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1 **Title:** Destructive disinfection of infected brood prevents systemic disease spread in
2 ant colonies
3
4 **Short title:** Colony-level disease protection in ants
5

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25 **Abstract**

26 Social insects colonies protect themselves against pathogens by collectively
27 performing disease defences that result in social immunity. As a first barrier to
28 disease, sanitary care between nestmates reduces individual-level exposure and
29 infection. However, if a pathogen overcomes these defences, social insects should
30 have evolved mechanisms to prevent individual infections causing systemic disease
31 spread in the colony. In this study, we investigated how ants prevent disease
32 outbreaks following the successful infection of their brood with a fungal pathogen.
33 We found that the ants can detect lethal infections early in the non-infectious
34 incubation period of the pathogen's lifecycle, using chemical 'sickness cues'
35 expressed by the infected pupae. Following detection, the pupae are unpacked from
36 their cocoons by the ants and bitten to make perforations in the cuticle, through which
37 the ants spray their antimicrobial poison. Since the cocoon and cuticle act as barriers
38 against the poison, this multicomponent behaviour permits the ants to use their poison
39 for internal disinfection of the pupal body, where it can act directly against the
40 proliferating pathogen. We termed this mechanism destructive disinfection as it killed
41 the pupae and prevented pathogen replication. Ultimately, the ants stopped the
42 pathogen from completing its lifecycle and transmitting to new hosts. Thus,
43 destructive disinfection acts as a second line of defence against disease in ant colonies
44 and is analogous to the elimination of infected cells by adaptive immune systems. Our
45 results therefore suggest that pathogen-induced selection can result in similar
46 solutions to common problems across the different levels of biological organisation.
47
48
49

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50 **Introduction**

51 Pathogen replication and transmission from infectious to susceptible hosts is key to
52 the success of contagious diseases [1]. Social animals are therefore expected to
53 experience a greater risk of disease outbreaks than solitary species, because their
54 higher number of within-group interactions will promote pathogen spread [2–4]. As a
55 consequence, traits that mitigate this cost should have been selected for in group-
56 living animals as an essential adaptation to social life [5,6].
57
58 Eusocial insects (termites, ants and the social bees and wasps) live in complex
59 societies that are ecologically successful and diverse. They are typically single-family
60 colonies comprising one or a few reproducing queens and many sterile workers. Both
61 of these castes are highly interdependent: the queens are morphologically specialised
62 for reproduction and cannot survive without the assistance of the workers; conversely,
63 the workers cannot reproduce, but gain inclusive fitness by raising the queen's
64 offspring [7]. Consequently, social insects societies have become single reproductive
65 units, where natural selection acts on the colony instead of its individual members
66 [8,9]. This has parallels to the evolution of complex multicellular organisms, where
67 sterile somatic tissue and germ line cells form a single reproducing body. Hence,
68 social insect colonies are often termed "superorganisms" and their emergence is
69 considered a major evolutionary transition [8–11]. Since evolution favours the
70 survival of the colony over its members, selection has resulted in a plethora of
71 cooperative and altruistic traits that workers perform to protect the colony from harm
72 [5,8,12,13]. In particular, social insects have evolved physiological and behavioural
73 adaptations that limit the colony-level impact of infectious diseases, which could
74 otherwise spread easily due to the intimate social interactions between colony

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75 members [12,14–16]. These defences are performed collectively by the workers and
76 form a layer of protection known as "social immunity" that, like the immune system
77 of a body, protects the colony from invading pathogens [12,17].
78
79 Our understanding of how social immunity functions is based mostly on the first line
80 of defence that reduces the probability of pathogen exposure and infection. It is well
81 known for example that social insects avoid pathogens, like fungal spores, in their
82 environment, and perform sanitary care when nestmates come into contact with them
83 [18–22]. In ants, sanitary care involves grooming and the use of antimicrobial
84 secretions to mechanically remove and chemically disinfect the pathogen, reducing
85 the likelihood that pathogen exposure leads to the development of an infection
86 [21,22]. However, what happens when sanitary care fails and a pathogen successfully
87 infects an ant, with the consequent potential to create an epidemic, remains poorly
88 understood. In a body, infected cells are eliminated by the immune system to prevent
89 the proliferation and systemic spread of pathogens through the tissue. Since infected
90 ants become highly contagious to their nestmates [23,24], we hypothesised that they
91 should have evolved an analogous mechanism to detect and contain lethal infections
92 in individuals as early as possible, to prevent disease outbreaks in the colony.
93
94 To test this hypothesis, we exposed pupae of the invasive garden ant, *Lasius*
95 *neglectus,* to a generalist fungal pathogen, *Metarhizium brunneum*. When the
96 infectious conidiospores of this fungus come into contact with insect cuticle, they
97 attach, germinate and penetrate the host cuticle within 48 h to cause internal
98 infections. After a short, non-infectious incubation period of a few days, a successful
99 fungal infection then induces host death, after which the fungus replicates and

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100 releases millions of new infectious conidiospores in a process called sporulation
101 [23,25]. Previous work found that brood infected with *Metarhizium* is removed from
102 the brood chamber, however, it is unknown how the ants then respond to the infection
103 [26,27]. Here we demonstrate that ants detect infected pupae during the pathogen's
104 non-infectious incubation period and react by performing a multicomponent
105 behaviour. To investigate this response we used a series of behavioural and chemical
106 experiments to determine its function and underlying mechanisms. Finally, we tested
107 the impact of the multicomponent behaviour on the pathogen's ability to complete its
108 lifecycle and cause a systemic colony infection.
109
110 **Results**
111 **Destructive disinfection of lethally infected pupae**
112 We exposed ant pupae to one of either three dosages of *Metarhizium* conidiospores or
113 a sham control. We observed that ants tending pathogen-exposed pupae prematurely
114 removed the pupae from their cocoons in a behaviour we termed "unpacking",
115 whereas control pupae were left cocooned (Fig 1A-B, Video S1; Cox proportional
116 hazards regression: likelihood ratio test (LR) $\chi^2 = 55.48$, df = 3, $P = 0.001$; post hoc
117 comparisons: control vs. low, $P = 0.004$; low vs. medium, $P = 0.006$; medium vs. high
118 = 0.024; all others, $P = 0.001$). Unpacking occurred between 2-10 d after pathogen
119 exposure, but sooner and more frequently at higher conidiospore dosages (Fig 1B). As
120 unpacking was a belated response to pathogen exposure and we were unable to
121 remove any conidiospores from the cocoon or the unpacked pupae (Fig S1), we
122 concluded that the ants were not performing unpacking to simply dispose of the
123 contaminated cocoons. Instead, we postulated that unpacking was a response to
124 successful infection. At the time of unpacking, the majority of pupae were still alive

