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## Defensive behavior is linked to altered surface chemistry following infection in a termite society

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1 Defensive behavior is linked to altered surface chemistry following infection in a  
2 termite society

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16

17 **Abstract**

18 The care-kill response determines whether a sick individual will be treated or eliminated from an insect  
19 society, but little is known about the underpinnings of this process. We exploited the stepwise  
20 infection dynamics of an entomopathogenic fungus in a termite to explore how care-kill transitions  
21 occur, and identify the chemical cues behind these shifts. We found collective responses towards  
22 pathogen-injected individuals to vary according to severity and timing of pathogen challenge, with  
23 elimination (via cannibalism) occurring sooner in response to a severe active infection. However,  
24 injection with inactivated fungal blastospores also resulted in increased albeit delayed cannibalism,  
25 even though injection did not universally cause host death. This indicates that the decision to eliminate  
26 an individual is triggered before pathogen viability or even terminal disease status is known. We then  
27 compared the surface chemistry of differently challenged individuals, finding increased amounts of  
28 long-chained methyl-branched alkanes with similar branching patterns in individuals injected with  
29 both dead or viable fungal blastospores, with the latter showing the largest increase. This coincided  
30 with the highest amounts of observed cannibalism as well as signs of severe moribundity. Our study  
31 provides new mechanistic insight into the emergent collective behaviours involved in the disease  
32 defence of a termite society.

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## 37 Introduction

38 The evolutionary and ecological success of social insects is widely appreciated. Although sociality offers  
39 many benefits, there are also associated costs<sup>1</sup>. On the one hand, cooperation facilitates division of  
40 labour, enabling the optimization of tasks such as brood care, foraging, nest building, information  
41 sharing and defense<sup>2-4</sup>. On the other hand, densely populated and closely related individuals inside a  
42 confined nest environment are at risk of disease transmission<sup>5,6</sup>. The risk of repeated pathogen  
43 exposures have shaped the evolution of defence mechanisms across insect societies<sup>7</sup>. Social insect  
44 individuals are not only able to fight infections through their own individual immune systems<sup>8,9,10</sup>, but  
45 also via social immune defenses<sup>2,3,11</sup>. Social immunity can be viewed as a distributed organ, similar to  
46 the immune system of metazoan animals<sup>12</sup> that builds on two principal adaptive pillars – behaviour  
47 and physiology – that together act to prevent the entrance, establishment, and spread of pathogens  
48 in a colony<sup>2,4,6,9,13,14</sup>.

49 Social insect groups, such as Hymenoptera (social bees, wasps, and ants) and Isoptera (termites)  
50 contain multiple lineages of species with advanced social living, often referred to as superorganisms<sup>15</sup>.  
51 Studies in these social groups have demonstrated the range of collective disease defenses that can be  
52 deployed against infected individuals<sup>3,16-20</sup>. Infected individuals can be groomed, which in combination  
53 with the use of antimicrobial secretions, serve to cure or rescue infected individuals and reduce the  
54 likelihood of internal infection. In contrast, once an internal infection is successfully established, it can  
55 pose a threat to the colony and may need to be eliminated from the colony<sup>3,6,17,21</sup>. Such radically  
56 different responses towards infected individuals have been described in terms of a care-kill  
57 dichotomy<sup>6,21,22</sup>. Allogrooming is one of the first lines of defense against external pathogens, while  
58 elimination typically occurs at a later stage<sup>17,19,23-25</sup>. Both strategies are thought to reduce the risk of  
59 disease outbreak and enhance colony survival<sup>3,6</sup>, but the latter imposes an additional cost to the colony  
60 through the loss of workforce members, depending on worker age as well as colony size and maturity.

61 Collective disease defenses are known to vary within and between species and across different levels  
62 of social organization<sup>3,12,26,27</sup>. Detection of pathogens and the regulation of the collective response  
63 depends on the ability of social organisms to accurately communicate disease-related information  
64 through behavioural or chemical cues<sup>6,28</sup>. Chemical communication is thought to play a central role in  
65 social immunity<sup>6</sup>. For instance, in the ant *Lasius neglectus*, destructive disinfection behaviours are  
66 employed, whereby ants initially detect fungus-infected pupae via sickness cues emitted by the pupae,  
67 followed by elimination<sup>19</sup>. Signalling and chemical communication in social insects are often mediated  
68 by cuticular hydrocarbons (CHCs)<sup>29</sup>. CHCs are the most abundant class of chemicals coating the cuticle  
69 of terrestrial insects and have a key role in inter- and intraspecific communication<sup>30,31</sup> potentially also  
70 serving to discriminate between healthy and sick nestmates of a colony<sup>29</sup>. CHC profiles can be altered

71 by activation of the immune system<sup>29,32-34</sup> and altered CHC profiles have been implicated as signals to  
72 eliminate infected individuals in ants and honeybees<sup>19,29,32,35</sup> in a manner analogous to body cells  
73 signalling their status to the immune system<sup>36</sup>.

74 Termites also employ several collective mechanisms to prevent disease outbreaks, largely using two  
75 complementary behaviours: allogrooming and cannibalism, reflecting the care-kill strategy mentioned  
76 above. The switch in the response depends on the progression of the infection, with cannibalism  
77 occurring when infected individuals display signs of overt sickness, such as slow movement<sup>17</sup>.  
78 Application of antimicrobial secretions, in addition to behavioural responses, can effectively remove  
79 external pathogens from the cuticle of infected individuals and inhibit pathogen growth during the  
80 early-stage of infection<sup>23,37-44</sup>. However, when a pathogen successfully initiates a potentially lethal  
81 internal infection<sup>45</sup>, such weak<sup>46</sup> or moribund but not yet dead<sup>47</sup> individuals are typically cannibalized.  
82 Cannibalism is thought to be ancestral to all termites, which may have evolved as a prophylactic  
83 mechanism against disease from an initial role in nitrogen recycling<sup>48</sup>. But the basis of the switch  
84 between social immune behaviours in termites remains largely unknown, and it is unclear what signals  
85 or chemical cues trigger elimination behaviours or to what extent they are linked with factors  
86 associated with moribundity<sup>17</sup>.

87 To address these gaps in our knowledge, we investigated how termites detect and respond to internal  
88 infection upon injection of either viable or dead blastospores of a generalist fungal pathogen. Fungal  
89 blastospores, which represent a within-host infectious form, play an important role in pathogenesis  
90 during disease development and their experimental injection can elicit a robust immune response<sup>49-52</sup>.  
91 Blastospores are a vegetative cell state that can quickly multiply and colonize the insect body or a  
92 nutrient-rich liquid culture<sup>53-55</sup>. Furthermore, blastospores reflect an intermediate stage in the  
93 infection process, which begins with conidia adhesion and germination on the insect cuticle, followed  
94 by cuticular penetration via appressoria to colonize the host's body. Within the host, the fungus  
95 proliferates as single-celled blastospores or hyphal bodies in the hemolymph ultimately killing the  
96 infected insect<sup>45,53,56,57</sup>. During infection, virulence factors are synthesized, such as destruxins  
97 (cyclodepsipeptidic mycotoxins) and even host miRNAs can be manipulated to inactivate the host and  
98 assist in avoidance of immune clearance<sup>49,52,54,58-60</sup>.

99 Here, we combine blastospore infection and behavioural experiments with chemical analyses of  
100 termite CHC profiles and fungal destruxins to expand our understanding of termite social immunity.  
101 Our rationale is that to better study the care-kill switch, it is useful to separate direct external pathogen  
102 cues from indirect host-mediated cues, allowing one to better investigate how termites detect and  
103 respond to an internal pathogen challenge. We explore how defensive behaviours are triggered by  
104 experimentally varying internal pathogen challenges, and correlate these with alterations to termite

105 surface chemistry. Our study sheds new light on a key collective defensive mechanism of termite  
106 societies.

