Behav Ecol Sociobiol (2014) 68:499–507
DOI 10.1007/s00265-013-1664-9

ORIGINAL PAPER

Sanitizing the fortress: protection of ant brood and nest material by worker antibiotics

C. Tranter · P. Graystock · C. Shaw · J. F. S. Lopes · W. O. H. Hughes

Received: 7 August 2013 / Revised: 25 November 2013 / Accepted: 28 November 2013 / Published online: 13 December 2013

Abstract Social groups are at particular risk for parasite infection, which is heightened in eusocial insects by the low genetic diversity of individuals within a colony. To combat this, adult ants have evolved a suite of defenses to protect each other, including the production of antimicrobial secretions. However, it is the brood in a colony that are most vulnerable to parasites because their individual defenses are limited, and the nest material in which ants live is also likely to be prone to colonization by potential parasites. Here, we investigate in two ant species whether adult workers use their antimicrobial secretions not only to protect each other but also to sanitize the vulnerable brood and nest material. We find that, in both leaf-cutting ants and weaver ants, the survival of the brood was reduced and the sporulation of parasitic fungi from them increased, when the workers nursing them lacked functional antimicrobial-producing glands. This was the case for both larvae that were experimentally treated with a fungal parasite (*Metarhizium*) and control larvae which developed infections of an opportunistic fungal parasite (*Aspergillus*). Similarly, fungi were more likely to grow on the nest material of both ant species if the glands of attending workers were blocked. The results show that the defense of brood and sanitization of nest material are important functions of the antimicrobial secretions of adult ants and that ubiquitous, opportunistic fungi may be a more important driver of the evolution of these defenses than rarer, specialist parasites.

Keywords Parasite · Social immunity · Social insect · Disease resistance · Metapleural gland · Venom gland · Nest hygiene · *Metarhizium* · *Aspergillus*

Communicated by D. Naug

Electronic supplementary material The online version of this article (doi:10.1007/s00265-013-1664-9) contains supplementary material, which is available to authorized users.

C. Tranter (✉) · P. Graystock · W. O. H. Hughes
School of Life Sciences, University of Sussex,
Brighton BN1 9QG, UK
e-mail: c.tranter@sussex.ac.uk

C. Shaw
School of Biology, University of Leeds, Leeds LS2 9JT, UK

J. F. S. Lopes
Pós-Graduação Comportamento e Biologia Animal, Instituto de Ciências Biológicas, Campus Universitário de Martelos,
36.036-330 Juiz de Fora, MG, Brazil

Introduction

Many species form social groups and, by doing so, benefit from greater resource exploitation, antipredator defense, and reproductive fitness (Dornhaus et al. 2010). However, such benefits come at the potential cost of increased parasite exposure (Alexander 1974; Krause and Ruxton 2002). Eusocial insects are one of the pinnacles of sociality, but their vulnerability to parasites is heightened by a homeostatic nest environment and low genetic diversity of individuals within a colony, which will facilitate parasite transmission and evolution (Schmid-Hempel 1998). To counter this, social insects, such as ants, have evolved a suite of behavioral and chemical defenses which physically remove or chemically kill parasites that contaminate their cuticle (Boomsma et al. 2005; Wilson-Rich et al. 2009). These first-line defenses are important for resistance to specialist entomopathogens and also the more common opportunistic parasites which abound in and around ant colonies (Milner et al. 1998; Schmid-Hempel 1998; Poulsen et al. 2006; Evans et al. 2011; Fountain and Hughes 2011; Reber and Chapuisat 2012; Andersen et al. 2012). Ants (Storey et al. 1991; Mackintosh et al. 1995; Zelezetsky et al.

2005; Mendonça et al. 2009), bees (Evans et al. 2006; Baracchi and Turillazzi 2010; Baracchi et al. 2011), wasps (Turillazzi et al. 2006; Baracchi et al. 2012), termites (Rosengaus et al. 2000, 2004), and eusocial thrips (Turnbull et al. 2011, 2012), as well as nonsocial insects (Bulet et al. 1999; Kuhn-Nentwig 2003; Haine et al. 2008; Stow and Beattie 2008), produce defensive compounds in their hemolymph and venom. In particular, most ants secrete antimicrobial compounds from their metapleural or venom glands onto their cuticle (Hölldobler and Wilson 1990). The secretions from both glands have been shown to inhibit the growth of parasites in vitro, and adult workers with nonfunctional glands are more susceptible to parasites (Storey et al. 1991; Blum 1992; Bot et al. 2001; Poulsen et al. 2002; Graystock and Hughes 2011; Tragust et al. 2013).

