Title: Destructive disinfection of infected brood prevents systemic disease spread in ant colonies

Short title: Colony-level disease protection in ants

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Abstract

Social insects protect their colonies from infectious disease through collective defences that result in social immunity. In ants, workers first try to prevent infection of colony members. Here, we show that if this fails and a pathogen establishes an infection, ants employ an efficient multicomponent behaviour – "destructive disinfection" – to prevent further spread of disease through the colony. Ants specifically target infected pupae during the pathogen's non-contagious incubation period, relying on chemical 'sickness cues' emitted by pupae. They then remove the pupal cocoon, perforate its cuticle and administer antimicrobial poison, which enters the body and prevents pathogen replication from the inside out. Like the immune system of a body that specifically targets and eliminates infected cells, this social immunity measure sacrifices infected brood to stop the pathogen completing its lifecycle, thus protecting the rest of the colony. Hence, the same principles of disease defence apply at different levels of biological organisation.

Introduction

Pathogen replication and transmission from infectious to susceptible hosts is key to the success of contagious diseases [1]. Social animals are therefore expected to experience a greater risk of disease outbreaks than solitary species, because their higher number of within-group interactions will promote pathogen spread [2–4]. As a consequence, traits that mitigate this cost should have been selected for in group-living animals as an essential adaptation to social life [5,6].

Eusocial insects (termites, ants and the social bees and wasps) live in complex societies that are ecologically successful and diverse. They are typically single-family

colonies comprising one or a few reproducing queens and many sterile workers. Both of these castes are highly interdependent: the queens are morphologically specialised for reproduction and cannot survive without the assistance of the workers; conversely, the workers cannot reproduce, but gain inclusive fitness by raising the queen's offspring [7]. Consequently, social insects societies have become single reproductive units, where natural selection acts on the colony instead of its individual members [8,9]. This has parallels to the evolution of complex multicellular organisms, where sterile somatic tissue and germ line cells form a single reproducing body. Hence, social insect colonies are often termed "superorganisms" and their emergence is considered a major evolutionary transition [8–11]. Since evolution favours the survival of the colony over its members, selection has resulted in a plethora of cooperative and altruistic traits that workers perform to protect the colony from harm [5,8,12,13]. In particular, social insects have evolved physiological and behavioural adaptations that limit the colony-level impact of infectious diseases, which could otherwise spread easily due to the intimate social interactions between colony members [12,14–16]. These defences are performed collectively by the workers and form a layer of protection known as "social immunity" that, like the immune system of a body, protects the colony from invading pathogens [12,17].

Our understanding of how social immunity functions is based mostly on the first line of defence that reduces the probability of pathogen exposure and infection. It is well known for example that social insects avoid pathogens, like fungal spores, in their environment, and perform sanitary care when nestmates come into contact with them [18–22]. In ants, sanitary care involves grooming and the use of antimicrobial secretions to mechanically remove and chemically disinfect the pathogen, reducing

the likelihood that pathogen exposure leads to the development of an infection [21,22]. However, what happens when sanitary care fails and a pathogen successfully infects an ant, with the consequent potential to create an epidemic, remains poorly understood. In a body, infected cells are eliminated by the immune system to prevent the proliferation and systemic spread of pathogens through the tissue. Since infected ants become highly contagious to their nestmates [23,24], we hypothesised that they should have evolved an analogous mechanism to detect and contain lethal infections in individuals as early as possible, to prevent disease outbreaks in the colony.

To test this hypothesis, we exposed pupae of the invasive garden ant, *Lasius neglectus*, to a generalist fungal pathogen, *Metarhizium brunneum*. When the infectious conidiospores of this fungus come into contact with insect cuticle, they attach, germinate and penetrate the host cuticle within 48 h to cause internal infections. After a short, non-infectious incubation period of a few days, a successful fungal infection then induces host death, after which the fungus replicates and releases millions of new infectious conidiospores in a process called sporulation [23,25]. Previous work found that brood infected with *Metarhizium* is removed from the brood chamber, however, it is unknown how the ants then respond to the infection [26,27]. Here we demonstrate that ants detect infected pupae during the pathogen's non-infectious incubation period and react by performing a multicomponent behaviour. To investigate this response we used a series of behavioural and chemical experiments to determine its function and underlying mechanisms. Finally, we tested the impact of the multicomponent behaviour on the pathogen's ability to complete its lifecycle and cause a systemic colony infection.

Results

Destructive disinfection of lethally infected pupae

We exposed ant pupae to one of either three dosages of *Metarhizium* conidiospores or a sham control. We observed that ants tending pathogen-exposed pupae prematurely removed the pupae from their cocoons in a behaviour we termed "unpacking", whereas control pupae were left cocooned (Fig 1A-B, Video S1; Cox proportional hazards regression: likelihood ratio test (LR) $\chi^2 = 55.48$, df = 3, $P = 0.001$; post hoc comparisons: control vs. low, $P = 0.004$; low vs. medium, $P = 0.006$; medium vs. high = 0.024; all others, $P = 0.001$). Unpacking occurred between 2-10 d after pathogen exposure, but sooner and more frequently at higher conidiospore dosages (Fig 1B). As unpacking was a belated response to pathogen exposure and we were unable to remove any conidiospores from the cocoon or the unpacked pupae (Fig S1), we concluded that the ants were not performing unpacking to simply dispose of the contaminated cocoons. Instead, we postulated that unpacking was a response to successful infection. At the time of unpacking, the majority of pupae were still alive (Fig S2) and fungal outgrowth had not yet occurred (Fig 1F). Hence, to test if the ants were reacting to early-stage infections, we removed both unpacked and non-unpacked pathogen-exposed cocooned pupae from the ants and incubated them under optimal conditions for fungal outgrowth. We found that, on average across the conidiospore dosages, 90% of unpacked pupae harboured infections that sporulated in the absence of the ants. In contrast, only 25% of non-unpacked pupae were infected (generalised linear model [GLM]: overall LR $\chi^2 = 21.52$, df = 3, $P = 0.001$; cocooned vs. unpacked pupae: LR $\chi^2 = 18.5$, df = 1, $P = 0.001$; conidiospore dose: LR $\chi^2 = 0.42$, df = 2, $P = 0.81$). We therefore concluded that the ants were detecting and unpacking pupae with lethal infections during the asymptomatic incubation period of the pathogen's

lifecycle. At this time point the fungus is non-infectious and so there is no risk of the ants contracting the disease.