## 107 **Results**

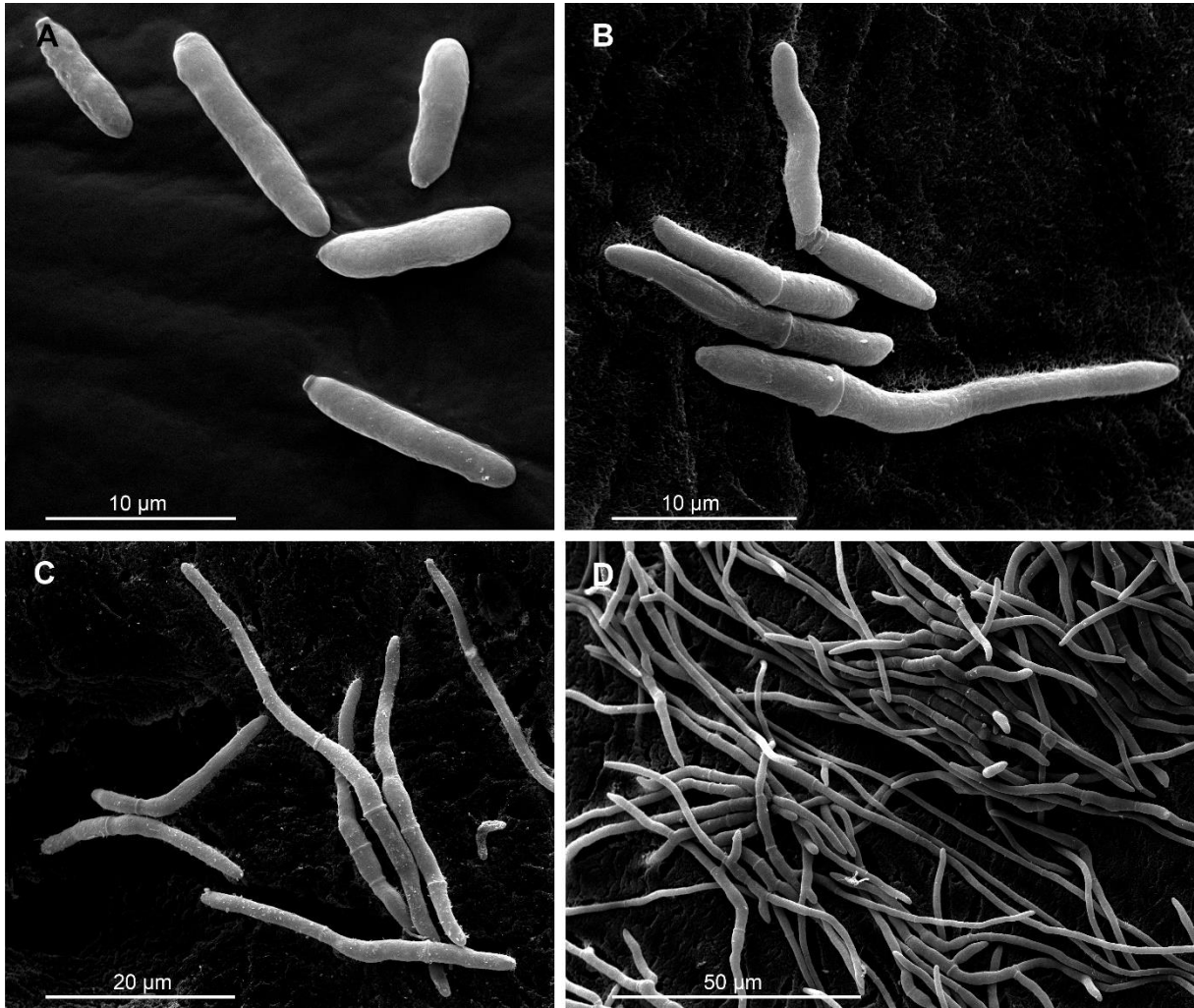
### 108 **Blastospores germinate quickly and synthesize destruxins**

109 We applied *Metarhizium robertsii* blastospores via injection to exclude the presence of external  
110 pathogen cues (cuticular conidiospore exposure) from internal host activity (infection initiation). Prior  
111 to injection experiments, we examined rates of blastospore germination. We used a scanning electron  
112 microscope (SEM) to quantify germination (Figure 1A, B, C, D) on potato dextrose agar (PDA) plates.  
113 Germination was observed as early as 4 hours after inoculation, as evidenced by the formation of germ  
114 tubes (Figure 1B, Supplementary Figure S1). The earliest time reported in the literature is 2.7 hours in  
115 the unrelated fungus *Isaria fumosorosea*<sup>61</sup>. At 8 hours, germination and elongation of germ tubes  
116 continued and by 12 hours a thick mass of elongated hyphae, forming the mycelium (Figure 1C, D)  
117 could be observed. Our observed rate of *in vitro* blastospore germination is faster than either *in vitro*  
118 or *in vivo* (on cuticle) conidia germination<sup>17,45</sup> (Supplementary Figure S1, S2). In addition to viable  
119 blastospores, we employed dead blastospores as an injection treatment. We confirmed the efficiency  
120 of inactivation following heat-treatment (autoclaving) by the absence of germination following plating.  
121 The presence of 5 major classes of destruxins: Destruxin A, B, C, D and Ed were also investigated in the  
122 filtered media (CD modified media) of cultured viable *M. robertsii* blastospores (Supplementary Figure  
123 S3). Comparisons of the UHPLC-MS/MS data with the elution order and the MS/MS schemes published  
124 in the literature<sup>62,63</sup> verified the retention times for the respective destruxins found in the  
125 chromatograms, confirming their presence. By contrast, no destruxins were detected in the filtrate  
126 derived from heat-inactivated (dead) blastospores (Supplementary Figure S4), indicating that  
127 destruxins were destroyed by autoclaving.

### 128 **Both viable and dead blastospore injection causes termite death**

129 Before embarking on behavioural experiments in our study species, the eastern subterranean termite  
130 *Reticulitermes flavipes*, we first measured survival of the termite when kept in isolation, in order to  
131 measure treatment lethality in the absence of group dynamics. Ringer-injection had no negative effect  
132 on termite survival, whereas viable blastospores caused 100% mortality by 2.5 days post injection, and  
133 dead blastospores resulted in a plateau at 40% mortality by 5 days post injection (Supplementary  
134 Figure S5), remaining unchanged for 10 days, at which point observations were terminated.

135



137  
 138 **Figure 1.** Germination process of *M. robertsii* 1490 blastospores. **A-D:** Scanning electron micrograph  
 139 (SEM) images of blastospore germination at different time-points post-inoculation *in vitro* (PDA  
 140 media). At 2 h blastospores are not yet germinated (A), while at 4 h, germination of blastospores can  
 141 already be observed (B), which continues to an advanced stage via germ tube elongation at 8 h (C). By  
 142 12 h, elongation of the fungal hyphae has continued, and mycelial networks are now visible (D).

