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Slowing them down will make them lose: a role for attine ant crop fungus in defending pupae against infections?

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Summary

1. Fungus-growing ants (Attini) have evolved an obligate dependency upon a basidiomycete fungus that they cultivate as their food. Less well known is that the crop fungus is also used by many attine species to cover their eggs, larvae and pupae.

2. The adaptive functional significance of this brood covering is poorly understood. One hypothesis to account for this behaviour is that it is part of the pathogen protection portfolio when many thousands of sister workers live in close proximity and larvae and pupae are not protected by cells, as in bees and wasps, and are immobile.

3. We performed behavioural observations on brood covering in the leaf-cutting ant *Acromyrmex echinatior,* and we experimentally manipulated mycelial cover on pupae and exposed them to the entomopathogenic fungus *Metarhizium brunneum* to test for a role in pathogen resistance.

4. Our results show that active mycelial brood covering by workers is a behaviourally plastic trait that varies temporally, and across life stages and castes. The presence of a fungal cover on the pupae reduced the rate at which conidia appeared and the percentage of pupal surface that produced pathogen spores, compared to pupae that had fungal cover experimentally removed or naturally had no mycelial cover. Infected pupae with mycelium had higher survival rates than infected pupae without the cover, although this depended upon the time at which adult sister workers were allowed to interact with pupae. Finally, workers employed higher rates of metapleural gland grooming to infected pupae without mycelium than to infected pupae with mycelium.

5. Our results imply that mycelial brood covering may play a significant role in suppressing the growth and subsequent spread of disease, thus adding a novel layer of protection to their defence portfolio.

Key-words: *Acromyrmex echinatior*, brood, fungal parasite, metapleural gland, *Metarhizium*, mycelial cover, public health

Introduction

Among animals, the younger immature stages are generally more vulnerable (Ricklefs & Miller 1999), which explains why some form of parental care has evolved repeatedly across diverse animal taxa (Tallamy 1984; Reynolds, Goodwin & Freckleton 2002). Parental care is common in vertebrates but proportionately rare in arthropods (Reynolds, Goodwin & Freckleton 2002). Eusocial Hymenoptera, however, have evolved extensive cooperative brood care, including sibling care. Similar to other holometabolous insects, eusocial hymenopteran pupae can be completely enveloped in protective cocoons that are not opened until adult eclosion (Craig 1997; Chapman 1998). The ants (Formicidae), however, have no brood cells, as found in some other Hymenoptera (e.g. Quiñones &

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Wcislo 2015 and references therein). Furthermore, in many ant lineages, the cocoon has been lost, so that pupae are exposed (Wheeler 1915; Armitage *et al.* 2012). Pupae may also be less frequently tended by adults than larvae, because they are not fed. Mass-rearing in open chambers may appear to facilitate spread of disease, yet ants have some of the largest and most long-lived colonies known (Dornhaus, Powell & Bengston 2012), such as the *Atta* fungus-growing leafcutter ants that can have millions of individuals per colony (Weber 1972). This contradiction has led to the increasing appreciation that ants have sophisticated collective prophylaxis and social immunity defences so they can control and/or eliminate generalist pathogens (Boomsma, Schmid-Hempel & Hughes 2005; Cremer, Armitage & Schmid-Hempel 2007). The mechanisms by which ants achieve their formidable hygienic successes have only just begun to be explored in detail (Fernández-Marín *et al.* 2006, 2015; Ugelvig & Cremer 2007; Tragust *et al.* 2013a).

The fungus-growing ants (Attini) have a mutualistic relationship with basidiomycete fungal symbionts that are used as food sources (Weber 1972; De Fine Licht, Boomsma & Tunlid 2014). Several microbes are also associated with this mutualism, such as *Escovopsis,* a fungal parasite of the fungus garden (Currie, Bot & Boomsma 2003); stable colony-specific actinomycete bacterial communities (Andersen *et al.* 2013), including *Pseudonocardia,* that grow on the ant cuticle (Currie *et al.* 2006) and that have antifungal activity (Currie *et al.* 1999; Sen *et al.* 2009); and black yeasts, which also grow on the ant cuticle (Little & Currie 2007) and seem to negatively affect the ants' ability to suppress a parasitic fungus, *Escovopsis* (Little & Currie 2008). Attine pupae do not spin cocoons and it has long been known that the fungal mycelia also cover brood (including eggs, larvae and pupae) (Wheeler 1907). Such covers occur in many but not all species that have been studied (for a review see Armitage *et al.* 2012). At least in some taxa, initiation of fungal growth on the larvae results from workers actively planting tufts of fungal hyphae on the larval cuticle (e.g. Lopes *et al.* 2005; Camargo *et al.* 2006; Fernández-Marín *et al.* 2013) rather than passive overgrowth. Once the tufts have been planted, they become interconnected with mycelium (Ramos Lacau *et al.* 2008), so brood appear as having a mycelial cocoon when fungal cover becomes extensive. Histological sections of larvae from phylogenetically more basal species showed that the fungal hyphae emit projections to the interior of the cuticle, suggesting that the relationship between the fungal covering and the larval cuticle is intimate (Ortiz, Mathias & Bueno 2012).

Several hypotheses have been proposed to explain the evolution of mycelial covering behaviours, but are untested. Ramos Lacau *et al.* (2008) hypothesized that the fungal mycelium might facilitate moulting by aiding degradation of the soon-to-be moulted cuticle. A mycelial brood cover may also protect against adverse abiotic environmental factors (Mueller, Ortiz & Bacci 2010) and may provide protection against predators such as army ants or macroparasites such as parasitoid wasps (LaPolla *et al.* 2002; Powell & Clark 2004; Fernández-Marín, Zimmerman & Wcislo 2006; Pérez-Ortega *et al.* 2010). However, none of these appear to be compelling general explanations: abiotic factors seem unlikely to vary in an underground nest beyond what ants can normally control by moving their brood around. Available information for mycelial cover and its correlation with predation and parasitism neither supports nor refutes this kind of protective function (see Discussion in Armitage *et al.* 2012).

Here, we explore whether the cover protects brood from microbial parasites (Lopes *et al.* 2005; Mueller, Ortiz & Bacci 2010; Armitage *et al.* 2012). Indeed, a number of animals use symbiotic microbes and fungi for protection against parasites (Flórez *et al.* 2015). If this is the case for fungus-growing ants, the defence could be prophylactic, induced or constitutively active. Furthermore, the barrier could be of a chemical or a physical nature, minimizing or blocking contact between pathogens and brood. In support of a potential chemical prophylaxis role, *Lepiota*, a fungal cultivar of *Cyphomyrmex costatus* fungus-growing ants, produces lepiochlorin, which has activity against the bacterium *Staphylococcus aureus* (Hervey & Nair 1979; Wang, Mueller & Clardy 1999). In addition, the fungal cultivar of *Atta colombica* inhibits the growth of endophytic fungi growing in the leaves that the ants cut (Van Bael *et al.* 2009). Finally, attine species that have lower mycelial cover on their brood more frequently use metapleural gland (MG) grooming as a defensive behaviour (Armitage *et al.* 2012), suggesting that these defences trade-off, but this study did not examine mycelial cover in the context of experimental parasite exposure, so the results remained correlative.

