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ORIGINAL PAPER

Home economics in an oak gall: behavioural and chemical immune strategies against a fungal pathogen in *Temnothorax* ant nests

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Abstract

Nest architecture is a fundamental character shaping immune strategies of social insects. The arboreal ant *Temnothorax unifasciatus* nests in cavities such as oak galls where the entire colony lives in a unique small chamber. In these conditions, physiological and behavioural strategies likely prevail over compartmentalisation and are presumably tuned with colony size. We designed two experiments to study chemical and behavioural immune strategies against the entomopathogenic fungus *Metarhizium anisopliae* in colonies of different sizes. First, we compared spore germination and length of germinal tubes inside artificial nests, designed to impede the contact between the ants and the fungus, in colonies of different size. In the absence of direct contact, *Temnothorax unifasciatus* colonies inhibit fungal growth inside their nests, presumably through volatile compounds. The analysis revealed a positive correlation between fungistatic activity and colony size, indicating that workers of smaller colonies do not invest a higher per capita effort in producing such substances compared to larger colonies. Second, we performed a removal experiment of contaminated and non-contaminated items introduced inside the nests of colonies of different size. Small colonies challenged with contaminated fibres showed an increased removal of all the items (both contaminated and non-contaminated) compared to small colonies challenged with non-contaminated fibres only. Conversely, larger colonies moved items regardless of the presence of the spores inside the nest. Colony size qualitatively affected removal of waste items showing a pathogen elicited reaction in small colonies to optimise the reduced workforce, while the removal behaviour in larger colonies revealed to be expressed constitutively.

Keywords *Temnothorax unifasciatus* · Waste removal · *Metarhizium anisopliae* · Alternative strategies · Colony size · Antimycotic

Introduction

Social insect colonies are faced with several consequences of sociality. Crowded nests, high rates of social interactions, and high relatedness among individuals increase the risk that pathogens spread among colony members (Schmid-Hempel 1998). To cope with these risks, social insects show individual immunity and a large array of behavioural, physiological, and organisational strategies, collectively known as social immunity (Cremer et al., 2007). Many ants build their nests with complex architectures that, together with the structure of the interaction network and division of labour, constitute the organisational component of the social immunity (Baracchi and Cini 2014; Stroeymeyt et al. 2014; Tranter and Hughes 2016). Many ant species show a much simpler nest architecture. In extreme cases, this is represented by a single chamber of a few cubic centimetres hosting all colony members. In these conditions, compartmentalisation is expected to play a secondary role and ants should mostly rely on physiological and behavioural responses to cope with parasites.

Colony size has a positive relationship with general colony efficiency (Hölldobler and Wilson 1990; Bourke et al. 1995;

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Luque et al. 2013; Colin et al. 2017). Social insects often employ self-organisation algorithms in collective behaviours requiring communication, task allocation, and consensus-building. Models predict that an efficient actuation of these algorithms can be strongly favoured in large groups (Anderson and Ratnieks 1999; Gautrais et al. 2002). Moreover, colonies of different size often use alternative strategies to solve the same problems (Dornhaus and Franks 2006) also in response to infection risk (Leclerc and Detrain 2018). Individuals can qualitatively change their behaviour depending on colony size (Jeanne and Bouwma 2002; Leclerc and Detrain 2018). Small colonies are expected to be more risk-averse and more likely to adopt behavioural strategies to optimise the efficiency of individuals compared to large colonies (Herbers 1981). Compared with solitary species, social insects heavily invest in antimicrobial compounds to reduce microbial growth (Stow et al. 2007; Hoggard et al. 2011; Wang et al. 2015; Penick et al. 2018), and many ant species have glands used mainly for this task (Yek and Mueller 2011). Across ant species, investment in the production of antimicrobials seems to be independent of colony size (Penick et al. 2018). However, among conspecific colonies, large colonies are expected to rely on antimicrobial production, due to a low cost per individual, while smaller colonies could invest more resources on behavioural responses, due to the high per-capita costs of producing sufficient quantities of antimicrobials (Karlik et al. 2016).

Temnothorax unifasciatus ants nest among rocks and under stones and they also frequently occur under bark, in hollow plant stems (Czechowski et al. 2002) and in other wooden tree structures like cynipid oak galls (Espadaler and Nieves-Aldrey 1983; Mackay 2000). The colonies are usually established in a single cavity where all the individuals live in close contact. In these conditions, strategies of compartmentalisation and worker relocation to peripheral tasks as quarantine methods are presumably unaffordable (Heinze and Walter 2010). Instead, *Temnothorax unifasciatus* exhibits several behavioural strategies to cope with the spread of infections among nestmates of the obligate-killer pathogen *Metarhizium anisopliae*. These include social withdrawal of dying workers (Heinze and Walter 2010), the removal of infected corpses and items (Colin et al. 2017) and brood reallocation in nests exposed to pathogens (Karlik et al. 2016). Some of these strategies have also been shown to be dependent on colony size (Colin et al. 2017).