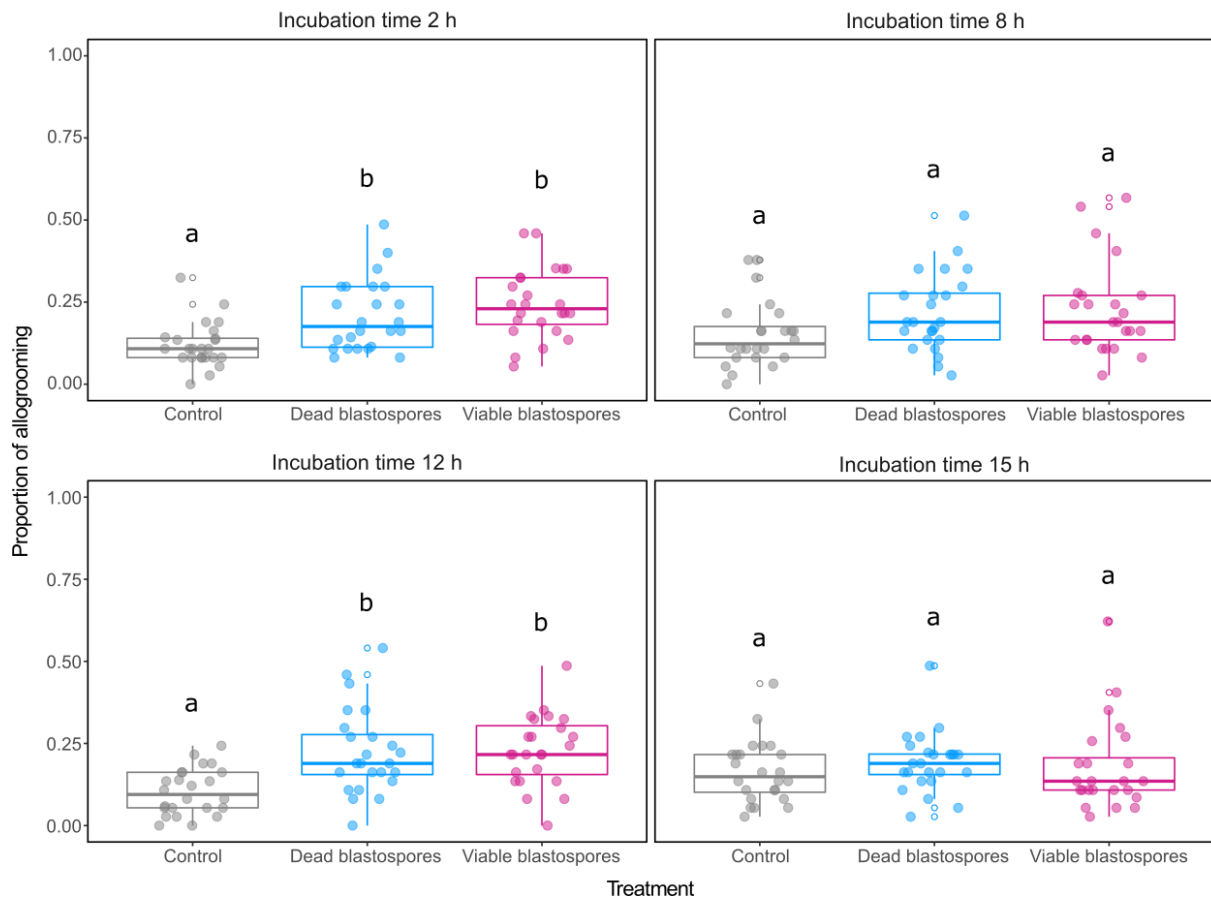
143

#### 144 **Termites slightly increase allogrooming following exposure to injected individuals**

145 In the first behavioural observation experiment, we compared the effects of injection with Ringer's  
 146 solution or blastospores (viable or dead). Injected focal termites were isolated for 2, 8, 12 and 15 hours  
 147 prior to introduction into experimental colonies. Behavioural patterns were then recorded in response  
 148 to Ringer's solution (Ringer), viable (Blastospores+) or dead blastospore (Blastospores-) injected focal  
 149 termites at different stages post-challenge. In general, observed behaviours in response to blastospore  
 150 injected focal termites consisted of low levels of grooming, high amounts of cannibalism, no  
 151 observations of burial (Supplementary Figure S6), as well as other behavioural states unrelated to  
 152 social immunity. Behavioural patterns in the control treatments were characterized mostly by other  
 153 states, low levels of grooming and one incident of cannibalism in the 15 hours incubation time.

154 The overall proportion of allogrooming in both viable and dead blastospore treatments were  
 155 significantly higher than control treatments, except at 8h and 15 hours (Supplementary Table S1) (2h,  
 156 Blastospores- vs Ringer  $z = 3.293$   $P = 0.0030$ , Blastospores+ vs Ringer  $z = 4.448$   $P = < 0.0001$ ; 12h,  
 157 Blastospores- vs Ringer  $z = 4.324$   $P = < 0.0001$ , Blastospores+ vs Ringer  $z = 4.515$   $P = < 0.0001$ ). The low  
 158 proportion of allogrooming observed at the later 15 hour timepoint is linked with a high proportion of  
 159 cannibalism (Figure 2, 3A, Supplementary Table S1, Supplementary Figure S7).

160



161 **Figure 2.** Allogrooming as a proportion of total states across treatments in the blastospore experiment.  
 162 The proportion of allogrooming states depends significantly on treatment. Significant *post*  
 163 *hoc* comparisons are indicated by different letters, derived from separate GLMMs from each time  
 164 point. Note that *post hoc* comparisons do not apply between incubation time points. Lower and upper  
 165 hinges correspond to first and third quartiles. Upper and lower whiskers extend to the largest/smallest  
 166 value if no greater/smaller than 1.5 times the inter-quartile range from the hinge, respectively.  
 167

168 In a follow-up experiment, we compared injection of dead blastospores with injection of an equivalent  
 169 solution filtered to remove blastospore debris (Filtrate). The overall proportion of allogrooming was at  
 170 its highest at 12 hours post-challenge and decreased over time for the two fungal treatments  
 171 (Blastospores- and Filtrate) while remaining unchanged for the control group, with no significant  
 172 differences detected between any of the time points. The overall proportion of allogrooming in both  
 173 dead blastospore and filtrate treatments was significantly higher over control treatments at two  
 174 incubation time points (12h, Blastospores- vs Ringer  $z = 7.636$   $P = 0.0001$ , Filtrate vs Ringer  $z = 5.349$   $P$

175 = 0.0001; 15h, Blastospores- vs Ringer  $z = 6.626$   $P = 0.0001$ , Filtrate vs Ringer  $z = 4.634$   $P = 0.0001$ )  
176 (Supplementary Figure S8). Allogrooming was significantly higher in dead blastospore versus filtrate  
177 treatment at 12 hours but at no other time point (12h, Blastospores- vs Filtrate  $z = -2.510$   $P = 0.0363$ ).  
178 A progressively lower level of grooming corresponded with an increasingly higher proportion of  
179 cannibalism for the 15 and 18 hour time points, with a high number of termites already being  
180 eliminated by 21 hours post infection (Supplementary Table S3 and Supplementary Figure S9, S10).

### 181 **Cannibalism does not completely depend on termite moribundity**

182 In the first experiment, the highest amounts of cannibalism were observed at 15 hours post injection  
183 in the viable blastospores treatment, with cannibalism beginning shortly after introduction of the focal  
184 termite, and completely replacing grooming before the end of the observation period (Supplementary  
185 Figure S6). Interestingly, we observed that nestmates cannibalized dead blastospore-injected focal  
186 individuals as well (Figures 3A, Supplementary Table S2). However, cannibalism occurred sooner and  
187 more frequently when infected focal termites were visibly ill and close to death (i.e. viable blastospores  
188 at 15h). All focal termites were alive at the time of cannibalism (beginning < 24 h after challenge),  
189 tallying with the survival of injected individual termites when kept in isolation, where blastospore-  
190 induced death began between 24-48 h post injection (Supplementary Figure S5). Control focal termites  
191 were left unharmed.

192 In survival analyses, significant differences were detected between viable blastospore and Ringer-  
193 injected control treatments (2h, Blastospores+ vs Ringer  $z = 3.312$   $P = 0.0028$ ; 8h, Blastospores+ vs  
194 Ringer  $z = 3.226$   $P = 0.0038$ ; 12h, Blastospores+ vs Ringer  $z = 2.808$   $P = 0.0150$ ; 15h, Blastospores+ vs  
195 Ringer  $z = 4.822$   $P = < 0.0001$ ). Cannibalism was observed in all dead-blastospore treatments and  
196 comparisons with Ringer-injected controls were also significant for all incubation timepoints (2h,  
197 Blastospores- vs Ringer  $z = 3.114$   $P = 0.0055$ ; 8h, Blastospores- vs Ringer  $z = 2.397$   $P = 0.0497$ ; 12h,  
198 Blastospores- vs Ringer  $z = 2.946$   $P = 0.0097$ ; 15h, Blastospores- vs Ringer  $z = 2.684$   $P = 0.0218$ ) (Figure  
199 3B, and Supplementary Table S2). Except for 1 individual in the 15h/Ringer treatment, cannibalism was  
200 not observed for any Ringer-injected focal termites (Figure 3A, and Supplementary Table S2).

201 In comparisons of viable and dead blastospore-injected treatments, the probability of remaining  
202 unharmed differed significantly only at the 15 hours incubation timepoint (15h, Blastospores+ vs  
203 Blastospores-  $z = 4.054$   $P < 0.0002$ ). At this incubation time point, focal termites infected with viable  
204 blastospores treatment were quickly dismembered, whereas dead blastospore-injected individuals  
205 were cannibalized more slowly, but nonetheless these were completely cannibalized by 24 h after  
206 introduction (Figure 3B). Complete elimination of individuals quantified at 24 hours after introduction  
207 of the focal individual into experimental colonies was significantly higher for both viable and dead  
208 blastospore treatments compared with controls: (time in hours after injection) (26h, Blastospores- vs