Social insects are characterized by cooperation, with workers acting to maximize the fitness of their colony in spite of costs to themselves on an individual level. As a result, the resistance of social insects to disease consists of individual immunity and group-level responses that produce a form of "social immunity" that can be adaptive and proactive (Rosengaus et al. 1998; Traniello et al. 2002; Hughes et al. 2002; Cremer et al. 2007; Chapuisat et al. 2007; Ugelvig and Cremer 2007; Walker and Hughes 2009; Morelos-Juárez et al. 2010; Reber et al. 2011; Hamilton et al. 2011; Konrad et al. 2012). Social immunity may be particularly important for the more vulnerable aspects of a colony, such as developing brood and nest substrates. Insect brood lack a fully developed physiological immune system (Gillespie et al. 1997; Lavine and Strand 2002; Wilson-Rich et al. 2008), are unable to self-groom, and do not have the important antimicrobial-producing glands (Hölldobler and Wilson 1990). The brood is, thus, extremely susceptible to disease and may consequently be particularly reliant on social immunity, including potentially the donation of antimicrobial secretions by adult workers. In an elegant study, Tragust et al. (2013) showed that nursing adult workers of *Lasius neglectus* donate venom to brood during grooming, both directly via the acidopore and indirectly through oral uptake, and that this then benefited brood defense against parasites. In addition to brood, the substrate in, on, or with which ants form their colony is also likely to be vulnerable to contamination or, in some cases, infection by potentially dangerous parasites (Currie et al. 1999; Keller et al. 2003; Hughes et al. 2004b; Fountain and Hughes 2011; Reber and Chapuisat 2011). This is particularly evident in the attine fungus-growing ants, which cultivate a mutualistic fungal crop that forms the central substrate of the colony and which is very vulnerable to infection by parasites (Mueller et al. 1998; Currie et al. 1999; Gerardo et al. 2006; Little et al. 2006). Consequently, fungus-growing ants will mechanically groom their gardens to remove potential threats, have large metapleural glands, and apply metapleural secretions onto the fungal crop (Currie and Stuart 2001; Sumner et al. 2003; Fernández-Marín et al. 2006, 2009; Little et al. 2006; Hughes et al. 2008, 2010). It is likely, therefore, that care, particularly the use of antimicrobial secretions, by worker ants is important to keep colony nest material hygienic.

Here, we use the entomopathogenic fungus *Metarhizium anisopliae* with a leaf-cutting ant and a weaver ant to test experimentally if, and how effectively, the antimicrobial secretions produced by the venom and metapleural glands of adult workers are utilized to aid in brood survival and how worker secretions may be used to keep nest material hygienic.

Methods

We studied two ant species: (1) the Brazilian leaf-cutting ant *Acromyrmex subterraneus subterraneus*, which has large antibiotic-producing metapleural glands (de Souza et al. 2006) as well as a venom gland, and (2) the Southeast Asian weaver ant *Polyrhachis dives*, which lacks the metapleural gland but produces venom with antimicrobial properties (Zenghe 1986; Graystock and Hughes 2011). In both species, the respective glands (metapleural and venom) have been shown to be important in the disease resistance of adult workers (Poulsen et al. 2002; Graystock and Hughes 2011). Workers and brood were collected from two colonies of weaver ants (Pd0701 and Pd0704) and three colonies of leaf-cutting ants (As085, As086, and As0811) that had been maintained in the laboratory at 26 °C and 80 % relative humidity (RH) for >6 months prior to use and showed no apparent signs of decline or infection. Due to the availability of brood at the time of the experiment, all leaf-cutting ant brood were pupae of approximately 5 mm in length, while all weaver ant brood were larvae of approximately 5 mm length. For each species, adult workers were selected of similar size (6–8 mm), cuticle melanization, and location in the colony (and thus inferred age; Armitage and Boomsma 2010). We confirmed in a preliminary experiment that workers of these sizes and ages successfully cared for brood over 14 days when kept in isolation (i.e., a single ant with a single pupa or larva). As our experimental parasite, we used a strain of the entomopathogenic fungus *M. anisopliae* (isolate 144467, CABI; isolated from the soil of a maize field in Canada) which was exotic to both of the ant species. Fungal conidia were harvested from freshly sporulating media plates, and viability was confirmed to be >92 % throughout the experiments by plating the conidia solutions onto Sabouraud dextrose agar plates, incubating for 24 h, and quantifying conidia germination. We applied 0.5 μl doses of species-specific concentrations of conidia in Triton X that we had determined in preliminary trials caused 50 % mortality to brood (weaver ant, 1×10^5 conidia/ml; leaf-cutting ant, 1×10^4 conidia/ml).