The first objective of our present study was to quantify the consequences of experimental removal of mycelial brood cover and to obtain observations of the behaviours that produce mycelial brood covering on larvae and pupae of a leaf-cutting ant, *Acromyrmex echinatior* Forel (Hymenoptera: Formicidae), which frequently covers its eggs, larvae and pupae with crop fungus (Armitage *et al.* 2012). Three experiments tested the hypothesis that the mycelial cover of pupae represents a defensive function after exposure to a generalist entomopathogenic fungus *Metarhizium brunneum* [Hypocreales: Clavicipitaceae; formerly known as *Metarhizium anisopliae* var. *anisopliae* (Bischoff, Rehner & Humber 2009)]. This fungus has been isolated from areas around leaf-cutting colonies (Hughes *et al.* 2004b) and is known to infect and kill leaf-cutting ants in the laboratory (Hughes *et al.* 2004a). *Metarhizium* has been used to address host-pathogen interactions in *A. echinatior* (e.g. Hughes & Boomsma 2004; Hughes *et al.* 2004a) and sanitary behaviours in other ant species (e.g. Ugelvig & Cremer 2007; Ugelvig *et al.* 2010; Tragust *et al.* 2013b). We first tested whether mycelial cover retards growth of *M. brunneum* in the absence of active brood care. The second experiment tested whether mycelial cover affected hygienic behaviour, that is the efficiency by which workers detect and remove diseased brood (Arathi, Burns & Spivak 2000; Wilson-Rich *et al.* 2009), following exposure to *M. brunneum*. Lastly, we tested whether pupal mycelial cover affected the use of MG grooming, a known alternative hygienic strategy in attine ants (Fernández-Marín *et al.* 2009, 2015) after *M. brunneum* exposure.

Materials and methods

For detailed materials and methods, please see Appendix S1, Supporting Information.

ANTS AND FUNGUS

For the behavioural observations and the first exposure experiment, we used *Acromyrmex echinatior* colonies that had been collected between 2001 and 2007 from Gamboa, Panama, and taken to Copenhagen, Denmark. For the second exposure experiment, we used nine *A. echinatior* colonies collected in 2008 in Panama and taken to Copenhagen and another seven colonies collected in 2011 and maintained in Panama. For experiment 3, 10 *A. echinatior* colonies were collected in 2011 and maintained in Panama. Ant colony codes are given in the raw data file (deposited in Dryad). *Acromyrmex echinatior* worker voucher specimens were deposited at the Museo de Invertebrado, Universidad de Panama. *Metarhizium brunneum* (KVL-0272: Hughes *et al.* 2004b) was isolated from the same area in Panama as where ant colonies were collected.

QUANTIFICATION OF MYCELIAL COVER AND BEHAVIOURAL OBSERVATIONS

Mycelical brood cover on larvae and pupae

We examined whether colonies differed consistently in the amount of mycelial brood cover that they applied after disturbance. Ten final instar large worker larvae were removed from each of 10 colonies (see Appendix S1 for details) to estimate their percentage mycelial cover under a stereomicroscope, after which they were transferred to the edge of a Petri dish containing ~180 mg of fungus garden. Two large, four medium and 10 small workers were added and every day the mycelial cover was scored for each larva or pupa using a dissecting microscope. Larvae were checked 18 h after the start of the experiment (day 1), and every subsequent day until 2 days after all larvae had pupated (day 10). Larval sample sizes decreased over time as individuals pupated; therefore, we present differences in cover between days 0, 1, 2, 3 and 10 (see raw data file for details). In one colony (Ae265), nine larvae died, and one became a pupa, but mortality was negligible in all other colonies. The data were analysed using JMP®, version 9.0.0 for Macintosh (SAS Institute Inc., Cary, NC, USA, 1989–2007).

Mycelical cover on sexual brood

Sexual brood (gynes and males) are more costly to produce given their larger size and are more valuable given their greater potential reproductive value for the colony than workers. In queenright colonies of *Acromyrmex subterraneus brunneus* planting of fungal hyphae on worker larvae occurred more frequently than planting on male (sexual) larvae, but sexual pupae or gynes were not examined (Camargo, Lopes & Forti (2006). We therefore quantified mycelial cover on reproductive brood of both sexes and tested whether it differed from that found on the workers. We estimated per cent mycelial cover for a minimum of 20 brood items (either male pupae, gyne pupae or older sexual larvae) from five colonies and also surveyed worker pupae from the same colonies where possible.

Behavioural observations on workers and mycelial cover in relation to pupal age

We asked the following questions: (i) How long it took for naked pupae to be covered in mycelia, (ii) Whether workers differentially covered pupae of different ages and (iii) What the frequency was with which tufts of mycelium were placed on the pupae? Two young white worker pupae and two older worker pupae with brown cuticle were removed from each of nine colonies. Petri dishes were prepared as described as above, and mycelial cover was recorded for all pupae and then removed with a fine dry paintbrush, which was washed thoroughly in 96% ethanol and allowed to dry between handling different colonies. The pupae were placed in the Petri dish away from the fungus garden fragment. Each Petri dish was observed constantly for 60 min after adding the pupae, and the time when the pupae were moved to the fungus garden was recorded. The percentage mycelial cover of each pupa was recorded every hour until 9 h after set-up and then observed again at 25 and 72 h. We analysed the differences in the mean cover per colony of young and old pupae before mycelial removal and then at 9, 25 and 72 h after mycelial removal (Appendix S1).

Brood covering behaviour has been previously described (Lopes *et al.* 2005; Camargo *et al.* 2006; Ortiz, Mathias & Bueno 2012) and is similar to fungal planting behaviour that occurs on the garden substrate (Lopes *et al.* 2005). Prior to planting fungus for brood covering, an immature is thoroughly licked (Lopes *et al.* 2005), after which a worker – usually a small one (Camargo *et al.* 2006) – picks up a mycelial tuft from the fungus garden close to the pupa with its mandibles. It then antennates the pupa before placing the tuft on the pupal body in an area that is clear of mycelium and secures it into position by alternate patting movements with the front legs. One small adult ant per subcolony was watched constantly for 10 min and the number of mycelial tufts placed on the pupa during this time was recorded ($n = 7$ subcolonies). For 10 tufts, the number of securing pats with the front legs were also recorded ($n = 8$ subcolonies). Our behavioural observations were made after the aforementioned 9-h observation period. By this time, the pupae already had considerable cover; therefore, in order to standardize cover across colonies, we observed the behaviour towards white pupae that already had 70% cover.