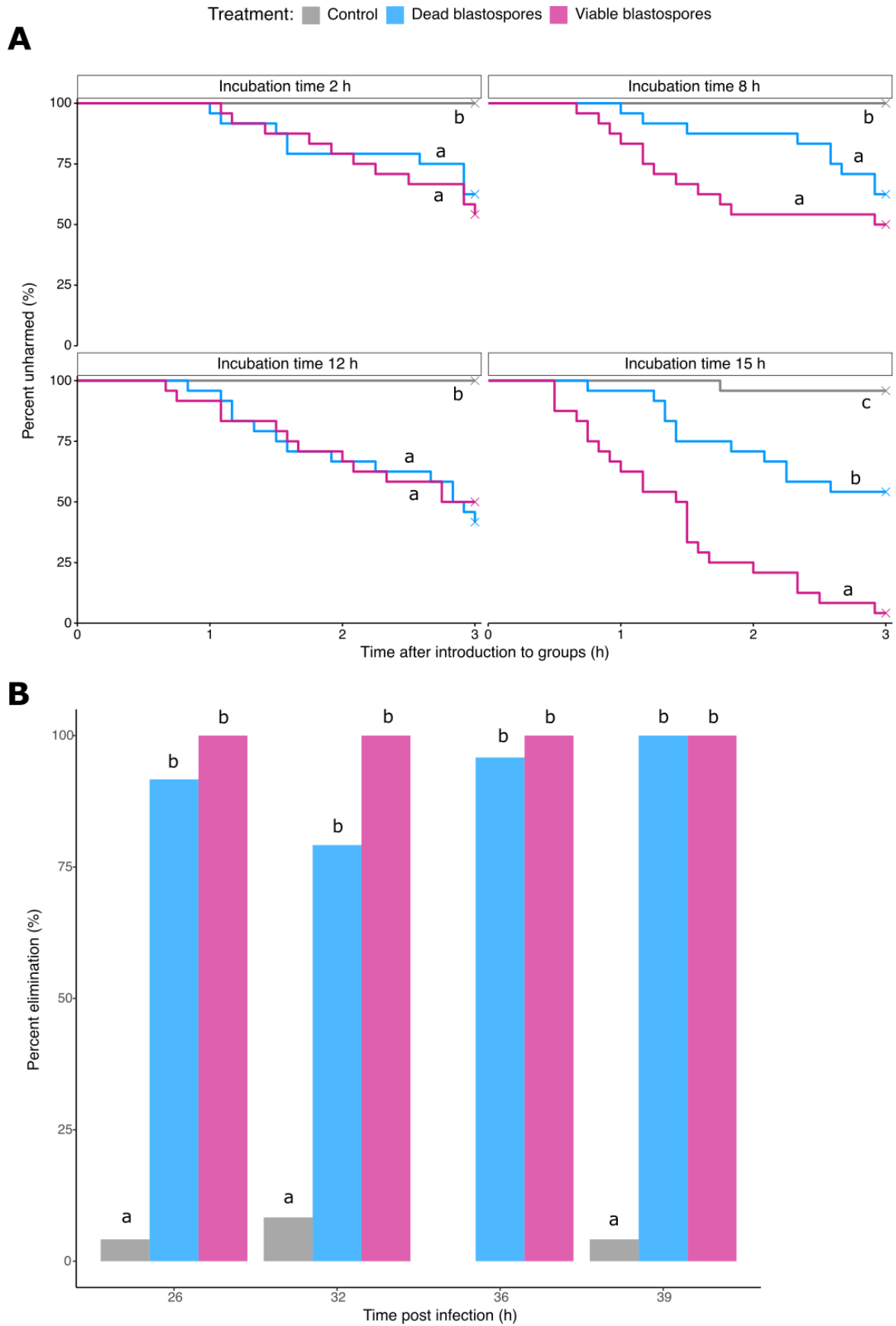
209 Ringer  $P = 0.0032$ , Blastospores+ vs Ringer  $P = 0.0018$ ; 32h, Blastospores- vs Ringer  $P = 0.0044$ ,  
210 Blastospores+ vs Ringer  $P = 0.0023$ ; 36h, Blastospores- vs Ringer  $P = 0.0018$ , Blastospores+ vs Ringer  $P$   
211  $= 0.0012$ ; 39h, Blastospores- vs Ringer  $P = 0.0012$ , Blastospores+ vs Ringer  $P = 0.0012$ ). Close to 100%  
212 of all focal individuals were eliminated in both viable and dead blastospore treatments. This is  
213 contrasted with the survival of the majority of termites following dead blastospore injection, when  
214 kept individually (Supplementary Figure S5). The percentage of eliminated focal individuals quantified  
215 at 24 hours did not significantly differ between viable and dead blastospore-injected individuals (26h,  
216 Blastospores+ vs Blastospores-  $P = 0.5150$ ; 32h, Blastospores+ vs Blastospores-  $P = 0.2244$ ; 36h,  
217 Blastospores+ vs Blastospores-  $P = 1$ ; 39h, Blastospores+ vs Blastospores-  $P = 1$ ) (Figure 3B).

218 In the blastospore filtrate experiment, significantly increased cannibalism was observed in both the  
219 dead blastospore and filtrate-injected treatments compared to the control (Blastospores- vs Ringer  $z$   
220  $= 3.587$   $P = 0.000838$ ; Filtrate vs Ringer  $z = 3.339$   $P = 0.0021$ ) (Supplementary Table S4 and  
221 Supplementary Figure S10). Termites challenged with dead blastospores were generally cannibalized  
222 at a higher rate, although the amount of cannibalism and overall survival probability was not  
223 significantly different between dead blastospore and filtrate injection (Supplementary Table S4).

#### 224 **Moribund termites display altered cuticular hydrocarbon profiles**

225 A total of 90 individual equivalents of CHC extracts from worker pools (3 individuals each) taken from  
226 3 different *R. flavipes* colonies were pre-defined into six groups for a discriminant analysis (DA)  
227 according to the different treatments: 12 and 15 hours after injection of viable and dead blastospores  
228 and Ringer's solution as a control, respectively (see above). Overall differentiation of the CHC profiles  
229 was significant according to the different treatments (Wilk's  $\lambda < 0.05$ ,  $P < 0.05$ ). Discriminant function  
230 1 accounted for 47.36 % and discriminant function 2 for 26.11 % of the total variation, amounting to  
231 73.47 % of total variance explained by the first two functions (Figure 4). Concerning the variations in  
232 specific CHC compounds according to treatments, 15 hours after injection, four methyl-branched  
233 alkanes consistently displayed significantly higher quantities in the viable blastospore treatment versus  
234 the control ( $P < 0.05$ , Benjamini-Hochberg corrected Mann-Whitney U tests, Figure 5). These  
235 compounds were 11-MeC35, 12-MeC36, 11-; 13-MeC37, and 11,15-DiMeC37. Injections with dead  
236 blastospores also led to a visible, albeit non-significant increase in overall quantities of these particular  
237 compounds compared to the controls. An overview of all identified CHCs including their respective  
238 absolute quantities per treatment is given in Table 1.

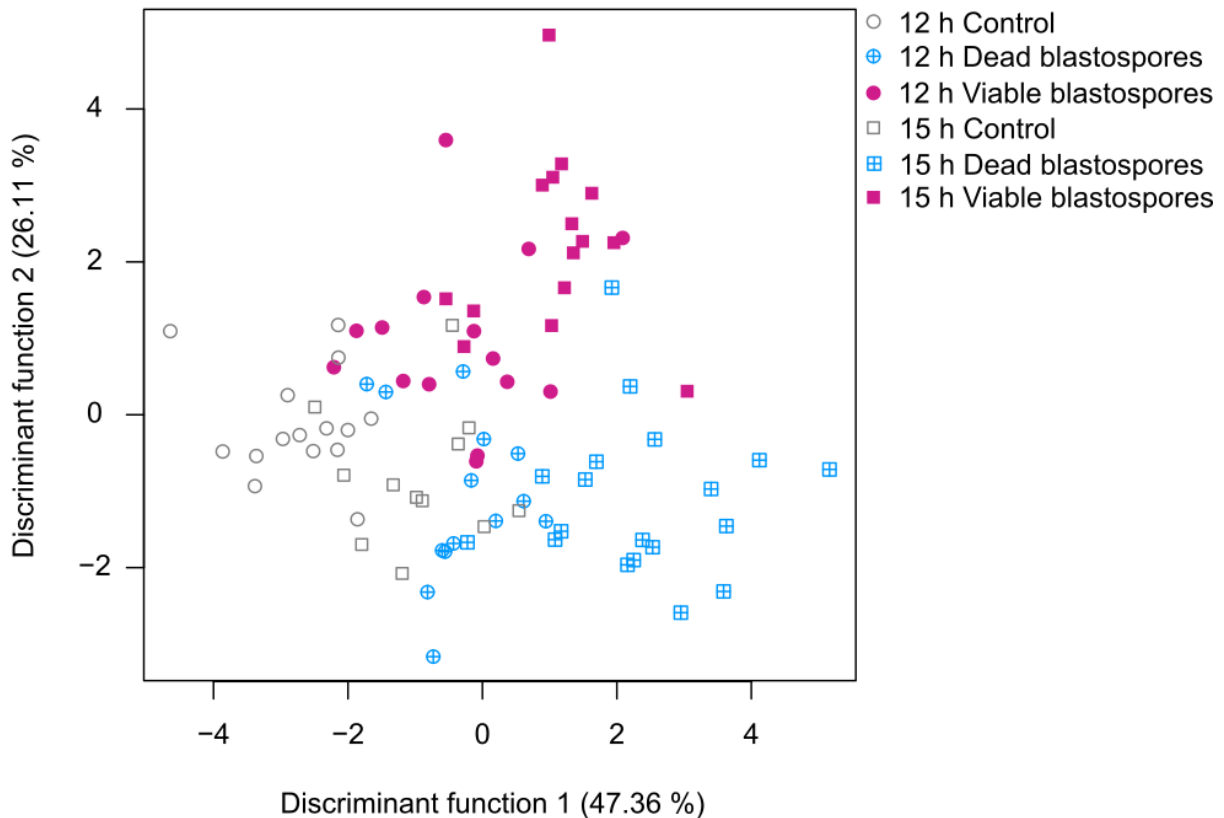
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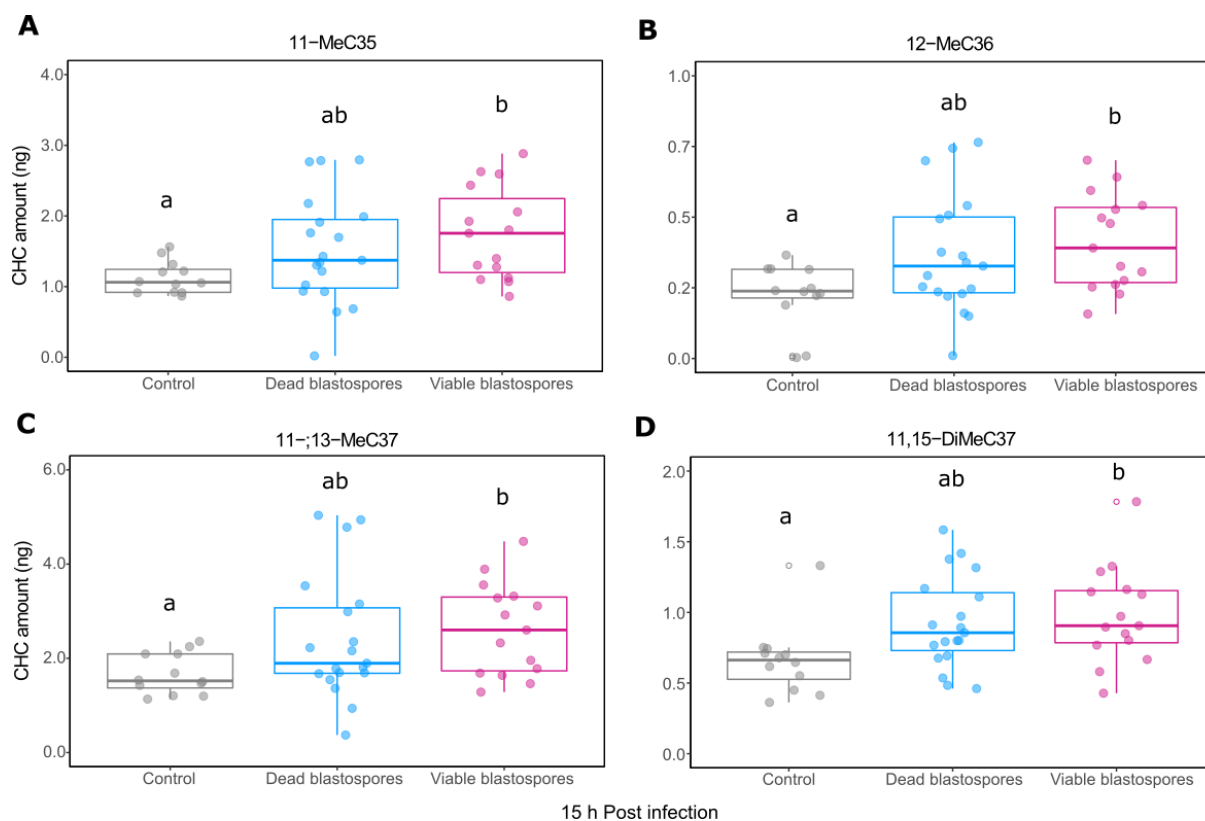
**Figure 3.** Survival of focal termites following introduction into experimental colonies. **A:** Percentage of focal termites that remain unharmed (due to cannibalism) after different incubation times (2, 8, 12, 15 h). Cannibalism states were recorded over a three-hour observation period following introduction into