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125 (Fig S2) and fungal outgrowth had not yet occurred (Fig 1F). Hence, to test if the ants
126 were reacting to early-stage infections, we removed both unpacked and non-unpacked
127 pathogen-exposed cocooned pupae from the ants and incubated them under optimal
128 conditions for fungal outgrowth. We found that, on average across the conidiospore
129 dosages, 90% of unpacked pupae harboured infections that sporulated in the absence
130 of the ants. In contrast, only 25% of non-unpacked pupae were infected (generalised
131 linear model [GLM]: overall LR $\chi^2 = 21.52$, df = 3, $P = 0.001$; cocooned vs. unpacked
132 pupae: LR $\chi^2 = 18.5$, df = 1, $P = 0.001$; conidiospore dose: LR $\chi^2 = 0.42$, df = 2, $P =$
133 0.81). We therefore concluded that the ants were detecting and unpacking pupae with
134 lethal infections during the asymptomatic incubation period of the pathogen's
135 lifecycle. At this time point the fungus is non-infectious and so there is no risk of the
136 ants contracting the disease.
137
138 Next, we filmed ants presented with pathogen-exposed pupae and compared their
139 behaviour before and after unpacking. Prior to unpacking, we observed the typical
140 sanitary care behaviours reported in previous studies [20,22,23,28]. Namely, the ants
141 groomed the pupae (Fig 1C), which has the dual function of removing the
142 conidiospores and applying the ants' antimicrobial poison [22]. In *L. neglectus*, the
143 poison is mostly formic acid and is emitted from the acidopore at the abdominal tip,
144 where the ants actively suck it up and transiently store it in their mouths until
145 application during grooming. Additionally, the ants can spray their poison directly
146 from the acidopore; yet, this behaviour is rarely expressed during sanitary care (about
147 once every 28 h; Fig 1D) [22]. However, after unpacking, we observed a set of
148 behaviours markedly different to sanitary care (Fig 1A, Video S1). The ants sprayed
149 the pupae with poison from their acidopore approx. 15-times more frequently than

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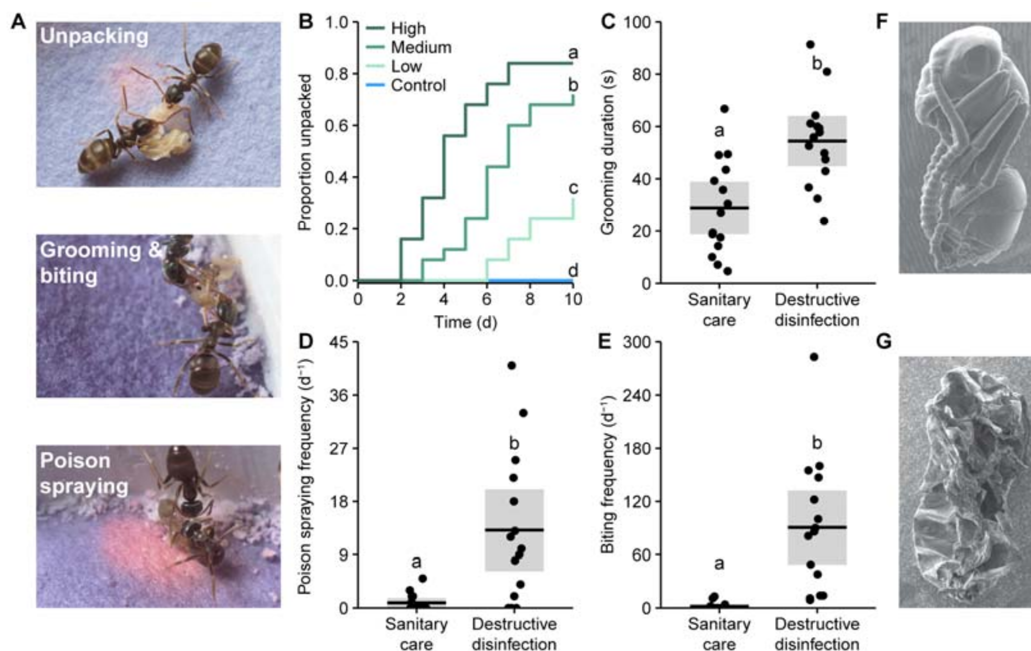
150 during sanitary care (~ 13-times/d; Fig 1D; generalised linear mixed model [GLMM]:
151 LR χ^2 = 17.04, df = 1, P = 0.001), and increased grooming by 50% (Fig 1C; linear
152 mixed effects regression [LMER]: LR χ^2 = 145.26, df = 1, P = 0.001). Given that
153 there was no fungus to remove at the time of unpacking, the increase in grooming
154 probably functioned solely to apply poison from the oral store [22]. Furthermore, the
155 ants repeatedly bit the pupae to make perforations in their cuticles (Fig 1E; GLMM:
156 LR χ^2 = 39.44, df = 1, P = 0.001). Together these three behaviours resulted in the
157 death of the pupae and left their corpses heavily damaged and coated in the ants'
158 poison (Fig 1G, Fig S2, Fig S3). Accordingly, we named the combination of
159 unpacking, grooming, poison spraying and biting "destructive disinfection", and
160 performed a series of experiments to determine its function.



161

162 **Fig 1. Ants perform destructive disinfection in response to lethal fungal**
163 **infections of pupae.**
164 (A) Destructive disinfection starts with the unpacking of pupae from their cocoons
165 and is followed by grooming, poison spraying and biting (ants housed on blue pH-

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166 sensitive paper to visualise acidic poison spraying, which shows up pink). (B)
167 Unpacking occurred significantly more in pupae exposed to fungal conidiospores and
168 was dose-dependent, occurring sooner and in higher amounts as the dose of
169 conidiospores increased (letters denote groups that differ significantly in Tukey post
170 hoc comparisons [$P < 0.05$]). (C-E) Comparison of the ants' behaviour between
171 sanitary care and destructive disinfection. Destructive disinfection is characterised by
172 increases in grooming duration, poison spraying frequency and biting frequency (all
173 data points displayed; lines ± shaded boxes show mean ± 95% confidence intervals
174 [CI]; letters denote groups that differ significantly in logistic regressions [$P < 0.05$]).
175 (F) Scanning electron micrographs (SEM) of an asymptomatic pupa immediately after
176 unpacking, and (G) of a destructively disinfected pupa 24 h later.
177

178 **Chemical detection of internal infections**

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

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191

192 Gas chromatography-mass spectrometry (GC–MS) analysis of the solvent wash
193 confirmed that unpacked pupae have distinct chemical profiles compared to non-
194 infected control pupae, whilst cocooned (non-unpacked) pathogen-exposed pupae
195 were intermediate (Fig 3B, Fig S5; perMANOVA: $F = 1.49$, df = 46, $P = 0.002$; post
196 hoc perMANOVA comparisons: unpacked vs. control, $P = 0.003$; unpacked vs.
197 cocooned, $P = 0.79$; cocooned vs. control, $P = 0.08$). Most chemical messages in
198 social insects are encoded by quantitative shifts of several compounds [29].
199 Correspondingly, we found that 8 out of the 24 CHCs identified (Table S1) had higher
200 relative abundances on unpacked pupae compared to control pupae (Fig 3C-F, Fig S5;
201 all Kruskal-Wallis [KW] test statistics and post hoc comparisons in Table S2).
202 Moreover, four of these CHCs were also present in relatively higher quantities on
203 unpacked pupae compared to the non-unpacked cocooned pupae. Hence, several
204 specific CHCs probably accumulate on infected pupae over time, eventually reaching
205 an amount that, relative to the other compounds, is sufficient to elicit destructive
206 disinfection. This corresponds to current models of social insect behaviour, where the
207 likelihood of a response depends on stimuli exceeding a certain threshold [30,31].
208 Interestingly, the four CHCs specifically increased on unpacked pupae were all long-
209 chained CHCs (carbon chain length $C_{33\text{-}35}$) with a low volatility, meaning that the ants
210 have to be close to or touching the pupae to detect them [32]. As ants keep pupae in
211 large piles, using low-volatility CHCs may be important so that the ants accurately
212 identify the sick pupae and do not mistakenly destroy healthy ones.

