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ORIGINAL ARTICLE

Acid, silk and grooming: alternative strategies in social immunity in ants?

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Abstract Parasites are an important force in evolution, driving the need for costly resistance mechanisms. The threat from disease is potentially high in group-living species such as social insects, which have accordingly evolved behavioural and chemical defences that vary between species depending on their life histories. Several ant genera have lost a key exocrine antimicrobial defence, the metapleural gland, and yet are still able to thrive in environments abundant with parasites. We investigate, in species lacking the metapleural gland, how the production of antimicrobial venom, grooming behaviours and the use of potentially antimicrobial larval silk may have evolved as alternative antiparasite defences. We focus on the Australasian weaver ant *Oecophylla smaragdina* and compare this to *Polyrhachis* weaver ants. We show that the production of venom by *O. smaragdina* workers is important for disease resistance but that the presence of larval silk is not, and that workers use their acidic venom to maintain nest hygiene. The grooming defences of *O. smaragdina* differ between castes, with minor workers allogrooming more and major workers showing greater upregulation of grooming in response to parasites. Chemical and behavioural defences differ interspecifically between *O. smaragdina* and *Polyrhachis*, with *O. smaragdina* appearing to rely primarily on its venom while *Polyrhachis* use higher rates of grooming. The results show how alternative investment strategies can evolve for disease defence, notably the highly effective application of acidic venom by *O. smaragdina*, and highlight the need for targeted comparative studies to understand how organisms respond to the ubiquitous threat from parasites.

Keywords Venom · Disease resistance · *Metarhizium* · Weaver ant · *Oecophylla* · *Polyrhachis*

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Introduction

The threat from disease is an important driver of host biology and population structures (Poulin and Morand 2000; Poulin 2007). Strong parasite pressures can result in the rapid evolution of host defences which are required to reduce the cost of this pressure on host fitness (Brockhurst et al. 2004; Duffy and Sivars-Becker 2007; Decaestecker et al. 2007). This threat has led to the evolution of a complex array of defence mechanisms, ranging from behavioural avoidance strategies to the complex adaptive immune system of vertebrates (Siva-Jothy et al. 2005; Thieltges and Poulin 2008; Wisenden et al. 2009; Tranter et al. 2015). Organisms that live in groups possess the additional benefit of group-level defences, such as allogrooming or shared use of antimicrobial secretions, which function in combination with individual-level defences against the threat from parasites (Krause and Ruxton 2002; Nunn and Altizer 2006). However, living in groups also potentially increases the threat of disease, because the greater density of individuals within a group can enhance intragroup transmission, and many social activities such as chemical communication or sharing of food (trophallaxis) put members in close physical proximity (Alexander 1974; Møller et al. 1993; Rose 1999; Altizer et al. 2003; Godfrey et al. 2009). Defence mechanisms against parasites are often costly

(Bowers et al. 1994; Poulsen et al. 2002; Rigby et al. 2002), and the great variety of strategies that organisms employ means that comparative studies are key to understanding the evolutionary biology of host disease resistance. In cases where the extensive datasets needed for full comparative analyses are lacking, targeted studies on species that differ in specific life-history traits that are predicted to affect the host-parasite relationship can be highly informative.

In social insects, the selection pressure from the threat of disease has resulted in the evolution of a suite of individual and group-level behavioural and chemical defences (Boomsma et al. 2005; Cremer et al. 2007; Wilson-Rich et al. 2009; Rosengaus et al. 2011) Social insect colonies are based on division of labour, and this can apply to disease resistance too, with worker and reproductive castes in leaf-cutting ants show differences in the use and effectiveness of disease resistance mechanisms (Hughes et al. 2002; Baer et al. 2005; Poulsen et al. 2006; Hughes et al. 2010). These defences include meticulous self-grooming and allogrooming that are effective, adaptive and proactive in removing parasites (Farish 1972; Basibuyuk and Quicke 1999; Traniello et al. 2002; Yanagawa et al. 2008; Morelos-Juárez et al. 2010). In particular, ants and termites will increase grooming when exposed to fungal conidia (Rosengaus et al. 1998; Yanagawa and Shimizu 2006; Yanagawa et al. 2008; Walker and Hughes 2009; Morelos-Juárez et al. 2010; Reber et al. 2011). This grooming can be combined with the use of antimicrobial secretions from exocrine glands by workers to sterilise themselves, their brood, their nest material and food (Fernández-Marín et al. 2009; Tragust et al. 2013a, 2013b; Tranter et al. 2014). Similarly, 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 non-social insects (Bulet et al. 1999; Kuhn-Nentwig 2003; Haine et al. 2008; Stow and Beattie 2008), produce defensive compounds in their haemolyph and secretions which can be used in the defence against pathogens. Of particular importance is the antimicrobial-producing exocrine metapleural gland (MG), which is unique to, and ancestral in, ants (Hölldobler and Engel-Siegel 1984; Veal et al. 1992; Ortius-Lechner et al. 2000; Quinet and Vieira 2012; Vieira et al. 2012). Antimicrobial compounds are likely often transferred between nestmates during grooming, and in at least one ant species by trophallaxis (Hamilton et al. 2011).