245 experimental colonies. In both survival analyses, crosses indicate the presence of right-censored data  
 246 (i.e. focal termites that were not dead (A) or harmed (B) during the observation period). Significant  
 247 differences between treatments derived from survival models are indicated by different letters. Note  
 248 that *post hoc* comparisons do not apply between incubation time points. **B:** Percentage of focal  
 249 termites that had been eliminated at 24 h following their introduction into experimental colonies,  
 250 corresponding to 26, 32, 36 and 39 h post-injection for incubation timepoints 2, 8, 12 and 15 h,  
 251 respectively. Note that at equivalent timepoints for dead blastospore-injected termites kept in  
 252 isolation (Supplementary Figure S5), mortality was lower than 20%. Treatments: Grey = Ringer  
 253 (control), Blue = dead blastospores, Pink: viable blastospores.  
 254



255  
 256 **Figure 4.** Linear discriminant analysis (LDA) based on 90 cuticular hydrocarbon (CHC) extracts from  
 257 worker pools (3 individuals each) taken from 3 different *R. flavipes* colonies. The workers were treated  
 258 with viable and dead blastospores, and Ringer solution as a control, and they were freeze-killed 12 or  
 259 15 h after injection. Overall differentiation was significant according to the different treatments (Wilk's  
 260  $\lambda < 0.05$ ,  $p < 0.05$ ), the respective contributions of the two discriminant functions to the total variation  
 261 are indicated in percentages. Different colors and symbols indicate different treatments and time  
 262 points, the different colony affiliations are not indicated due to them not resulting in consistently  
 263 discernable differentiation.

264  
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 267  
 268



269  
 270 **Figure 5.** Quantitative comparison of a subset of CHC compounds (three mono- and one di-methyl-  
 271 branched alkane) 15 h post injection of viable and dead blastospores, or Ringer solution as a control.  
 272 CHC amounts were measured in ng as individual equivalents from extracts of worker pools (3  
 273 individuals each) taken from 3 different *R. flavipes* colonies. The absolute quantities of each of these  
 274 compounds were significantly increased 15 h after injection of active blastospores as compared to the  
 275 Ringer control (Benjamini-Hochberg corrected Mann-Whitney U tests). Although injection with  
 276 inactive blastospores did not lead to significant increases after 15 h, an upward trend is observable.

277  
 278 **Table 1.** Retention indices (RI), compound identifications and the respective absolute quantities as well  
 279 as standard deviations (in ng) for all CHCs compared between treatments of *R. flavipes* workers: 12  
 280 and 15 h post injection of viable and dead blastospores and Ringer solution as a control, respectively.  
 281 Retention indices were calculated according to the position of detected *n*-alkanes in our samples and,  
 282 where not available, with a C21-40 *n*-alkane standard run under the same conditions. Significantly  
 283 different compound quantities are indicated in bold, accessed through Benjamini-Hochberg corrected  
 284 Mann-Whitney U tests.

RI	Compound ID	12 h Dead blastospores	12 h Viable blastospores	12 h Control	15 h Dead blastospores	15 h Viable blastospores	15 h Control
2207	n-C22	0.12 ± 0.07	0.11 ± 0.08	0.09 ± 0.08	0.12 ± 0.06	0.15 ± 0.1	0.08 ± 0.05
2270	3-MeC22	0.09 ± 0.26	0.04 ± 0.05	0.02 ± 0.02	0.03 ± 0.02	0.04 ± 0.04	0.1 ± 0.27
2280	C23-ene_1	1.15 ± 0.69	0.96 ± 0.59	0.84 ± 0.69	1.4 ± 0.71	1.64 ± 1.35	0.86 ± 0.66
2287	C23-ene_2	0.07 ± 0.03	0.07 ± 0.04	0.05 ± 0.03	0.09 ± 0.03	0.11 ± 0.06	0.05 ± 0.02
2310	n-C23	9.2 ± 5.76	8.28 ± 5.93	8.04 ± 6.3	9.77 ± 4.21	11.69 ± 7.68	7.34 ± 4.79
2343	11-,13-MeC23	0.77 ± 0.47	0.66 ± 0.41	0.59 ± 0.44	0.92 ± 0.42	1.04 ± 0.72	0.63 ± 0.43
2371	4-MeC23	1.61 ± 1.08	1.54 ± 1.09	1.27 ± 0.87	1.87 ± 0.84	2.21 ± 1.46	1.33 ± 0.84
2380	C24-ene	0.57 ± 0.33	0.53 ± 0.37	0.43 ± 0.3	0.67 ± 0.23	0.82 ± 0.53	0.45 ± 0.29
2409	n-C24	5.66 ± 2.97	5.23 ± 3.11	5.03 ± 2.89	5.93 ± 1.61	6.89 ± 3.6	4.85 ± 2.28
2442	12-,14-MeC24	1.45 ± 0.77	1.36 ± 0.79	1.12 ± 0.68	1.82 ± 0.65	1.96 ± 1	1.27 ± 0.73
2463	C25-ene_1	0.1 ± 0.04	0.12 ± 0.04	0.1 ± 0.05	0.12 ± 0.04	0.16 ± 0.08	0.1 ± 0.03