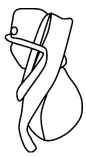
DOES MYCELIAL BROOD COVER PROVIDE PROTECTION FROM A GENERALIST PATHOGEN?

Exposure experiment 1: Does mycelial cover slow growth of Metarhizium brunneum?

Four small Petri dishes were prepared for each colony ($n = 11$ colonies; Appendix S1). Twelve young pupae with over 75% mycelial cover and four young pupae with no mycelial cover were removed from the fungus garden of each colony. The four pupae 'Without natural' mycelial cover ($WO^{Natural}$; Table 1) were randomly assigned to one of the four Petri dishes (Fig. S1). The twelve pupae with mycelial cover were randomly allocated to one of three degrees of mycelial cover: 'Without Removed' ($WO^{Removed}$) were manipulated so that the pupae had the mycelial cover on their gaster (hymenopteran 'abdomen') gently removed with a brush. Only the gaster was treated because it is exposed and hence easy to apply fungal conidia to it, and the appendages of the pupae can be easily damaged if manipulated. 'With Natural' ($W^{Natural}$) were unmanipulated so that mycelial cover remained all over the pupae, and 'With Sham' (W^{Sham}) were sham treated so that mycelial cover remained all over the pupae, but the mycelial cover on the gaster was touched gently with a brush as a control for the mechanistic part of the removal of mycelium in $WO^{Removed}$. One pupa from each category ($WO^{Natural}$, $WO^{Removed}$, $W^{Natural}$ and W^{Sham}) was then added to each Petri dish. For each colony, two Petri dishes were randomly assigned as controls and two were randomly assigned as treatments.

Infections were done under a stereomicroscope: dry *M. brunneum* spores were removed from a freshly sporulating agar plate and, using a sterile dissection needle, approximately 31 500 ± 2256 spores (±1 SE) were gently dispersed across an area (~1 × 1 mm) of the gaster – on a tergite near the junction with the sternite (Fig. S1). The control pupae were gently touched with a sterile dissection needle in the same place. Pupae were left without workers and were checked daily for fungal germination and fungal sporulation on the cuticle. After 1–3 days, dry *Metarhizium* spores germinate into white hyphae, and 3–4 days later the new conidia (or spores) start to grow progressively over the pupae. After 10 days, the percentage cover with *M. brunneum*

Table 1. Mycelial cover manipulation for each of the four treatment groups used in the *M. brunneum* exposure experiments

	Mycelial cover	Mycelia manipulated
	$WO^{Natural}$ No cover anywhere (natural)	No
	$WO^{Removed}$ Cover on gaster experimentally removed	Yes
	$W^{Natural}$ Natural cover everywhere	No
	W^{Sham} Natural cover everywhere, gaster sham treated	Yes

conidia was recorded for each pupa. We only analysed the treatment pupae because no control pupae showed evidence of *M. brunneum* conidia. Time until conidia appearance was analysed using the R statistical package (R Core Team 2014) version 2.13.0 using Cox models. Per cent spore cover 10 days after application was analysed using linear mixed-effects models (Appendix S1).

Exposure experiment 2: Does mycelial cover increase survival of pupae after Metarhizium brunneum *exposure?*

We tested whether mycelial cover affected hygienic behaviour, that is worker detection and removal of diseased brood after *M. brunneum* exposure. Sixteen colonies were used to determine the survivorship of *M. brunneum*-exposed pupae isolated temporarily from adult workers. Twenty-four small Petri dishes were prepared per colony, as detailed above. From each colony, we removed 48 white pupae that had over 75% mycelial cover. Half of these pupae were randomly allocated to the $WO^{Removed}$ treatment and half to W^{Sham} treatment (Fig. S2). Within $WO^{Removed}$ and W^{Sham}, half of the pupae were left as controls, and the other half had dry spores of *M. brunneum* applied to their gaster as detailed for exposure Experiment 1. We allocated the pupae to Petri dishes, such that six Petri dishes contained two $WO^{Removed}$ spore-exposed pupae, six contained two W^{Sham} unexposed pupae, six contained two $WO^{Removed}$ spore-exposed pupae and six contained two W^{Sham} unexposed pupae. One of each of these four treatment combinations was allocated to one of six time periods: 0, 12, 24, 36, 48 and 60 h. These times correspond to the hour after set-up at which a group of workers, including two large, two medium and eight small, were added to each Petri dish. Once the workers had been added we recorded every 12 h for 4 days whether the pupae remained in the fungus garden, or in the dump area where they would almost certainly die, which is an assay of hygienic behaviour (Tragust *et al.* 2013b). The removal of control pupae was rare (Appendix S1), so we only analysed the data for the removal of the *M. brunneum*-exposed pupae, using mixed-effects Cox models (Appendix S1).

Exposure experiment 3: Does mycelial cover affect metapleural gland grooming in the presence of Metarhizium brunneum?

Ten colonies were used to test whether mycelial cover affects the number of workers attending brood and their metapleural gland use when pupae had been exposed to *M. brunneum*. Twelve small Petri dishes were prepared for each colony, as detailed for the previous experiment. From each colony, we removed 36 white pupae that had over 95% mycelial cover. Half of the pupae were randomly allocated to the $WO^{Removed}$ treatment and the other half to the W^{Sham} treatment. We allocated the pupae to Petri dishes such that there were three pupae of the same treatment in each Petri dish (Fig. S3). Eighteen pupae (six Petri dishes) were infected with dry *M. brunneum* conidia (~30 000 conidia), and 18 pupae were controls. Ten workers from the same colony as the pupae, and of only one size class (large, medium or small), were added to each Petri dish and allowed to acclimatize for 5 min prior to behavioural observations (Fernández-Marín *et al.* 2009). Once every 10 min for 1 h, we recorded the number of ants attending the pupae, and for the entire hour, we recorded the number of times that pupae were contacted following metapleural gland grooming (Fernández-Marín *et al.* 2006; Fernández-Marín *et al.* 2009). The data were analysed in R using generalized linear models with quasibinomial errors. We tested (i) whether there was an effect of spore exposure, mycelial cover presence and worker size on the mean number of workers attending pupae per 10-min 'snapshot' and (ii) whether there was an effect of these factors on the total number of metapleural gland grooming events (Appendix S1).