The most extreme evolutionary transition in antibiotic use, however, is shown by some formicine ant taxa in the genera *Polyrhachis*, *Camponotus* and *Oecophylla* which have secondarily lost the MG entirely, suggesting either a substantial relaxation in parasite pressure negating the requirement for maintaining an energetically costly gland, or the development of alternative forms of defence (Yek and Mueller 2011). The losses of the MG in these genera are correlated with arboreality and the evolution of weaving nests from larval silk, a behaviour which is unique to certain species in these genera (Johnson et al. 2003; Robson and Kohout 2005, 2007). It has been suggested that arboreal ants, such as *Oecophylla* and many *Polyrhachis* species, may be able to invest less in costly defences such as the MG because they are less exposed to the fungal parasites that are abundant in soil (Boomsma et al. 2005), although supporting evidence for this hypothesis is still lacking (Walker and Hughes 2011). It may also be the case that the silk substrate of weaver ant nests provides an aseptic habitat for these species (Johnson et al. 2003), or that the silk contains antimicrobial compounds, as in other invertebrates (Wright and Goodacre 2012), which may transfer to ants. In the only direct test of this, Fountain and Hughes (2011) failed to find any benefit from silk for defence against pathogenic fungi in the weaver ant *Polyrhachis dives*, with the silk in fact carrying viable opportunistic fungal parasites. However, silk weaving in *Oecophylla* appears to be more derived and complex than in *Polyrhachis* (Crozier and Newey 2010); so, it is possible that there may be stronger benefits from silk for *Oecophylla* than for the weaver ants investigated previously (Fountain and Hughes 2011; Graystock and Hughes 2011). Alternatively, the loss of the MG may be associated with the evolution of different defence mechanisms. The venom of many social insects may possess antimicrobial properties, but it is in ants where we see the use of acidic venom diversified beyond stinging behaviours (Moreau 2013). Along with other formicines, *Oecophylla* and *Polyrhachis* produce acidic venom, composed principally of formic acid (Bradshaw 1979; Hölldobler and Wilson 1990; Blum 1992). *Polyrhachis dives*, as well as other formicines, are known to use their venom during grooming, spreading venom on themselves, their brood and their nest material (Graystock and Hughes 2011; Tragust et al. 2013a, 2013b; Tranter et al. 2014; Otti et al. 2014). The use of venom in *Oecophylla* and *Polyrhachis* may be particularly important because they have lost their MG and the antimicrobial secretion it produces. Self-grooming also appears to be upregulated in *Polyrhachis* compared to other ants so may be another mechanism to compensate for the lack of an MG (Graystock and Hughes 2011; Tranter et al. 2015). Ants may also alter the amount of trophallaxis, which may reduce the transmission of pathogens through minimising physical contact, or, in at least one species of ant, transfer antimicrobial proteins to nestmates (Hamilton et al. 2011; Reber and Chapuisat 2012). These behaviours may provide an alternative form of protection in species which lack antimicrobial defences.

In this study, we investigate the defences employed for disease resistance, but little studied green weaver ant *Oecophylla smaragdina*, a species which has complex

societies with advanced nest weaving and polymorphic workers, and compare this with three *Polyrhachis* weaver ant species that also lack the MG, but which have monomorphic workers and less advanced nest weaving. Specifically, we test the following: (i) the importance of antimicrobial venom and the presence of potentially antimicrobial larval silk for the ability of *O. smaragdina* workers to resist parasite infection; (ii) whether polymorphism in *O. smaragdina* allows division of labour in disease resistance, by examining whether the major and minor worker castes of *O. smaragdina* differ in their grooming defence against parasites; (iii) how the grooming response of *O. smaragdina* to parasites compares with that of three *Polyrhachis* weaver ant species; (iv) the effectiveness of venom in maintaining acidic nest conditions in both *O. smaragdina* and *P. delecta*.

Methods

We used four different species of weaver ant in the experiments. *Oecophylla smaragdina* was our primary study species and used in all four experiments. This is an ecologically dominant, polymorphic species of weaver ant found across Asia and Australia which shows the most advanced form of nest weaving, making nests in trees and vegetation using leaves woven together with larval silk (Crozier and Newey 2010). We compared the behavioural and chemical defences of *O. smaragdina* in experiments 3 and 4 with those of three species of *Polyrhachis* weaver ants: (2) *Polyrhachis* (*Myrmhopla*) *dives* (in experiment 3), a monomorphic arboreal species from Asia and Australia which forms characteristic carton nests from twigs, foliage and general detritus bound with larval silk; (3) *Polyrhachis* (*Myrma*) *foreli* (in experiment 3), a large monomorphic *Polyrhachis* species which tends to be terrestrial and nest in rotting wood or ground-level epiphytes (Robson and Kohout 2007; Kohout 2012); (4) *Polyrhachis* (*Cyrtomyrma*) *delecta* (in experiments 3 and 4), a monomorphic shiny black weaver ant from Queensland, Australia, which builds carton nests from larval silk between the lower leaves of trees and shrubs (Kohout 2006). Other than in experiment 2, we used major workers of *O. smaragdina* throughout as they were most similar in size to the *Polyrhachis* workers. Fungi appear to be the most common parasite threat for ants and, while particularly abundant in the soil environment, are also present in the arboreal habitat as well (Boomsma et al. 2005; Griffiths and Hughes 2010). Fungal disease threats to ants include specialist parasites of ants such as *Ophiocordyceps*, generalist entomopathogens such as *Metarhizium* and opportunistic parasites such as *Aspergillus* (Jouvenaz et al. 1972; Alves and Sosa-Gómez 1983; Humber 1992; Schmid-Hempel 1998; Hughes et al. 2004; Rodrigues et al. 2010; Lacerda et al. 2010; Ribeiro et al. 2012). Specialist ant parasites are likely to show strong coevolution with their specific host species making them unsuitable for comparative experiments; so, we here use *Metarhizium* as the experimental parasite because it has been reported parasitising a wide diversity of ant species (Allen and Buren 1974; Lofgren and Vander Meer 1986; Sanchez-Pena and Thorvilson 1992; Quiroz et al. 1996; de Zarzuela et al. 2007, 2012; Castilho et al. 2010) and should be less likely to exhibit species-specific coevolution with the ants investigated here.

