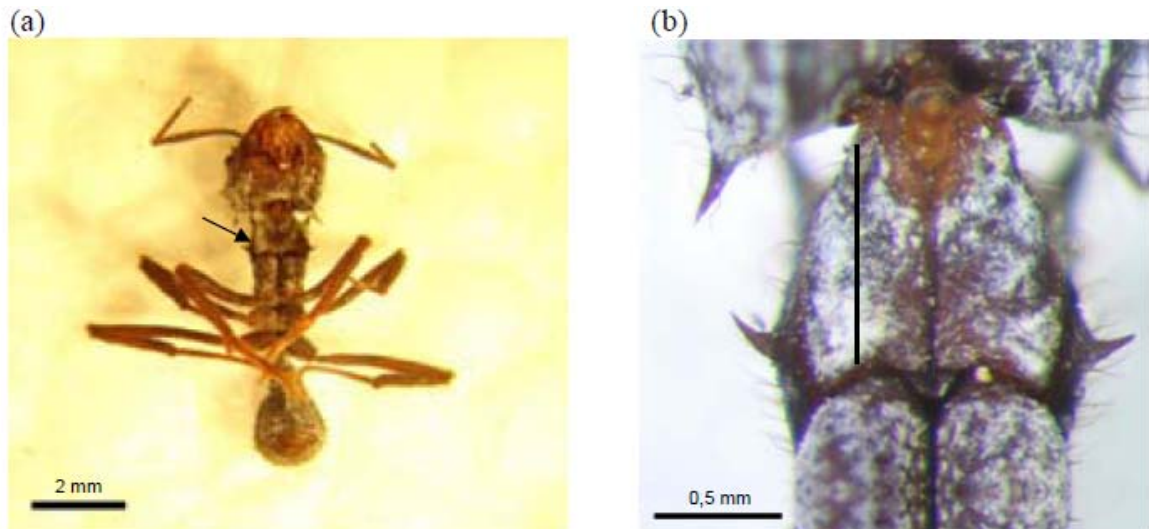


Figure 2. Colonies of symbiotic actinomycete bacteria on the propleura of the thorax of the leafcutter ant *Acromyrmex subterraneus brunneus*. Ventral views. Arrow (a) indicates region measured and solid line (b) indicates extent and orientation of measurements taken.

Statistical Analyses

All analyses were conducted using the program R (Crawley 2007) with discriminatory significance levels (α) of 0.05. The first set of analyses were of covariance (ANCOVA) and began with the construction of a full model using \log_{10} bulla volume as the dependent variable and \log_{10} worker weight as the covariable (i.e. the actinomycete on *Ac. subterraneus brunneus* was considered separately, below). Explanatory variables were location of collection (i.e. the individual was described as a forager, waste worker or gardener) and colony number (1-3; 1-2 for *Ac. disciger*).

Initially, the four leafcutter species were included in a single model, with species as a further explanatory variable, in the hope that patterns could be seen across species. Simplification of this model were based upon attempts to join intercepts of similar values for the different species but data for no two species could be joined. From this point on, each species was considered in isolation.

For each species, a full model was constructed as described above for ANCOVA, with the all of the pertinent interactions terms included. To simplify the model, non-significant terms ($\alpha = 0.05$) were excluded, beginning with the

most complex interactions. At each exclusion step, the simplified model was compared by ANOVA with the previous model, non-significance indicating that the simplification (the term elimination) could be accepted and a new term selected for exclusion. This procedure was followed until the minimal models were obtained. At this juncture, a further simplification was attempted in which different levels of the explanatory variables might be joined (for example joining colony 1 with colony 3 or forager with waste worker).

To determine if there was a correlation in between the estimates of actinomycete colony sizes on the cuticles of *Ac. subterraneus brunneus* and the estimated volumes of these ants' bulla, a Spearman correlation (Beasley 2004) was conducted. As a significant correlation was detected (see Results), a final ANCOVA was conducted as described above but with estimates of actinomycete colony size as the dependent variable. As the aim was to determine if there was a pattern beyond the observed correlation, the estimated volume of the bulla was included as a covariable, rather than weight (this latter variable now excluded). Explanatory variables (location and colony number) were included as above and model simplification was as above.