Results

QUANTIFICATION OF MYCELIAL COVER AND BEHAVIOURAL OBSERVATIONS

Mycelial brood cover on larvae and pupae

Seventy per cent of the larvae had no cover, and the average cover across all laboratory colonies was less than 1% when removed from their mother colonies (Fig. 1a). However, we observed a monotonic increase in the amount of mycelial cover after removal from the mother colony (Overall effect of day: $\chi^2 = 28{\cdot}44$, df = 4, $P < 0{\cdot}0001$; for statistics see Appendix S2, Table S1), and in comparisons with day 0, all days had significantly more mycelial cover. However, after excluding Ae265 from the analysis (see Methods), and after sequential Bonferroni corrections, the day 0 vs. days 1 and 2 comparisons were no longer statistically significant (Appendix S2, Table S2). There were also significant differences between colonies in the degree of cover (day 0: $\chi^2 = 34{\cdot}52$, df = 9, $P < 0{\cdot}0001$; day 10: $\chi^2 = 56{\cdot}72$, df = 9, $P < 0{\cdot}0001$; Appendix S2, Table S3); some colonies had no cover on the larvae at day 0 whereas all colonies had a minimum of 50% pupal cover by the end of the experiment at day 10. Finally, there were significantly different variances in mycelial cover across colonies (day 0: $F_{9,90} = 12{\cdot}86$, $P < 0{\cdot}0001$; day 10: $F_{8,79} = 5{\cdot}24$, $P < 0{\cdot}0001$; Appendix S2, Table S4).

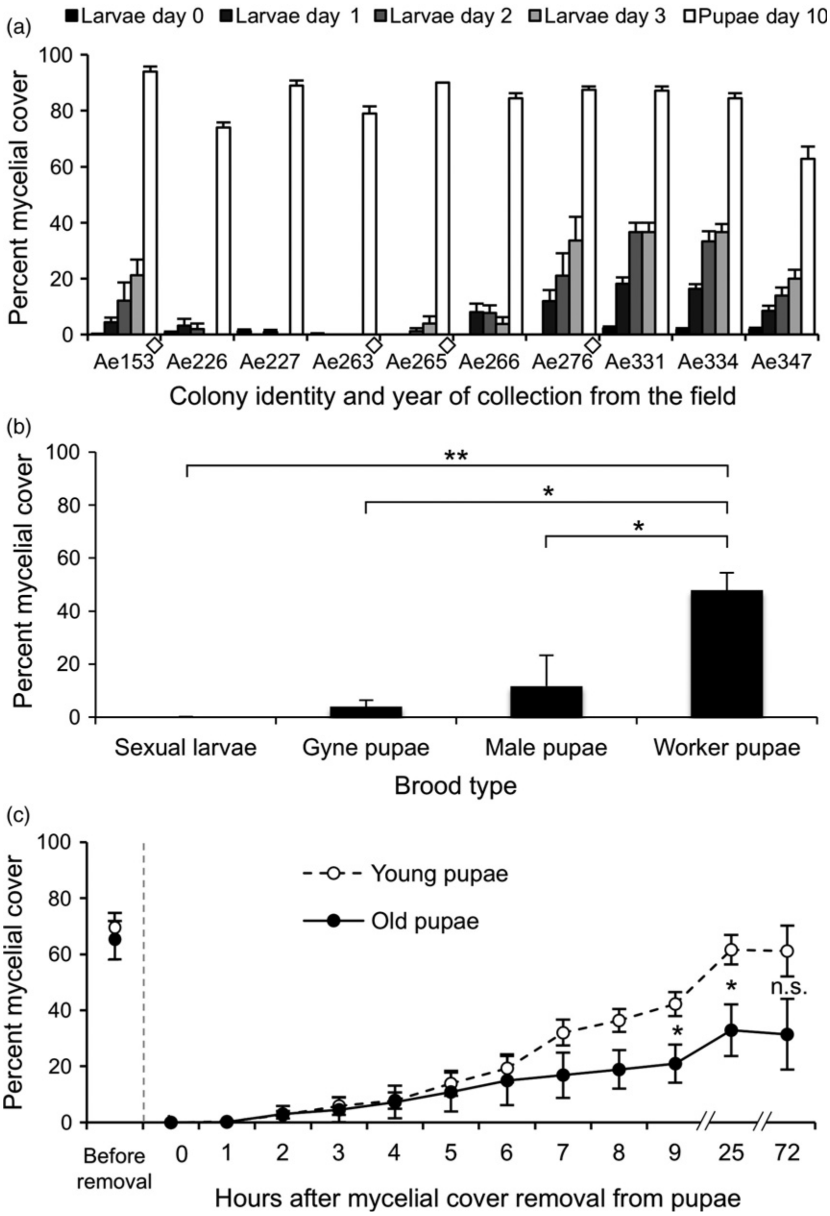


Fig. 1. Quantification of mycelial cover and behavioural observations. (a) Mycelial brood cover on larvae and pupae in response to disturbance. Mean (±1 SE) mycelial brood cover after removal from the mother colony. Ten larvae were observed from each colony, but as some pupated the means across days are derived from sequentially fewer observations of larvae. Diamonds next to colony names indicate where the mycelial cover on pupae in the mother colony was on average <25% at the time of larval collection. (b) Mycelial cover on sexual larvae, and gyne, male and worker pupae. The numbers of colonies examined for each caste were 5, except for male pupae where it was 4. Significant differences are given as: *$P < 0{\cdot}05$, **$P < 0{\cdot}01$. (c) The increase in mycelial brood cover over a 72-h post-removal period, for younger (white) and older (brown) pupae. Each point represents the mean for two pupae from nine colonies, except for old pupae after 25 h ($N = 17$ individuals from nine colonies) and after 72 h ($N = 11$ individuals from seven colonies), because some pupae had emerged into adults. *indicates significant differences ($P < 0{\cdot}05$) and n.s. indicates no significant difference. The means to the left of the dashed grey line show cover before removal of the mycelial cover at the start of the experiment.

Mycelical cover on sexual brood

There was a significant overall effect of brood caste on the degree of mycelial cover ($\chi^2 = 12{\cdot}61$, df = 3, $P = 0{\cdot}0056$; Fig. 1b; Appendix S2, Table S5). Worker pupae were covered with significantly more mycelium than sexual larvae (Z = 2·59, $P = 0{\cdot}0097$; Appendix S2, Table S6), female pupae (Z = 2·51, $P = 0{\cdot}0122$) and male pupae (Z = 2·09, $P = 0{\cdot}0365$).