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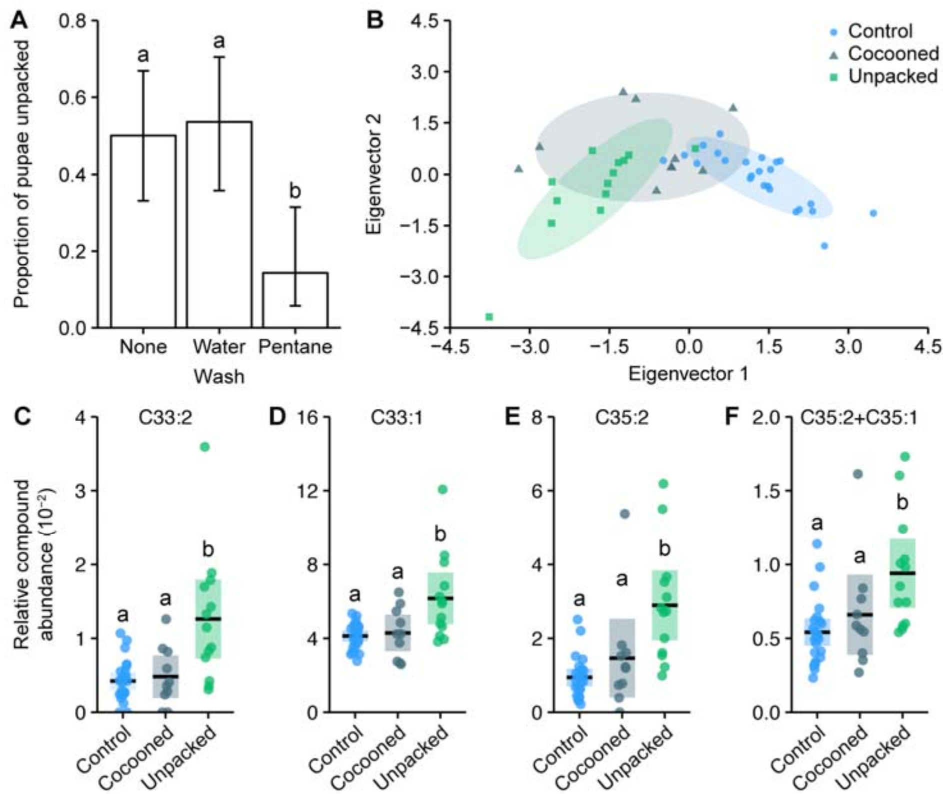

213

214 **Fig 2. Destructive disinfection is induced by changes in the chemical profile of**

215 **infected pupae.**

216 (A) Pupae washed in pentane solvent to reduce the abundance of their cuticular

217 hydrocarbons (CHCs) were unpacked less than unwashed or water-washed pupae

218 (positive and handling controls, respectively; error bars show ± 95% CI; letters

219 specify significant Tukey post hoc comparisons [$P < 0.05$]). (B) Unpacked pathogen-

220 exposed pupae have distinct chemical profiles compared to sham-treated control

221 pupae. Pathogen-exposed pupae that were not unpacked (cocooned group) have

222 intermediate profiles (axes show discriminant analysis of principle components

223 eigenvectors). (C-F) The four CHCs with higher relative abundances on unpacked

224 pupae compared to both control and cocooned pupae, (C) Tritriacontadiene, C33:2

225 (D), Tritriacontene, C33:1 (E), Pentatriacontadiene, C35:2 (F) co-eluting

226 Pentatriacontadiene and Pentatriacontene, C35:2+C35:1 (all data points displayed;

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227 line ± shaded box show mean ± 95% CI; letters specify groups that differ significantly
228 in KW test post hoc comparisons [$P < 0.05$]).
229

230 **Destructive disinfection prevents pathogen replication**

231 We next tested if destructive disinfection prevents pupal infections from replicating
232 and becoming infectious. Pathogen-exposed pupae were kept with groups of ants (8
233 ants per pupae per group) until unpacking. They were then left with the ants for a
234 further 1 or 5 d before being removed and incubated for fungal growth. We compared
235 the number that subsequently sporulated to pathogen-exposed pupae kept without
236 ants. Whilst 90% of pupae contract infections, destructive disinfection significantly
237 reduced the proportion of pupae that sporulated and hence became infectious (Fig 3A;
238 GLM: LR $\chi^2 = 40.47$, df = 2, $P = 0.001$; Tukey post hoc comparisons: 1 vs. 5 d, $P =$
239 0.04; all others, $P = 0.001$). After only 1 d, the number of destructively disinfected
240 pupae that sporulated decreased by 65%. With more time, the ants could reduce the
241 number of pupae sporulating even further by 95%. Since the pupae were removed
242 from the ants for fungal incubation, we can conclude that destructive disinfection
243 permanently prevents pathogen replication. We repeated this experiment with a
244 smaller number of ants (3 ants per pupae per group) to investigate how group size
245 influences the success of destructive disinfection. Smaller groups of ants were less
246 efficient than larger ones: although they could still inhibit > 90% of pupal infections
247 within 5 d of unpacking, pupae tested for infection after 1 d still sporulated 70% of
248 the time (Fig S6; GLM: LR $\chi^2 = 35.23$, $P = 0.001$; Tukey post hoc comparisons: 0 vs.
249 1 d, $P = 0.2$; 0 vs. 5 d, $P = 0.001$; 1 vs. 5 d, $P = 0.002$). As the effectiveness of
250 destructive disinfection increased with the amount of time the ants had, as well as

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251 with the number of ants present, we inferred that there must be a limiting factor
252 affecting the inhibition the pathogen.
253
254 To study the underlying mechanisms of destructive disinfection, we performed its
255 different components – unpacking, biting and poison spraying – *in vitro* to test for
256 their relative importance and potential synergistic effects. We simulated unpacking by
257 removing the cocoons of the pupae manually, and the cuticle damage caused by biting
258 using forceps. Previous work establishing the composition of *L. neglectus* poison [22]
259 allowed us to create a synthetic version for use in this experiment (60% formic acid
260 and 2% acetic acid, in water; applied at a dose equivalent to what ants apply during
261 destructive disinfection; Fig S8), with water as a sham control. We then performed
262 these 'behaviours' in different combinations in a full-factorial experiment. We found
263 that all three behaviours must be performed in the correct order and interact to prevent
264 pathogen replication (overview graph showing odds ratios of sporulation in Fig 3B,
265 full data dataset displayed in Fig S7; GLM: overall LR $\chi^2 = 79.9$, df = 5, $P = 0.001$;
266 interaction between behaviours LR $\chi^2 = 20.6$, df = 2, $P = 0.001$; all post hoc
267 comparisons in Table S3). As in sanitary care, the poison was the active antimicrobial
268 compound that inhibited fungal growth (Fig S7, Table S3 [21,22]). However, for the
269 poison to function the pupae had to be removed from their cocoons and their cuticles
270 damaged. Firstly, this is because the cocoon itself is hydrophobic and thus prevents
271 the aqueous poison from reaching the pupae inside (Fig S9). Secondly, as the
272 infection is growing internally at the time of unpacking, the cuticle must be broken in
273 order for the poison to enter the hemocoel of the pupae. This is achieved with the
274 perforations created by the ants biting the pupal cuticle. As the active antimicrobial
275 component, we concluded that the poison is probably the limiting factor determining