Experiment 1: brood care

To determine the importance of adult worker antimicrobial secretions for brood survival, we collected 120 leaf-cutting ant workers and 160 weaver ant workers, split into two cohorts. The leaf-cutting ant cohorts were each formed of 60 ants, with 20 ants from each of the three colonies, while the weaver ant cohorts consisted of 80 ants, with 40 ants from each of the two colonies used. Half the ants from each colony had their main antimicrobial-producing glands (the metapleural gland in leaf-cutting ants and the venom gland in weaver ants) blocked using nail varnish, and the remaining workers had nail varnish applied to the pronotum as a control (Poulsen et al. 2002; Graystock and Hughes 2011). After 24 h, we collected 60 leaf-cutting ant pupae and 80 weaver ant larvae, for each of the two cohorts, and surface-treated half of them with the *Metarhizium* parasite and the other half with 0.5 μl of a 0.05 % Triton X control solution using a micropipette. Each pupa or larva was then placed in a pot (40 mm diameter) with a single "nurse" worker ant from the same colony to give four combinations of infected/uninfected brood and workers with functional/nonfunctional glands, in a full factorial design, with a total of 30 leaf-cutting ant and 40 weaver ant replicates of each (Fig. S1). Ants were maintained in the pots with moistened cotton wool to supply water and sucrose solution ad libitum. Any workers which died during the experiment were replaced with an identically treated worker. The survival of the brood was monitored for 14 days. Dead brood were each placed on moistened filter paper in a Petri dish at 26 °C and 80 % RH and checked daily for the appearance of fungal conidia and conidiophores diagnostic of a *Metarhizium* infection. To confirm that the blockage treatment did not affect normal brood care behaviors, we also compared the behavior of nurse workers for 20 ants of each species. Half the ants in each species had their respective glands blocked and the other half had the control treatment applied to the pronotum. The ants were placed in a Petri dish with a single item of brood (pupae for leaf-cutting ants and larvae for weaver ants) and (a) the duration of any non-grooming interaction between nurse and brood (e.g., carrying, antennation), (b) the frequency of physical contact between nurse and brood, and (c) the frequency of brood grooming by the nurse ant were recorded for a 10-min period.

Experiment 2: nest hygiene

Sixty weaver ants (30 ants per colony) were collected from within the nest. Half of the ants from each colony had their venom gland blocked with nail varnish and half had a control treatment on the pronotal spines, for a total of 30 replicates per treatment. One hundred twenty leaf-cutting ant workers (40 ants per colony) were collected from the outer surface of the fungal crop. The ants from each colony were divided evenly into the four blockage treatments as follows: (1) varnish applied to the pronotal spines as a control, (2) metapleural gland blocked, (3) venom gland blocked, or (4) both venom and metapleural glands blocked, with a total of 30 replicates per treatment. Each ant was placed in a pot with a 10-mm^2 section of either the silk nest material of weaver ants or the fungal garden of leaf-cutting ants, from their original nest, and balls of cotton wool moistened with water and sucrose solution at 26 °C and 80 % RH. Thirty further 10 mm^2 sections of nest material were set up identically for each species, except no ant was placed in the pot (Fig. S2). The nest substrate was monitored for 15 days for the appearance of any foreign fungus and death of the fungal crop. If a worker died during the experiment, then it was replaced with an identically treated worker.

To identify the fungi that developed in the leaf-cutting ant trials, three representative samples of each fungal morphotype (based on external morphology, spore structure, and color) were isolated on malt extract agar plates at 30 °C until the fungi produced conidia, and then stored at 4 °C. DNA was extracted from the samples by adding 200 μl of 5 % Chelex solution (in 10 mM Tris buffer) and 0.05 g of 0.1 mm silica beads to approximately 0.05 g of the sample fungus and placed in a QIAGEN TissueLyser BeadBeater for 4 min at 50 oscillations/s. Samples were then incubated at 90 °C before being centrifuged for 30 min at 4 °C. Supernatant from the samples was cleaned with OneStep-96 Polymerase Chain Reaction (PCR) Inhibitor Removal Kit (Zymo Research) prior to PCR amplification of the internal transcribed spacer regions 1 and 2 with the primers ITS1 and ITS4 (Henry et al. 2000; Foley et al. 2012). PCR products were sequenced and fungi were identified by BLASTn searches of the resulting sequences.