Behavioural observations on workers and mycelial cover in relation to pupal age

There was no significant difference in mycelial cover between young and old pupae when they were removed from the maternal colonies (Z = 0·23, $P = 0{\cdot}822$; Fig. 1c; Appendix S2, Table S7). Just over sixty per cent of the pupae were moved to the fungus garden in the first hour of observations and for these pupae it took on average 17 min (±SE 16 min 51 s) to be placed in the fungus garden; the remaining pupae had all been moved within 2¾ h. There was an increase in cover of both young and old pupae from 0 to 72 h (Fig. 1c). Young pupae had significantly more cover compared to old pupae at both 9 and 25 h after experimental removal from their natural garden environment to Petri dishes (9 h: $t = 2{\cdot}64$, df = 16, $P = 0{\cdot}0178$; 25 h: $t = 2{\cdot}70$, df = 16, $P = 0{\cdot}0157$), but there was only a non-significant trend in this direction after 72 h (Z = −1·91, $n = 9$ and 7, $P = 0{\cdot}0564$). Covering of the brood with mycelium was carried out almost exclusively by small workers: tens of observations vs. only two observations of a medium worker performing this behaviour (large workers never covered brood). The mean number of tufts added to a young pupa in 10 min per worker was 23·9 (±4·9), so the average time per tuft was 29·3 s (±5·3). The mean number of pats with the front legs to secure one tuft was 20·9 (±1·8).

DOES MYCELIAL BROOD COVER PROVIDE PROTECTION FROM A GENERALIST PATHOGEN?

Exposure experiment 1: Does mycelial cover slow growth of Metarhizium brunneum?

Spores appeared significantly more quickly on pupae with their mycelial cover experimentally removed (WO$^{\text{Removed}}$) than on pupae with mycelial cover intact (comparison with W$^{\text{Natural}}$: $Z = -5{\cdot}23$, $P < 0{\cdot}0001$ and with W$^{\text{Sham}}$: $Z = -5{\cdot}12$, $P < 0{\cdot}0001$; Fig. 2a; Appendix S2, Table S8). However, there was no difference in the time to spore appearance between WO$^{\text{Natural}}$ and WO$^{\text{Removed}}$ ($Z = 0{\cdot}5$, $P = 0{\cdot}84$), implying that mycelial removal from the gaster is sufficient for eliciting a similar increase in pathogen efficiency as pupae that naturally lacked cover. Treatment significantly affected the *M. brunneum* spore cover ($F_{3,39} = 44{\cdot}28$, $P < 0{\cdot}0001$; Appendix S2, Table S9): WO$^{\text{Removed}}$ had significantly higher spore covers than W$^{\text{Natural}}$ and W$^{\text{Sham}}$, but significantly lower cover than WO$^{\text{Natural}}$ (Fig. 2b).

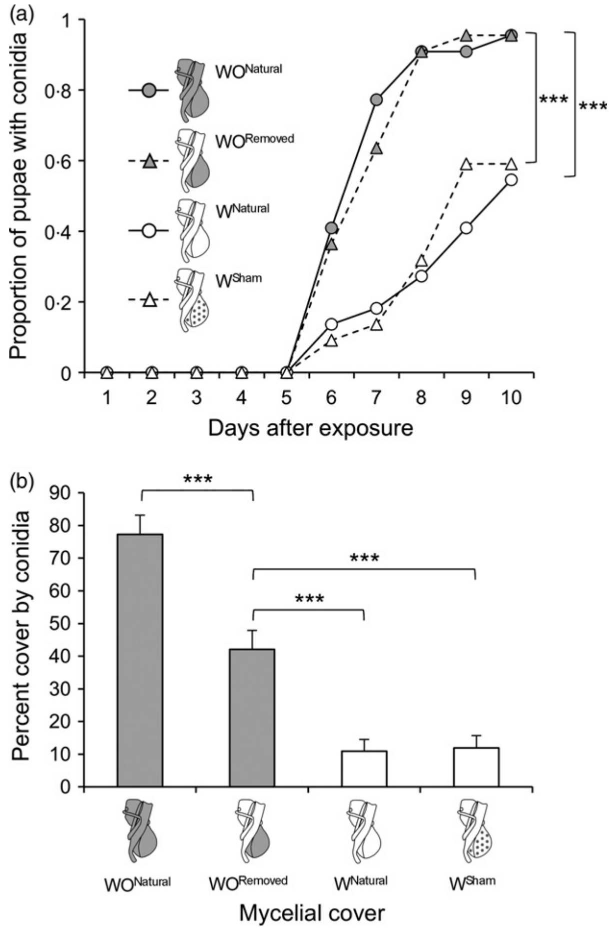


Fig. 2. First conidia exposure experiment: Pupal mycelial cover was left in the natural state (WO$^{\text{Natural}}$ or W$^{\text{Natural}}$), removed (WO$^{\text{Removed}}$) or sham treated (W$^{\text{Sham}}$) after which *M. brunneum* spores were applied and the time at which conidia appeared was recorded. (a) Cumulative proportion of pupae with visible *M. brunneum* conidia. A survival analysis was performed but the data are presented as the inverse for clarity. ***indicate significant differences ($P < 0{\cdot}001$) between WO$^{\text{Natural}}$ and W$^{\text{Natural}}$, and WO$^{\text{Natural}}$ and W$^{\text{Sham}}$. (b) Mean percentage cover with *M. brunneum* spores 10 days after infection for the four treatment groups. Figures are based on means from 11 colonies.

Exposure experiment 2: Does mycelial cover increase survival of pupae after Metarhizium brunneum *exposure?*

When workers were added 24 h after *M. brunneum* exposure, the removal of mycelial cover (WO$^{\text{Removed}}$) significantly increased the proportion of pupae that workers removed from the fungus garden and disposed of, compared to W$^{\text{Sham}}$ pupae ($Z = 2{\cdot}36$, $P = 0{\cdot}018$, Fig. 3a; Appendix S2, Table S10): the absence of mycelial cover doubled the percentage of pupae removed from the fungus garden 96 h after adding workers (WO$^{\text{Removed}}$: 25% in fungus garden, W$^{\text{Sham}}$ 56% in fungus garden; Fig. 3a, b). However, mycelial cover treatment had no significant