RESULTS

Between-species variation in the volume of the bulla of the metapleural gland

As described above, it was not possible to simplify the statistical model so as to remove species as an explanatory factor, implying a statistically significant difference in the estimated volume of the metapleural gland bulla between species (ANCOVA, $F_{3,1747} = 38,71$, $P < 0.0001$, Table 1). It can be seen in Figure 3a-d that the bulla of the two *Atta* species were larger than the two *Acromyrmex* species.

Table 1: Minimal model obtained by ANCOVA of the volume of the bulla of the metapleural gland of four species of leafcutter ants (see text for details), with individuals' weights as a covariable.

	d.f.	SS	MS	F	P
Regression	1	97.89	97.89	8580.2	0.0001
Species	3	63.67	21.22	1860.3	0.0001
Weight: Species	3	1.32	0.44	38.71	0.0001
Residual	1747	19.93			
Total	1754				

Variation in the volume of the bulla of the metapleural gland between workers from different areas of work

The ANCOVA's conducted for the four species individually showed that the work area explained none of the variation in bulla volume for the two species *A. sexdens rubropilosa* and *Ac. disciger* (Table 2 b&c). This variable did, however, contribute to the variation observed in bulla volume for the species *A. laevigata* and *Ac. subterraneus brunneus* ($F_{2,283} = 10,92$, $P < 0,0001$; $F_{2, 492} = 5,03$, $P = 0,0068$, respectively, Table 2a&d). In Figure 3b&d it can be seen that the intercepts and gradients of the curves for foragers and gardeners were greater, for both species, than were these parameters for waste workers.

Table 2: Minimal models for each of four species of leafcutter ants, obtained from ANCOVA's of the volume of the bulla of the metapleural gland (see text for details), with individuals' weights as a covariable.

a) *Atta laevigata*

	d.f.	SS	MS	F	P
Regression	1	20.16	20.16	889.37	0.0001
Work area	2	0.03	0.01	0.70	0.4944
Weight: Work area	2	0.49	0.24	10.92	0.0001
Residual	283	6.41	0.02		
Total	288	27.09			

b) *Atta sexdens rubropilosa*

	d.f.	SS	MS	F	P
Regression	1	30.03	30.03	4847.8	0.0001
Work area	2	0.03	0.01	2.62	0.0736
Colony	2	0.38	0.19	31.40	0.0001
Weight: Colony	2	0.06	0.03	5.30	0.0052
Residual	590	3.65	0.00		
Total	597	34.15			

c) *Acromyrmex disciger*

	d.f.	SS	MS	F	P
Regression	1	6.57	6.57	808.74	0.0001
Residual	363	2.94	0.00		
Total	364	9.51			

d) *Acromyrmex subterraneus brunneus*

	d.f.	SS	MS	F	P
Regression	1	14.50	14.50	1483.2	0.0001
Colony	2	0.57	0.28	29.49	0.0001
Work area	2	0.19	0.09	9.99	0.0001
Weight: Colony	2	0.15	0.07	7.77	0.0004
Weight: Work area	2	0.09	0.04	5.03	0.0068
Colony: Work area	4	0.09	0.02	2.55	0.0382
Residual	488	4.77	0.00		
Total	501	20.36			

d.f. = degrees of freedom; SS = Sum of Squares; MS = Mean Square.