Experiment 1: the effect of venom gland blockage and nest silk on the disease resistance of *O. smaragdina* workers

Forty-eight major workers were collected from immediately outside the nest entrances for each of five colonies of *O. smaragdina*. The ants from each colony were divided into eight treatment groups representing a full-factorial combination of trials with ants either (i) treated or untreated with *Metarhizium pingshaense* [MT02_73 isolated from Panama (Hughes et al. 2004; Pull et al. 2013)], (ii) with venom glands blocked with nail varnish or unblocked, and (iii) with or without a section of nest silk to test whether ant workers may gain antimicrobial compounds from the silk. The *Metarhizium*-treated ants had 0.5 μl of a 1.5×10^6 conidia per millilitre suspension of *Metarhizium* conidia in a 0.05 % solution of Triton-X surfactant applied directly to the mesosoma and gaster of the ant with a micropipette. Similar doses have produced approximately 50 % mortality in *Polyrhachis* and other formicine ants (Graystock and Hughes 2011; C Tranter unpublished data). Control ants had 0.5 μl of a 1.5×10^6 particle per millilitre suspension of talcum powder in 0.05 % Triton-X solution applied in the same way as a particulate control. Ants were held with soft forceps during the treatment procedure. *Metarhizium* conidia were harvested from freshly sporulating media plates, and viability was confirmed to be >90 % throughout the experiments by quantifying conidia germination 24 h after plating onto Sabouraud dextrose agar (Siegel 2012). Venom glands were blocked by placing a drop of quick-dry nail varnish over the acidopore with a needle, with a drop of nail varnish being applied on to the dorsal surface of the gaster in control ants. The nail varnish was checked daily to ensure that it was still intact, but in all trials remained present for the duration of the experiment. Fresh white nest silk was obtained from the outer sections of the nest-of-origin for each ant, and cut into 1-cm^2 squares, before being paired with each ant. After treatment, ants were kept individually at 22 °C in pots (height 100 mm, diameter 22 mm), with two small balls of cotton wool soaked in water and 20 % sucrose solution, and their survival monitored daily for a period of 2 weeks.

Experiment 2: caste differences in *O. smaragdina* behavioural defences and trophallaxis

In order to investigate whether *O. smaragdina* worker castes may differ in their self-grooming behavioural defences, 10 major and 10 minor workers were collected from each of five colonies of *O. smaragdina.* Half of the ants of each caste were treated with *Metarhizium* and half with a control treatment of talcum powder, as in experiment 1. Each individual was placed in an individual pot, in the conditions described above, and observed for 15 min, with the length of time the ant spent self-grooming being recorded. In addition, 12 minor and 12 major workers were collected from each of four colonies of *O. smaragdina* to look at differences in allogrooming and trophallaxis between pairs of ants. For this, the ants from each colony were split into three pairings: major-major, major-minor and minor-minor, with each pair of ants being placed together in a pot. The duration of any allogrooming or trophallaxis between the two ants was then recorded over a period of 15 min. Whilst it was possible to discriminate the direction of allogrooming, e.g. a major grooming a minor, this directionality was not accurately determinable for trophallaxis.

Experiment 3: comparative behavioural defences of four species of weaver ant

Ten major worker ants from each of three colonies were collected from the exterior nest surface of each of the four weaver ant species (*P. delecta*, *P. dives*, *P. foreli*, *O. smaragdina*). Half of the ants from each colony were treated with *Metarhizium* and the other half with a talcum powder control suspension, as in experiment 1, placed in individual pots, and their self-grooming observed for 15 min. Data from *P. dives* was collected prior to the rest of the experiment in 2012 from colonies kept in the UK, using a different stock suspension of the same strain of *Metarhizium*, but with the concentration, delivery methods and rest of the protocol being identical.

Experiment 4: the use and effectiveness of acidic venom for nest hygiene

We investigate directly whether worker presence affected the pH of nest material in four ways. First, nest silk from colonies of *O. smaragdina* and *P. delecta* was cut into 1-cm^2 sections, and the pH tested by soaking the nest material samples in 0.5 ml of distilled water and adding universal indicator solution (Fluka Universal indicator solution; pH 4–10). The nest material was sampled broadly from both towards the inside and outside the colony. This was repeated 15 times (five samples from each of three colonies) from each of the two species for the following: (1) nest material freshly sampled (less than 2 h since colony collection); (2) nest material which had been left on its own in a pot for 24 h; (3) nest material which had been paired with a worker ant and left in a pot for 24 h. *Oecophylla* majors were used for the pairings, and all ants were sampled from those found immediately external to the nest. Second, the ability of ants to maintain alterations in pH in their environment was investigated by placing ants with a section of pH indicator paper (Fluka indicator paper; pH 0.5–7). Individual ants were placed into a pot with the bottom lined with two sections of indicator paper for 48 h. At time points 2, 12, 24 and 48 h, both indicator sections of the dish were observed and the pH recorded based on the colour of the indicator paper. At each of the time points, the left hand section of the indicator paper was replaced to account for any variation in the result due to time affecting the indicator paper, but both papers gave the same (±<0.5 pH) results throughout. After 48 h, the ant was removed, and the paper left for a further 24 h before a final recording was taken. This was repeated for five ants from each of three colonies for *P. delecta* and five ants from each of five colonies for *O. smaragdina*. Third, in order to confirm that venom use was responsible for changes in pH, we blocked the venom gland of 15 ants with nail varnish and applied nail varnish to the dorsal surface of the gaster for 15 control ants from five colonies of *O. smaragdina* and recorded the pH of the environment within a pot in which they had been kept for 48 h using pH indicator paper. Fourth, the antifungal effect of pH was tested by measuring conidia viability on media at three acidities. Twenty agar media plates (Sabarose dextrose agar medium plus yeast) were prepared, and 100 μl of a 1.5×10^6 suspension containing freshly harvested *Metarhizium* conidia in Triton-X was pipetted and spread evenly over the surface of the plate. The plates were left for 10 min for any excess liquid to evaporate before 100 μl of one of a series of sequentially diluted pH treatments was applied. Doses were as follows: undiluted formic acid solution (pH 3.7), 1:10 dilution of formic acid/ddH_20 (pH 4.7), 1:100 dilution of formic acid/ddH_20 (pH 5.7) and pure ddH_20. Plates were then incubated for 16 h at 28 °C before the percentage of viable conidia (counted as those where the germ tube produced was longer than the diameter of the conidia) were counted under a stereo microscope at ×200 magnification (Siegel 2012).