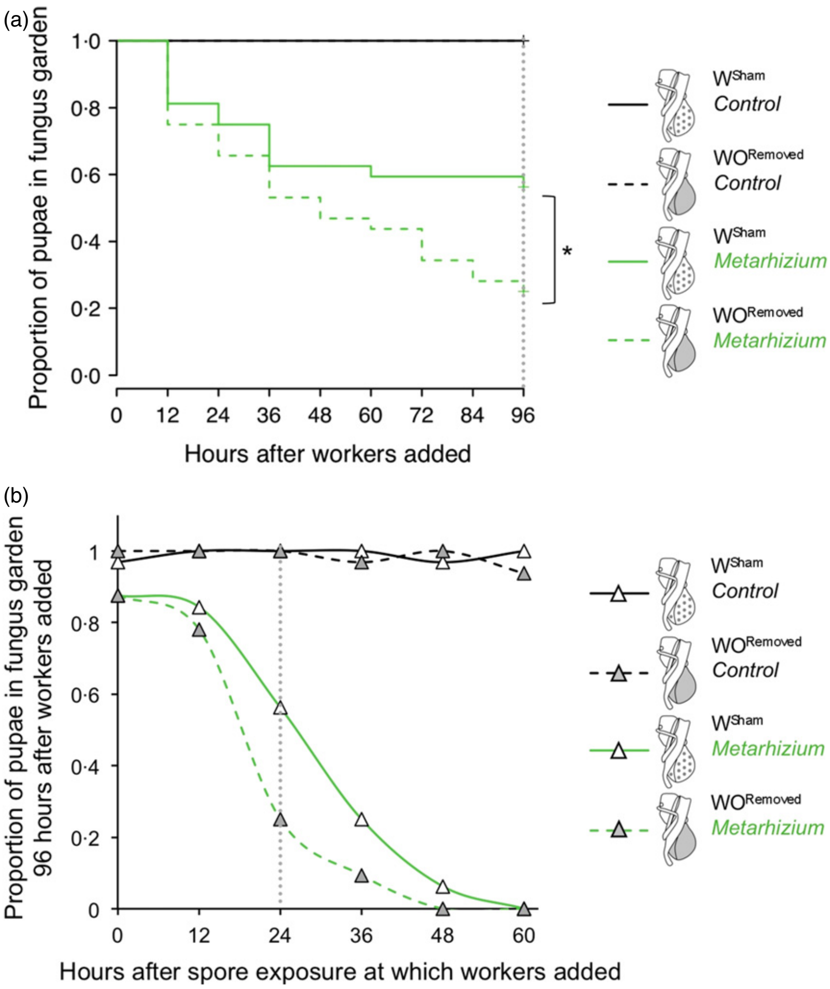


Fig. 3. Second conidia exposure experiment: (a) The proportion of pupae found in the fungus garden vs. the dump when workers were added 24 h (t = 24 h, see Fig. S2) after the pupae had been exposed to *M. brunneum* conidia and followed for 96 h. Each curve is from two pupae ($WO^{Natural}$ or W^{Sham}) from each of 16 colonies. None of the pupae from the two control treatments were removed to the dump; hence, both control treatment lines appear as one line. *indicates a significant difference between survival curves (P < 0·05). The dotted vertical line corresponds to figure (b) below. For figures of other times at which workers were added see Fig. S4. (b) Summary figure of the proportion of pupae found in the fungus garden versus the dump, including all time points at which the workers were added (i.e. t = 0, 12, 24, 36, 48 and 60 h), but the status at only 96 h after adding the workers, that is the last census point, is shown. The lines do not indicate survival curves, rather they aim to aid interpretation by joining data points of the same mycelial/exposure treatment. The dotted vertical line corresponds to the same time point after adding workers as shown in survival figure (a).

effect on the proportion of pupae found in the fungus garden for any of the other times (0, 12, 36, 48 and 60 h) at which workers were added (P > 0·081 in all cases, Appendix S2, Table S10; Figs 3b and S4), meaning that both $WO^{Removed}$ and W^{Sham} treatment pupae were removed from the fungus garden at the same rate.

Exposure experiment 3: Does mycelial cover affect metapleural gland grooming in the presence of Metarhizium brunneum?

Significantly more workers attended pupae exposed to *M. brunneum* than unexposed pupae ($F_{1,118}$ = 165·47, P < 0·0001; Fig. 4a; Appendix S2, Table S11), but there was no difference in the number of workers attending $WO^{Removed}$ compared to W^{Sham} pupae ($F_{1,117}$ = 1·774, P = 0·186). There was a significant difference in the number of attending ants relative to caste-specific body size: small workers attended more than medium workers, which attended more than large workers (Fig. 4a; Appendix S2, Table S11). We observed only one incident of MG grooming on a control pupa (n = 180), whereas exposed pupae (n = 180) received 262 MG grooming events over 60 min for all colonies, suggesting that this behaviour is related to the spore exposure. We therefore only tested MG use on pupae that had been exposed to *M. brunneum*. In the presence of *M. brunneum,* the MG was used more than twice as often when pupae had no mycelial cover compared to sham-treated pupae ($F_{1,58}$ = 4·97, P = 0·029; Fig. 4b; Appendix S2, Table S12), and there was a significant effect of worker size on the number of MG grooming events (Appendix S2, Table S12), with smaller workers grooming more frequently.

Discussion

Our behavioural observations showed that mycelial cover is a plastic trait: it varied across castes, time, life stages and across time within colonies. The fungal spore exposure experiments clearly indicate that a cover with cultivar mycelium provides some degree of protection against a fungal entomopathogen, so that the ants work less to sanitize their brood.

QUANTIFICATION OF MYCELIAL COVER AND BEHAVIOURAL OBSERVATIONS

Laboratory colonies had very little or no mycelial cover on the larvae, and at the time at which they were collected from their colonies, it was lower than what we