Next, we filmed ants presented with pathogen-exposed pupae and compared their behaviour before and after unpacking. Prior to unpacking, we observed the typical sanitary care behaviours reported in previous studies [20,22,23,28]. Namely, the ants groomed the pupae (Fig 1C), which has the dual function of removing the conidiospores and applying the ants' antimicrobial poison [22]. In *L. neglectus*, the poison is mostly formic acid and is emitted from the acidopore at the abdominal tip, where the ants actively suck it up and transiently store it in their mouths until application during grooming. Additionally, the ants can spray their poison directly from the acidopore; yet, this behaviour is rarely expressed during sanitary care (about once every 28 h; Fig 1D) [22]. However, after unpacking, we observed a set of behaviours markedly different to sanitary care (Fig 1A, Video S1). The ants sprayed the pupae with poison from their acidopore approx. 15-times more frequently than during sanitary care (~ 13-times/d; Fig 1D; generalised linear mixed model [GLMM]: LR χ^2 = 17.04, df = 1, P = 0.001), and increased grooming by 50% (Fig 1C; linear mixed effects regression [LMER]: LR χ^2 = 145.26, df = 1, P = 0.001). Given that there was no fungus to remove at the time of unpacking, the increase in grooming probably functioned solely to apply poison from the oral store [22]. Furthermore, the ants repeatedly bit the pupae to make perforations in their cuticles (Fig 1E; GLMM: LR χ^2 = 39.44, df = 1, P = 0.001). Together these three behaviours resulted in the death of the pupae and left their corpses heavily damaged and coated in the ants' poison (Fig 1G, Fig S2, Fig S3). Accordingly, we named the combination of

unpacking, grooming, poison spraying and biting "destructive disinfection", and performed a series of experiments to determine its function.

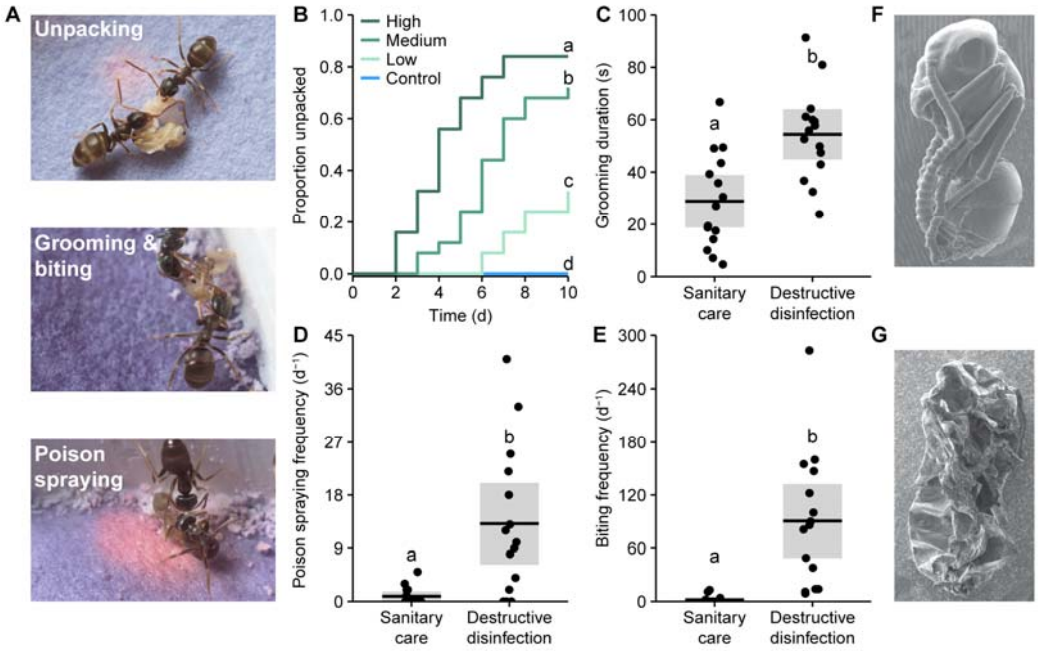


Fig 1. Ants perform destructive disinfection in response to lethal fungal infections of pupae.

(A) Destructive disinfection starts with the unpacking of pupae from their cocoons and is followed by grooming, poison spraying and biting (ants housed on blue pH-sensitive paper to visualise acidic poison spraying, which shows up pink). (B) Unpacking occurred significantly more in pupae exposed to fungal conidiospores and was dose-dependent, occurring sooner and in higher amounts as the dose of conidiospores increased (letters denote groups that differ significantly in Tukey post hoc comparisons [$P < 0.05$]). (C-E) Comparison of the ants' behaviour between sanitary care and destructive disinfection. Destructive disinfection is characterised by increases in grooming duration, poison spraying frequency and biting frequency (all data points displayed; lines ± shaded boxes show mean ± 95% confidence intervals [CI]; letters denote groups that differ significantly in logistic regressions [$P < 0.05$]).

(F) Scanning electron micrographs (SEM) of an asymptomatic pupa immediately after unpacking, and (G) of a destructively disinfected pupa 24 h later.

Chemical detection of internal infections

Firstly, we wanted to know how the ants identify internal infections during the pathogen's non-contagious incubation period, when pupae were still alive and showed no external signs of disease. As ants use chemical compounds on their cuticles to communicate complex physiological information to nestmates [29], we speculated that infected pupae may produce olfactory sickness cues. We washed infected pupae in pentane solvent to reduce the abundance of their cuticular hydrocarbons (CHCs). When pentane-washed pupae were presented to ants, there was a 72% reduction in unpacking compared to both non- and water-washed infected pupae (Fig 2A; GLM: LR $\chi^2 = 12.2$, df = 2, $P = 0.002$; Tukey post hoc comparisons: water-washed vs. non-washed, $P = 0.79$; all others, $P = 0.009$). As pentane-washed pupae had lower abundances of CHCs (Fig S4), this result indicates that the ants use one or more cuticle compounds to detect the infections.

Gas chromatography-mass spectrometry (GC–MS) analysis of the solvent wash confirmed that unpacked pupae have distinct chemical profiles compared to non-infected control pupae, whilst cocooned (non-unpacked) pathogen-exposed pupae were intermediate (Fig 3B, Fig S5; perMANOVA: $F = 1.49$, df = 46, $P = 0.002$; post hoc perMANOVA comparisons: unpacked vs. control, $P = 0.003$; unpacked vs. cocooned, $P = 0.79$; cocooned vs. control, $P = 0.08$). Most chemical messages in social insects are encoded by quantitative shifts of several compounds [29]. Correspondingly, we found that 8 out of the 24 CHCs identified (Table S1) had higher

relative abundances on unpacked pupae compared to control pupae (Fig 3C-F, Fig S5; all Kruskal-Wallis [KW] test statistics and post hoc comparisons in Table S2). Moreover, four of these CHCs were also present in relatively higher quantities on unpacked pupae compared to the non-unpacked cocooned pupae. Hence, several specific CHCs probably accumulate on infected pupae over time, eventually reaching an amount that, relative to the other compounds, is sufficient to elicit destructive disinfection. This corresponds to current models of social insect behaviour, where the likelihood of a response depends on stimuli exceeding a certain threshold [30,31]. Interestingly, the four CHCs specifically increased on unpacked pupae were all long-chained CHCs (carbon chain length C_{33-35}) with a low volatility, meaning that the ants have to be close to or touching the pupae to detect them [32]. As ants keep pupae in large piles, using low-volatility CHCs may be important so that the ants accurately identify the sick pupae and do not mistakenly destroy healthy ones.