Statistical analyses

All data was tested for normality prior to analyses in order to determine the correct test, and model distributions were chosen based on the best fit using AIC scores and the structure of the non-normal data. No over-dispersion was observed based on model deviance/df values. The survival of *Oecophylla smaragdina* ants in experiment 1 were analysed using a Cox regression with fungal treatment, the presence of silk, gland blockage and colony included as factors. Non-significant interaction terms were removed stepwise to achieve the minimum adequate model. Pairwise comparisons of treatments

were made using Kaplan-Meier tests with the Breslow statistic. In experiment 2, the effect of caste on the duration of self-grooming for *O. smaragdina* workers was examined using a linear mixed model, with caste-pairings and fungal treatment included as fixed factors. Allogrooming and trophallaxis rates in experiment 2 and the differences between self and allogrooming rates between the four species of weaver ants with and without fungal treatment in experiment 3 were analysed using generalised linear mixed models (GLMM) with gamma distributions and log link functions. The pH levels of the environment shared by an individual ant in experiment 4 were analysed using a GLMM with gamma distribution and log link function, with time and species included as fixed factors. The pH levels of nest silk paired with different ant species for different periods, differences in conidia viability between pH treatments and the effect of gland blockage on pH were analysed using linear mixed model. Post hoc comparisons for all models were adjusted using the sequential Bonferroni method, and colony-of-origin was included as a random factor for all linear mixed models and GLMMs. Results of all statistical tests can be viewed in online supplementary material (S1).

Results

Exp. 1: the effect of venom gland blockage and nest silk on the disease resistance of *O. smaragdina* workers

None of the control ants which died sporulated with the *Metarhizium* parasite, whereas approximately 75 % of the dead *Metarhizium*-treated ants sporulated with the parasite. There was a significant interaction between the effects of gland blockage and *Metarhizium* treatment on survival (Wald=44.7, d.f.=1, $p<0.001$). Ants treated with control solution survived similarly well regardless of whether their venom gland was blocked and all ants exposed to *Metarhizium* parasite suffered greater mortality, but ants exposed to the *Metarhizium* parasite survived significantly less well when their venom gland was non-functional (Fig. 1). The presence or absence of nest silk had no effect on worker survival (Wald=0.42, d.f.=1, $p=0.52$). There were differences in the survival rates of ants from different colonies (Wald=7.08, d.f.=1, $p=0.008$) but no interactions between the colony-of-origin and the effects of treatments ($p>0.05$ in all cases).

Exp. 2: caste differences in *O. smaragdina* behavioural defences and trophallaxis

There was no effect of colony of origin on self-grooming, allogrooming or trophallaxis rates (z=2.12, $p=0.07$; z=0.72, $p=0.47$; z=0.703, $p=0.48$, respectively), and therefore, data across colonies were analysed together. There was a significant interaction between the effects of caste and fungal treatment on both self-grooming rates (F=5.57, d.f.=1, $p=0.02$) and allogrooming rates ($F_{3,88}=2.85$, $p=0.042$). Major workers, but not minor workers, significantly increased their self-grooming rates in response to fungal exposure (Fig. 2a). Minor workers had higher baseline allogrooming levels overall but did not alter their allogrooming rates in response to a fungal threat (Fig. 2b). Major workers, however, significantly up-regulated allogrooming rates, both of major and minor workers, in response to fungal exposure. The increase in allogrooming by majors after exposure to the fungi resulted in levels comparable to minors. Trophallaxis rates differed significantly between the caste combinations, with the presence of majors leading to higher trophallaxis rates ($F_{2,66}=61.4$, $p<0.001$; Fig. 2c). Overall, there was less trophallaxis

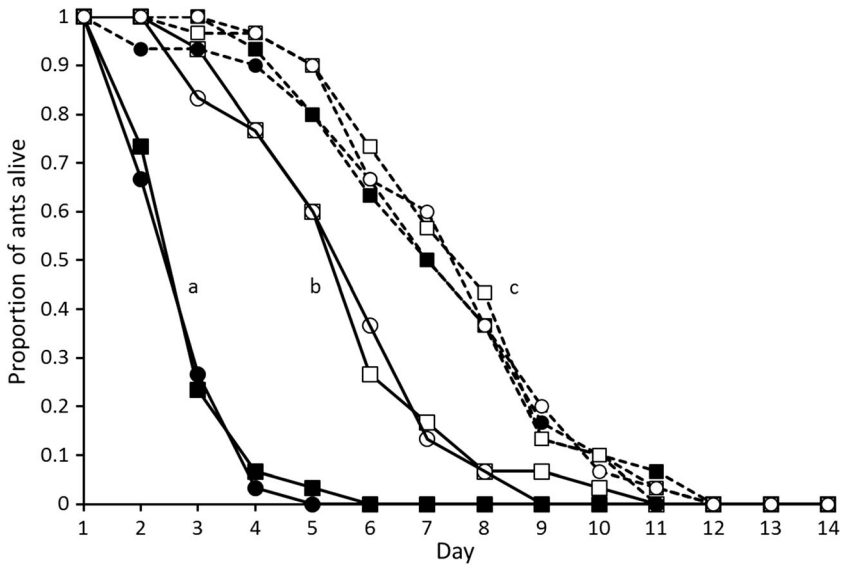

Fig. 1 The survival of individual *O. smaragdina* workers exposed to the *Metarhizium* parasite (*solid lines*) or control solution (*dashed lines*), with venom glands blocked (*black symbols*) or unblocked (*white symbols*), and kept with nest silk (*squares*) or without silk (*circles*). *Different letters* indicate three sets of treatments which differed significantly from one another in pairwise comparisons ($p<0.05$)