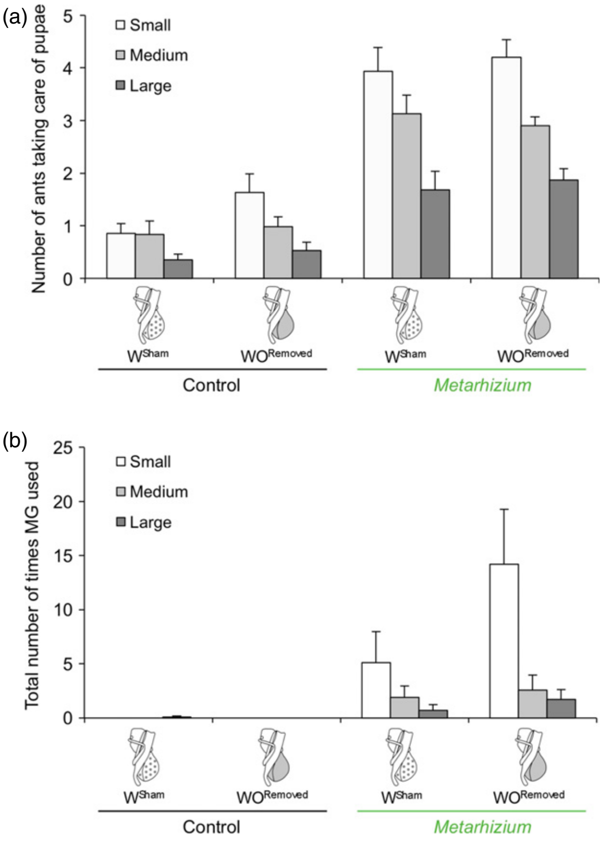


Fig. 4. Third conidia exposure experiment: Pupal mycelial cover was sham treated (W^{Sham}) or removed ($WO^{Removed}$), after which pupae were either not exposed (Control) or exposed to *M. brunneum* spores before 10 workers of a specific size class (indicated in the legend in the top left of the figures) were added and their metapleural gland grooming behaviours observed. (a) The mean number of workers of each size attending pupae per 10-min 'snapshot'. (b) The total number of times that secretion from the metapleural gland was used for disinfecting pupae in 60 min. Bars are means from 10 colonies (for statistics see text).

previously reported for *A. echinatior* (1% this study vs. 34% in Armitage *et al.* 2012). This might be explained by the fact that we used established laboratory colonies in this experiment, whereas Armitage *et al.* (2012) used recently collected (disturbed) field colonies, in which brood might have been recovered in direct response to the disturbance. The workers generally increased larval cover the longer that larvae were kept in artificial subcolonies: by day 3 after collection some colonies had more than 35% cover, comparable to Armitage *et al.* (2012). On average pupae in Petri dishes had >80% cover, irrespective of the fact that pupae examined from four of the colonies before the start of the experiment had less than 25% cover on their pupae, suggesting again that this could be a response to disturbance. In addition to the general increase in cover over the course of the experiment, there was significant variation among colonies, which might be due to overall behavioural differences or to differences in the vigour with which different symbiont strains grow on brood cuticle. Overall, these results indicate that the extent of mycelial cover is plastic over relatively short time spans and that mechanical disturbance and exposure to stressful conditions (such as being placed in Petri dishes) might be one of the cues that cause more complete mycelial cocoons, although further experimental tests are needed.

Pupal caste influenced the degree of mycelial cover in laboratory-kept colonies: both gyne and male pupae had significantly less cover than worker pupae. Sexual larvae also had less cover than worker pupae, but we did not test whether they differed from worker larvae because it is difficult to discriminate between worker and young (small) sexual larvae. Our results for pupae mirror the results of Camargo, Lopes & Forti (2006) from queen-right colonies, where workers tended to plant fungal hyphae on worker larvae more frequently than on male sexual larvae. Despite clear differences, such as the material from which the covering is made, the mycelial cover is similar to a pupal cocoon, creating partial to complete envelopes around the pupae (Armitage *et al.* 2012). In this respect, it is interesting to note that caste dimorphism in cocoon spinning has also been observed in *Neivamyrmex* and *Aenictus* army ants, but here worker pupae are naked (Brady & Ward 2005) and sexual pupae spin their own cocoons (Wheeler & Wheeler 1976); the functional significance of this behavioural difference is not known. We do not have data to address this point, but speculate that *A. echinatior* adult workers may attend sexual brood more frequently than worker brood and hence keep sexuals healthy using other defensive mechanisms.

Both young and old naked pupae were actively covered in mycelium by adult workers, and in some cases, this began within an hour after pupae were moved to Petri dishes. This shows that the response can be modulated relatively rapidly, supporting the findings of Lopes *et al.* (2005) who observed an increase in fungal planting behaviour 90–120 min after adding *Acromyrmex* workers to isolated pupae. Workers tended to cover older pupae to a lesser degree than younger pupae: if the mycelium indeed protects the pupae in some way, it may be that older pupae with darker harder cuticles are better protected and require less cover due to shorter times until emergence, implying that cues to cover the pupae with mycelia are age-linked, but this remains to be tested. The younger and older pupae had similar mycelial cover upon removal from the mother colony at the start of the experiments, but it might be possible that the mycelia establish themselves less readily on older pupae. Similar to Camargo *et al.*'s (2006) observations on *A. subterraneus brunneus*, the small workers predominantly engaged in brood covering behaviour. The number of small tufts of mycelia to be added to each brood item will depend upon the size of the item. Considering that mature *A. echinatior* colonies contain approximately 40 000 workers (Baer *et al.* 2009), the numbers of brood to be taken care of by colonies for fungal brood covering represent an intense collective behaviour of small workers and thus a significant social cost. Our behavioural observations hint that it might be worthwhile to explore the role of disturbance in mycelial cover. There are many outstanding questions remaining about this intriguing behaviour – Is the mycelia growing on the brood? Does it need nutrients for growth, and if so are the nutrients coming from secretions from the cuticle, or is the saliva a fungal growth promoter that is applied to the brood whilst grooming and before the mycelia are added (Weber 1972; Lopes *et al.* 2005)?

DOES THE MYCELIAL BROOD COVER PROVIDE PROTECTION FROM A FUNGAL PATHOGEN?

In the absence of adult workers, the mycelial cover on pupae slowed, but did not stop, the growth of the entomopathogen. For example, by 6 days after exposure, around 40% of $WO^{Removed}$ and $WO^{Natural}$ pupae showed *M. brunneum* spores, whereas it took 8–9 days for $W^{Natural}$ and W^{Sham} to reach this percentage. We also found that a lower proportion of the body of $W^{Natural}$ and W^{Sham} had spore cover compared to $WO^{Natural}$. Interestingly, the spore cover on the body of the $WO^{Removed}$ group was lower than the $WO^{Natural}$ group. This is consistent with the manipulation that we applied (i.e. removing mycelial covers only from the gaster), because the cover on the rest of the pupae might have still afforded some protection from entomopathogenic fungal growth, especially if the protection is chemical in nature. If we mark the start of the fungal pathogen life history once it has infected a host (Schmid-Hempel 2011), then the stage at which the fungal pathogen becomes transmissible will be delayed when the pupae are covered in mycelia. The cover might therefore affect the rate of parasite transmission, one of the four main factors governing the dynamics of the spread of disease from infected to susceptible hosts (Anderson & May 1979).