Statistical analysis

The effects of *Metarhizium* exposure, gland closure, and ant species on brood survival and the effects of gland closure and ant species on the appearance of foreign fungi on nest material were analyzed using Cox proportional hazards regression models. Colony of origin and cohort (in experiment 1) were included in the models to account for the structured nature of the data. Pairwise Kaplan–Meier tests were used to test for pairwise differences between treatment groups. The effects of blockage on the duration of behavioral interactions of nurse ant and brood were examined using Mann–Whitney U tests, and the survival of the nurses was analyzed using Cox proportional hazards regression models. The proportions of brood sporulating with fungi were examined with χ^2 tests, and the proportions of nest material sporulating with fungi were analyzed with Fisher's exact tests.

Results

Experiment 1: brood care

Workers of both species tended to the brood throughout the experiment and the survival of brood that were cared for by a replacement worker did not differ from those that were cared for by the same worker ant throughout (leaf-cutting ants: Wald=2.54, $p=0.111$; weaver ants: Wald=0.19, $p=0.67$). Nurse worker ants with blocked or unblocked glands did not differ in their behaviors when attending to brood or in their survival throughout the experiment (Fig. S3). There were significant effects of both exposure to *Metarhizium* and of gland blockage on brood survival (Wald=17.8, $p<0.001$ and Wald=27.2, $p<0.001$, respectively), but no overall difference between the ant species (Wald=1.84, $p=0.1$) or significant interactions between these effects ($p>0.2$ in all cases). There was no difference in brood survival between leaf-cutting ant cohorts (Wald=0.54, $p=0.817$), but mortality was higher in the second, compared with the first, cohort of weaver ants tested (Wald=8.52, $p=0.004$), and there were no significant differences between colonies ($p>0.1$ in both species). In both ant species, gland blockage reduced brood survival regardless of treatment, while the effect of *Metarhizium* exposure was less consistent (Fig. 1). Compared to the control brood cared for by nurse ants with functioning glands, the hazard ratio for the leaf-cutting ant brood was increased to 2.7 by blocking the metapleural gland, to 3.7 by exposure to *Metarhizium* when the metapleural gland was functional, and to 5.5 by both exposure to *Metarhizium* and blocking the gland. For the weaver ant brood, the hazard ratio was increased to 1.9 by exposure to *Metarhizium* with the venom gland of nurse ants functional, to 3.4 by blocking the venom gland, and to 4.7 by both exposure to *Metarhizium* and blocking the gland.

Significantly fewer of the *Metarhizium*-exposed weaver ant brood sporulated with *Metarhizium* when the venom glands of their nurse ants were functional than when the glands were blocked ($\chi^2=8.25$, $p=0.04$), while there was no effect of gland blockage on *Metarhizium* sporulation from leaf-cutting ant brood ($\chi^2=1.07$, $p=0.3$; Fig. 2). A substantial number of brood of both ant species sporulated with the opportunistic fungal parasite *Aspergillus* sp. (Fig. 2). The proportion sporulating with this fungus was significantly greater when nurse ants had blocked glands, both for the weaver ants and leaf-cutting ants ($\chi^2=12.5$, $p<0.001$ and $\chi^2=13.1$, $p<0.001$, respectively). Few brood sporulated with *Aspergillus* when the nursing workers had functioning glands, but 48 % of the weaver ant brood and 50 % of the leaf-cutting ant brood did so when the glands were blocked (Fig. 2). Gland blockage, therefore, significantly increased the proportion of brood exposed to *Metarhizium* that then sporulated with this parasite and also significantly increased the proportion of brood, either treated with *Metarhizium* or not, that sporulated with opportunistic *Aspergillus* fungi.