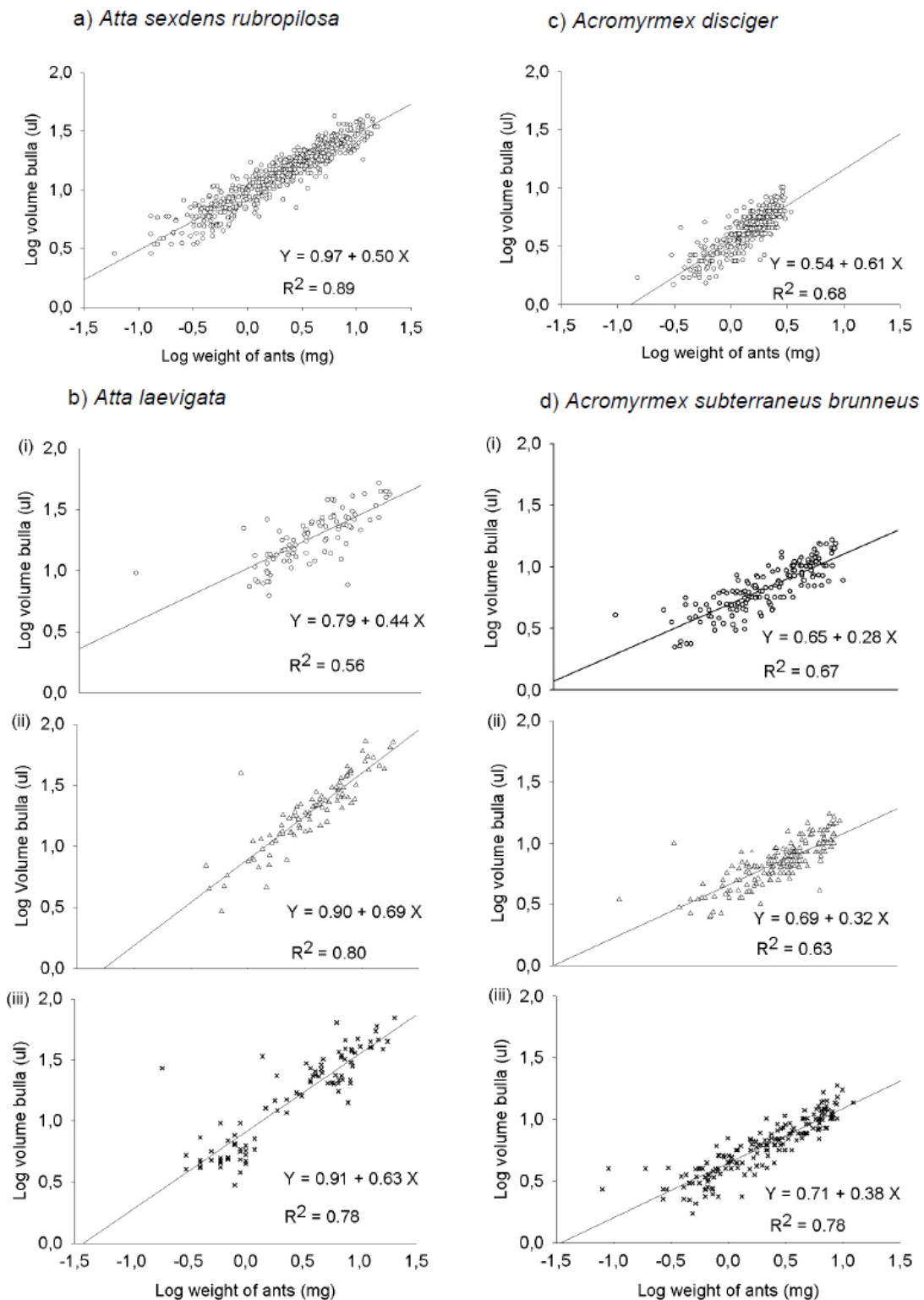


Figure 3 – Between-species variation in volume of the bulla of the metapleural gland of workers of four species of leafcutter ants, with variation between areas of work. For (a) *Atta sexdens rubropilosa* and (c) *Acromyrmex disciger*, no variation was found between work areas so data are pooled. For (b) *Atta laevigata* and (d) *Acromyrmex subterraneus brunneus*, variation was found between work areas (see table 2) so respective work areas are shown: (i) waste workers, (ii) foragers, and (iii) gardeners.

Between-colony variation in the volume of the bulla of the metapleural gland

According to the ANCOVA's (Table 2), the colony of origin explained a significant amount of the variation in bulla volume for the two species *A. sexdens rubropilosa* and *Ac. subterraneus brunneus* ($F_{2,590} = 5,30$, $P = 0.0052$; $F_{2, 492} = 5,03$, $P = 0.0004$, respectively) but not for the other two species. The regressions in Fig. 4 show that, in spite of the significances of the differences found between colonies, these differences were actually minor.