Our second exposure experiment tested whether mycelial cover affected hygienic behaviour after *M. brunneum* spore exposure, that is, where workers detect and remove diseased brood (Arathi, Burns & Spivak 2000; Wilson-Rich *et al.* 2009). By exposing pupae to *M. brunneum* spores and adding adult workers at different times after exposure, we found that there was a critical window within which pupae were either kept in the fungus garden or taken to the dump area and that the addition of workers 24 h after exposure is potentially late enough for them to detect a difference due to *M. brunneum* treatment yet early enough to make a difference in rescuing some of the pupae. When the workers were added shortly after *M. brunneum* exposure (0 or 12 h), the pupae had relatively low overall probabilities of being removed to the dump; if workers were added after 36 h or later then exposed pupae had a 75% or higher chance of being discarded overall, indicating that *M. brunneum* is particularly virulent at this stage, and that the presence or absence of mycelial cover made little difference. One possible explanation for the critical time window is that *M. anisopliae* conidia require 12–24 h to adhere to and germinate on a host cuticle (Vestergaard *et al.* 1999; Arruda *et al.* 2005), consistent with observations that fungal spores did not penetrate the cuticle within the first 24 h after spore exposure in larvae of the ant *Cardiocondyla obscurior* (Ugelvig *et al.* 2010). It seems likely that workers added before 24 h would have been able to groom some of the spores off the pupae. These findings suggest that there is a window of time in which infections can be brought under control, potentially by grooming (e.g. Ugelvig *et al.* 2010) before spores have had a chance to germinate and that infections may be incurable after 24 h unless slowed by mycelial cover. Tragust *et al.* (2013b) tested brood removal behaviour in response to *M. brunneum* exposure for non-attine ant pupae, either with or without cocoons, and found that naked pupae were discarded more frequently. As described above, although there are clear differences between a silken cocoon and mycelial pupal covering, they potentially both offer a barrier against microbes circulating in the local environment. Interestingly, and by analogy, adult *Acromyrmex subterraneus subterraneus* workers derive survival benefits after infection by *Metarhizium anisopliae* from another of their symbiotic partners – the *Pseudonocardia* bacteria (Mattoso, Moreira & Samuels 2012).

Metapleural glands are unique to ants (Hölldobler & Wilson 1990; Yek & Mueller 2011). In Attini, they secrete antimicrobial compounds that are deployed to combat disease agents such as fungal conidia (e.g. Fernández-Marín *et al.* 2006, 2009), employing specific chemical compounds that inhibit the growth of fungal pathogens (Fernández-Marín *et al.* 2015). The metapleural gland extract from a sister species to *A. echinatior*, *A. octospinosus*, has been shown to have powerful sanitary action against fungi, including *M. brunneum* (Yek *et al.* 2012). We hypothesized that the mycelial cover provides additional protection against *M. brunneum* and tested whether the presence of mycelial cover affects the number of ants attending the pupae and MG grooming rates. As expected, *M. brunneum* spores increased the number of workers attending the pupae, but $WO^{Removed}$ and W^{Sham} pupae were attended to a similar degree. In ants, and specifically in *Acromyrmex*, MG grooming was earlier shown to be almost exclusively used on fungal entomopathogen-exposed brood, but without taking into account brood mycelial cover (e.g. Fernández-Marín *et al.* 2006). In our study, MG grooming was used significantly more frequently on *M. brunneum*-exposed $WO^{Removed}$ pupae than on W^{Sham} pupae. The workers therefore appeared to modulate their MG behaviour at this time after exposure, not only in response to a fungal pathogen, but also to whether an alternative defence is present. As with the mycelial covering behaviour, small workers were largely responsible for MG grooming on the pupae.

Two other insect clades have evolved agricultural mycophagy: some beetle species (Farrell *et al.* 2001) and macrotermitine termites (Aanen & Eggleton 2005). Unlike ants, where the eggs, larvae and pupae are immobile, only the eggs and pupae of beetles and the eggs of termites (hemimetabolous insects without a pupal stage) are immobile. To the best of our knowledge, there are no reported cases where farmed fungi from these beetle or termite species are placed or grow on the cuticle of immature or adult individuals, but two antibiotics have been isolated from a fungal strain associated with the ambrosia beetle *Xyleborus validus* (Nakashima *et al*. 1982) and numerous plants make use of symbiotic fungi such as endophytes for defensive purposes, including making leaves tougher through increased lignification and chemical defences (Van Bael, Estrada & Wcislo 2011). From a broader perspective, it is interesting to note that female European beewolves, *Philanthus triangulum*, apply *Streptomyces* bacteria from their antennal glands to the brood cell before they oviposit (Kaltenpoth *et al*. 2005). Larval beewolves subsequently incorporate the bacteria into their pupal cocoons, which provide protection against fungal infestation (Kaltenpoth *et al*. 2005). The protection comes from nine cocoon-derived antibiotic substances produced by the bacteria that have activity against different entomopathogenic fungi and bacteria (Kroiss *et al*. 2010). Our results suggest that the fungal symbiont is used in more than one way by *A. echinatior*, that is, as a food source and brood cover. There is also evidence from other organisms that symbionts may play more than one role with their partner. For example, the community of symbionts in lower termite guts break down dietary lignocellulose and are important for nitrogen fixation (reviewed in Brune 2014), and in *Zootermopsis angusticollis,* the hindgut protozoa, and/or their associated bacteria, produce β-1,3-glucanases that break down ingested fungal cell walls and are proposed to help protect the termites against fungal pathogens (Rosengaus *et al*. 2014). Furthermore, entomopathogenic nematodes harbour symbiotic bacteria, which can assist the nematode in killing its insect host (reviewed in Forst & Nealson 1996) and subsequently protect the cadaver from microbial competitors via the production of defensive chemicals (Flórez *et al*. 2015).

In conclusion, the functionality of mycelial brood covering in fungus-growing ants appears to be a rewarding target for further study of the multilayered intricacies of disease defence in complex symbioses. Our results clearly suggest that mycelial brood covering behaviour slows down the spread of disease, either as a physical defence that prevents or slows pathogenic fungal spore germination, or the result of fungistatic properties of the cultivar or one of the other microbes associated with the ant-fungus mutualism. The cover delays death from a generalist insect pathogen at a critical point during infection and disease progression, so the cover buys the ants time to respond by making their social immune defences more secure. Therefore, in addition to the more familiar personal immunity (Siva-Jothy, Moret & Rolff 2005; Cotter & Kilner 2010), the fungus-growing ants have co-opted their crop fungus to provide a novel social level of immunity (Cremer, Armitage & Schmid-Hempel 2007; Cotter & Kilner 2010; Meunier 2015), that is symbiont-derived defence for their brood.

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Data accessibility

Data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.6rn29 (Armitage *et al*. 2016).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Experimental design for first conidia exposure experiment.

Fig. S2. Experimental design for second conidia exposure experiment.

Fig. S3. Experimental design for third conidia exposure experiment.

Fig. S4. Survival curves for all time points for second conidia exposure experiment.

Appendix S1. Supplementary materials and methods.

Appendix S2. Detailed statistical results.