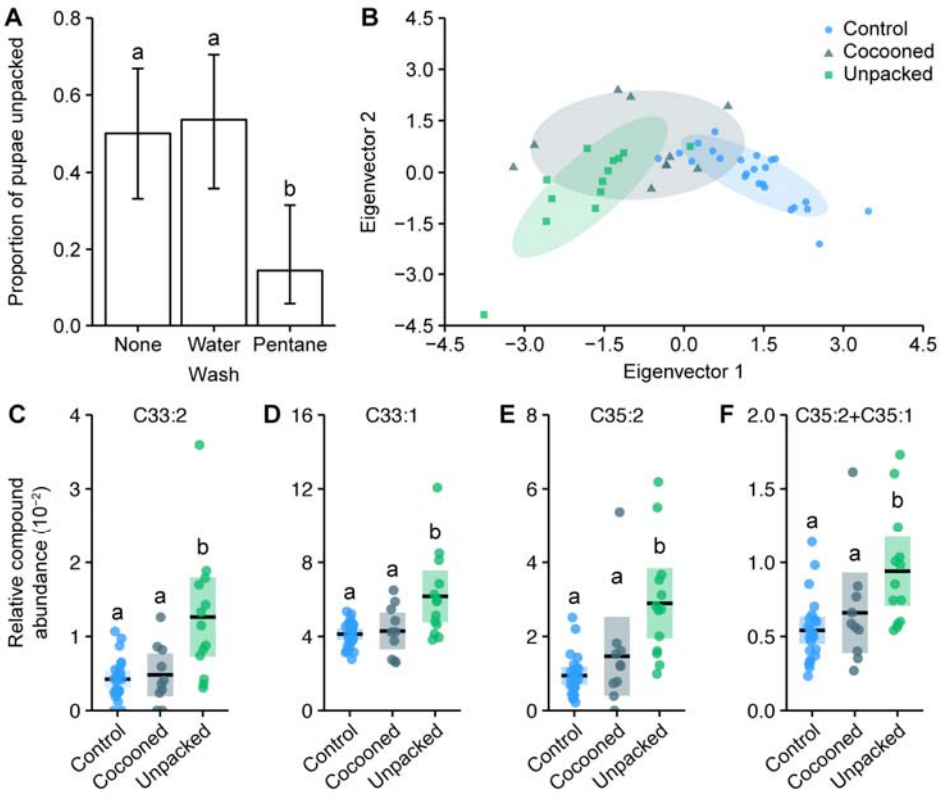


Fig 2. Destructive disinfection is induced by changes in the chemical profile of infected pupae.

(A) Pupae washed in pentane solvent to reduce the abundance of their cuticular hydrocarbons (CHCs) were unpacked less than unwashed or water-washed pupae (positive and handling controls, respectively; error bars show ± 95% CI; letters specify significant Tukey post hoc comparisons [$P < 0.05$]). (B) Unpacked pathogen-exposed pupae have distinct chemical profiles compared to sham-treated control pupae. Pathogen-exposed pupae that were not unpacked (cocooned group) have intermediate profiles (axes show discriminant analysis of principle components eigenvectors). (C-F) The four CHCs with higher relative abundances on unpacked pupae compared to both control and cocooned pupae, (C) Tritriacontadiene, C33:2 (D), Tritriacontene, C33:1 (E), Pentatriacontadiene, C35:2 (F) co-eluting Pentatriacontadiene and Pentatriacontene, C35:2+C35:1 (all data points displayed; line ± shaded box show mean ± 95% CI; letters specify groups that differ significantly in KW test post hoc comparisons [$P < 0.05$]).

Destructive disinfection prevents pathogen replication

We next tested if destructive disinfection prevents pupal infections from replicating and becoming infectious. Pathogen-exposed pupae were kept with groups of ants (8 ants per pupae per group) until unpacking. They were then left with the ants for a further 1 or 5 d before being removed and incubated for fungal growth. We compared the number that subsequently sporulated to pathogen-exposed pupae kept without ants. Whilst 90% of pupae contract infections, destructive disinfection significantly reduced the proportion of pupae that sporulated and hence became infectious (Fig 3A; GLM: LR $\chi^2 = 40.47$, df = 2, $P = 0.001$; Tukey post hoc comparisons: 1 vs. 5 d, $P =$

229 0.04; all others, $P = 0.001$). After only 1 d, the number of destructively disinfected
230 pupae that sporulated decreased by 65%. With more time, the ants could reduce the
231 number of pupae sporulating even further by 95%. Since the pupae were removed
232 from the ants for fungal incubation, we can conclude that destructive disinfection
233 permanently prevents pathogen replication. We repeated this experiment with a
234 smaller number of ants (3 ants per pupae per group) to investigate how group size
235 influences the success of destructive disinfection. Smaller groups of ants were less
236 efficient than larger ones: although they could still inhibit > 90% of pupal infections
237 within 5 d of unpacking, pupae tested for infection after 1 d still sporulated 70% of
238 the time (Fig S6; GLM: LR $\chi^2 = 35.23$, $P = 0.001$; Tukey post hoc comparisons: 0 vs.
239 1 d, $P = 0.2$; 0 vs. 5 d, $P = 0.001$; 1 vs. 5 d, $P = 0.002$). As the effectiveness of
240 destructive disinfection increased with the amount of time the ants had, as well as
241 with the number of ants present, we inferred that there must be a limiting factor
242 affecting the inhibition the pathogen.
243
244 To study the underlying mechanisms of destructive disinfection, we performed its
245 different components – unpacking, biting and poison spraying – *in vitro* to test for
246 their relative importance and potential synergistic effects. We simulated unpacking by
247 removing the cocoons of the pupae manually, and the cuticle damage caused by biting
248 using forceps. Previous work establishing the composition of *L. neglectus* poison [22]
249 allowed us to create a synthetic version for use in this experiment (60% formic acid
250 and 2% acetic acid, in water; applied at a dose equivalent to what ants apply during
251 destructive disinfection; Fig S8), with water as a sham control. We then performed
252 these 'behaviours' in different combinations in a full-factorial experiment. We found
253 that all three behaviours must be performed in the correct order and interact to prevent

pathogen replication (overview graph showing odds ratios of sporulation in Fig 3B, full data dataset displayed in Fig S7; GLM: overall LR χ^2 = 79.9, df = 5, P = 0.001; interaction between behaviours LR χ^2 = 20.6, df = 2, P = 0.001; all post hoc comparisons in Table S3). As in sanitary care, the poison was the active antimicrobial compound that inhibited fungal growth (Fig S7, Table S3 [21,22]). However, for the poison to function the pupae had to be removed from their cocoons and their cuticles damaged. Firstly, this is because the cocoon itself is hydrophobic and thus prevents the aqueous poison from reaching the pupae inside (Fig S9). Secondly, as the infection is growing internally at the time of unpacking, the cuticle must be broken in order for the poison to enter the hemocoel of the pupae. This is achieved with the perforations created by the ants biting the pupal cuticle. As the active antimicrobial component, we concluded that the poison is probably the limiting factor determining whether destructive disinfection is successful. Because the poison has a slow biosynthesis and each ant can only store a limited amount [22,33], it would explain why destructive disinfection was more likely to be successful the longer the ants had to treat the pupae, and as the number of ants increased (Fig 3A, Fig S6). By sharing the task of poison synthesis and application, the ants probably increase their chances of preventing the pathogen becoming infectious.