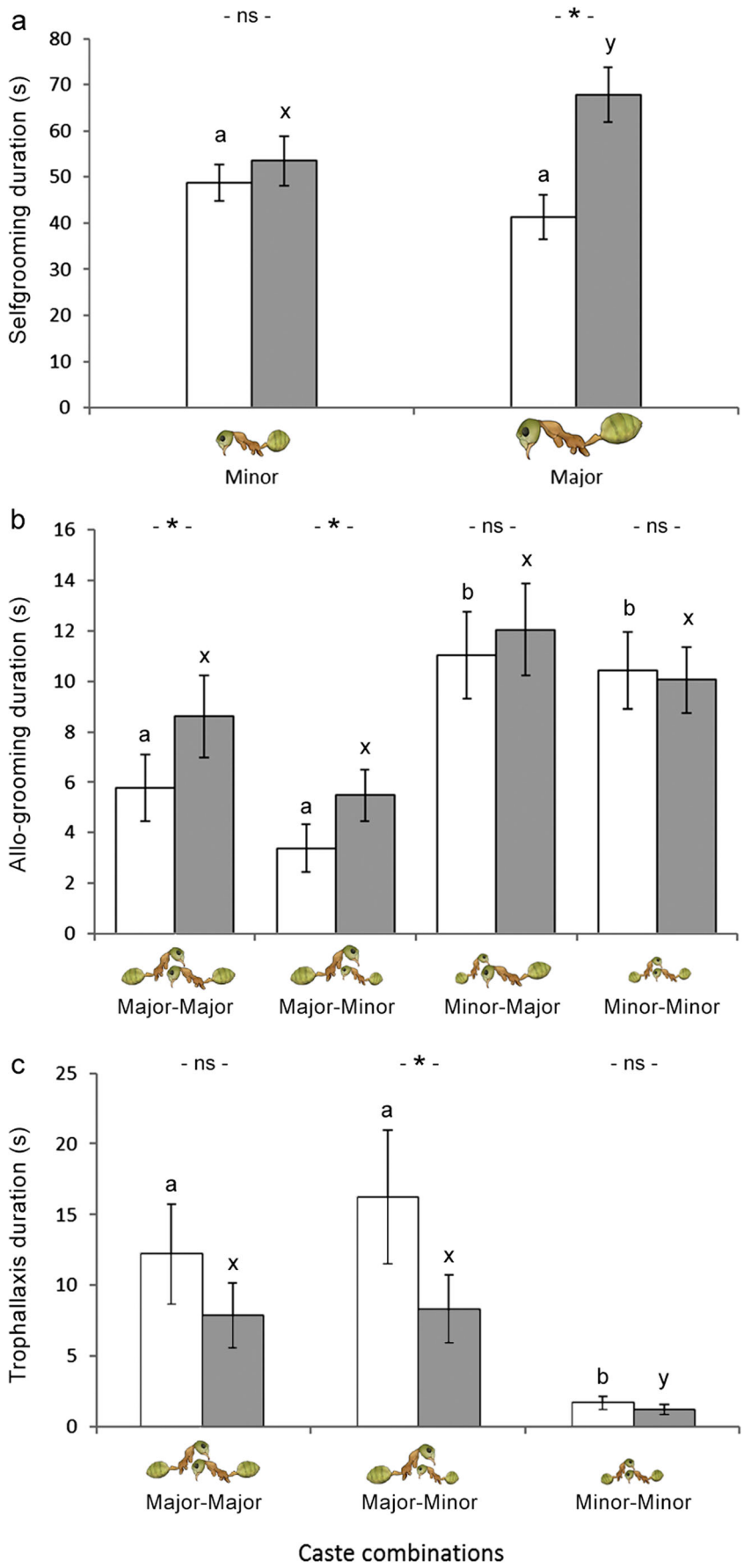

Fig. 2 The mean±lengths of time spent engaged in **a** self-grooming, **b** allogrooming and **c** trophallaxis for *Oecophylla smaragdina* major or minor workers that were either treated with the *Metarhizium* parasite (*grey*) or Triton-X control (*white*). Self-grooming observations were made on individual minor or major workers, whilst allogrooming and trophallaxis rates were quantified for pairs of ants in all four combinations of major and minor castes. In **b**, the first caste listed in the *x*-axis label indicates the ant carrying out allogrooming and the second caste listed indicates the ant receiving allogrooming. It was not possible to accurately determine the direction of trophallaxis. An *asterisk above columns* indicates a significant difference between the *Metarhizium* and control treatments, while *different letters above columns* indicate caste combinations which differed significantly from one another (talcum powder: *a–c*, *Metarhizium*: *x–z*; $p<0.05$)

when an ant had been treated with *Meta rhizium* ($F_{1,66}$=15.4, p<0.001), although this difference was only significant in the major-minor pairing (Fig. 2c), and there was no interaction between terms (F=0.25, d.f.=2,66, p=0.77).

Exp. 3: comparative behavioural defences of four species of weaver ant

The four weaver ant species differed significantly in their baseline self-grooming levels ($F_{7,112}$=25.6, p<0.001), and all the species groomed significantly more when exposed to *Metarhizium* ($F_{1,112}$=58.3, p<0.001). There was no interaction between factors indicating that they responded in similar ways ($F_{3,112}$=0.88, p=0.454), and no effect of colony (z=0.33, p=0.75). *Oecophylla smaragdina* had significantly lower baseline self-grooming and up-regulation of grooming rates compared with the *Polyrhachis* species, of which *P. foreli* had the highest baseline grooming rates (Fig. 3a). Overall, there was a significant up-regulation of allogrooming in ants treated with *Metarhizium* compared to those treated with talcum powder ($F_{1,112}$=4.89, p=0.029; Fig. 3b), but no significant difference between species ($F_{3,112}$=1.46, p=0.225), interaction between effects ($F_{3,112}$=0.515, p=0.673) or effect of colony (z=0.725, p=0.469).