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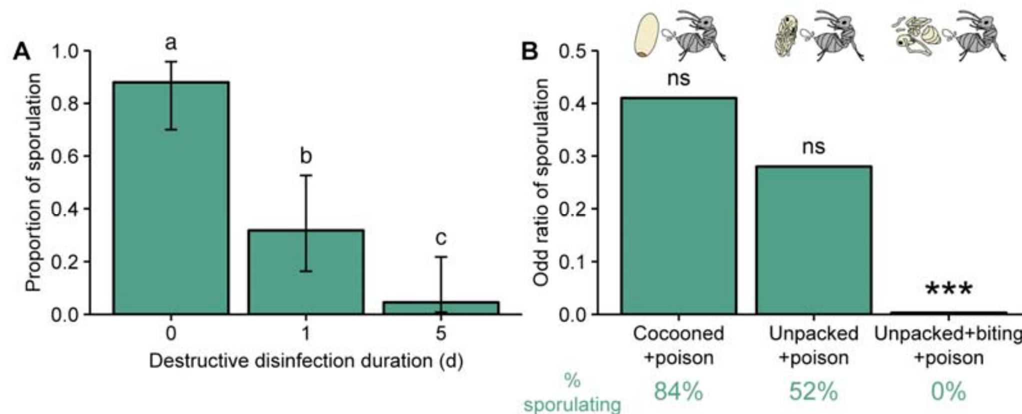
276 whether destructive disinfection is successful. Because the poison has a slow
277 biosynthesis and each ant can only store a limited amount [22,33], it would explain
278 why destructive disinfection was more likely to be successful the longer the ants had
279 to treat the pupae, and as the number of ants increased (Fig 3A, Fig S6). By sharing
280 the task of poison synthesis and application, the ants probably increase their chances
281 of preventing the pathogen becoming infectious.



282

283 **Fig 3. Destructive disinfection by ants prevents pathogen replication.**
284 (A) Destructive disinfection greatly reduced the probability of pupae sporulating
285 compared to pupae that received no destructive disinfection (time point 0), and its
286 effectiveness increased with the length of time ants could perform destructive
287 disinfection (1 vs. 5 d; error bars show ± 95% CI; letters denote groups that differ
288 significantly in Tukey post hoc comparisons [$P < 0.05$]). (B) The individual
289 components of destructive disinfection (unpacking, biting and poison spraying)
290 interacted to inhibit pathogen replication (% of pupae sporulating in each treatment
291 shown under graph in green). The odds of sporulation for cocooned and unpacked
292 pupae treated with poison were not significantly different to those of control pupae
293 (cocooned pupae treated with water). But when unpacking, biting and poison spraying
294 were combined the odds of sporulation were significantly reduced (logistic regression;

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295 ns = non-significant deviation from control, *** = $P < 0.001$; complete data set of full
296 factorial experiment displayed in Fig S7 and all statistics in Table S3).
297
298 **Disruption of the pathogen lifecycle stops disease transmission**
299 Finally, we investigated the impact of destructive disinfection on disease transmission
300 within a social host group. We created mini-nests comprising two chambers and a
301 group of ants (5 ants per group). Into one of the chambers we placed an infectious
302 sporulating pupa – simulating a failure of the ants to detect and destroy the infection –
303 or a pupa that had been destructively disinfected, and was thus non-infectious. The
304 ants groomed, moved around and sprayed both types of corpses with poison. In the
305 case of the sporulating pupae, all conidiospores were removed from the corpse by the
306 ants. As in previous studies, sporulating corpses were highly virulent [23,24] and
307 caused lethal infections that became contagious after host death in 42% of ants (Fig
308 4A). However, there was no disease transmission from destructively disinfected pupae
309 (Fig 4A; GLM: LR $\chi^2 = 31.32$, df = 1, $P = 0.001$). We therefore concluded that by
310 preventing the pathogen from completing its lifecycle destructive disinfection stops
311 intra-colony disease transmission (Fig 4B).

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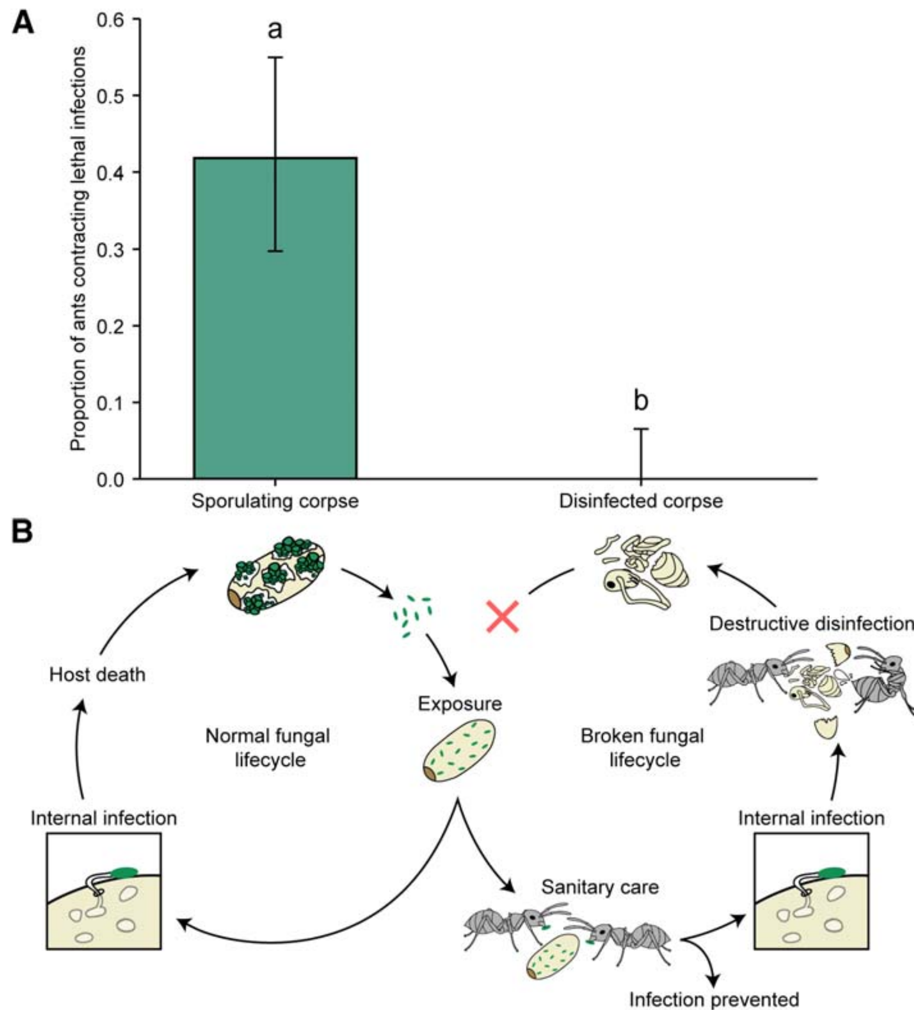