We used *Temnothorax unifasciatus* as a model to investigate the integration between physiological and behavioural strategies adopted in coping with parasites in an ant species with extremely reduced compartmentalisation. In particular, we tested whether *Temnothorax unifasciatus* displays waste removal toward items contaminated by the entomopathogenic fungus *Metarhizium anisopliae* and production of antimicrobial compounds to reduce the growth of the same pathogen. Furthermore, we evaluated the effect of colony size in the expression of distinct strategies and in determining the different effort invested by the workforce.

Material and methods

Study species and laboratory conditions

In spring 2017 and 2018, we collected colonies of *Themonthorax unifasciatus* in oak galls generated by cynipids of the genus *Andricus* spp. (mainly *A. quercustozae*). In the lab, we opened the galls and relocated the colonies to artificial nests composed of two rounded plastic containers organised in an upper colony chamber (2 cm Ø) and a lower peripheral one (5 cm Ø), containing ad libitum water, sugar and proteins (finely chopped dry dog food). We connected the two chambers by a hole (2–3 mm Ø). All colonies contained the queen, the brood and a number of workers ranging from about ten to a maximum of approximately two hundred. We kept the colonies in a thermostatic room (20–25 °C temperature, 80% humidity).

Waste management experiment

Colonies spent at least three days in laboratory conditions before the beginning of the experiment. To study the management of pathogen-contaminated material, we introduced differently treated nylon fibres (2-mm length, 0.3 mm Ø) in the upper colony chamber. As a pathogen, we used commercial spores (Met52© Monsanto) of the entomopathogenic fungus *Metarhizium anisopliae*. To verify the virulence of *Metarhizium anisopliae* spores against ants, we refreshed the spores on ants as described in Bordoni et al. (2018). We rinsed some foundresses of *Crematogaster scutellaris* in a suspension of the commercial product. After the death of the ants, we collected the fungal spores by washing the ant bodies showing growth of mycelium in Triton solution (0.01% in distilled water). The conidiospore suspension was plated on Maltose Extract Agar (MEA, OXOID) in Petri dishes and incubated at 30 °C for some days. Conidia from individual colonies were recognised as belonging to *Metarhizium anisopliae* under microscope according to their morphology and then collected in Triton solution. Dilutions of spore suspension were plated on Malt Extract Agar (MEA, Oxoid) and incubated at 30 °C. The number of colony-forming units (CFU) were counted to determine the spore viable titre (CFU/ml). We then used a final spore suspension in a concentration of 10^8 CFU/ml. To distinguish treatments during the observations, we used fibres with different colours (blue, red, black, brown and grey). Once sterilised, we subjected experimental nylon fibres to two treatments in groups of 200: (i) immersion in 1 ml of the *Metarhizium anisopliae* spore suspension (contaminated

fibres) and (ii) immersion in 1 ml of the water and Triton solution (0.01%) (non-contaminated fibres). We dried contaminated and non-contaminated fibres by evaporation for 48 h. To discern if ants managed differently exposed, non-contaminated fibres and non-contaminated fibres co-occurring in the nest with exposed ones, we introduced 20 fibres to upper colony chambers in two different combinations: (i) 10 contaminated fibres of one colour and 10 non-contaminated fibres of another colour or (ii) 10 non-contaminated fibres of one colour and 10 non-contaminated fibres of another colour. This resulted in three kinds of fibres involved in different experiments: (i) contaminated fibres (m fibres), (ii) non-contaminated fibres co-occurring with contaminated fibres (cm fibres) and (iii) non-contaminated fibres only (cc fibres).

We tested a total number of 54 *Temnothorax unifasciatus* colonies in two different experimental trials (24 colonies for the first trial, 30 different colonies for the second). We recorded the dimension of the colonies (1, $n < 50$ workers, small; 2, $50 < n < 100$ medium; 3, $n > 100$ large) and allocated fibres of each colour to distinct fibre treatments in different colonies. Using different colours allowed us to check the number of fibres belonging to distinct treatments moved from the upper colony chamber to peripheral ones after 1, 2, 4, 6 and 24 h.

Fungal growth inhibition experiment

To test whether *Temnothorax unifasciatus* affects microbiological growth in the nest environment, we designed specific artificial nests. These nests allowed the growth of *Metarhizium anisopliae* in the nest environment without any physical contact with ants (Fig. 3a). Artificial nests were composed of a single plastic container (2 cm Ø), divided in an upper and a lower chamber (0.5 cm and 1.8 cm height, respectively) by a nylon net (300 μm mesh). We sterilised all nest components with alcohol and UV light (30-min exposure).