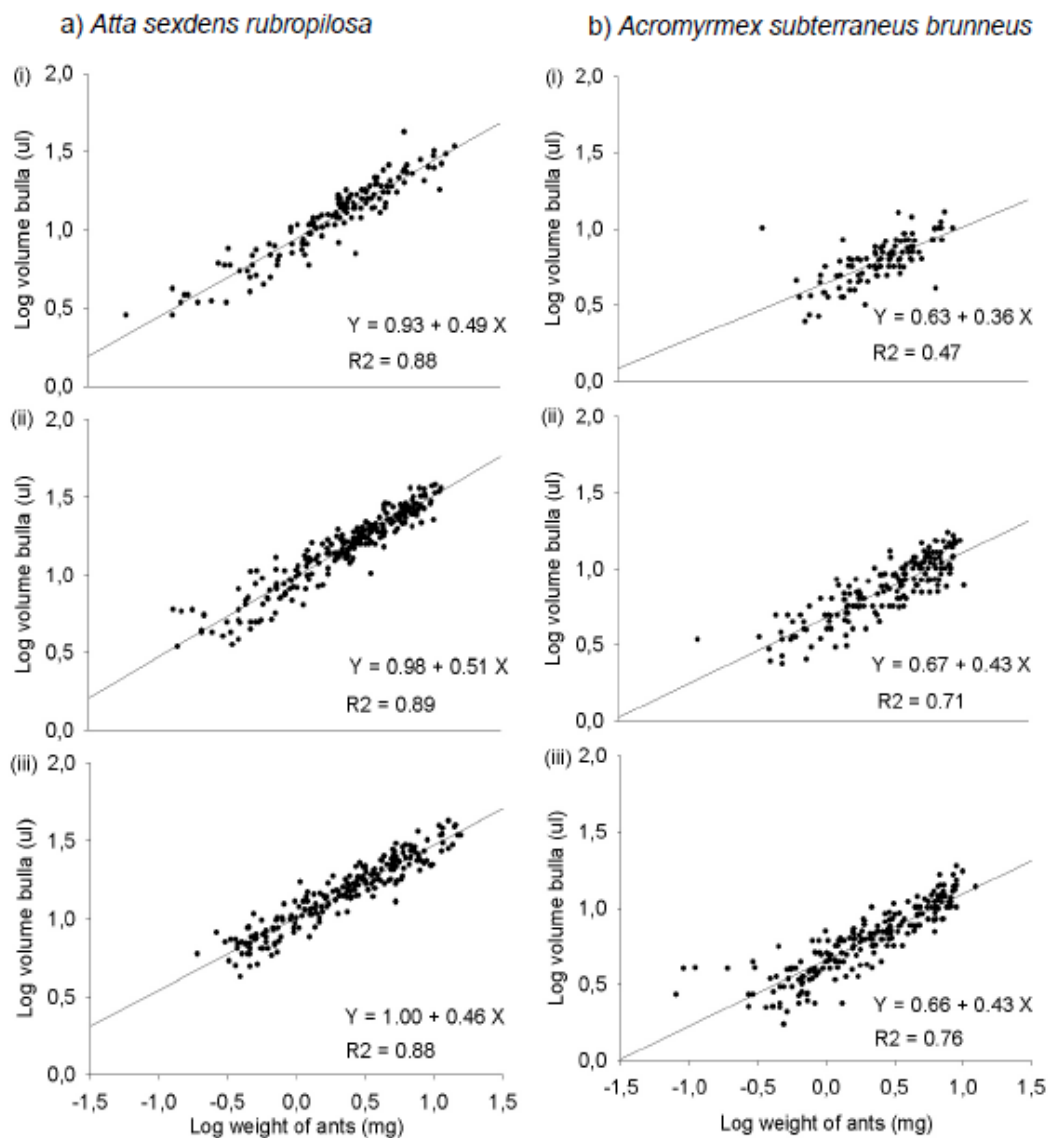


Figure 4 – Between-colony variation in volume of the bulla of the metapleural gland workers of two species of leafcutter ants, (a) *Atta sexdens rubropilosa* and (b) *Acromyrmex subterraneus brunneus*. Labels i to iii represent separation of data for the three colonies of each species. Note that no significant between colony variation was found for the other two study species.

Weight distributions of leafcutter workers according to task

The distributions of weights of workers can be seen in Figure 5. The principal differences were the skews in gardeners towards the smaller size categories, though it is noticeable that there was a similar skew in waste workers in the case of *Acromyrmex subterraneus brunneus*. Perhaps the most striking features throughout are the similarity in the upper size percentiles between the areas and the skew in gardener sizes towards smaller ants.