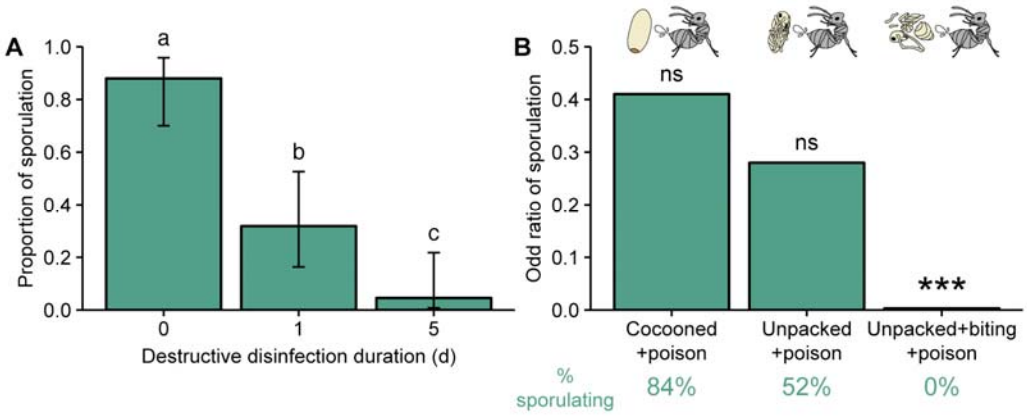


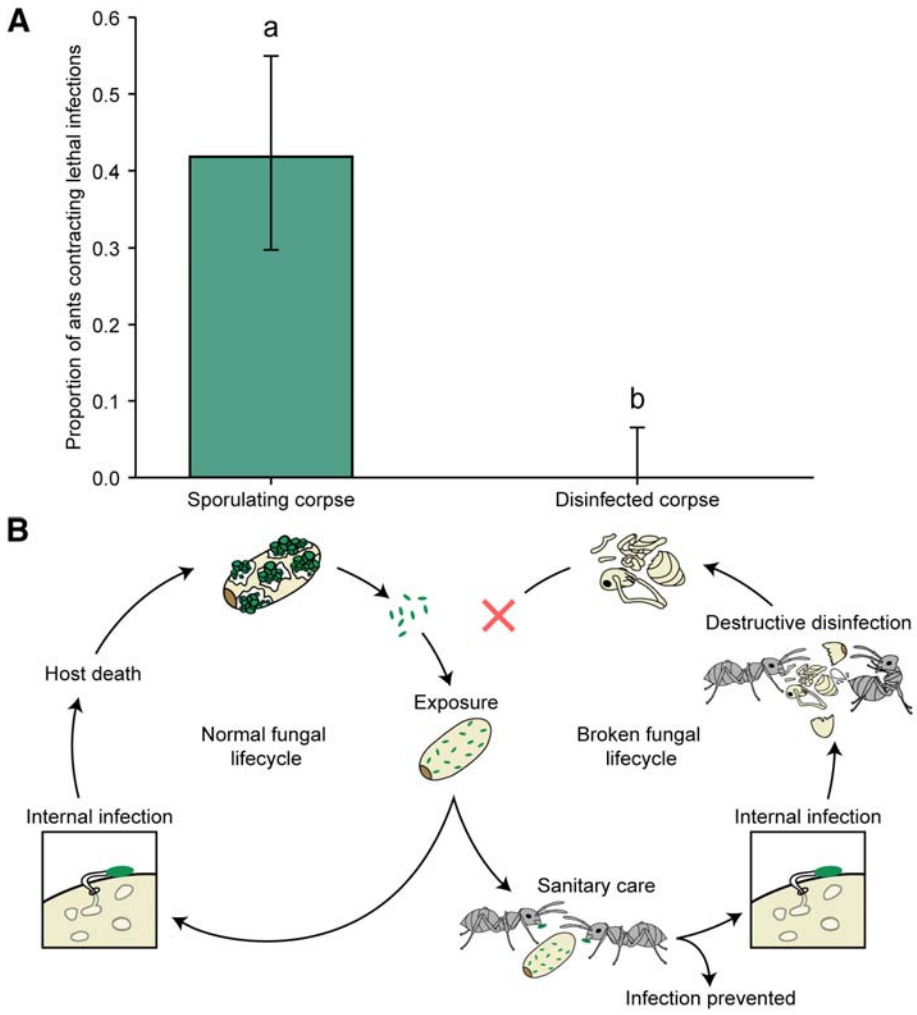
Fig 3. Destructive disinfection by ants prevents pathogen replication.

(A) Destructive disinfection greatly reduced the probability of pupae sporulating compared to pupae that received no destructive disinfection (time point 0), and its effectiveness increased with the length of time ants could perform destructive disinfection (1 vs. 5 d; error bars show ± 95% CI; letters denote groups that differ significantly in Tukey post hoc comparisons [$P < 0.05$]). (B) The individual components of destructive disinfection (unpacking, biting and poison spraying) interacted to inhibit pathogen replication (% of pupae sporulating in each treatment shown under graph in green). The odds of sporulation for cocooned and unpacked pupae treated with poison were not significantly different to those of control pupae (cocooned pupae treated with water). But when unpacking, biting and poison spraying were combined the odds of sporulation were significantly reduced (logistic regression; ns = non-significant deviation from control, *** = $P < 0.001$; complete data set of full factorial experiment displayed in Fig S7 and all statistics in Table S3).

Disruption of the pathogen lifecycle stops disease transmission

Finally, we investigated the impact of destructive disinfection on disease transmission within a social host group. We created mini-nests comprising two chambers and a group of ants (5 ants per group). Into one of the chambers we placed an infectious sporulating pupa – simulating a failure of the ants to detect and destroy the infection – or a pupa that had been destructively disinfected, and was thus non-infectious. The ants groomed, moved around and sprayed both types of corpses with poison. In the case of the sporulating pupae, all conidiospores were removed from the corpse by the ants. As in previous studies, sporulating corpses were highly virulent [23,24] and caused lethal infections that became contagious after host death in 42% of ants (Fig 4A). However, there was no disease transmission from destructively disinfected pupae

299 (Fig 4A; GLM: LR χ^2 = 31.32, df = 1, P = 0.001). We therefore concluded that by
300 preventing the pathogen from completing its lifecycle destructive disinfection stops
301 intra-colony disease transmission (Fig 4B).