2473	4-MeC24	16.46 ± 8.66	16.46 ± 9.71	13.92 ± 7.83	18.29 ± 5.39	22.33 ± 11.84	13.99 ± 6.49
2483	C25-ene_2	9.58 ± 5.23	9.19 ± 6.17	7.3 ± 4.86	11.7 ± 4.15	14.79 ± 8.41	7.7 ± 4.07
2483	C25-diene_1	6.02 ± 3.87	5.97 ± 4.53	4.49 ± 2.77	6.97 ± 3.69	8.9 ± 6.29	4.64 ± 3.12
2509	n-C25	25.88 ± 12.72	24.05 ± 13.28	23.74 ± 12.34	27 ± 6.45	31.66 ± 15.7	22.96 ± 9.55
2542	9-;11-;13-;15-MeC25	21.06 ± 11.28	19.47 ± 11.1	16.07 ± 9.25	24.94 ± 7.95	27.99 ± 14.37	18.62 ± 10.35
2555	C25-diene_2	3.29 ± 1.75	3.12 ± 1.81	2.72 ± 2.17	3.59 ± 1.61	4.74 ± 3.51	2.48 ± 1.39
2557	5-MeC25	2.58 ± 1.87	2.14 ± 1.68	1.68 ± 1.16	2.96 ± 1.99	4.09 ± 3.49	2.11 ± 1.52
2575	4-MeC25	3.37 ± 1.59	3.22 ± 1.59	2.7 ± 1.48	3.89 ± 1.05	4.67 ± 2.17	2.78 ± 1.19
2577	C25-diene_3	3.19 ± 1.95	3.15 ± 2.31	2.7 ± 1.5	3.66 ± 1.53	4.37 ± 2.72	2.86 ± 1.63
2581	3-MeC25	6.34 ± 3.15	6.24 ± 3.48	5.41 ± 3.11	7.06 ± 2.21	8.7 ± 4.72	5.59 ± 2.89
2641	12-;14-;16-MeC26	0.69 ± 0.45	0.63 ± 0.44	0.49 ± 0.31	0.73 ± 0.36	0.9 ± 0.51	0.58 ± 0.33
2651	6-MeC26	0.21 ± 0.09	0.2 ± 0.1	0.18 ± 0.12	0.25 ± 0.11	0.34 ± 0.28	0.2 ± 0.12
2669	4-MeC26	0.78 ± 0.42	0.71 ± 0.51	0.65 ± 0.39	0.89 ± 0.34	1.05 ± 0.56	0.69 ± 0.36
2681	3-MeC26 + C27ene	0.15 ± 0.1	0.14 ± 0.08	0.23 ± 0.2	0.18 ± 0.11	0.27 ± 0.17	0.14 ± 0.09
2711	n-C27	0.22 ± 0.11	0.18 ± 0.12	0.47 ± 0.36	0.28 ± 0.37	0.76 ± 2.11	0.28 ± 0.24
2742	11-;13-;15-MeC27	0.11 ± 0.05	0.08 ± 0.05	0.07 ± 0.04	0.12 ± 0.05	0.14 ± 0.07	0.1 ± 0.06
2744	C27diene1	0.29 ± 0.17	0.25 ± 0.17	0.21 ± 0.2	0.29 ± 0.18	0.45 ± 0.4	0.21 ± 0.15
2756	C27diene2	0.11 ± 0.05	0.09 ± 0.07	0.09 ± 0.05	0.13 ± 0.07	0.14 ± 0.06	0.11 ± 0.04
2792	5,17-DiMeC27	0.06 ± 0.03	0.05 ± 0.03	0.05 ± 0.04	0.07 ± 0.04	0.09 ± 0.11	0.05 ± 0.04
3531	<b>11-MeC35</b>	1.33 ± 0.62	1.44 ± 0.8	1.07 ± 0.41	1.52 ± 0.76	<b>1.75 ± 0.65</b>	<b>1.13 ± 0.23</b>
3629	<b>12-MeC36</b>	0.32 ± 0.15	0.34 ± 0.21	0.24 ± 0.12	0.37 ± 0.21	<b>0.41 ± 0.17</b>	<b>0.22 ± 0.11</b>
3729	<b>11-;13-MeC37</b>	2.12 ± 0.9	2.21 ± 1.16	1.7 ± 0.6	2.42 ± 1.33	<b>2.62 ± 0.98</b>	<b>1.66 ± 0.43</b>
3751	<b>11,15-DiMeC37</b>	0.77 ± 0.32	0.79 ± 0.38	0.6 ± 0.18	0.89 ± 0.38	<b>0.96 ± 0.33</b>	<b>0.61 ± 0.25</b>
3774	5,15-;5,17-DiMeC37	0.91 ± 0.47	0.93 ± 0.53	0.7 ± 0.22	1.07 ± 0.56	1.11 ± 0.48	0.67 ± 0.24
3927	13-MeC39	0.81 ± 0.39	0.83 ± 0.51	0.62 ± 0.22	0.94 ± 0.62	0.98 ± 0.46	0.61 ± 0.25
3949	11,15-DiMeC39	0.62 ± 0.31	0.63 ± 0.35	0.49 ± 0.12	0.73 ± 0.39	0.73 ± 0.3	0.52 ± 0.1
3973	5,15-;5,17-DiMeC39	0.54 ± 0.28	0.56 ± 0.34	0.4 ± 0.13	0.65 ± 0.39	0.63 ± 0.31	0.43 ± 0.1

285

286

## 287 Discussion

288 Our study shows that the collective response towards pathogen-exposed termites varies according to  
289 the severity and timing of internal challenge, and furthermore, that these are associated with  
290 alterations to the surface chemistry of the infected individual. Notably, we demonstrate that the  
291 decision to eliminate an individual via cannibalism is triggered at an early stage after pathogen  
292 exposure, before pathogen viability or even terminal disease status is known.

293 Individuals that were very moribund at 15 hours following viable blastospore injection were  
294 cannibalized at a higher rate than individuals injected with an equivalent dose of dead blastospores or  
295 a control solution. These observations complement previous findings from fungus-exposed individuals

296 that were cannibalized when *R. flavipes* termites were close to death<sup>17</sup>. Surprisingly, however, we  
297 observed increased cannibalism in response to both viable and dead blastospore challenge as early as  
298 2 hours post injection (p.i.), when termites did not yet display overt signs of illness. This means that  
299 cannibalism is triggered in response to individuals that are not necessarily terminally ill. This can be  
300 seen in focal termites injected with dead blastospores, which induced 40% mortality at the individual  
301 level yet almost all were eventually cannibalized by 24 hours p.i. when placed inside experimental  
302 colonies. Dead blastospore-injected termites did not display severe signs of sickness even at later  
303 experimental time points, and generally died much later than viable blastospore-injected individuals  
304 when kept in isolation. A similar pattern was observed when termites were injected with a dead  
305 blastospore solution lacking both fungal cells and intact destruxins, showing that even subcellular  
306 fungal components lacking active toxins are sufficient to elicit a strong cannibalism response. The  
307 cause of dead blastospore induced death remains unclear but represents a worthy topic for future  
308 investigation.

309 These results reveal that cannibalism is triggered before the onset of moribundity. This could reflect  
310 the need to react rapidly to the natural course of disease progression of entomopathogenic fungi such  
311 as *Metarhizium*, indicating that *R. flavipes* might employ a better safe than sorry strategy towards  
312 individuals suspected of internal fungal infection. *Metarhizium* deploys a multi-component infection  
313 strategy to kill the host and complete the infection cycle, including toxin release after the fungus  
314 breaches the cuticular barrier<sup>49</sup>, so once this stage has been reached, host death is likely unavoidable  
315 under normal circumstances. There are several points during internal infection that could activate  
316 endogenous signalling pathways, which in turn may communicate a change in disease status to  
317 interacting nestmates. The first may be as soon as when blastospores have gained access to the  
318 hemocoel, as this could feasibly be linked to the rapid activation of innate immune signalling pathways.  
319 The second stage is associated with fungal acquisition of nutrients for growth and reproduction, during  
320 which time, termite hosts gradually begin to show visible signs of sickness (from 12 h p.i.). Similar  
321 symptoms have been described from the higher termite *Nasutitermes exitiosus*<sup>53</sup>. Our results indicate  
322 that elimination cues are transmitted to nestmates from an early stage following cuticular breach, but  
323 also that reinforcement cues may be generated that correlate with the progression and severity of  
324 infection. Correlated with the phase of fungal proliferation, is the ramping up of toxin  
325 biosynthesis<sup>49,54,57,59,64</sup> which likely accelerates host death<sup>46,53,57</sup>. Blastospores might evade innate  
326 insect immune responses through reduction of the number of pathogen-associated molecular patterns  
327 (PAMPs), by camouflaging or  $\beta$ -glucan modification<sup>65,66</sup>, but in general their interaction with the  
328 termite immune system is poorly understood, and represents an interesting target for future research.  
329 With respect to inactivated blastospores, these harbour several components that could activate the  
330 immune system of the host, including denatured cellular contents and cell wall constituents. One

331 factor to consider is that heat-inactivated blastospores did not contain intact destruxins. Such missing  
332 factors could represent an important form of danger signal, either via direct interaction with signalling  
333 receptors, or indirectly via their negative influence on host cells, which may then produce signals of  
334 damage<sup>67</sup>. It is possible that the faster cannibalism response to viable blastospore-injected termites is  
335 linked to the presence and active synthesis of virulence factors such as destruxins, although this also  
336 requires further testing.