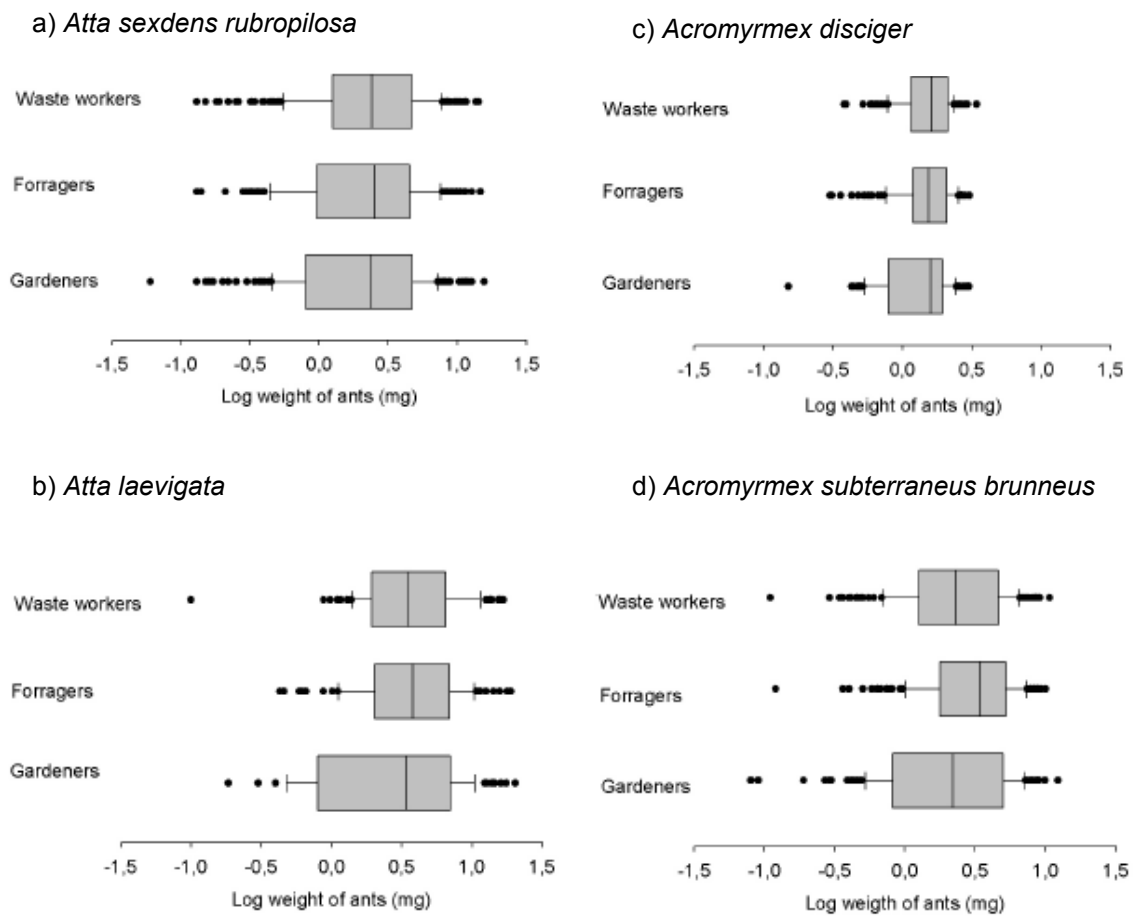


Figure 5 – Weight distributions of worker of four species of leafcutter ants, in which workers were collected from four work areas of the nest.

Variation in the quantity of actinomycete symbiont and its relation with volume of the bulla of the metapleural gland

The quantity of the actinomycete bacterial symbiont was estimated only for workers of the subspecies *Acromyrmex subterraneus brunneus*. There was a strong positive correlation between the two parameters (Spearman Correlation: $R=0,7649$, $S=3180940$, $P < 10^{-15}$; Figure 6). The ANCOVA conducted (with bacterial colony size as the dependent variable and log volume as the covariable in the place of weight) revealed no statistically significant contribution of the independent variables to the dependent variable, so it is not presented here.