Colonies used for this experiment spent 1 day of adaptation in these experimental structures before the beginning of the treatment. The lower chamber hosted either the whole ant colony (experimental group) or nothing (control group), while in the upper chamber, we placed fungal spores. We applied a thin layer of 10 μl of Maltose Extract Agar on a glass cover slide where we plated the spores (1 μl of suspension of 4×10^8 CFU/ml of *Metarhizium anisopliae* spores in 0.01% Triton solution). We made a hole of 1 cm of diameter on the ceiling of the upper chamber (see Fig. 3a for nest structure) and applied the cover slide over the hole by orienting the fungus upside-down, facing the colony and exposing it to the air of the colony. We fixed the slide with Parafilm® and let the spores grow in experimental and control nests in controlled conditions (20–25 °C temperature, 90% humidity) for 24 h. After 24 h, we collected the slides and took two photographs of the central area of the structure by a camera connected to a microscope (× 600 magnification) were at least 10 spores occurred. We carried out the experiment in two sessions for a total of 40 (20+20) colonies of ants and 40 (20+20) controls.

The photographs were analysed by an experimenter who was unaware of the experimental group assignment of the images. This experimenter took two measurements. First, he counted germinated spores and non-germinated spores in each picture (Fig. S1 in supplementary figures as an example). He excluded from the count the spores which could not be attributed to either group (e.g. in areas out of focus, in the presence of optical artefacts or in the occurrence of clustered spores). We then calculated the germination ratio between number of germinated spores and total spores. Second, to evaluate the length of the germinal tubes, we examined from each picture a maximum of 10 randomly chosen germinated spores. By using ImageJ, we measured the length in pixels by using a segmented line (usually 1–6 segments). For each photo, we calculated the average length between measured spores. We scored the dimension of each colony as in the waste management experiment above (small = 1, medium = 2, large = 3), while we attributed size equal to zero to empty control nests. We replicated the experiment on further 40 artificial nests, and pictures were taken after 48 h (Fig. S1 in supplementary figures).

Statistical analyses

The count variable of moved fibres showed a negative binomial distribution. For this reason, we applied the "glmmadmb" function of the "glmmADMB" R package to fit a generalised linear mixed model (GLMM). As predictor variables, we used (i) treatment of the fibres (m, cm, cc); (ii) time of observation (1, 2, 4, 6, 24 h); (iii) colour of the fibres; (iv) size of the colony as an ordered variable among small, medium and large; and (iv) the interaction between time, treatment and colony size. We used colony ID for data collected at different times as a random factor. We used type III sum of squares as returned by the "Anova" function of the "car" R package. We calculated Tukey post hoc tests among main effects, estimated marginal means and their standard errors with the "emmeans" function of the "emmeans" package. In the presence of significant interactions between variables, the influence of the main effects cannot be interpreted. For this reason, in case of significant interactions, we ran individual glmmADMB models for individual classes of colony (time, size, treatment) and compared their efficiency of fibre removal using Tukey post hoc tests. The germination ratio and the square root transformed average length of germination tubes were normally distributed, and we included them as a response variables in two GLMMs ("glmmPQL" function of the MASS library) using size as an ordered predictor (which also included the zero value for controls) and replicate as a random factor. Scripts and original data are available in Appendix S1.

Results

Waste management experiment

The mixed linear model comparing the removal of different kinds of fibres carried out by 54 colonies of different size returned a significant effect for colour, treatment, colony size and for the interaction between size and treatment (Table 1). Among colours, the overall significant effect was due to higher removal of blue fibres compared to the other colours, while the other comparisons did not show significant effects (Fig. 1, Table 2). The significant interaction between colony size and treatment (Table 1) made it impossible to assess the influence of the two determinants independently. For this reason, we ran three separated models for colony of different size by comparing the effect of colours, time and treatment. Among the three GLMMs, treatment showed a significant effect only among small colonies (Chisq = 8.642, P = 0.013), and post hoc comparisons showed that in small colonies cc fibres were removed less frequently than cm and m fibres (Table 2 and Fig. 2). In the individual GLMMs using single treatments (cc, cm, m), a significant effect for size occurred in cc and m fibres. Pairwise comparisons showed that cc fibres were removed less frequently by small colonies compared to medium and large ones and m fibres were removed less frequently by small compared to large colonies (Fig. 2, Table 2). These results showed that the significant interaction between size and treatment was produced by small colonies differentially moving cm and m fibres more than cc fibres, while medium and large colonies expressed a similar effort in moving the three kinds of fibres (Fig. 2).