302

303 **Fig 4. Destructive disinfection stops disease transmission.**

304 (A) Ants that interacted with sporulating pupae contracted lethal infections and died
305 from fungal infection in 42% of the cases, whilst there was no disease transmission
306 from destructively disinfected pupae (error bars show ± 95% CI; letters denote groups
307 that differ significantly in a logistic regression [$P < 0.05$]). (B) Overview of normal
308 fungal lifecycle resulting in infectious, sporulating corpses (left) and a broken
309 lifecycle due to the interference of the ants (right). When sanitary care fails to prevent

infection in pathogen-exposed individuals, the ants switch to colony-level disease control, i.e. destructive disinfection to stop pathogen replication, resulting in non-infectious corpses.

Discussion

Ants are extremely hygienic and frequently perform sanitary behaviours to prevent microbial infection of themselves and colony members [12]. However, if these behaviours fail, the colony faces a problem because infections can become highly contagious and cause disease outbreaks [23,24]. In this study, we have characterised a multicomponent behaviour that ants use to fight lethal infections of a common fungal pathogen. Our results show that ants detect infected pupae using chemical signatures whilst the pathogen is still in its non-transmissible incubation period (Fig 2). In contrast to the simple removal of infected brood in honeybees [15], the ants then performed destructive disinfection, utilising their antimicrobial poison for internal disinfection of the host body to stop the pathogen from replicating and completing its lifecycle (Fig 1, Fig 3). Ultimately, this prevented the fungus from infecting new hosts and effectively reduced its fitness to zero (Fig 4). These findings extend our current understanding of collective disease defence in ants, showing that they not only avoid [18], groom [20–22] and isolate pathogens [22,26], but can even interfere with the infectious cycle of the pathogen to actively arrest its establishment and replication in the colonies (Fig 4b). This will have important implications for the evolution of host-pathogen interactions in social insects, as the pathogen is unable to reproduce. More generally, our results reveal the remarkable adaptations that can evolve in superorganisms to avoid disease outbreaks.

We found that destructive disinfection acts like a second line of defence for the colony, when the first, sanitary care, fails to prevent infection. This has parallels to the immune system of the body where defences are layered to prevent pathogen establishment and replication at multiple levels [17]. The first line of defence in the body is made up of mechanical and chemical defences, such as ciliated cells in the lung that move pathogens trapped in mucus out of the body [17]. In ants, grooming and chemical disinfection during sanitary care play an analogous role [20–22]. However, if a pathogen circumvents these defences and a cell is infected, the second line of defence is often a targeted elimination of the cell. This starts with immune cells detecting an infection and then transporting cell death-inducing and antimicrobial compounds into the infected cell by creating pores in its membrane [34–36]. Likewise, our experiments revealed that ants detect sick pupa using chemical compounds on their cuticle. They then unpack the pupa and make perforations in its cuticle, enabling the ants to spray their poison directly into the pupa's body. In both cases, the second line of defence destroys the infected cell/insect, along with the infection, to prevent transmission [37]. Since the loss of somatic cells and individual insect workers can be tolerated with negligible effects on fitness [17], these analogous strategies are a unique way to clear infections and avoid any further damage to the body and colony, respectively.

Previous studies have suggested that ants might use chemical cues to detect sick colony members, but evidence to support this hypothesis has been lacking [26,38,39]. To our knowledge, we have therefore discovered the first known instance of ants using chemical information to identify and specifically target infected individuals. The chemical compounds with increased abundances on infected pupae are distinct

360 from those that induce the removal of corpses in ants [40–42], and, like in tapeworm-
361 infected ants [43], are not pathogen-derived because they are also present in lower
362 amounts on healthy pupae. This alteration of the hosts' chemical profile may arise
363 during infection from the breakdown of hydrocarbons by *Metarhizium* penetration
364 [44] or after infection due to an immune response affecting the synthesis of specific
365 hydrocarbons [45,46]. The latter is more likely as the ants only display destructive
366 disinfection once the fungus is growing inside the pupae. Interestingly, two of the four
367 CHCs that were increased on infected pupae also had higher abundances on virus-
368 infected honeybees (Tritriacontadiene [47]) and their brood experiencing a simulated
369 bacterial infection (Tritriacontene [46]). As these compounds belong to the same
370 hydrocarbon substance class – unsaturated hydrocarbons – their common biosynthetic
371 pathway might be upregulated upon infection. This raises the possibility that these
372 hydrocarbons are evolutionarily conserved sickness cues in Hymenopteran social
373 insects. Such cues may have evolved into general sickness signals in social insects as
374 they alert nestmates to the presence of an infection that will harm the colony if it
375 spreads [48]. Similar to the "find-me/eat-me" signals expressed by infected cells in a
376 body [49,50], they will be selected for as they enhance colony fitness by preventing a
377 systemic infection. Hence, altruistic displays of sickness can evolve in
378 superorganisms, even if this results in the destruction of the individual that expresses
379 them.
380
381 It is well established that social insects use glandular secretions with antimicrobial
382 properties as external surface disinfectants [51]. However, because these compounds
383 can also harm the host, they should be used with caution inside the colony. For
384 example, the acidic poison *L. neglectus* and other Formicine ants produce is extremely

385 caustic and is used to attack conspecifics [22,33,52]. During sanitary care they apply
386 this poison via grooming because it is probably more accurate and less wasteful than
387 spraying [22]. Moreover, as pathogen-exposed insects typically survive when they
388 receive sanitary care [20–23], conservatively applying the poison may also reduce the
389 damage it causes to individuals that can then continue contributing to the colony. This
390 is supported by our observation that *L. neglectus* will apply large quantities of poison
391 onto pupae only when they become infected. Remarkably, we found that, in addition
392 to being external disinfectants, ants use antimicrobial secretions as internal
393 disinfectants against infections within the bodies of nestmates. Since infected pupae
394 are moribund there is no risk that the ants' poison is harming individuals with a future
395 role in the colony. Taken together, these observations suggest that ants adjust their
396 behaviours in response to the risk presented to the colony. It would be interesting to
397 explore further how social immunity defences are regulated to prevent collateral
398 damage, or 'social immunopathology', within the colony.
399
400 Our experiments show that destructive disinfection was highly effective and
401 prevented 95% of infections becoming transmissible. Destructive disinfection will
402 thus keep the average number of secondary infections caused by an initial infection
403 small and the disease will die out within the colony [3]. This may explain why
404 infections of *Metarhizium* and other generalist entomopathogenic fungi like
405 *Beauveria*, though common in the field [53–56], do not seem to cause colony-wide
406 epidemics in ants, but are more numerous in solitary species that lack social immunity
407 [57–59]. Behaviours like destructive disinfection that are able to reduce pathogen
408 fitness to zero could have selected for host manipulation in fungi that specialise on
409 infecting ants, e.g. *Ophiocordyceps* and *Pandora* [60-62]. These fungi force their ant

hosts to leave the nest and climb plant stems near foraging trails. There they die and become infectious, releasing new spores that infect ants foraging below. However, ants infected with *Ophiocordyceps* that were experimentally placed back into the nest disappeared [61]. Our study suggests that these ants could have been eliminated through destructive disinfection. Consequently, ant-specialist fungi like *Ophiocordyceps* and *Pandora* may have evolved host manipulation as a means to complete their lifecycle outside of the nest and avoid destructive disinfection [61,62]. In contrast to specialists, generalist pathogens like *Metarhizium* infect a broad range of solitary and social hosts, making it less likely that they evolve strategies to escape social immunity defences [63]. Future work that investigates how social immunity disrupts typical host-pathogen dynamics will shed light on the co-evolution of pathogens and their social hosts [3].