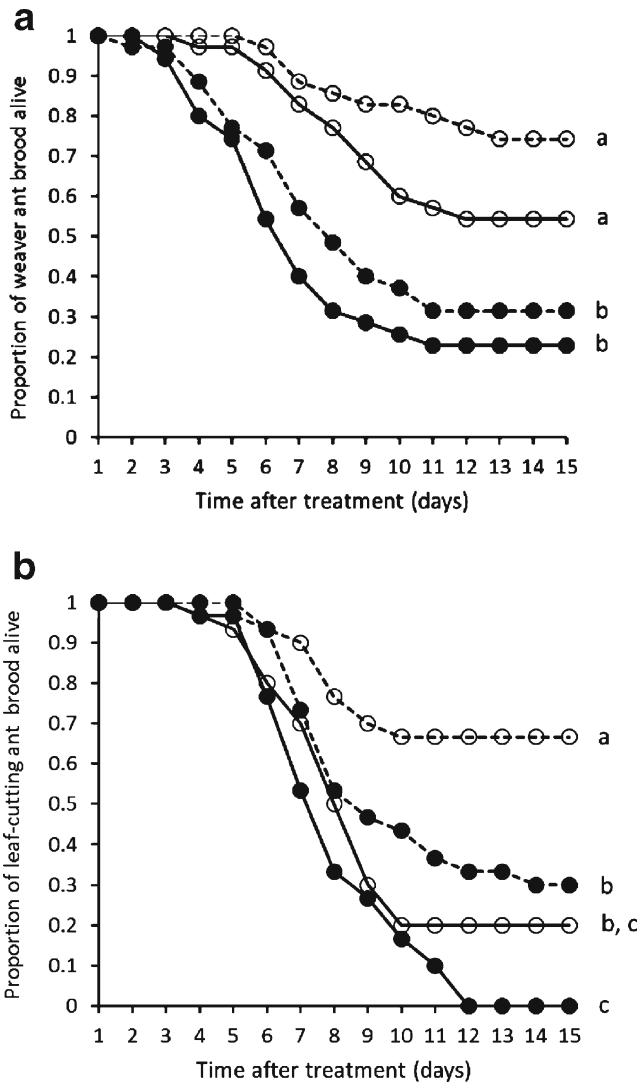

Fig. 1 Survival of **a** weaver ant pupae and **b** leaf-cutting ant larvae that were treated with either *Metarhizium* parasite (*solid lines*) or control solution (*dashed lines*) and cared for by workers either with (*open circles*) or without (*black circles*) functional antimicrobial glands (the venom gland for weaver ants and the metapleural gland for leaf-cutting ants). For each species, *different letters* indicate treatments which differed significantly from one another at $p<0.05$ in pairwise comparisons with Kaplan–Meier tests

Experiment 2: nest hygiene

There was a significant effect of both gland blockage and ant species on the appearance of fungi on nest material, but no interaction between them (Wald=35.9, $df=4$, $p<0.001$; Wald=55.9, $df=1$, $p<0.001$; and Wald=5.46, $df=2$, $p=0.65$, respectively). There were no significant differences between colonies ($p>0.2$ in both species). Both weaver ants and leaf-cutting ants experienced fungal growth sooner if one or both glands were blocked (Fig. 3). For leaf-cutting ants,

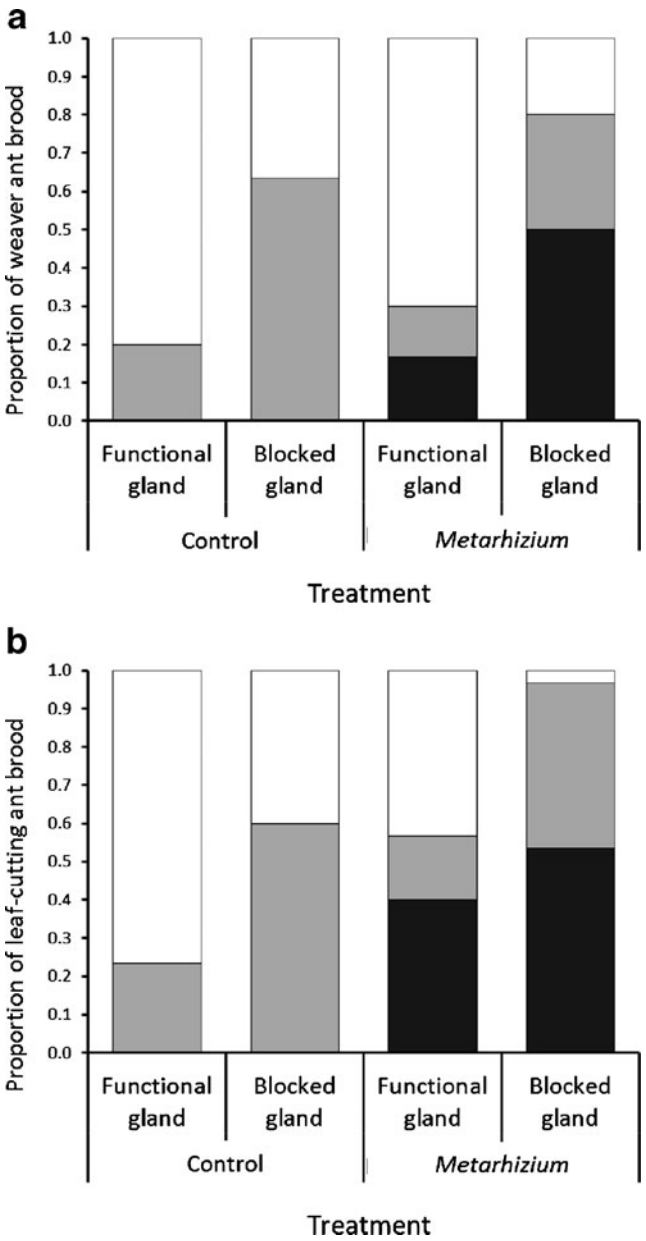

Fig. 2 Proportions of **a** weaver ant larvae and **b** leaf-cutting ant pupae that produced conidia of the *Metarhizium* experimental parasite (*black*), the opportunistic *Aspergillus* fungus (*gray*), or remained uninfected (*white*). Brood were either treated with *Metarhizium* parasite or control solution and kept with workers either with or without functional antibiotic-producing glands (the venom gland for weaver ants and the metapleural gland for leaf-cutting ants)