Table 1 ANOVA table (type III sum of squares) for a generalised linear mixed model comparing the number of fibres moved by *Temnothorax unifasciatus* against fibre colour (Colour), time of check from the beginning of the experiment (Time), treatment with *Metarhizium anisopliae* and ant colony size (Size) for the 54 tested colonies

Variable	*df*	Chisq	*P*
Colour	3	336.507	< 0.0001
Time	1	3.825	0.0504
Treatment	2	8.127	0.0172
Size	2	11.947	0.0025
Time × treatment	2	5.406	0.0670
Time × size	2	1.677	0.4324
Treatment × size	4	13.141	0.0106
Treatment × size × time	4	3.716	0.4458

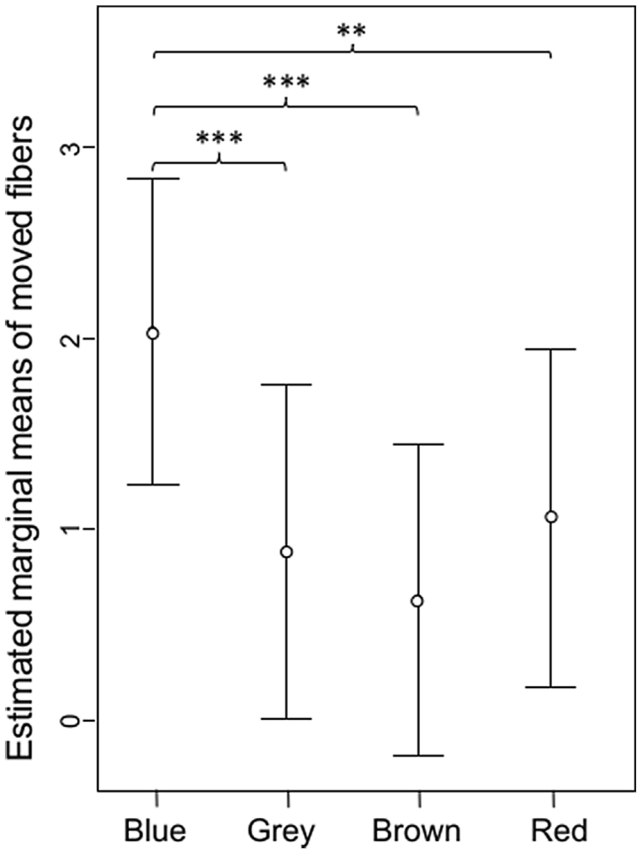

Fig. 1 Estimated marginal means (circles) with standard error bars obtained from a generalised linear mixed model of the number of fibres of different colours removed by *Temnothorax unifasciatus*. Asterisks indicate P values obtained after post hoc comparisons (*0.050 > P > 0.010; **0.010 > P > 0.001; ***P < 0.001)

Fungal growth inhibition experiment

The GLMMs comparing the germination ratio and average length of germination tubes revealed that colony size significantly affected both germination rate (Chisq = 16.477, df = 3, P < 0.001) and the average length of germination tubes (Chisq = 56.949, df = 3, P < 0.001). Post hoc tests for germinating ratios revealed differing patterns. Small colonies did not differ when compared to empty nests (estimate = 0.129, SE = 0.065, df = 72, t-ratio = 1.996, P = 0.199), while medium colonies showed a trend (estimate = 0.142, SE = 0.056, df = 72, t-ratio = 2.540, P = 0.062) and large colonies showed a significant difference (estimate = 0.181, SE = 0.053, df = 72, t-ratio = 3.416, P = 0.005) (Fig. 3b).

Post hoc tests revealed that tubes were longer in empty nests than in all ant nests (control vs small: estimate = 2.035, SE = 0.740, df = 72, t-ratio = 3.117, P = 0.014; control vs medium: estimate = 3.158, SE = 0.640, df = 72, t-ratio = 4.935, P < 0.001; control vs large: estimate = 3.893, SE = 0.608, df = 72, t-ratio = 6.402, P < 0.001), while no significant differences emerged among ant nests (Fig. 3c). Fungal growth advanced very rapidly after 24 h, and examination of

Table 2 Tukey post hoc comparisons for removal by *Temnothorax unifasciatus* among fibres of different colours in the full generalised linear mixed model; among fibres of different treatments in small colonies (m: fibres contaminated by *Metharhizium anisopliae*; cm: non-contaminated occurring together with contaminated fibres; cc: non-contaminated fibres); among colonies of different size for cc and m fibres. Other generalised linear mixed models for individual sizes (medium and large) did not show a significant effect for treatment, and the generalised linear mixed model for cm fibres did not show a significant effect for colony size

Model	Contrast	Estimate	SE	*z* ratio	*P* value
Colours (full model)	blue-gray	1.147	0.302	3.795	0.0009
	blue-brown	1.404	0.156	8.98	< 0.0001
	brown-red	0.971	0.301	3.223	0.0069
	gray-brown	0.257	0.426	0.603	0.9310
	gray-red	− 0.175	0.213	− 0.822	0.8441
	brown-red	− 0.433	0.427	− 1.014	0.7412
Treatment (size, small)	cc-cm	− 2.383	0.818	− 2.912	0.0100
	cc-m	− 1.982	0.811	− 2.443	0.0387
	cm-m	0.402	0.579	0.694	0.7667
Size (treatment, cc)	1-2	− 2.615	0.514	− 5.091	< 0.0001
	1-3	− 1.996	0.354	− 5.633	< 0.0001
	2-3	0.619	0.452	1.369	0.3573
Size (treatment, m)	1-2	− 0.403	0.338	− 1.192	0.4581
	1-3	− 0.794	0.309	− 2.568	0.0276
	2-3	− 0.391	0.333	− 1.175	0.4680

germination and tube length was impossible after 48 h in both control and experimental nests (Fig. S2 in supplementary figures)