Destructive disinfection has probably evolved in ants because the removal of corpses from the colony alone does not guarantee that disease transmission is prevented [61]. This is because ants place corpses onto midden (trash) sites that are located inside or outside near the nest and regularly visited by midden workers [64–66]. Consequently, midden sites represent a potential source for disease transmission back into the colony. In contrast to ants, honeybees have no middens and corpses are dumped randomly outside of the hive [15]. But because honeybees forage on the wing, it is unlikely that corpses are re-encountered and so removal is sufficient to prevent disease transmission [67]. Termites on the other hand perform a different behaviour, whereby the dead are cannibalised [19,68]. Cannibalism is effective because the termite gut neutralises ingested pathogens [69–71] and has likely evolved because dead nestmates are a source of valuable nitrogen in their cellulose-base diet [72]. The

same selective pressure has driven this suite of independently evolved innovations – the need to eliminate or remove infected individuals early in the infectious cycle – with the ants expressing a particularly complex behavioural repertoire. This seems to be a general principle in disease defence as cells are also rapidly detected and destroyed shortly after infection to prevent pathogen spread in multicellular organisms [17]. Understanding how natural selection can result in similar traits at different levels of biological organisation and in organisms with different life histories is a central question in evolutionary biology [8]. Studying the similarities and differences between organismal immunity and social immunity could therefore lead to new insights about how disease defences evolve [17]. For example, the results of our study suggest that equivalent selection pressures can result in convergent defences that protect multicellular organisms and superorganismal insect societies from systemic disease spread. Future work that can link the performance of social immunity defences to colony fitness will therefore provide useful insights into how such traits are selected for over evolutionary time.

Materials and Methods

Ant host

We studied the unicolonial invasive garden ant, *Lasius neglectus*, collected in Seva, Spain (41.809000, 2.262194) [55]. Stock colonies were kept at a constant temperature of 23°C with 70% humidity and a day/night cycle of 14/10 h. All experiments were conducted in plastered petri dishes (Ø = 33, 55 or 90 mm) with 10% sucrose solution provided *ad libitum* and environmental conditions were controlled throughout (23°C; 70% RH; 14/10 h light/dark cycles). Care of animals was in accordance with institutional guidelines.

460

461 **Fungal pathogen**

462 As a model pathogen, we used the entomopathogenic fungus *Metarhizium brunneum*
463 (strain MA275, KVL 03-143). Multiple aliquots were kept in long-term storage at –
464 80°C. Prior to each experiment the conidiospores were grown on sabaroud dextrose
465 agar at 23°C until sporulation and harvested by suspending them in 0.05% sterile
466 Triton X-100 (Sigma). The germination rate of conidiospore suspensions was
467 determined before the start of each experiment and was > 90% in all cases.

468

469 **Pupal pathogen exposure**

470 Conidiospores were applied in a suspension of 0.05% autoclaved Triton-X 100 at 10^6
471 conidia/ml in all experiments unless otherwise stated. Throughout the study, we used
472 cocooned worker pupae of approximately the same age, which was determined by
473 assessing the melanisation of the eyes and cuticle. Single pupae were exposed by
474 gently rolling them in 1 µl of the conidiospore suspension using sterile soft forceps.
475 Pupae were then allowed to air dry for 5-10 min before being used in experiments.
476 This exposure procedure resulted in pupae receiving ~ 1800 conidiospores, of which
477 5% (~ 95 conidiospore) passed through the cocoon and came into contact with the
478 pupa inside (Fig S1).

479

480 **Statistical Analysis**

481 Statistical analyses were carried out in R version 3.3.2 [73]. All statistical tests were
482 two-tailed. General(ised) linear and mixed models were compared to null (intercept
483 only) and reduced models (for those with multiple predictors) using Likelihood Ratio
484 (LR) tests to assess the significance of predictors [74]. We controlled for the number

of statistical tests performed per experiment to protect against a false discovery rate using the Benjamini-Hochberg procedure ($\alpha = 0.05$). Moreover, all post hoc analyses were corrected for multiple testing using the Benjamini-Hochberg procedure ($\alpha = 0.05$) [75,76]. We checked the necessary assumptions of all tests i.e. by viewing histograms of data, plotting the distribution of model residuals, checking for non-proportional hazards, testing for unequal variances, testing for the presence of multicollinearity, testing for over-dispersion, and assessing models for instability and influential observations. For mixed effects modelling, we used the packages 'lme4' to fit models [77], 'influence.ME' to test assumptions [78], and, for LMERs, 'lmerTest' to obtain *P* values [79]. All logistic regressions were performed using either generalised linear models (GLMs) or generalised linear mixed models (GLMMs), which had binomial error terms and logit-link function. The Cox proportional hazards regression was carried out using the 'coxphf' package with post hoc comparisons achieved by re-levelling the model and correcting the resulting *P* values [80]. For Kruskal-Wallis (KW) tests and subsequent post hoc comparisons we used the 'agricolae' package, which implements the Conover-Iman test for multiple comparisons using rank sums [81]. For the perMANOVA, we used the package 'vegan' and performed pairwise perMANOVAs for post hoc comparisons [82]. All other post hoc comparisons were performed using the 'multcomp' package [83]. Finally, all graphs were made using the 'ggplot2' package [84]. Individual descriptions of statistical analyses are given for all experiments below.

Unpacking behaviour

To study how ants respond to infections, we exposed pupae to a low (10^4/ml), medium (10^6/ml) or high (10^9/ml) dose of conidiospores or autoclaved Triton X as a

sham control (sham control, $n = 24$; all other treatments, $n = 25$). The pupae were then placed into individual petri dishes with two ants and inspected hourly for 10 h/d for 10 d. When the ants unpacked a pupa, it was removed and surface-sterilised [85] to ensure that any fungal outgrowth was the result of internal infections and not residual conidiospores on the cuticle. After sterilisation, we transferred the pupae to a petri dish lined with damp filter paper at 23°C and monitored them for 2 weeks for *Metarhizium* sporulation to confirm the presence of an internal infection (low dose, $n = 8$; medium dose, $n = 18$; high, $n = 21$). In addition, any cocooned pupae that were not unpacked after 10 d were removed from the ants, surface sterilised and observed for sporulation, as above (low dose, $n = 11$; medium dose, $n = 4$; high, $n = 4$). We analysed the effect of treatment on unpacking using a Cox proportional hazards model with Firth's penalized likelihood, which offers a solution to the monotone likelihood caused by the complete absence of unpacking in the sham control treatment. We followed up this analysis with post hoc comparisons (model factor re-levelling) to test unpacking rates between treatments (Fig 1B). We compared the number of unpacked and cocooned pupae sporulating using a logistic regression, which included pupa type (cocooned, unpacked), conidiospore dose (low, medium, high) and their interaction as main effects. The interaction was non-significant (GLM: LR $\chi^2 = 5.0$, df = 2, $P = 0.084$); hence, it was removed to gain better estimates of the remaining predictors.