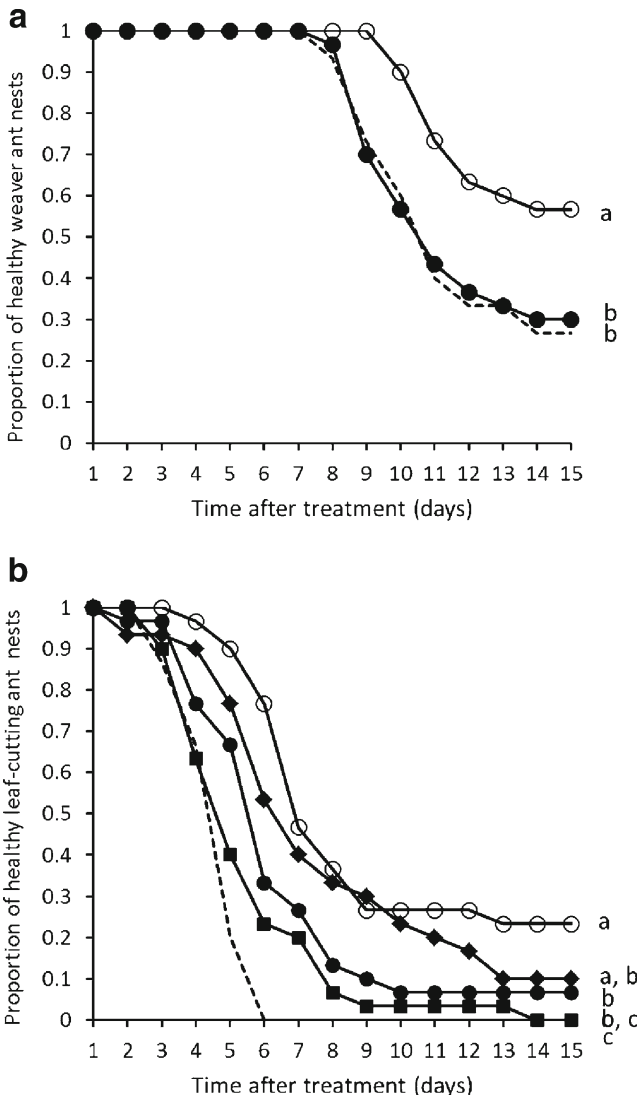

Fig. 3 Proportion of **a** weaver ant silk material and **b** leaf-cutting ant fungal crop material that was free of contaminant fungal growth when cared for by workers with functional glands (*white circles*), blocked venom gland (*black circles*), blocked metapleural gland (*black diamonds*), both glands blocked (*black squares*), or where the worker ant was absent (*dashed line*). For each species, *different letters* indicate treatments which differed significantly from one another at $p<0.05$ in pairwise comparisons with Kaplan–Meier tests

compared to nest material attended by an ant with unblocked glands, the hazard ratio for nest material attended by workers with blocked metapleural glands increased to 1.4; with workers with blocked venom glands, it increased to 1.99; when workers had both glands blocked, it increased to 2.93; and when no worker ant was present, it increased to 5.01. Blocking of the venom gland in weavers increased the hazard ratio to 2.29, and an absence of the worker ant to 2.39. Both results were significantly different ($p<0.05$) when compared to nest silk attended to by a worker with a functional gland, but not when compared to each other, in post hoc pairwise comparisons. Sporulation of fungi on the weaver ant silk resulted in only a sparse emergence of lightly filamentous fungi, which appeared morphologically similar across all trials and was not successfully isolated and cultured. In those leaf-cutting ant trials where the fungal crop developed other fungi, it was overgrown quickly. *Escovopsis* was found most commonly in the trials where worker ants possessed functioning glands ($p=0.007$; Fig. 4). The appearance of *Escovopsis* was relatively lower, and of other fungi relatively higher, when the glands of the attendant workers were blocked. *Aspergillus*