Discussion

In our experiments, *Temnothorax unifasciatus* exhibited different immune strategies in dealing with the occurrence of entomopathogenic *Metarhizium anisopliae* spores inside nests. The growth of this fungus was inhibited in ant colonies in terms of both germination ratios and length of germinal tubes and, as predicted, such inhibition was stronger in large colonies when compared to small ones. In parallel, only small colonies tested with contaminated fibres showed an increased removal of all fibre types (both contaminated and non-contaminated) compared to colonies presented with non-contaminated fibres. Interestingly, a differential removal of fibres of different colours was also observed, possibly due to differential detectability of blue fibres by the ants. This could occur as a result of different UV reflection of the blue fibres compared to natural objects. Leaves and soil reflect light dominated by green and yellow light, and typically lack an intense UV light component (Menzel, 1979). This did not significantly impact the results of the experiments as blue fibres were attributed to both control and exposed groups in different experimental trials.

Inhibition of microorganism growth has been described in several social insects, mainly through the application of antimicrobial substances on the surfaces of nests. In many cases, these substances form part of the nest structure. These substances can be represented by resins collected in the environment, exhibited by many ant (Brühl 2003; Christe et al. 2003; Chapuisat et al. 2007) and bee species (Ghisalberti 1979; Velikova et al. 2000; Simone et al. 2009), chemical compounds such as naphthalene (Chen et al. 1998) and faeces, as used by some termites (Rosengaus et al. 1998). Antimicrobial substances are also produced by exocrine glands, and, in ants, the metapleural gland plays a fundamental role in their production (Schluns and Crozier 2009). Alternatively, in the fire ant *Solenopsis invicta*, venom alkaloids have been shown to produce antimycotic activity against the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* (Storey et al. 1991). Sting secretion of the common acrobat ant *Crematogaster scutellaris* also has antimicrobial activity against Gram-positive and Gram-negative bacteria and the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Perito et al. 2018). The use of volatile secretion can be highly efficient, mostly in a very restricted nest. In fact, whereas non-volatile compounds work passively and require direct contact with the pathogen to be effective, the fumigation of the entire nest with volatile compounds can largely increase the effectiveness of target substances (Chen et al. 1998). For example, Storey et al. (1991) hypothesised that fire ants can diffuse venom alkaloids as an aerosol, which was later demonstrated by Wang and collaborators (Wang et al. 2015), who showed that fire ants inhibited the germination and the growth of the fungus *Beauveria bassiana* by fumigating their

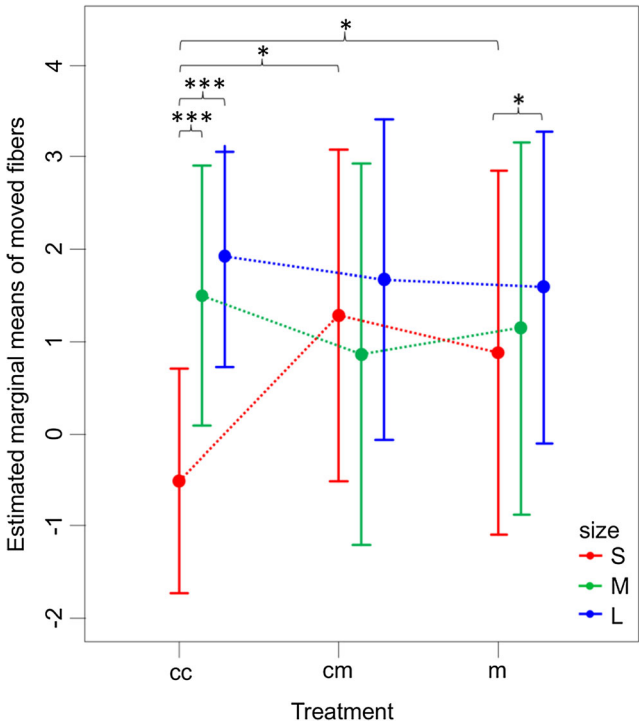

Fig. 2 Estimated marginal means (circles) with standard error bars of fibres moved by *Temnnothorax unifasciatus* and belonging to different treatments (m: fibres contaminated by *Metharhizium anisopliae*; cm: non-contaminated occurring together with contaminated fibres; cc: non-contaminated fibres) and moved by individual from colonies of different size (S = small, red line; M = medium, green line; L = large, blue line). Asterisks indicate *P* values obtained after Tukey post hoc comparisons from individual analyses of size and treatments (*0.050 > *P* > 0.010; **0.010 > *P* > 0.001; ***$P < 0.001$)