Images and scanning electron micrographs (SEMs) of destructive disinfection

Photographs of destructive disinfection were captured (Nikon D3200) and aesthetically edited (Adobe Photoshop) to demonstrate the different behaviours (Fig 1A). They were not used in any form of data acquisition. We also made representative SEMs of a pupa directly after unpacking and one after destructive disinfection (24 h

after unpacking; Fig 1F-G). As the pupae were frozen at – 80ºC until the SEMs were made, we also examined non-frozen pupae taken directly from the stock colony and confirmed that freezing itself does not cause damage to the pupa (not shown).

Comparison of sanitary care and destructive disinfection behaviours

To observe how the behavioural repertoire of the ants changes between sanitary care and destructive disinfection, we filmed three individually colour-marked ants tending a single pathogen-exposed pupa with a USB microscope camera (Di-Li ® 970-O). To characterise the sanitary care behaviours of the ants, we analysed the first 24 h of the videos following the introduction of the pupa. To study destructive disinfection behaviours, we analysed the 24 h period that immediately followed unpacking. Videos were analysed using the behavioural-logging software JWatcher™ [86]. For each ant (n = 15), we recorded the duration of its grooming bouts, the frequency of poison application and the frequency of biting. Grooming duration was analysed using a LMER, having first log-transformed the data to fulfil the assumption of normality (Fig 1C). The frequency of poison application and biting (Fig 1D-E) were analysed using separate GLMMs with Poisson error terms for count data and logit-link function. We included an observation-level random intercept effect to account for over-dispersion in the data [87]. In all three models, we included petri dish identity as a random intercept effect because ants from the same dish are non-independent. Additionally, a random intercept effect was included for each ant as we observed the same individuals twice (before and after unpacking).

Chemical bioassay

559 We determined whether ants detect infected pupae through potential changes in the
560 pupae's cuticular chemical profile. We established internal infections in pupae by
561 exposing them to the pathogen and leaving them for 3 d in isolation. In pilot studies,
562 approx. 50% of these pupae were then unpacked within 4 h of being introduced to
563 ants. After 3 d, pupae were washed for 2.5 min in 300 µl of either pentane solvent to
564 reduce the abundance of all CHCs present on the pupae (n = 28), or in autoclaved
565 water as a handling control (n = 28). After washing, pupae were allowed to air dry on
566 sterile filter paper. Additionally, non-washed pupae were used as a positive control (n
567 = 30). Pupae were placed individually with a pair of ants in petri dishes and observed
568 for unpacking for 4 h. We used GC–MS (see below for methodology) to confirm that
569 washing was effective at removing cuticular compounds, by comparing the total
570 amount of chemicals present on pupae washed in pentane to non- and water-washed
571 pupae (n = 8 per treatment; Fig S4). The number of pupae unpacked between the
572 different treatments was analysed using a logistic regression (Fig 2A). As several
573 researchers helped to wash the pupae, we included a random intercept for each person
574 to control for any potential handling effects. Additionally, the experiment was run in
575 two blocks on separate days, so we included a random intercept for each block to
576 generalise beyond any potential differences between runs. The total peak area from
577 the GC–MS analysis was compared between treatments using a KW test with post hoc
578 comparisons.
579
580 **Chemical analysis of pupal hydrocarbon patterns**
581 To confirm that infected pupae had chemical profiles that are different from pathogen-
582 exposed cocooned and control pupae, we exposed pupae to the pathogen or a sham
583 control. Pupae were then isolated for 3 d to establish infections in the pathogen-

584 exposed treatment (as above). Following isolation, pupae were individually placed
585 with ants and observed for unpacking for 4 h. Unpacked pupae were immediately
586 frozen at – 80 °C with the removed cocoons (n = 13) and we also froze cocooned
587 pathogen-exposed pupa that had not yet been unpacked (n =10). Furthermore, we
588 froze a pair of control pupae, of which one was cocooned (n = 12), whilst the other
589 was first experimentally unpacked (to test if the cocoon affects cuticular compound
590 extraction; n =12). Cuticular chemicals were extracted from individual pupae and
591 their cocoons in glass vials (Supelco; 1.8 ml) containing 100 µl n-pentane solvent for
592 5 min under gentle agitation. The vials were then centrifuged at 3000 rpm for 1 min to
593 spin down any fungal conidiospores that might be remaining, and 80 µl of the
594 supernatant was transferred to fresh vials with 200 µl glass inserts and sealed with
595 Teflon faced silicon septa (both Supelco). The pentane solvent contained four internal
596 standards relevant for our range of hydrocarbons (C_{27} – C_{37}); n-Tetracosane, n-
597 Triacontane, n-Dotriacontane and n-Hexatriacontane (Sigma Aldrich) at 0.5 µg/ml
598 concentration, all fully deuterated to enable spectral traceability and separation of
599 internal standards from ant-derived substances. We ran extracts from the different
600 groups in a randomised manner, intermingled with blank runs containing only
601 pentane, and negative controls containing the pentane plus internal standards (to
602 exclude contaminants emerging e.g. from column bleeding), on the day of extraction,
603 using GC–MS (Agilent Technologies; GC7890 coupled to MS5975C).
604
605 A liner with one restriction ring filled with borosilicate wool (Joint Analytical
606 Systems) was installed in the programmed temperature vaporisation (PTV) injection
607 port of the GC, which was pre-cooled to -20 °C and set to solvent vent mode. 50 µl of
608 the sample extractions were injected automatically into the PTV port at 40 µl/s using

609 an autosampler (CTC Analytics, PAL COMBI-xt; Axel Semrau, CHRONOS 4.2
610 software) equipped with a 100 µl syringe (Hamilton). Immediately after injection, the
611 PTV port was ramped to 300 °C at 450 °C/min, and the sample transferred to the
612 column (DB-5ms; 30 m × 0.25 mm, 0.25 µm film thickness) at a flow of 1 ml/min.
613 The oven temperature program was held at 35 °C for 4.5 min, then ramped to 325 °C
614 at 20 °C/min, and held at this temperature for 11 min. Helium was used as the carrier
615 gas at a constant flow rate of 3 ml/min. For all samples, the MS transfer line was set
616 to 325 °C, and the MS operated in electron ionisation mode (70 eV; ion source 230
617 °C; quadrupole 150 °C, mass scan range 35-600 amu, with a detection threshold of
618 150). Data acquisition was carried out using MassHunter Workstation, Data
619 Acquisition software B.07.01 (Agilent Technologies).
620
621 Analytes were detected by applying deconvolution algorithms to the total ion
622 chromatograms of the samples (MassHunter Workstation, Qualitative Analysis
623 B.07.00). Compound identification (Table S1) was performed via manual
624 interpretation using retention indices and spectral information, and the comparison of
625 mass spectra to the Wiley 9th edition/NIST 11 combined mass spectral database
626 (National Institute of Standards and Technologies). As the molecular ion was not
627 detectable for all analytes based on electronic ionisation, we in addition performed
628 chemical ionisation on pools of 20 pupae in 100 µl n-pentane solvent with 0.5 µg/ml
629 internal standards. The higher extract concentration was needed to counteract the loss
630 in ionisation efficiency in chemical ionisation mode. A specialised chemical
631 ionisation source with methane as the reagent gas was used with the MS, while the
632 chromatographic method was the same as in electronic ionisation mode. Use of
633 external standards (C_7-C_{40} saturated alkane mixture [Sigma Aldrich]) enabled