Exp. 4: the use and effectiveness of acidic venom for nest hygiene

In the first assay when the pH of silk was measured directly, there was a significant interaction in the effects of ant species and whether the silk had been kept with attending workers on the pH of silk ($F_{5,84}$=4.41, p<0.001). The rise in pH when silk was kept without ants was greater for silk from *O. smaragdina* nests than for silk from *P. delecta* nests, and the reduction in the pH of silk when kept for 24 h with an ant was also much greater with *O. smaragdina* than *P. delecta*, becoming more

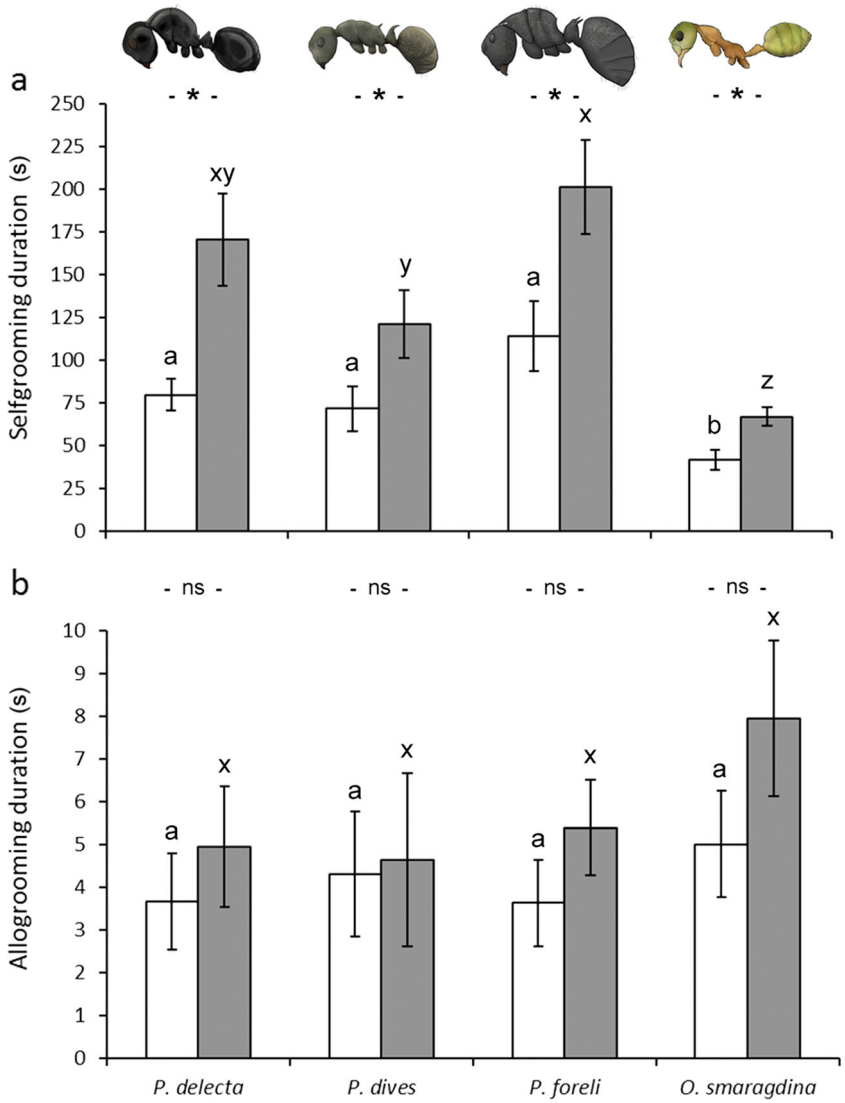

Fig. 3 The mean±lengths of time spent **a** self-grooming and **b** allogrooming by workers from four species of weaver ants (*Polyrhachis delecta*, *Polyrhachis dives*, *Polyrhachis foreli*, *Oecophylla smaragdina*), after exposure to the *Metarhizium* parasite (*grey*) or Triton-X control (*white*), in a 15-min period. An *asterisk above columns* indicates a significant difference between the *Metarhizium* and control treatments, while *different letters above columns* indicate caste combinations which differed significantly from one another (talcum powder: *a–c*, *Metarhizium*: *x–z*; p<0.05)