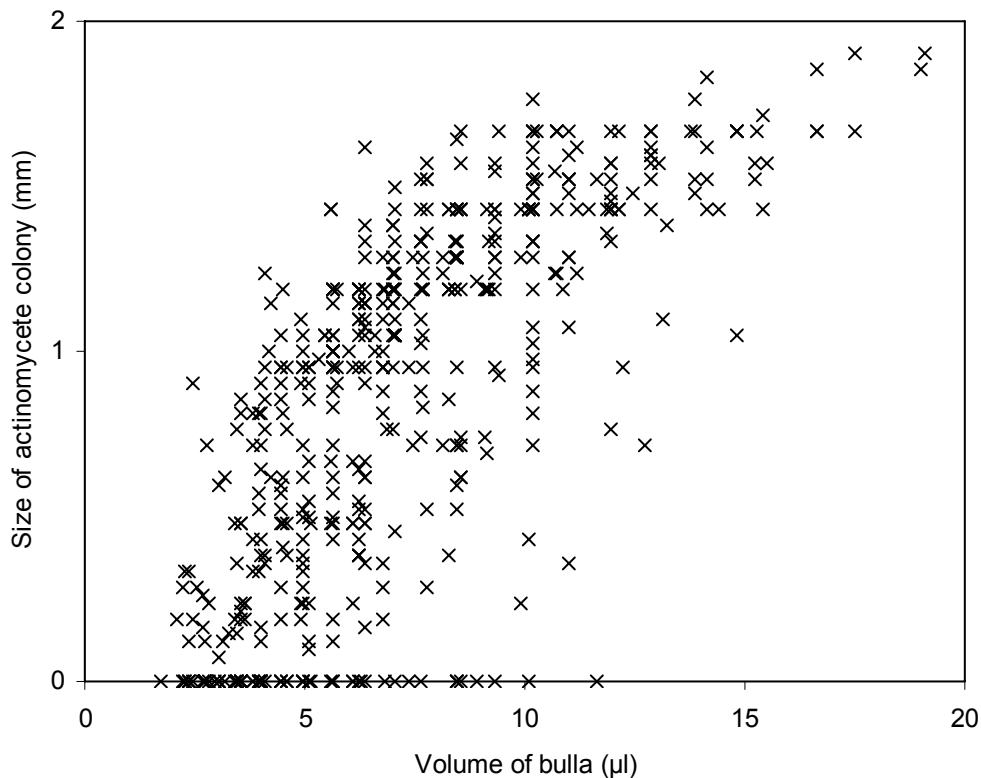


Figure 6 – Correlation between volume of the bulla of the metapleural gland and estimated amount of symbiotic bacteria present on the cuticle, using workers of the leafcutter ant *Acromyrmex subterraneus brunneus*.

DISCUSSION

In this study, two forms of investment in defence against disease were evaluated in workers of leafcutter ant species. The first of these was the size of the external part, the bulla, of the reservoir of the metapleural gland. This structure has previously been used as a means to estimate investment of an individual in production of antimicrobial secretions (Hughes *et al.* 2008) as its size has previously been shown to correlate positively with the size of the gland and the number of secretory cells present (Bot *et al.* 2001). The second measure was an estimate of the size of the colonies of the symbiotic acitnomycete present on worker cuticles in the subspecies *Acromyrmex brunneus subterraneus*.

It was initially hypothesized that species which construct more superficial nests in the soil and which have simpler social systems (i.e. *Acromyrmex* spp.) would invest more in defence due to greater disease risk, when compared with other species (i.e. *Atta* spp.). It was further hypothesized that workers involved in tending the fungus garden would also show greater investment in these defences than those foraging or working on the waste, due to the importance of the fungus garden to the colony. It was further hypothesized that there would be observable variation between colonies in these parameters. Finally, it was hypothesized that there would be a negative correlation between bulla volume and amount of symbiotic bacterium, representing a degree of specialisation of individuals for different forms of defence.

Between-species variation in investment in defence

Contary to expectations, observable investment in defence (measured as bulla size) was greater in the two *Atta* species than the two *Acromyrmex* species. This conclusion was, however, supported by a recent study by Hughes *et al.* (2008) in which it was shown that it is the more basal attine species which invest less in this form of defence. As the disease risk experienced by ant colonies has never been measured, even at the level of an individual colony, efforts to define patterns across species can be no more than speculative.