312

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

314 (A) Ants that interacted with sporulating pupae contracted lethal infections and died
315 from fungal infection in 42% of the cases, whilst there was no disease transmission
316 from destructively disinfected pupae (error bars show ± 95% CI; letters denote groups
317 that differ significantly in a logistic regression [$P < 0.05$]). (B) Overview of normal
318 fungal lifecycle resulting in infectious, sporulating corpses (left) and a broken
319 lifecycle due to the interference of the ants (right). When sanitary care fails to prevent
320 infection in pathogen-exposed individuals, the ants switch to colony-level disease
321 control, i.e. destructive disinfection to stop pathogen replication, resulting in non-
322 infectious corpses.

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323

324 **Discussion**

325 Ants are extremely hygienic and frequently perform sanitary behaviours to prevent
326 microbial infection of themselves and colony members [12]. However, if these
327 behaviours fail, the colony faces a problem because infections can become highly
328 contagious and cause disease outbreaks [23,24]. In this study, we have characterised a
329 multicomponent behaviour that ants use to fight lethal infections of a common fungal
330 pathogen. Our results show that ants detect infected pupae using chemical signatures
331 whilst the pathogen is still in its non-transmissible incubation period (Fig 2). In
332 contrast to the simple removal of infected brood in honeybees [15], the ants then
333 performed destructive disinfection, utilising their antimicrobial poison for internal
334 disinfection of the host body to stop the pathogen from replicating and completing its
335 lifecycle (Fig 1, Fig 3). Ultimately, this prevented the fungus from infecting new hosts
336 and effectively reduced its fitness to zero (Fig 4). These findings extend our current
337 understanding of collective disease defence in ants, showing that they not only avoid
338 [18], groom [20–22] and isolate pathogens [22,26], but can even interfere with the
339 infectious cycle of the pathogen to actively arrest its establishment and replication in
340 the colonies (Fig 4b). This will have important implications for the evolution of host-
341 pathogen interactions in social insects, as the pathogen is unable to reproduce. More
342 generally, our results reveal the remarkable adaptations that can evolve in
343 superorganisms to avoid disease outbreaks.

344

345 We found that destructive disinfection acts like a second line of defence for the
346 colony, when the first, sanitary care, fails to prevent infection. This has parallels to the
347 immune system of the body where defences are layered to prevent pathogen

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348 establishment and replication at multiple levels [17]. The first line of defence in the
349 body is made up of mechanical and chemical defences, such as ciliated cells in the
350 lung that move pathogens trapped in mucus out of the body [17]. In ants, grooming
351 and chemical disinfection during sanitary care play an analogous role [20–22].
352 However, if a pathogen circumvents these defences and a cell is infected, the second
353 line of defence is often a targeted elimination of the cell. This starts with immune
354 cells detecting an infection and then transporting cell death-inducing and
355 antimicrobial compounds into the infected cell by creating pores in its membrane [34–
356 36]. Likewise, our experiments revealed that ants detect sick pupa using chemical
357 compounds on their cuticle. They then unpack the pupa and make perforations in its
358 cuticle, enabling the ants to spray their poison directly into the pupa's body. In both
359 cases, the second line of defence destroys the infected cell/insect, along with the
360 infection, to prevent transmission [37]. Since the loss of somatic cells and individual
361 insect workers can be tolerated with negligible effects on fitness [17], these analogous
362 strategies are a unique way to clear infections and avoid any further damage to the
363 body and colony, respectively.
364
365 Previous studies have suggested that ants might use chemical cues to detect sick
366 colony members, but evidence to support this hypothesis has been lacking [26,38,39].
367 To our knowledge, we have therefore discovered the first known instance of ants
368 using chemical information to identify and specifically target infected individuals.
369 The chemical compounds with increased abundances on infected pupae are distinct
370 from those that induce the removal of corpses in ants [40–42], and, like in tapeworm-
371 infected ants [43], are not pathogen-derived because they are also present in lower
372 amounts on healthy pupae. This alteration of the hosts' chemical profile may arise

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373 during infection from the breakdown of hydrocarbons by *Metarhizium* penetration
374 [44] or after infection due to an immune response affecting the synthesis of specific
375 hydrocarbons [45,46]. The latter is more likely as the ants only display destructive
376 disinfection once the fungus is growing inside the pupae. Interestingly, two of the four
377 CHCs that were increased on infected pupae also had higher abundances on virus-
378 infected honeybees (Tritriacontadiene [47]) and their brood experiencing a simulated
379 bacterial infection (Tritriacontene [46]). As these compounds belong to the same
380 hydrocarbon substance class – unsaturated hydrocarbons – their common biosynthetic
381 pathway might be upregulated upon infection. This raises the possibility that these
382 hydrocarbons are evolutionarily conserved sickness cues in Hymenopteran social
383 insects. Such cues may have evolved into general sickness signals in social insects as
384 they alert nestmates to the presence of an infection that will harm the colony if it
385 spreads [48]. Similar to the "find-me/eat-me" signals expressed by infected cells in a
386 body [49,50], they will be selected for as they enhance colony fitness by preventing a
387 systemic infection. Hence, altruistic displays of sickness can evolve in
388 superorganisms, even if this results in the destruction of the individual that expresses
389 them.
390
391 It is well established that social insects use glandular secretions with antimicrobial
392 properties as external surface disinfectants [51]. However, because these compounds
393 can also harm the host, they should be used with caution inside the colony. For
394 example, the acidic poison *L. neglectus* and other Formicine ants produce is extremely
395 caustic and is used to attack conspecifics [22,33,52]. During sanitary care they apply
396 this poison via grooming because it is probably more accurate and less wasteful than
397 spraying [22]. Moreover, as pathogen-exposed insects typically survive when they

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398 receive sanitary care [20–23], conservatively applying the poison may also reduce the
399 damage it causes to individuals that can then continue contributing to the colony. This
400 is supported by our observation that *L. neglectus* will apply large quantities of poison
401 onto pupae only when they become infected. Remarkably, we found that, in addition
402 to being external disinfectants, ants use antimicrobial secretions as internal
403 disinfectants against infections within the bodies of nestmates. Since infected pupae
404 are moribund there is no risk that the ants' poison is harming individuals with a future
405 role in the colony. Taken together, these observations suggest that ants adjust their
406 behaviours in response to the risk presented to the colony. It would be interesting to
407 explore further how social immunity defences are regulated to prevent collateral
408 damage, or 'social immunopathology', within the colony.
409
410 Our experiments show that destructive disinfection was highly effective and
411 prevented 95% of infections becoming transmissible. Destructive disinfection will
412 thus keep the average number of secondary infections caused by an initial infection
413 small and the disease will die out within the colony [3]. This may explain why
414 infections of *Metarhizium* and other generalist entomopathogenic fungi like
415 *Beauveria*, though common in the field [53–56], do not seem to cause colony-wide
416 epidemics in ants, but are more numerous in solitary species that lack social immunity
417 [57–59]. Behaviours like destructive disinfection that are able to reduce pathogen
418 fitness to zero could have selected for host manipulation in fungi that specialise on
419 infecting ants, e.g. *Ophiocordyceps* and *Pandora* [60-62]. These fungi force their ant
420 hosts to leave the nest and climb plant stems near foraging trails. There they die and
421 become infectious, releasing new spores that infect ants foraging below. However,
422 ants infected with *Ophiocordyceps* that were experimentally placed back into the nest