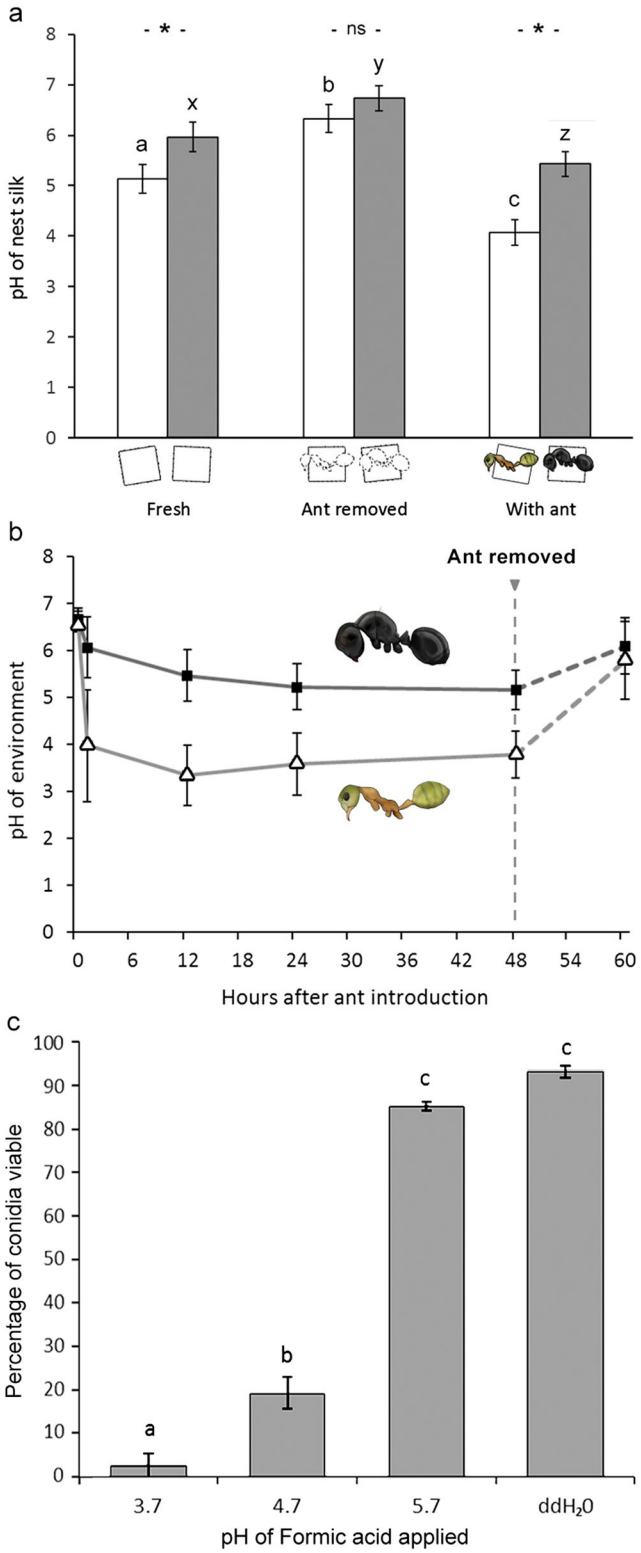

Fig. 4 **a** The pH of nest silk that had been freshly removed from the inside of the colony (*left*), left for 24 h without an attending ant (*middle*), or kept for 24 h with an attending ant (*right*), from colonies of either *O. smaragdina* (*white bars*) or *P. delecta* (*grey bars*). An *asterisk above columns* indicates a significant difference between the *Metarhizium* and control treatments, while *different letters above columns* indicate caste combinations which differed significantly from one another (talcum powder: *a–c*, *Metarhizium*: *x–z*; $p<0.05$). **b** The effect of the introduction of an individual ant on the pH of its environment at six time points over a 60-h period. pH readings were taken at 0, 2, 12, 24, 48 and 60 h, and each data point represents a mean±s.e. of 25 individuals of either *Polyrhachis delecta* (*black squares*) or *Oecophylla smaragdina* (*white triangles*). At 48 h (indicated by the *dashed vertical line*), the ant was removed. **c** The percentage of *Metarhizium* conidia germinating on media in the presence of three dilutions of formic acid, resulting in pH values of 3.7, 4.7, 5.7, or a control treatment of ddH_20. *Different letters* indicate treatments which differed significantly from one another at $p<0.05$

acidic than silk from the nest in the former but becoming identical to silk from the nest in the latter (Fig. 4a). The pH of *O. smaragdina* silk was significantly lower when compared to silk from colonies of *P. delecta* for both fresh silk and silk that had been kept with an ant, but there was no difference in the pH of silk from the two species when it had been left without an attending ant for 24 h. In the second assay, the presence of ants resulted in a significant reduction in the environmental pH over time ($F_{5,228}=47$, $p<0.001$). Two hours after introduction of the ants, the pH was substantially reduced to a low level at which it was maintained for the 48 h that the ant was present (Fig. 4b). After the ant was removed after 48 h, the pH returned to near the initial value by 60 h. The reduction in pH was much stronger with *O. smaragdina* than *P. delecta* ($F_{1,228}=203$, $p<0.001$), but the change over time was similar for the two species ($F_{3,112}=0.88$, $p=0.454$; Fig. 4b). In the third assay, the pH of the environment within pots was significantly lower when *O. smaragdina* ants had functional venom glands, at pH 4.5±0.2, compared to pH 6.1±0.1 when the gland was blocked ($F_{1,15}=54.96$, $p=0.002$), and there was no effect of colony-of-origin on the pH ($F_{1,4}=0.28$, $p=0.56$). Additionally, ants were observed spraying acid from their venom glands during trials which could be seen as distinct spots on the pH paper, indicating areas that had undergone significant reductions in their pH. In the fourth assay, the application of formic acid in vitro had a significant effect on conidia viability ($F_{3,16}=354.7$, $p<0.001$), with a pH of 4.7 reducing conidial viability by approximately 80 % and a pH of 3.7 reducing it to almost to zero (Fig. 4c).

Discussion

The results shed light on the behavioural and, in particular, chemical defences that *Oecophylla* weaver ants have against disease, and that the strength of these defences varies interspecifically when compared to *Polyrhachis* weaver ants. *O. smaragdina* workers were more resistant to the entomopathogenic fungus *Metarhizium* when they could secrete venom. Their acidic venom was strongly antimicrobial, and application improved the hygiene of their nest material. There

was no evidence for *O. smargdina* workers gaining antimicrobial compounds from silk, although other benefits of silk are possible. The behavioural defences of *O. smaragdina* varied phenotypically, with minor workers showing lower self-grooming, higher allogrooming and lower up-regulation in response to fungal threat than major workers. When compared with *Polyrhachis* weaver ant species, *O. smaragdina* exhibited lower grooming rates, but more active use of a more acidic venom.

The use of venom in *O. smaragdina* seems to be very important for disease resistance, with individuals surviving exposure to the *Metarhizium* parasite significantly better when their venom gland was functional, as opposed to being blocked. The presence of *O. smaragdina* workers with functional venom glands also significantly reduced the pH of the ant's environment and nest silk. The original nest material sampled from within the intact colony was found to be maintained at slightly acidic levels of around pH 5 which may represent the natural baseline pH within a colony. When the nest material was removed and left unattended, pH levels rose towards neutral after a short space of time (<24 h). The acidity of less than pH 4 was rapidly regained when silk was kept with a single attending worker. This acidity appears likely to be key in the general sterilisation of the ant colony against parasitic fungi because the highly acidic environments, generated through venom use in these ants, significantly reduced the viability of *Metarhizium* conidia. Fungi have been previously found to suffer decreased growth and viability at low pH or in the presence of generally acidic compounds (Do Nascimento and Schoeters 1996; Rousk et al. 2009, 2010), and we confirm this here using formic (methanoic) acid that forms the major component of *Oecophylla*, and other formicine, ant venom (Bradshaw 1979; Blum 1992). Many fungal parasites benefit from a mildly alkaline environmental pH, and *Metarhizium* itself will even raise the pH of its environment in order to promote its own fitness (St Leger et al. 1991, 1999). Thus, the use of acidic venom to lower environmental pH may be a beneficial adaptation to help ants combat the efficacy of fungal parasites. However, Tragust et al. (2013a) found that dilutions of both hydrochloric and sulphuric acid set to the same pH (pH 2.5) as formic acid did not result in the same inhibition of fungal germination. Therefore, formic acid may be antifungal for reasons other than pH, or the antifungal activity be due to the high concentrations of formic acid used. One other major component of *Oecophylla* venom is undecane which makes up around 40 % of the Dufor gland secretions (Bradshaw 1979; Keegans et al. 1991), and when combined with formic acid results in a strong behavioural alarm response in many insects. Undecane can produce antifungal effects synergistically with other gland components, but on its own has no documented antifungal activity (Bradshaw 1979; Dani et al. 2000; Fernando et al. 2005; Tragust et al. 2013a). Acids such as formic acid when found in combination with other short-chained acids can amplify the effective pH changes, or act as wetting agents promoting the delivery or effect, which may have compounding effects on their antifungal actions (Schildknecht and Koob 1971; Ortius-Lechner et al. 2000; Mendonça et al. 2009). Therefore, whilst it seems likely that a large part of the sterilising power of *Oecophylla* venom is due to the presence of formic acid, the interaction of this component with other compounds in the venom and neighbouring glands is likely to be important. Formic acid has also been identified as a major component of the cephalic exocrine secretions of *Oxytrigona* 'firebees', which use the acidic secretions as an effective defence against vertebrates (Roubik et al. 1987), and it would be interesting to discover whether the antimicrobial benefits of formic acid may extend beyond ants to other social insects.