nest. This study represented the first evidence for the use of antimicrobial volatiles in ants; although the experiment involved attests of a constant number of ants, thus precluding measurements of the potential effects of colony size. In addition, ants were removed from their nests during the experiment and this could have altered their responses. We carried out experiments on whole colonies with a period of habituation in artificial nests, reducing the effects of stress on the experiment. Interestingly, the fungistatic activity of venom alkaloids of fire ant is temporary in *Beauveria bassiana* and *Metarhizium anisopliae*, and the inhibition of germination rate and tube growth is only significant in the first 24 h (Storey et al. 1991). We also observed that the efficacy of nest fumigation by *Temnothorax unifasciatus* against *Metarhizium anisopliae* is limited to the first 24 h. Since our experimental nests were not sealed, we can conclude that reduced fungal growth was not due to high concentrations of carbon dioxide and low concentration of oxygen. Besides, the effect was no longer detectable after 48 hours (supplementary materials). The peculiar simplified natural nest of *Temnothorax* ants, with its unique chamber that hosts the whole colony, is therefore likely to offer a suitable environment to maximise the effectiveness of fumigation with antimicrobial volatiles. In our experiments colony size showed a positive correlation with fungal growth inhibition, and we found no evidence suggesting increased per-capita production of antimycotic substances in small colonies. This observation is in line with the proposals by Karlik and collaborators (Karlik et al. 2016) that smaller colonies should be unable to sustain the increased per-capita cost of producing a sufficient quantity of antimicrobials and that they should rely

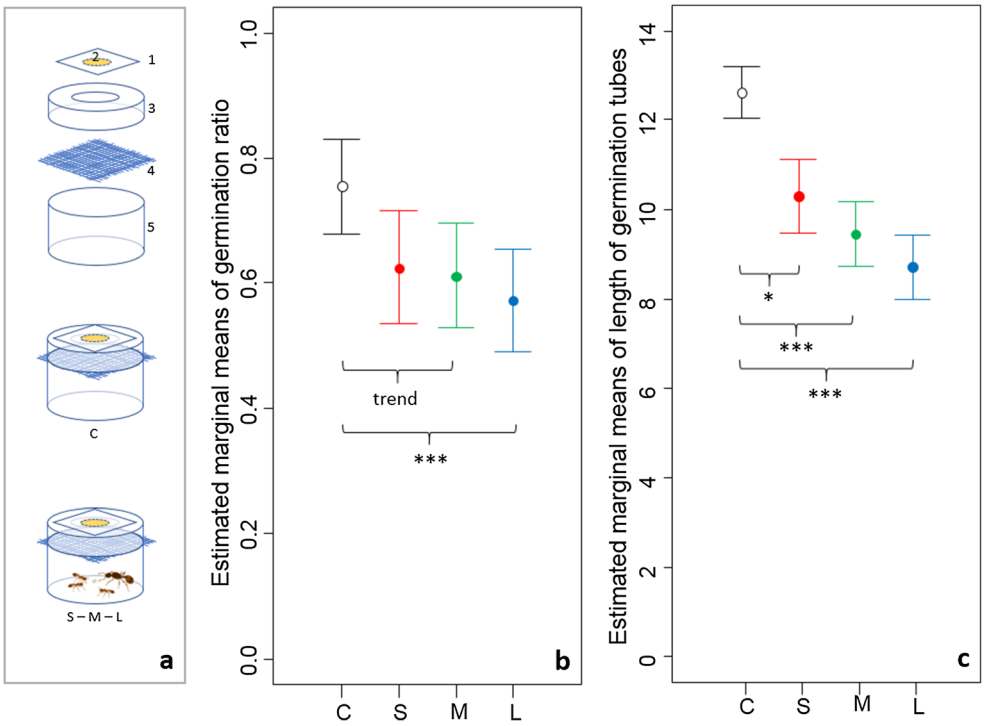

Fig. 3 The experimental nests used for estimation of reduction of fungal growth (**a**); 1, glass cover slide; 2, agar solution with *Metarhizium anisopliae* spores; 3, upper chamber; 4, nylon net; 5, lower colony chamber; C, control (empty) nests; S-M-L: small, medium and large colonies. Estimated marginal means (circles) and standard error bars obtained after running a generalised linear mixed model of germination ratio of *Metarhizium anisopliae* spores (**b**) and of length of germination tubes (**c**) in empty nests (C) and in small (S), medium (M) and large (L) colonies. Asterisks indicate *P* values obtained after Tukey post hoc comparisons (*0.050 > *P* > 0.010; **0.010 > *P* > 0.001; ***$P < 0.001$)

more on behavioural responses in immune defence. Future studies are necessary to understand which substances are involved in the fungistatic effect. It is also unknown whether the immune defence is expressed continuously or elicited by the occurrence of the pathogen inside the nest.