337 We hypothesized that activation of endogenous host pathways would result in external changes that  
338 may be perceptible by interacting nestmates. We examined cuticular hydrocarbon (CHC) profiles of  
339 internal pathogen-challenged individuals to understand whether they could serve as chemical cues  
340 indicating infection. Injections with both viable and inactivated blastospores shifted overall CHC  
341 profiles, with a trend towards more clear differentiation after a longer incubation time. We detected  
342 significant increases in four exclusively methyl-branched CHCs 15 hours after injection with viable  
343 blastospores compared to control-injected individuals. Interestingly, these four compounds are  
344 structurally related, long-chained mono- and di-methyl-branched alkanes with a similar chain length  
345 range (C35-C37) and internal methyl-branches at positions 11, 12, 13 and 15. A trend towards an  
346 increase in these compounds was also clearly discernible albeit non-significant in individuals injected  
347 with dead blastospores. These results indicate a common pattern for encoding and conveying a  
348 chemical cue associated with infection status, which would be in line with the high potential of methyl-  
349 branched alkanes to encode chemical information<sup>67-69</sup>. However, whether these compounds are  
350 directly responsible for eliciting the observed cannibalism response requires further experimental  
351 validation. Similar findings have been reported in challenged *Lasius* ants where infection has been  
352 linked with changes in CHCs of a similar chain length range (C33-35), albeit of non-methyl branched  
353 compounds<sup>19</sup> whereas in *Myrmica* ants, straight-chain alkane quantities increased upon infection  
354 while methyl-branched alkane quantities decreased<sup>34</sup>. Studies reporting cuticular profile changes  
355 associated with disease have also been carried out in honeybees<sup>29,32,35</sup>. In line with these findings, our  
356 study points to host condition-dependent cues as playing an important role, with elimination  
357 behaviours potentially being activated when changes in CHC compounds exceed a given quantitative  
358 threshold<sup>70</sup>. Our observations of increased amounts of long-chained mono- and di-methyl-branched  
359 alkanes over time following viable blastospore injection, as well as a trend towards higher quantities  
360 of these compounds in dead blastospore injected termites, may be consistent with a threshold  
361 hypothesis.

362 With respect to other behaviours, in comparison to previous work employing conidia on the termite  
363 cuticle<sup>17,18,23,40,42,57,71,72</sup>, our observed rates of allogrooming are lower across comparable time points,  
364 suggesting that direct contact with external pathogens are more important in generating an  
365 allogrooming response. The benefits of allogrooming for enhancing group survival in the presence of

366 external pathogens are well documented<sup>14,40-42</sup>, which makes intuitive sense as allogrooming can  
367 remove potentially infectious microbes on the surface before they breach the cuticle. The general  
368 absence of burial in our experiments supports the idea that termites preferentially eliminate harmful  
369 individuals through cannibalism<sup>47,73</sup>, although burial may be a more important defensive mechanism  
370 for older or decomposing cadavers<sup>73-75</sup>.

371 Overall, our results show that termites are capable of readily detecting and eliminating individuals  
372 displaying signs of internal fungal challenge. Our findings also indicate that elimination response  
373 behaviours are triggered before individuals enter a terminal disease state, and furthermore, that  
374 individuals that could theoretically recover will nonetheless be cannibalized by their nestmates. These  
375 findings may reflect the need for a vigilant social immune system that has evolved to recognize early  
376 signs of a typically highly virulent fungal infection. In terms of the care-kill paradigm, internal challenge  
377 with fungal blastospores appears to be uniformly associated with a kill response. Trade-offs in immune  
378 system vigilance have been explored in the context of beneficial versus pathogenic microbiota<sup>76</sup> and  
379 similar trade-offs may also operate with regards to social immunity. Here, the level of social immune  
380 vigilance might be determined by pathogen hazard on the one hand, and cost of social immunity  
381 (damage to the colony due to elimination of constituent members as well as risk to the survival of  
382 intervening nestmates), on the other. In comparisons across species, factors such colony size and  
383 maturity, as well as individual age could play a role in shaping this trade-off. As in individual immunity,  
384 a positive correlation between pathogen hazard and social immune vigilance may be expected. It is  
385 perhaps unsurprising that *R. flavipes* does not differentiate between viable and inactivated  
386 blastospores or associated moribundity effects arising from an internal fungal challenge, because such  
387 a situation would not arise under natural conditions. Although we have not experimentally verified the  
388 cuticular chemical cues or underlying mechanisms used to trigger elimination, we speculate that they  
389 may involve crosstalk between immune and other physiological processes such as metabolism and the  
390 regulation of biosynthetic pathways involved in cuticular chemistry variation. Expansion of chemical  
391 analyses to other volatile compound classes such as alcohols (e.g. Butanol, 2,3-Butanediol) and ketones  
392 (e.g. 2-Pentanone) released by entomopathogenic fungi<sup>77,78</sup> may also be warranted. Another  
393 interesting question to consider is whether modification of the cuticular profile in pathogen-challenged  
394 individuals is sufficient to induce antifungal immunity in interacting nestmates<sup>19,29,32,35,79</sup>. Other targets  
395 for future work could include functional experiments to test whether specific compounds, chemical  
396 combinations, or general alterations in overall CHC abundance are sufficient to trigger changes in social  
397 immune behaviour.

398

399 **Methods**

400 **Termites**



401 Five *R. flavipes* colonies were used in these experiments: colonies 1, 5, 11+13, E and X. Additional  
402 information regarding host organisms are given in the supplementary material.

### 403 **Entomopathogenic fungus**

404 We used the semelparous fungal entomopathogen *Metarhizium robertsii* (DSM 1490), previously  
405 classified as *Metarhizium anisopliae* by the German Collection of Microorganisms and Cell Cultures  
406 GmbH (DSMZ) (<https://www.dsmz.de/collection/catalogue/details/culture/DSM-1490>). *M. anisopliae*  
407 and *M. robertsii* are closely-related obligate-killing entomopathogens of *R. flavipes*, and are detected  
408 in soils in close proximity to colonies<sup>80</sup>. Details on fungal conidia germination and harvesting methods  
409 are given in the supplementary material.

410 To prepare cultured blastospores, we added 1 mL of  $1 \times 10^8$  conidia/mL suspension to 100 mL  
411 quantities of two different liquid media in 300 mL Erlenmeyer flasks: (i) 40g/L yeast extract, 80g/L  
412 glucose and 0.1% Tween 80; (ii) 40g/L yeast extract, 40g/L glucose, 30g/L corn steep liquor and 0.1%  
413 Tween 80<sup>81</sup>. Erlenmeyer flasks were incubated in a shaking incubator for 3 days (exponential phase)  
414 at 25 °C and 290 rpm. Blastospores were then harvested by combining the two cultured media and  
415 filtering them through two layers of a sterile miracloth (Merck KGaA, Darmstadt, D) to remove the  
416 mycelia. The filtrate was centrifuged for 5 min at 2000g at 4 °C and the pellet containing the  
417 blastospores was washed and resuspended three times in Ringer's  $\frac{1}{4}$  solution. As with conidia,  
418 blastospore concentrations were determined in a Thoma counting chamber and adjusted to  $5 \times 10^8$   
419 blastospores/mL. Experimental injections consisted of two pathogen and one control treatment. The  
420 two pathogen treatments were: viable challenge, using live blastospores ( $5 \times 10^8$  blastospores/mL  
421 viable blastospores; 96% germination rate at 10 hours) or inactivated challenge, using dead  
422 blastospores ( $5 \times 10^8$  heat-inactivated blastospores/mL; 0% germination). The control treatment  
423 consisted of sterile Ringer's  $\frac{1}{4}$  solution only. To prepare the dead blastospore treatment, an aliquot of  
424 the viable blastospore suspension was autoclaved. Germination was assessed on two PDA plates  
425 streaked with 50  $\mu$ L of the autoclaved blastospore solution, followed by incubation in the dark at 25  
426 °C for 24 hours. As expected, no fungal growth was observed. Blastospores were considered to have  
427 germinated when an elongating germ tube of any size was visible at 200-400 $\times$  magnification<sup>82</sup>.

### 428 **Preparation of experimental colonies**

429 Experimental *R. flavipes* colonies were set up inside Petri dishes as described elsewhere<sup>17</sup> and as  
430 summarized in the supplementary material.