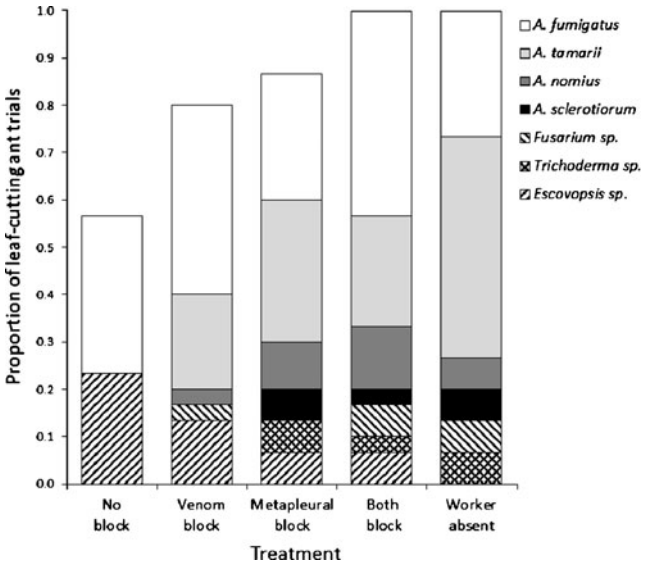

Fig. 4 Proportion of trials where foreign fungus overgrew leaf-cutting ant nest material grouped by treatment. Foreign fungal species were *A. fumigatus* (*white*), *Aspergillus tamarii* (*light gray*), *Aspergillus nomius* (*dark gray*), *Aspergillus sclerotiorum* (*black*), *Fusarium* sp. (*leftward diagonals*), *Trichoderma* sp. (*cross-hatched*), and *Escovopsis* sp. (*rightward diagonals*)

fumigatus was common regardless of whether the ants had functional or blocked glands, while all other fungi grew only when the fungal crop was not tended by a worker with functional glands.

Discussion

Previous work investigating the social immunity and antimicrobial secretions of ants has focused on their protection of other adults ants against parasites. The results presented here show that antimicrobial secretions produced by adult ant workers can also help increase the survival of both control and parasite-treated brood and reduce fungal growth on nest material. Importantly, the secretions in these contexts appear to be particularly significant for sanitizing against opportunistic fungi. In both leaf-cutting ants and weaver ants and regardless of experimental exposure to the *Metarhizium* parasite, brood suffered higher mortality and growth of the opportunistic *Aspergillus* fungus when the workers nursing them did not have functional antimicrobial-producing glands. Brood exposed to the specialist fungal parasite *Metarhizium* were also more likely to sporulate with this parasite when nursing workers lacked functional glands. Similarly, in both ant species, nest material was more likely to be overgrown by fungi when tended by workers without functional glands. This effect was most substantial in the weaver ants where blocking the venom gland was sufficient to result in fungal growth on nest material comparable to when no tending ant was present at all. Leaf-cutting ants required the blocking of both metapleural and venom glands to show a similar result. While adult insects, including ants, wasps, bees, termites, and eusocial thrips, utilize antibiotic secretions to protect themselves (Rosengaus et al. 2000; Bot et al. 2001; Turillazzi et al. 2006; Turnbull et al. 2011; Baracchi et al. 2011, 2012), it has recently been shown that *Lasius* ants transfer antimicrobial venom to enhance the resistance of brood to disease (Tragust et al. 2013). Our results indicate that this is also the case for *Acromyrmex* and *Polyrhachis* ants. There has been much interest in the role of social immunity in the disease resistance of adult social insects, but their lack of individual immunity is likely to make brood the most vulnerable life stage (Hölldobler and Wilson 1990; Cremer et al. 2007; Gillespie et al. 1997). Social immunity may, therefore, be especially essential for brood protection.