Removal and disposal of waste material is a virtually ubiquitous behaviour in ants, in particular when potentially contaminated items occur in the nest (Hölldobler and Wilson 1990; Diez et al. 2012; Leclerc and Detrain 2018). Removal of contaminated items is so essential that ants have evolved different behavioural strategies to optimise this task. Many of these have been found to depend on colony size. Large colonies of *Myrmica rubra* react faster to items contaminated with *Metarhizium brunneum* and are more efficient in active removal from the nest in comparison to smaller colonies (Leclerc and Detrain 2018). On the other hand, a consistent fraction of small colonies display the alternative strategy of evacuating the entire nest, sanitising it and reintegrating the colony (Leclerc and Detrain 2018).

Removal of waste material is expected to be a particularly urgent task in small *Temnothorax* nests. Accordingly, we found that medium and large colonies removed contaminated and non-contaminated items with the same efficiency, while workers of small colonies removed fewer fibres from the nest when contaminated fibres did not occur. This alternative strategy suggests that smaller colonies need to optimise their reduced workforce compared to medium and larger colonies, resulting in non-contaminated items being disregarded in the absence of infection risk. Optimisation of workforce has been found to occur in *Temnothorax* ants. Dornhaus and collaborators (2008) observed that, during colony emigration, small colonies of *Temnothorax albipennis* dedicated a small fraction of workers to item transportation. This restricted workforce transported a higher number of items per-capita and showed higher overall efficiency in transport (more items carried per-capita/time). The manner in which individuals in colonies of differing sizes can exhibit varying behavioural strategies is still unclear and largely depends on the context. Dornhaus and Franks (2006) provided three possible mechanisms: (i) self-organisation may result in different collective patterns if fewer nestmates participate in them, like in the establishment and maintenance of pheromone trails; (ii) individuals could assess the size of their colony and adjust their behaviour accordingly, as it occurs in the decrease of per-capita defensive effort with the increase of colony size in *Polybia occidentalis* (London and Jeanne 2003); (iii) correlation between a trait and the colony size may not be due to direct causation and colony size is correlated with other factors determining the observed pattern. Further studies will help to elucidate the mechanisms involved.

Due to their peculiar ecology, nesting habit and social structure, *Temnothorax* ants exhibit several behavioural strategies to deal with pathogens (Heinze and Walter 2010; Karlik et al. 2016). Our experiments confirmed that behavioural and chemical immune strategies can be combined to contrast pathogen establishment and transmission inside the reduced dimensions of the nests. *Temnothorax unifasciatus* also showed strong plasticity in optimising behavioural strategies to remove infected items, and this was dependent on the number of workers present. This plasticity is likely to have a fundamental role during colony growth and development, especially in the first phase of colony growth following foundation by queens. At this stage, the colony is small, and the reduced workforce must be optimised to maintain colony health in an environment showing a high risk of infection.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval *Temnothorax unifasciatus* was used for this study. All applicable international, national and/or institutional guidelines for the care and use of animals were followed. This article does not contain any study with human participants.

References

Anderson C, Ratnieks FL (1999) Task partitioning in foraging: general principles, efficiency and information reliability of queueing delays. In: Detrain C, Deneubourg JL, Pasteels JM, Pasteels JM (eds) Information processing in social insects. Birkhäuser, Basel, pp 31–50

Baracchi D, Cini A (2014) A socio-spatial combined approach confirms a highly compartmentalised structure in honeybees. Ethology 120: 1167–1176

Bordoni A, Dapporto L, Tatini I, Celli M, Bercigli M, Ressurrección Barrufet S, Perito B, Turillazzi S (2018) Trans-generational immunization in the acrobat ant *Crematogaster scutellaris*. Biol Lett 14: 20170761

Bourke AF, Franks NR, Franks NR (1995) Social evolution in ants. Princeton University Press, Princeton

Brühl C (2003) Vollenhovia sp. aff. acanthina: an ant species that build nests using resin. ANeT Newslett 6:21–22

Chapuisat M, Oppliger A, Magliano P, Christe P (2007) Wood ants use resin to protect themselves against pathogens. Proc R Soc Lond B 274:2013–2017

Chen J, Henderson G, Grimm CC et al (1998) Termites fumigate their nests with naphthalene. Nature 392:558

Christe P, Oppliger A, Bancalà F et al (2003) Evidence for collective medication in ants. Ecol Lett 6:19–22

Colin T, Doums C, Péronnet R, Molet M (2017) Decreasing worker size diversity does not affect colony performance during laboratory challenges in the ant *Temnothorax nylanderi*. Behav Ecol Sociobiol 71: 92

Cremer S, Armitage SA, Schmid-Hempel P (2007). Social immunity. Current biology, 17(16), R693-R702.