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423 disappeared [61]. Our study suggests that these ants could have been eliminated
424 through destructive disinfection. Consequently, ant-specialist fungi like
425 *Ophiocordyceps* and *Pandora* may have evolved host manipulation as a means to
426 complete their lifecycle outside of the nest and avoid destructive disinfection [61,62].
427 In contrast to specialists, generalist pathogens like *Metarhizium* infect a broad range
428 of solitary and social hosts, making it less likely that they evolve strategies to escape
429 social immunity defences [63]. Future work that investigates how social immunity
430 disrupts typical host-pathogen dynamics will shed light on the co-evolution of
431 pathogens and their social hosts [3].
432
433 Destructive disinfection has probably evolved in ants because the removal of corpses
434 from the colony alone does not guarantee that disease transmission is prevented [61].
435 This is because ants place corpses onto midden (trash) sites that are located inside or
436 outside near the nest and regularly visited by midden workers [64–66]. Consequently,
437 midden sites represent a potential source for disease transmission back into the
438 colony. In contrast to ants, honeybees have no middens and corpses are dumped
439 randomly outside of the hive [15]. But because honeybees forage on the wing, it is
440 unlikely that corpses are re-encountered and so removal is sufficient to prevent
441 disease transmission [67]. Termites on the other hand perform a different behaviour,
442 whereby the dead are cannibalised [19,68]. Cannibalism is effective because the
443 termite gut neutralises ingested pathogens [69–71] and has likely evolved because
444 dead nestmates are a source of valuable nitrogen in their cellulose-base diet [72]. The
445 same selective pressure has driven this suite of independently evolved innovations –
446 the need to eliminate or remove infected individuals early in the infectious cycle –
447 with the ants expressing a particularly complex behavioural repertoire. This seems to

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448 be a general principle in disease defence as cells are also rapidly detected and
449 destroyed shortly after infection to prevent pathogen spread in multicellular organisms
450 [17]. Understanding how natural selection can result in similar traits at different levels
451 of biological organisation and in organisms with different life histories is a central
452 question in evolutionary biology [8]. Studying the similarities and differences
453 between organismal immunity and social immunity could therefore lead to new
454 insights about how disease defences evolve [17]. For example, the results of our study
455 suggest that equivalent selection pressures can result in convergent defences that
456 protect multicellular organisms and superorganismal insect societies from systemic
457 disease spread. Future work that can link the performance of social immunity
458 defences to colony fitness will therefore provide useful insights into how such traits
459 are selected for over evolutionary time.
460
461 **Materials and Methods**
462 **Ant host**
463 We studied the unicolonial invasive garden ant, *Lasius neglectus*, collected in Seva,
464 Spain (41.809000, 2.262194) [55]. Stock colonies were kept at a constant temperature
465 of 23°C with 70% humidity and a day/night cycle of 14/10 h. All experiments were
466 conducted in plastered petri dishes (Ø = 33, 55 or 90 mm) with 10% sucrose solution
467 provided *ad libitum* and environmental conditions were controlled throughout (23°C;
468 70% RH; 14/10 h light/dark cycles). Care of animals was in accordance with
469 institutional guidelines.
470
471 **Fungal pathogen**

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472 As a model pathogen, we used the entomopathogenic fungus *Metarhizium brunneum*
473 (strain MA275, KVL 03-143). Multiple aliquots were kept in long-term storage at –
474 80°C. Prior to each experiment the conidiospores were grown on sabaroud dextrose
475 agar at 23°C until sporulation and harvested by suspending them in 0.05% sterile
476 Triton X-100 (Sigma). The germination rate of conidiospore suspensions was
477 determined before the start of each experiment and was > 90% in all cases.
478
479 **Pupal pathogen exposure**
480 Conidiospores were applied in a suspension of 0.05% autoclaved Triton-X 100 at 10^6
481 conidia/ml in all experiments unless otherwise stated. Throughout the study, we used
482 cocooned worker pupae of approximately the same age, which was determined by
483 assessing the melanisation of the eyes and cuticle. Single pupae were exposed by
484 gently rolling them in 1 µl of the conidiospore suspension using sterile soft forceps.
485 Pupae were then allowed to air dry for 5-10 min before being used in experiments.
486 This exposure procedure resulted in pupae receiving ~ 1800 conidiospores, of which
487 5% (~ 95 conidiospore) passed through the cocoon and came into contact with the
488 pupa inside (Fig S1).
489
490 **Statistical Analysis**
491 Statistical analyses were carried out in R version 3.3.2 [73]. All statistical tests were
492 two-tailed. General(ised) linear and mixed models were compared to null (intercept
493 only) and reduced models (for those with multiple predictors) using Likelihood Ratio
494 (LR) tests to assess the significance of predictors [74]. We controlled for the number
495 of statistical tests performed per experiment to protect against a false discovery rate
496 using the Benjamini-Hochberg procedure ($\alpha = 0.05$). Moreover, all post hoc analyses

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497 were corrected for multiple testing using the Benjamini-Hochberg procedure (α =
498 0.05) [75,76]. We checked the necessary assumptions of all tests i.e. by viewing
499 histograms of data, plotting the distribution of model residuals, checking for non-
500 proportional hazards, testing for unequal variances, testing for the presence of
501 multicollinearity, testing for over-dispersion, and assessing models for instability and
502 influential observations. For mixed effects modelling, we used the packages 'lme4' to
503 fit models [77], 'influence.ME' to test assumptions [78], and, for LMERs, 'lmerTest'
504 to obtain *P* values [79]. All logistic regressions were performed using either
505 generalised linear models (GLMs) or generalised linear mixed models (GLMMs),
506 which had binomial error terms and logit-link function. The Cox proportional hazards
507 regression was carried out using the 'coxphf' package with post hoc comparisons
508 achieved by re-levelling the model and correcting the resulting *P* values [80]. For
509 Kruskal-Wallis (KW) tests and subsequent post hoc comparisons we used the
510 'agricolae' package, which implements the Conover-Iman test for multiple
511 comparisons using rank sums [81]. For the perMANOVA, we used the package
512 'vegan' and performed pairwise perMANOVAs for post hoc comparisons [82]. All
513 other post hoc comparisons were performed using the 'multcomp' package [83].
514 Finally, all graphs were made using the 'ggplot2' package [84]. Individual
515 descriptions of statistical analyses are given for all experiments below.
516