traceability of all peaks, and thus comparison to runs of single pupae extracts made in electronic ionisation mode. Modified Kovats retention indices for the peaks in question were calculated based on those standards. To further aid identification, we separated the substances based on polarity using solid phase extraction fractionation. For this purpose, pools of 20 pupae were extracted in 500 µl n-pentane containing 0.2 µg/ml internal standard, and separated on unmodified silica cartridges (Chromabond ® SiOH, 1ml, 100 mg) based on polarity. Prior to use, the cartridges were conditioned with 1 ml dichloromethane followed by 1 ml n-pentane. The entire extraction volume was loaded onto the silica and the eluent (fraction 1, highly apolar phase) collected. A wash with 1 ml pure n-pentane was added to fraction 1. Fraction 2 contained all substances washed off the silica with 1 ml 25 % dichloromethane in n-pentane, and finally a pure wash with 1 ml dichloromethane eluted all remaining substances (fraction 3). The polarity thus increased from fraction 1 through 3, but no polar substances were found. All fractions were dried under a gentle nitrogen stream and re-suspended in 70 µl n-pentane followed by vigorous vortexing for 45 s. GC–MS analysis of all fractions was performed in electronic ionisation mode under the same chromatographic conditions as before.

To quantify the relative abundances of all compounds found on each pupa, analyte-characteristic quantifier and qualifier ions were used to establish a method enabling automatized quantification of their integrated peak area relative to the peak area of the closest internal standard. For each analyte, the relative peak area was normalised, i.e. divided by the total sum of all relative peak areas of one pupa, to standardise all pupa samples. Only analytes, which normalised peak area contributed more than 0.05% of the total peak area, were included in the statistical analysis. We compared the

chemical profiles of the pupae using a perMANOVA analysis of the Mahalanobis dissimilarities between pupae, with post hoc perMANOVA comparisons. Since there was no difference between cocooned and unpacked control pupae we combined them into a single control group for the final analysis (perMANOVA: $F = 1.09$, df = 23, $P = 0.1$). We also performed a discriminant analysis of principle components (Fig 2B) to characterise the differences between the pupal treatments [88,89]. To identify the compounds that differ between treatments, we performed a conditional random forest classification (*n* trees = 500, *n* variables per split = 4) [88,90,91]. Random forest identified 9 compounds that were important in classifying the treatment group, of which 8 were significant when analysed using separate KW tests (results for significant compounds in Table S2). We followed up the KW tests with individual post hoc comparisons for each significant compound (Fig 2C-F, post hoc comparisons in Table S2).

Effect of destructive disinfection on pathogen replication

To test if destructive disinfection prevents *Metarhizium* from successfully replicating, we kept single pathogen-exposed pupae in petri dishes containing groups of 3 or 8 ants. This allowed us to assess how group size affects the likelihood of fungal inhibition. For the following 10 d, we observed the pupae for unpacking. When a pupa was unpacked, we left it with the ants for a further 1 or 5 d so that they could perform destructive disinfection. This allowed us to assess how the duration of destructive disinfection affects the likelihood of fungal inhibition. The destructively disinfected pupae were then removed and placed into petri dishes on damp filter paper at 23 ºC (8 ants 1 d and 5 d, *n* = 22 pupae each; 3 ants 1 and 5 d, *n* = 18 pupae each). We did not surface sterilise the pupae as this might have interfered with the

684 destructive disinfection the ants had performed. Removed pupae were observed daily
685 for *Metarhizium* sporulation for 30 d. To determine how many pupae sporulate in the
686 absence of destructive disinfection, we kept pathogen-exposed pupae without ants as a
687 control and recorded the number that sporulated for 30 d (n = 25). We compared the
688 number of pupae that sporulated after 1 d, 5 d and in the absence of ants using logistic
689 regressions and Tukey post hoc comparisons, separately for the two ant group sizes
690 (Fig 3A, Fig S6).
691
692 ***In vitro* investigation of destructive disinfection**
693 We examined the individual effects of unpacking, biting and poison application on
694 destructive disinfection by performing these behaviours *in vitro*. Pathogen-exposed
695 pupae were initially kept with ants so that they could perform sanitary care. After 3 d,
696 we removed the pupae and split them up into three groups: (i) pupae that we left
697 cocooned, (ii) experimentally unpacked and (iii) experimentally unpacked and bitten.
698 We simulated the damage the ants achieve through biting by damaging the pupal
699 cuticle and removing their limbs with forceps. The pupae were then treated with either
700 synthetic ant poison (60% formic acid and 2% acetic acid, in water; applied at a dose
701 equivalent to what ants apply during destructive disinfection; Fig S8) or autoclaved
702 distilled water as a control, using pressurised spray bottles (Lacor) to evenly coat the
703 pupae in liquid. The pupae were allowed to air dry for 5 min before being rolled over
704 and sprayed again and allowed to dry a further 5 min. All pupae were then placed into
705 separate petri dishes and monitored daily for *Metarhizium* sporulation (cocooned +
706 poison, n = 24; unpacked + poison + biting, n = 24; all other treatments, n = 25). The
707 number of pupae sporulating was analysed using a logistic regression with Firth's
708 penalised likelihood, which offers a solution to the monotone likelihood caused by the

complete absence of sporulation in one of the groups (R package 'brglm' [92]). Pupal manipulation (cocooned/unpacked only/unpacked and bitten), chemical treatment (water or poison) and their interaction were included as main effects (Fig 3B, Fig S7). We followed up this analysis with Tukey post hoc comparisons (Table S3).

Disease transmission from infectious and destructively disinfected pupae

We tested the impact of destructive disinfection on disease transmission within groups of ants by keeping them with sporulating pupae or pupae that had been destructively disinfected. Infections were established in pupae (as above) and half were allowed to sporulate (n = 11), whilst the other half were experimentally destructively disinfected (as above; n = 11). Pupae were then kept individually with groups of 5 ants in mini-nests (cylindrical containers [Ø = 90 mm] with a second, smaller chamber covered in red foil [Ø = 33 mm]). Ant mortality was monitored daily for 30 d. Dead ants were removed, surface sterilised and observed for *Metarhizium* sporulation. The number of ants dying from *Metarhizium* infections in each treatment was compared using a logistic regression (Fig 4A). Mini-nest identity was included as a random intercept effect as ants from the same group are non-independent.

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Competing financial interests

The authors declare no competing financial interests.

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