Thus, while it makes sense that a species which constructs more superficial nests would experience greater pressure from disease, this is several steps away from investment in defence and a number of other variables can be considered in order to explain the difference. One possibility is that we are observing a phylogenetic constraint. This argument is undermined somewhat, however, if we remember that it is essentially the same structure we are considering in the diverse species. As the size of this structure is fairly plastic at the individual level, we expect it to be even more so at the phylogenetic level when we compare species. It is important to note, of course, that no comparison has been made to date of the relative antimicrobial properties of the metapleural secretions of the diverse species and it is entirely feasible that a species with an apparently smaller reservoir actually makes greater use of these secretions. A second possible explanation for the observed differences is that *Acromyrmex* spp. do indeed invest less in defence due to a different life history. Thus, as *Acromyrmex* colonies reproduce after only a few years, *versus* the decade or more it takes for colonies of *Atta* spp. to reproduce, we may be observing different life history strategies – the former invests in rapid reproduction while the latter invests in sustained growth and so needs greater levels of defence to ensure it achieves reproductive age. It is also worth remembering that these defences are only part of a complex of defences, including behavioural defences such as division of labour (Schmid-Hempel 1998).

Variation in investment in defence with area of work

Significant variation in bulla size was found between workers sampled from different areas of the colony in the two species *Atta laevigata* and *Acromyrmex subterraneus brunneus* but not the other two species. In both instance, foragers and gardeners were highly similar (after accounting for body size), with waste workers found to have smaller bullas (considering either intercept or slope of the curves in Figure 3), assumed to represent a lesser antimicrobial capacity. This result is particularly interesting as it is the waste workers which will come into most contact with pathogens (Lacerda *et al.* 2006) so one might, *a priori*, expect these individuals to have a greater capacity for

defence against pathogens. Once committed to the task of waste management, however, these individuals may well never return to the colony (Hart *et al.* 2002). Under what is effectively a death sentence, it matters little to the colony if these individuals succumb to disease and this may explain their reduced defence. It is entirely possible that individuals which are found to have smaller bulla are assigned to the task of waste management rather than other more sensitive tasks such as tending the fungus garden (Maschwitz 1974; Veal *et al.* 1992; Nascimento *et al.* 1996; Bot *et al.* 2002; Poulsen *et al.* 2002b; Fernandez-Marin *et al.* 2006; Poulsen *et al.* 2006; Hughes *et al.* 2008).

The apparent similarity between gardeners and foragers requires explanation. In all four species, the distribution in sizes of individuals is skewed such that there are more smaller gardeners than small foragers (Figure 5). Allometric considerations have shown that smaller workers may be more resistant to disease as they have a relatively larger metapleural gland (Poulsen *et al.* 2006; Hughes *et al.* 2008). Thus, the smaller gardeners, considered as a group, would have a greater disease resistance than the larger foragers, thus ensuring that the most important area of the colony, the fungus garden, is tended by the workers which are best equipped for disease defence.

Between-colony variation in investment in defence

Between-colony variation in bulla size was found for the two subspecies *A. sexdens rubropilosa* and *Ac. subterraneus brunneus*. This variable was, in fact, the one which explained most of the variation in the data (Table 2), although the differences appear marginal in Figure 4. As it is lab colonies which are being considered, and these colonies were collected and reared together, it is important to note that there is variation to be found between colonies. This variation could represent underlying genetic variation, but it is likely that, even reared under similar conditions, the experience of each colony has been slightly different, leading to the observed phenotypic variation.

Perhaps the more interesting feature to note is that the within-colony variation in bulla size appears to be greater for the *Acromyrmex* subspecies than the *Atta* subspecies. We can only speculate why this would be the case,

but an interesting possibility is that, despite the greater range in worker morphology found in *Atta* species, natural selection has favoured less variation in disease defence as it needs only a few susceptible individuals to allow disease to enter a colony. Another explanation, also worth investigating, is that *Atta* colonies have greater homeostasis and so greater control over the development of individual workers. It may also represent greater underlying variation within the *Acromyrmex* colonies, perhaps due to polygyny.

The actinomycete symbiont in *Acromyrmex brunneus subterraneus*

Although a negative correlation was predicted between the size of actinomycete symbiont colonies and size of the metapleural gland bulla, a positive relation was found. The conclusion is therefore that individuals which have elevated levels of defence show this in more than one parameter, although it would be worth using more precise quantifications of the colony sizes.

FINAL CONSIDERATIONS

Extensive variation has been found in a parameter which has previously been shown to correlate with disease resistance. Variation has been shown at: between-species, between-colony, between-caste and between-individual levels. Perhaps the most striking feature of this variation is the limited investment in this form of defence in waste workers for two of the observed species. Future work should chart the variations in efficacy of metapleural gland secretions between species and castes and also chart these parameters in field populations and following exposure to disease.

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