517 **Unpacking behaviour**

518 To study how ants respond to infections, we exposed pupae to a low (10^4/ml),
519 medium (10^6/ml) or high (10^9/ml) dose of conidiospores or autoclaved Triton X as a
520 sham control (sham control, n = 24; all other treatments, n = 25). The pupae were
521 then placed into individual petri dishes with two ants and inspected hourly for 10 h/d

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522 for 10 d. When the ants unpacked a pupa, it was removed and surface-sterilised [85]
523 to ensure that any fungal outgrowth was the result of internal infections and not
524 residual conidiospores on the cuticle. After sterilisation, we transferred the pupae to a
525 petri dish lined with damp filter paper at 23°C and monitored them for 2 weeks for
526 *Metarhizium* sporulation to confirm the presence of an internal infection (low dose, n
527 $= 8$; medium dose, $n = 18$; high, $n = 21$). In addition, any cocooned pupae that were
528 not unpacked after 10 d were removed from the ants, surface sterilised and observed
529 for sporulation, as above (low dose, $n = 11$; medium dose, $n = 4$; high, $n = 4$). We
530 analysed the effect of treatment on unpacking using a Cox proportional hazards model
531 with Firth's penalized likelihood, which offers a solution to the monotone likelihood
532 caused by the complete absence of unpacking in the sham control treatment. We
533 followed up this analysis with post hoc comparisons (model factor re-levelling) to test
534 unpacking rates between treatments (Fig 1B). We compared the number of unpacked
535 and cocooned pupae sporulating using a logistic regression, which included pupa type
536 (cocooned, unpacked), conidiospore dose (low, medium, high) and their interaction as
537 main effects. The interaction was non-significant (GLM: LR $\chi^2 = 5.0$, df = 2, $P =$
538 0.084); hence, it was removed to gain better estimates of the remaining predictors.
539

540 **Images and scanning electron micrographs (SEMs) of destructive disinfection**

541 Photographs of destructive disinfection were captured (Nikon D3200) and
542 aesthetically edited (Adobe Photoshop) to demonstrate the different behaviours (Fig
543 1A). They were not used in any form of data acquisition. We also made representative
544 SEMs of a pupa directly after unpacking and one after destructive disinfection (24 h
545 after unpacking; Fig 1F-G). As the pupae were frozen at – 80ºC until the SEMs were

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546 made, we also examined non-frozen pupae taken directly from the stock colony and
547 confirmed that freezing itself does not cause damage to the pupa (not shown).
548

549 **Comparison of sanitary care and destructive disinfection behaviours**

550 To observe how the behavioural repertoire of the ants changes between sanitary care
551 and destructive disinfection, we filmed three individually colour-marked ants tending
552 a single pathogen-exposed pupa with a USB microscope camera (Di-Li ® 970-O). To
553 characterise the sanitary care behaviours of the ants, we analysed the first 24 h of the
554 videos following the introduction of the pupa. To study destructive disinfection
555 behaviours, we analysed the 24 h period that immediately followed unpacking.
556 Videos were analysed using the behavioural-logging software JWatcher™ [86]. For
557 each ant (n = 15), we recorded the duration of its grooming bouts, the frequency of
558 poison application and the frequency of biting. Grooming duration was analysed using
559 a LMER, having first log-transformed the data to fulfil the assumption of normality
560 (Fig 1C). The frequency of poison application and biting (Fig 1D-E) were analysed
561 using separate GLMMs with Poisson error terms for count data and logit-link
562 function. We included an observation-level random intercept effect to account for
563 over-dispersion in the data [87]. In all three models, we included petri dish identity as
564 a random intercept effect because ants from the same dish are non-independent.
565 Additionally, a random intercept effect was included for each ant as we observed the
566 same individuals twice (before and after unpacking).
567

568 **Chemical bioassay**

569 We determined whether ants detect infected pupae through potential changes in the
570 pupae's cuticular chemical profile. We established internal infections in pupae by

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571 exposing them to the pathogen and leaving them for 3 d in isolation. In pilot studies,
572 approx. 50% of these pupae were then unpacked within 4 h of being introduced to
573 ants. After 3 d, pupae were washed for 2.5 min in 300 µl of either pentane solvent to
574 reduce the abundance of all CHCs present on the pupae (n = 28), or in autoclaved
575 water as a handling control (n = 28). After washing, pupae were allowed to air dry on
576 sterile filter paper. Additionally, non-washed pupae were used as a positive control (n
577 = 30). Pupae were placed individually with a pair of ants in petri dishes and observed
578 for unpacking for 4 h. We used GC–MS (see below for methodology) to confirm that
579 washing was effective at removing cuticular compounds, by comparing the total
580 amount of chemicals present on pupae washed in pentane to non- and water-washed
581 pupae (n = 8 per treatment; Fig S4). The number of pupae unpacked between the
582 different treatments was analysed using a logistic regression (Fig 2A). As several
583 researchers helped to wash the pupae, we included a random intercept for each person
584 to control for any potential handling effects. Additionally, the experiment was run in
585 two blocks on separate days, so we included a random intercept for each block to
586 generalise beyond any potential differences between runs. The total peak area from
587 the GC–MS analysis was compared between treatments using a KW test with post hoc
588 comparisons.
589

590 **Chemical analysis of pupal hydrocarbon patterns**

591 To confirm that infected pupae had chemical profiles that are different from pathogen-
592 exposed cocooned and control pupae, we exposed pupae to the pathogen or a sham
593 control. Pupae were then isolated for 3 d to establish infections in the pathogen-
594 exposed treatment (as above). Following isolation, pupae were individually placed
595 with ants and observed for unpacking for 4 h. Unpacked pupae were immediately

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596 frozen at – 80 °C with the removed cocoons (*n* = 13) and we also froze cocooned
597 pathogen-exposed pupa that had not yet been unpacked (*n* =10). Furthermore, we
598 froze a pair of control pupae, of which one was cocooned (*n* = 12), whilst the other
599 was first experimentally unpacked (to test if the cocoon affects cuticular compound
600 extraction; *n* =12). Cuticular chemicals were extracted from individual pupae and
601 their cocoons in glass vials (Supelco; 1.8 ml) containing 100 µl n-pentane solvent for
602 5 min under gentle agitation. The vials were then centrifuged at 3000 rpm for 1 min to
603 spin down any fungal conidiospores that might be remaining, and 80 µl of the
604 supernatant was transferred to fresh vials with 200 µl glass inserts and sealed with
605 Teflon faced silicon septa (both Supelco). The pentane solvent contained four internal
606 standards relevant for our range of hydrocarbons (C_{27} – C_{37}); n-Tetracosane, n-
607 Triacontane, n-Dotriacontane and n-Hexatriacontane (Sigma Aldrich) at 0.5 µg/ml
608 concentration, all fully deuterated to enable spectral traceability and separation of
609 internal standards from ant-derived substances. We ran extracts from the different
610 groups in a randomised manner, intermingled with blank runs containing only
611 pentane, and negative controls containing the pentane plus internal standards (to
612 exclude contaminants emerging e.g. from column bleeding), on the day of extraction,
613 using GC–MS (Agilent Technologies; GC7890 coupled to MS5975C).
614
615 A liner with one restriction ring filled with borosilicate wool (Joint Analytical
616 Systems) was installed in the programmed temperature vaporisation (PTV) injection
617 port of the GC, which was pre-cooled to -20 °C and set to solvent vent mode. 50 µl of
618 the sample extractions were injected automatically into the PTV port at 40 µl/s using
619 an autosampler (CTC Analytics, PAL COMBI-xt; Axel Semrau, CHRONOS 4.2
620 software) equipped with a 100 µl syringe (Hamilton). Immediately after injection, the