Additionally, within *O. smaragdina* colonies, there appears to be some differentiation in grooming and trophallaxis levels, depending on caste. Major workers, which carry out most extranidal work and also predominate at the nest entrances, showed a greater up-regulation in their self-grooming and allogrooming levels in response to treatment with *Metarhizium*, whereas minor workers that stay exclusively within the social environment of the nest had higher baseline allogrooming rates. Trophallaxis levels between minor workers were generally very low, but interestingly, whilst there was no significant difference in trophallaxis rates between majors when exposed to *Metarhizium*, there was a significant reduction between majors and minors. Previously, ants have been found to show increases (de Souza et al. 2008; Hamilton et al. 2011), decreases (Aubert and Richard 2008) and no change (Konrad et al. 2012) in trophallaxis rate in response to parasite challenge; so, it appears that the interaction with this behaviour is quite variable. As minor workers are found almost exclusively within the nest, the reduction we see here in trophallaxis may represent a behavioural adaptation to try and stop the spread of parasites via trophallaxis into the colony. Wilson (1984) and Sempo and Detrain (2004), looking at the behavioural repertoires of castes in *Pheidole* found, as in this study, that minor workers performed the majority of allogrooming whilst majors performed more self-grooming. In leaf-cutting ants, it is the minor workers that play the major role in parasite defence, having relatively large metapleural glands, high grooming rates and resistance to parasites (Hughes et al. 2002, 2010; Poulsen et al. 2006; Griffiths and Hughes 2010; Abramowski et al. 2011). In *Oecophylla*, in contrast, it appears to be the major workers that may have the major role in defending the colony against parasites on incoming material or ants.

Between the different weaver ant species, there were notable differences in grooming rates and venom use. While *Polyrhachis delecta* used its venom in a similar manner to *O. smaragdina*, in order to increase the acidity of nest silk, it did not produce as large a reduction in pH. However, the

Polyrhachis weaver ants in general exhibited much higher baseline and up-regulated self-grooming rates than *O. smaragdina*, and indeed have higher rates of self-grooming than other ants too (Graystock and Hughes 2011; Tranter et al. 2015). The results of experiment 4 suggest that pH needs to be reduced to around 4.7 to gain a significant benefit in terms of anti-fungal effect. A reduction to pH 3.7 results in very low conidia viability and is even better for ants trying to prevent the spread of pathogenic fungi. *O. smaragdina* ants were consistently able to lower, and maintain, their environment to less than pH 4, whereas the *Polyrhachis* species tested was not. It appears then that the venom of *Polyrhachis* may be less effective, or used less effectively, when compared with *O. smaragdina*, or alternatively that it is used more sparingly in specific contexts such as to defend brood (Tragust et al. 2013a; Tranter et al. 2014). This may represent a differential investment into parasite defences between the genera, with *Polyrhachis* using behavioural removal of fungal conidia, instead of relying on the chemical sterilisation used by *O. smaragdina*. However, it is difficult to determine which traits are drivers of evolutionary change and which are the result of trade-offs. For example, *Polyrhachis* species may have evolved increased self-grooming because their venom is less effective, or evolved less effective venom because they do more self-grooming. Conversely, *Oecophylla* may have relaxed investment into grooming as they possess potent venom, or evolved more effective venom as they groom less. It seems likely, however, that there are fewer evolutionary constraints on behavioural self-grooming, making it most probable that there is a constraint on *Polyrhachis* venom production and that this then forces workers to use self-grooming to compensate.

In conclusion, we show that *Oecophylla* weaver ants are able to improve their resistance to the fungal parasite *Metarhizium*, and the hygiene of both themselves and their nest, through application of acidic venom. There was no difference in the survival of ants if they were paired with nest silk and so no evidence that any antimicrobial compounds in silk transfer to adult ants, although it remains possible that silk may have other benefits such as being an antiseptic nest material or blocking the entry of parasites in the environment. There was also evidence of trade-offs between chemical and behavioural defences, with different weaver ant species relying on alternative responses to parasites to protect themselves, and variations in the expression of grooming between castes in *Oecophylla*. It will be interesting to see in future work on more species whether grooming and acid production vary within the diverse *Polyrhachis* and in other genera with weaving habits, whether this is tied to their nesting habits or some other aspect of their biology, and whether venom use in *Polyrhachis* is more sparing or just less effective. We show that ant species which lack the antimicrobial secretions supplied by the metapleural gland, can use the application of acidic venom and grooming to help to defend themselves and their nest material against the threat of fungal parasites which are a ubiquitous and serious threat to ant societies. This demonstrates how differential investment in parasite defences can occur in species which share many ecological characteristics, further highlighting the complexity of disease resistance mechanisms, and the need for comparative studies to help understand how organisms have evolved in response to the threat of parasites.

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