Surprisingly, there was no significant interaction in either ant species between gland blockage and *Metarhizium* exposure. Antimicrobial secretions have previously been shown to be very important for protecting adult leaf-cutting ants and weaver ants against exposure to the *Metarhizium* parasite (Poulsen et al. 2002; Graystock and Hughes 2011), as well as for protecting brood of *L. neglectus* ants (Tragust et al. 2013). The lack of a significant interaction here between gland blockage and *Metarhizium* exposure is likely to be for two reasons. First, both the probability of parasite infection success and the effects of antimicrobial secretions are dose-dependent (Ebert et al. 2000; Hughes et al. 2004a; Stow et al. 2007; Turnbull et al. 2012). The greater the dose of the parasite strain, the more likely an infection is to be successful, and it may be that the dose of the parasite strain used here was too high for the antimicrobial secretions that were transferred from the adult ants to be fully effective in defending brood against the *Metarhizium* parasite. In addition, lower doses of antimicrobial compounds are less likely to be effective against a parasite and it may be that the dose of antimicrobial secretions transferred to the brood was too low to fully defend the brood against *Metarhizium* and, thus, too low for a strong effect of gland blockage on resistance to *Metarhizium* to be seen. Second, the effect of gland blockage on the mortality of even the control brood was relatively high. Both here and in other studies (Poulsen et al. 2002; Graystock and Hughes 2011), little impact of gland blockage on control adult ants themselves has been found, but it appears that control brood are far more susceptible to the impact of being with nursing workers with blocked glands. The behavior of the nursing workers, including their grooming of the brood, was unchanged by gland blockage, and there is no known nutritional role for the glandular secretions, so it seems most probable that this impact relates to the infections by opportunistic fungal parasites which developed.

As with all organisms, ant colonies coexist with a wide diversity of opportunistic microbes that can be parasitic, such as the *Aspergillus* fungus found in this experiment and in ant

colonies studied previously (Pereira and Stimac 1997; Schmid-Hempel 1998; Hughes et al. 2004b; Poulsen et al. 2006; Lacerda et al. 2010; Fountain and Hughes 2011). Adult ants appear to suffer relatively little from these opportunistic parasites even when their production of antimicrobial secretions is prevented (Poulsen et al. 2002; Graystock and Hughes 2011), presumably due to their well-developed immune system and grooming behavior. We also found this to be the case here for brood and nest material when the antibiotic-producing glands of nurses were functioning. However, when the antimicrobial secretions of nurses were lacking, most brood succumbed to infection by opportunistic *Aspergillus* fungi and most nest material became overgrown. It cannot be excluded that some of the fungal growth may have been opportunistic growth on larvae that died from another cause. However, even if this is the case, then the results nevertheless demonstrate the importance of antimicrobial secretions from nursing workers for sanitizing the cuticles of larvae. It, therefore, appears that the antimicrobial secretions of adult ants are essential to protect the vulnerable brood against opportunistic parasites and to prevent nest material becoming overgrown by contaminant fungi. It may indeed be the case that the protection of larvae against ubiquitous opportunistic microbes is of greater importance for ant fitness than protection against more specialist parasites such as *Metarhizium*, which tend to be rarer, and may potentially have driven the evolution of antimicrobial secretions in ants.

The leaf-cutting ant nest samples in this study were found to host at least seven species of fungi ranging from generalist, opportunistic *Aspergillus* spp. to *Escovopsis*, which specializes in parasitizing the fungal crop of leaf-cutting ants (Currie 2001). Workers with functioning glands reduced both the number and diversity of fungi found compared to treatments with blocked glands. Only *Escovopsis* and the hyperabundant *A. fumigatus* (Latgé 1999) were found in treatments where the attending workers had functioning antimicrobial-producing glands. Other fungi only occurred when the fungal crop was not tended by workers with functional glands. *Escovopsis* has evolved to be highly successful in natural leaf-cutting ant nest environments (Currie 2001; Currie and Stuart 2001) and, as our results show, is able to grow on the fungal crop even when workers are producing antimicrobial compounds from their metapleural glands. In this antimicrobial-rich setting, *Escovopsis* is then able to exclude most of the opportunistic fungi found in this study. Interestingly, however, our results suggest that the specialist *Escovopsis* may be less dominant if the antimicrobial secretions of the ants are reduced, through blocking of the metapleural gland, in which setting other fungi are far more competitive against *Escovopsis*. Consequently, antimicrobial secretions may be more important for protection against more opportunistic fungal pathogens than previously thought.

The results show how social immunity provided by the altruistic provision of antimicrobial secretions from adult ants has evolved to play an important role in brood survival and maintaining hygienic nest conditions and, thus, the fitness of their colony. In addition, we show that these social secretions are important, not just to combat specialist parasites like *Metarhizium* and *Escovopsis* but also in the everyday defense against opportunistic microbes which are ubiquitous in and around nest sites. This not only highlights the vulnerability of brood and nest material to disease but also their reliance on social care and provides a compelling explanation for how immobile brood with immature immunity survives in a world abundant with pathogens.

Acknowledgments We thank P. Chappell, C. Frost, R. Mitchell, and J. Parkinson for the technical assistance, V. Norman and two anonymous reviewers for their constructive comments which improved the manuscript, IBAMA for the permission to collect and export the leaf-cutting ant colonies, Martin Sebesta for providing the weaver ant colonies, and the Royal Society, BBSRC, and Leverhulme Foundation for the funding.

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