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

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646 question were calculated based on those standards. To further aid identification, we
647 separated the substances based on polarity using solid phase extraction fractionation.
648 For this purpose, pools of 20 pupae were extracted in 500 µl n-pentane containing 0.2
649 µg/ml internal standard, and separated on unmodified silica cartridges (Chromabond
650 ® SiOH, 1ml, 100 mg) based on polarity. Prior to use, the cartridges were conditioned
651 with 1 ml dichloromethane followed by 1 ml n-pentane. The entire extraction volume
652 was loaded onto the silica and the eluent (fraction 1, highly apolar phase) collected. A
653 wash with 1 ml pure n-pentane was added to fraction 1. Fraction 2 contained all
654 substances washed off the silica with 1 ml 25 % dichloromethane in n-pentane, and
655 finally a pure wash with 1 ml dichloromethane eluted all remaining substances
656 (fraction 3). The polarity thus increased from fraction 1 through 3, but no polar
657 substances were found. All fractions were dried under a gentle nitrogen stream and re-
658 suspended in 70 µl n-pentane followed by vigorous vortexing for 45 s. GC–MS
659 analysis of all fractions was performed in electronic ionisation mode under the same
660 chromatographic conditions as before.
661
662 To quantify the relative abundances of all compounds found on each pupa, analyte-
663 characteristic quantifier and qualifier ions were used to establish a method enabling
664 automatized quantification of their integrated peak area relative to the peak area of the
665 closest internal standard. For each analyte, the relative peak area was normalised, i.e.
666 divided by the total sum of all relative peak areas of one pupa, to standardise all pupa
667 samples. Only analytes, which normalised peak area contributed more than 0.05% of
668 the total peak area, were included in the statistical analysis. We compared the
669 chemical profiles of the pupae using a perMANOVA analysis of the Mahalanobis
670 dissimilarities between pupae, with post hoc perMANOVA comparisons. Since there

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671 was no difference between cocooned and unpacked control pupae we combined them
672 into a single control group for the final analysis (perMANOVA: $F = 1.09$, df = 23, P
673 = 0.1). We also performed a discriminant analysis of principle components (Fig 2B)
674 to characterise the differences between the pupal treatments [88,89]. To identify the
675 compounds that differ between treatments, we performed a conditional random forest
676 classification (n trees = 500, n variables per split = 4) [88,90,91]. Random forest
677 identified 9 compounds that were important in classifying the treatment group, of
678 which 8 were significant when analysed using separate KW tests (results for
679 significant compounds in Table S2). We followed up the KW tests with individual
680 post hoc comparisons for each significant compound (Fig 2C-F, post hoc comparisons
681 in Table S2).
682
683 **Effect of destructive disinfection on pathogen replication**
684 To test if destructive disinfection prevents *Metarhizium* from successfully replicating,
685 we kept single pathogen-exposed pupae in petri dishes containing groups of 3 or 8
686 ants. This allowed us to assess how group size affects the likelihood of fungal
687 inhibition. For the following 10 d, we observed the pupae for unpacking. When a
688 pupa was unpacked, we left it with the ants for a further 1 or 5 d so that they could
689 perform destructive disinfection. This allowed us to assess how the duration of
690 destructive disinfection affects the likelihood of fungal inhibition. The destructively
691 disinfected pupae were then removed and placed into petri dishes on damp filter paper
692 at 23 ºC (8 ants 1 d and 5 d, $n = 22$ pupae each; 3 ants 1 and 5 d, $n = 18$ pupae each).
693 We did not surface sterilise the pupae as this might have interfered with the
694 destructive disinfection the ants had performed. Removed pupae were observed daily
695 for *Metarhizium* sporulation for 30 d. To determine how many pupae sporulate in the

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696 absence of destructive disinfection, we kept pathogen-exposed pupae without ants as a
697 control and recorded the number that sporulated for 30 d (n = 25). We compared the
698 number of pupae that sporulated after 1 d, 5 d and in the absence of ants using logistic
699 regressions and Tukey post hoc comparisons, separately for the two ant group sizes
700 (Fig 3A, Fig S6).
701

702 ***In vitro* investigation of destructive disinfection**

703 We examined the individual effects of unpacking, biting and poison application on
704 destructive disinfection by performing these behaviours *in vitro*. Pathogen-exposed
705 pupae were initially kept with ants so that they could perform sanitary care. After 3 d,
706 we removed the pupae and split them up into three groups: (i) pupae that we left
707 cocooned, (ii) experimentally unpacked and (iii) experimentally unpacked and bitten.
708 We simulated the damage the ants achieve through biting by damaging the pupal
709 cuticle and removing their limbs with forceps. The pupae were then treated with either
710 synthetic ant poison (60% formic acid and 2% acetic acid, in water; applied at a dose
711 equivalent to what ants apply during destructive disinfection; Fig S8) or autoclaved
712 distilled water as a control, using pressurised spray bottles (Lacor) to evenly coat the
713 pupae in liquid. The pupae were allowed to air dry for 5 min before being rolled over
714 and sprayed again and allowed to dry a further 5 min. All pupae were then placed into
715 separate petri dishes and monitored daily for *Metarhizium* sporulation (cocooned +
716 poison, n = 24; unpacked + poison + biting, n = 24; all other treatments, n = 25). The
717 number of pupae sporulating was analysed using a logistic regression with Firth's
718 penalised likelihood, which offers a solution to the monotone likelihood caused by the
719 complete absence of sporulation in one of the groups (R package 'brglm' [92]). Pupal
720 manipulation (cocooned/unpacked only/unpacked and bitten), chemical treatment

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721 (water or poison) and their interaction were included as main effects (Fig 3B, Fig S7).
722 We followed up this analysis with Tukey post hoc comparisons (Table S3).
723

724 **Disease transmission from infectious and destructively disinfected pupae**

725 We tested the impact of destructive disinfection on disease transmission within groups
726 of ants by keeping them with sporulating pupae or pupae that had been destructively
727 disinfected. Infections were established in pupae (as above) and half were allowed to
728 sporulate ($n = 11$), whilst the other half were experimentally destructively disinfected
729 (as above; $n = 11$). Pupae were then kept individually with groups of 5 ants in mini-
730 nests (cylindrical containers [Ø = 90 mm] with a second, smaller chamber covered in
731 red foil [Ø = 33 mm]). Ant mortality was monitored daily for 30 d. Dead ants were
732 removed, surface sterilised and observed for *Metarhizium* sporulation. The number of
733 ants dying from *Metarhizium* infections in each treatment was compared using a
734 logistic regression (Fig 4A). Mini-nest identity was included as a random intercept
735 effect as ants from the same group are non-independent.
736

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750

751 **Competing financial interests**

752 The authors declare no competing financial interests.

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