Czechowski W, Radchenko A, Czechowska W (2002) The ants (Hymenoptera, Formicidae) of Poland. Museum and Institute of Zoology Warszawa, Warszawa

Diez L, Deneubourg J-L, Detrain C (2012) Social prophylaxis through distant corpse removal in ants. Naturwissenschaften 99:833–842

Dornhaus A, Franks NR (2006) Colony size affects collective decision-making in the ant *Temnothorax albipennis*. Insect Soc 53:420–427

Dornhaus A, Holley JA, Pook VG., Worswick G, Franks NR (2008). Why do not all workers work? Colony size and workload during emigrations in the ant Temnothorax albipennis. Behavioral Ecology and Sociobiology, 63(1), 43–51

Espadaler X, Nieves-Aldrey JL (1983) Hormigas (Hymenoptera, formicidae) pobladoras de agallas abandonadas de cinipidos (Hymenoptera, cynipidae) sobre Quercus SP en la Peninsula Iberica. Bol Estacion Cent Ecol 12:89–93

Gautrais J, Theraulaz G, Deneubourg J-L, Anderson C (2002) Emergent polyethism as a consequence of increased colony size in insect societies. J Theor Biol 215:363–373

Ghisalberti EL (1979) Propolis: a review. Bee World 60:59–84

Heinze J, Walter B (2010) Moribund ants leave their nests to die in social isolation. Curr Biol 20:249–252

Herbers JM (1981) Reliability theory and foraging by ants. J Theor Biol 89:175–189

Hoggard SJ, Wilson PD, Beattie AJ, Stow AJ (2011) Social complexity and nesting habits are factors in the evolution of antimicrobial defences in wasps. PLoS One 6:e21763

Hölldobler B, Wilson EO (1990) The ants. Harvard University Press, Cambridge

Jeanne RL, Bouwma AM (2002) Scaling in nests of a social wasp: a property of the social group. Biol Bull 202:289–295

Karlik J, Epps MJ, Dunn RR, Penick CA (2016) Life Inside an Acorn: How Microclimate and Microbes Influence Nest Organization in *Temnothorax* Ants. Ethology 122:790–797

Leclerc J-B, Detrain C (2018) Impact of colony size on survival and sanitary strategies in fungus-infected ant colonies. Behav Ecol Sociobiol 72:3

London KB, Jeanne RL (2003) Effects of colony size and stage of development on defense response by the swarm-founding wasp *Polybia occidentalis*. Behav Ecol Sociobiol 54:539–546

Luque GM, Giraud T, Courchamp F (2013) Allee effects in ants. J Anim Ecol 82:956–965

Mackay WP (2000) A review of the New World ants of the subgenus Myrafant, (genus Leptothorax)(Hymenoptera: Formicidae). Sociobiology 36

Menzel R (1979) Spectral Sensitivity and Color Vision in Invertebrates. In: Autrum H (ed) Handbook of Sensory Physiology, Springer, Berlin, 503–566

Penick CA, Halawani O, Pearson B et al (2018) External immunity in ant societies: sociality and colony size do not predict investment in antimicrobials. R Soc Open Sci 5:171332

Perito B, Cremonini M, Montecchi T, Turillazzi S (2018) A preliminary study on the antimicrobial activity of sting secretion and gastral glands of the acrobat ant *Crematogaster scutellaris*. Bull Insectol 71:97–101

Rosengaus RB, Guldin MR, Traniello JF (1998) Inhibitory effect of termite fecal pellets on fungal spore germination. J Chem Ecol 24: 1697–1706

Schluns H, Crozier RH (2009) Molecular and chemical immune defenses in ants (Hymenoptera: Formicidae). Myrmecol News 12:237–249

Schmid-Hempel P (1998) Parasites in social insects. Princeton University Press, Princeton

Simone M, Evans JD, Spivak M (2009) Resin collection and social immunity in honey bees. Evol Int J Org Evol 63:3016–3022

Storey GK, Vander Meer RK, Boucias DG, McCoy CW (1991) Effect of fire ant (*Solenopsis invicta*) venom alkaloids on the in vitro germination and development of selected entomogenous fungi. J Invertebr Pathol 58:88–95

Stow A, Briscoe D, Gillings M, Holley M, Smith S, Leys R, Silberbauer T, Turnbull C, Beattie A (2007) Antimicrobial defences increase with sociality in bees. Biol Lett 3:422–424

Stroeymeyt N, Casillas-Pérez B, Cremer S (2014) Organisational immunity in social insects. Curr Opin Insect Sci 5:1–15

Tranter C, Hughes WH (2016) A preliminary study of nest structure and composition of the weaver ant Polyrhachis (Cyrtomyrma) delecta (Hymenoptera: Formicidae). J Nat Hist 50:1197–1207

Velikova M, Bankova V, Tsvetkova I, Kujumgiev A, Marcucci MC (2000) Antibacterial ent-kaurene from Brazilian propolis of native stingless bees. Fitoterapia 71:693–696

Wang L, Elliott B, Jin X et al (2015) Antimicrobial properties of nest volatiles in red imported fire ants, *Solenopsis invicta* (hymenoptera: formicidae). Sci Nat 102:66

Yek SH, Mueller UG (2011) The metapleural gland of ants. Biol Rev 86: 774–791