### 431 **Injection of focal termites**

432 We marked focal termites with Nile blue, a fat-soluble stain that has been used in several studies to  
433 mark termites and has not been reported to affect termite behaviour<sup>17,83</sup>. It dyes the termites blue,

434 making them easily distinguishable from their otherwise colourless nestmates. Nile blue dyeing was  
435 carried out following a rapid method for marking termites as described previously<sup>17,84</sup>. Each focal  
436 termite was anaesthetized with CO<sub>2</sub>, then injected with 41.4 nL of either viable blastospores, dead  
437 blastospores, Ringer's solution or fungal filtrate directly into the hemocoel using a Nanoject II  
438 (Drummond Scientific Company, USA). The needle was inserted into the side of the thorax. With this  
439 method, the first stages of the *Metarhizium* infection process, that is, adhesion, germination of fungal  
440 conidia and cuticle penetration<sup>85,86</sup> were bypassed. As described above, this allowed us to test the  
441 effect of indirect cues triggered by internal pathogen challenge. As expected from previous studies  
442 <sup>49,55,82</sup>, germination and killing rates are faster when using blastospores as compared to conidia  
443 (Supplementary Figures S1, S11)<sup>17</sup>. By carrying out further experiments with dead blastospore and  
444 fungal filtrate treatments, we were able to test whether active infection and terminal sickness were  
445 necessary to induce cannibalism.

446 Injected focal termites were kept individually in small Petri dishes (35 Ø mm), each containing a Pall  
447 cellulose pad moistened with 1 mL of distilled water and then incubated for either 2, 8, 12 or 15 hours  
448 at 27 °C and 70% humidity post injection (p.i.) before use in behavioural observation experiments.  
449 Representative images and videos of focal termite and nestmate interactions inside experimental  
450 colonies were captured with a smartphone (Samsung galaxy S7) and adjusted for brightness and size  
451 (Adobe Photoshop [Adobe Systems, San Jose, California]) to highlight different social immune  
452 behaviours (Supplementary Videos). Pathogen dose and incubation times were determined based on  
453 blastospore germination and initial survival assays of termites injected with different concentrations  
454 of *M. robertsii* blastospores (Supplementary Figures S1, S11). The effect of the selected dose of viable  
455 and dead blastospores on termite survival when kept individually was examined by injecting one  
456 hundred and eighty *R. flavipes* termites from three different colonies, each assigned to one of the  
457 following treatments: (i) 60 individuals: Viable blastospores (injected 5 x 10<sup>8</sup> blastospores/mL); (ii) 60  
458 individuals: Dead blastospores (injected 5 x 10<sup>8</sup> heat-killed blastospores/mL); (iii) 60 individuals:  
459 control (injected Ringer's solution). Survival of each individual was recorded every 12 hours for a total  
460 of 240 hours (Supplementary Figure S5).

#### 461 **Experimental design**

462 In our observations, termites injected with the selected dose of viable blastospores were moribund by  
463 16 hours p.i., with death beginning between 22 and 26 hours (Supplementary Figure S5, S10 and Table  
464 S5). Knowledge of the germination and infection timeline in *R. flavipes* (Supplementary Figure S1, S2  
465 and Table S1) was used to select four incubation timepoints for behavioural observations experiments:  
466 (i) 2 h: blastospores have gained access to the hemocoel but have not yet germinated; (ii) 8 h:  
467 blastospores have germinated but the host appears healthy; (iii) 12 h: the host shows first signs of

468 sickness, as indicated by slower movements; and (iv) 15 h: the host is moribund (limited to no mobility).  
469 For individuals injected with dead blastospores, symptoms do not appear until 22 h p.i., and only  
470 among individuals that subsequently died (Supplementary Figure S1 and Table S1).

471 **Blastospore experiment.** Focal termites were injected with either a suspension containing  $5 \times 10^8$   
472 viable blastospores per mL, a suspension containing  $5 \times 10^8$  dead blastospores per mL, or Ringer's  
473 solution as a control. Following experimental challenge with *M. robertsii* blastospores, focal termites  
474 were kept individually for the pre-assigned incubation times (2, 8, 12 and 15 h) prior to introduction  
475 into experimental colonies. For each of the four incubation time points, we conducted 24 replicates  
476 from 5 independent colonies for each treatment. Colonies X and E contributed 3 plate replicates per  
477 incubation time point and treatment, while colonies 1, 5 and 11+13 each contributed 6. Data for  
478 colonies X, E, 5 and 11+13 were collected over 2 experimental repeats (with colony E replacing X in the  
479 second repeat) while data for colony 1 were collected from a third experimental repeat. Plates from  
480 each incubation time were randomly assigned to one of the three treatment groups after the 15-day  
481 colony establishment period had elapsed (Total N=288 plates). Randomisation was achieved by using  
482 a random number generator to produce double-digit number combinations, where the first digit  
483 corresponded to a colony and the second to one of the treatments.

484 **Blastospore filtrate experiment.** To further narrow down the pathogen components that may be  
485 responsible for inducing termite social immunity, we tested the effect of heat-inactivated fungal  
486 filtrate (lacking blastospores) on nestmate behaviour. Filtrates consisted of the final Ringer's  $\frac{1}{4}$  solution  
487 used to wash dead blastospores, so should contain subcellular components and metabolites but not  
488 larger blastospore-sized structural components, which were removed by filtration. Fifty-four  
489 experimental plates of *R. flavipes* from colonies 2, 5, and X (6 plates per colony) and the respective  
490 focal termites were randomly assigned to one of the three treatment groups (Ringer, dead  
491 blastospores, or fungal filtrate) after the 15-day colony establishment period had elapsed (N=18 per  
492 treatment). Focal termites were injected either with a suspension containing dead blastospores, fungal  
493 filtrate or Ringer's solution, which served as a control. Challenged termites were all isolated for 12  
494 hours prior to introduction into the experimental colonies. Challenged and control termites were  
495 introduced individually into the experimental colonies, which were then resealed with parafilm. The  
496 observation period began immediately after the last experimental colony was sealed.

#### 497 **Behavioural recording**

498 In the blastospore experiment, after 2, 8, 12, or 15 hours of incubation, injected focal termites were  
499 added individually to the Petri dish nests. To minimize vibrational stimuli that *R. flavipes* are known to  
500 be quite sensitive to<sup>87</sup>, Petri dishes were not opened or moved after sealing. This process took  
501 approximately 10 minutes, and the observation period began immediately after the last dish was

502 sealed. For observations, we adopted a scan sampling method used previously<sup>17</sup>. In the blastospore  
503 experiment, we performed scan sampling of each Petri dish nest every 5 minutes for a total of 3  
504 hours<sup>88</sup>. For the blastospore filtrate experiment, behavioural responses were recorded later, due to  
505 the delayed onset of cannibalism in the dead blastospore treatment. Here scan sampling was carried  
506 out for 5 minutes every 2 hours every at 12, 15, 18 and 21 hours post-injection. Scanning typically took  
507 around 1 minute. When necessary, a magnifying glass was used (up to 3x magnification) to better  
508 distinguish between observed behaviours and a Samsung S7 smartphone was employed as a digital  
509 voice recorder. Observations were performed under constant overhead light at 27 °C and 70%  
510 humidity. To minimize observer bias, the experiment was blinded by randomly assigning a treatment  
511 to each Petri dish nest prior to the observation period as mentioned in the above section. In both  
512 experiments, focal termites were counted 24 hours after introduction into the groups to quantify  
513 survivorship. Behaviours of interest were defined *a priori* and classified into visually distinguishable  
514 and non-overlapping categories relevant to social immunity. We focused on five different behavioural  
515 categories:

516 **Groomed by n (gb):** Focal termite is being groomed by N nestmates with no evidence of biting.

517 **Cannibalism (c):** Focal termite is being bitten by one or more nestmates and/or focal termite body is  
518 no longer intact.

519 **Buried (b):** Focal termite has had pieces of paper or faeces placed on top of it. Although increasingly  
520 difficult to assess, the termite may still be alive. Note that this behaviour did not get exhibited once  
521 during the observation period, which led to it being excluded from the analysis.

522 **Not visible (nv):** Focal termite is in a part of the nest where it cannot be observed.

523 **Other (o):** Focal termite is alive, intact, and unburied, but nestmates are not interacting with it. This  
524 reflects behavioural states unrelated to social immunity.

## 525 **Destruxin analysis**

526 Blastospore cultures were prepared as described above, except that Czapek Dox liquid medium was  
527 used instead (modified, Oxoid, England)<sup>89</sup>. Viable blastospore cultures were subjected to vacuum  
528 filtration (Thermo Scientific™ Nalgene™ Rapid-Flow™, USA) to remove all fungal cells and analysed  
529 directly. Dead blastospores were generated as described above. Autoclaved blastospores in Ringer's  
530 solution (as per the dead blastospore treatment) were then subjected to vacuum filtration to remove  
531 remaining fungal cells and used in the subsequent ultra-high pressure liquid chromatography coupled  
532 with tandem mass spectrometry (UHPLC-MS/MS) analysis for confirmation of the presence of  
533 respective destruxins. For this purpose, a Synapt G2-S HDMS, equipped with an Acquity UPLC system  
534 (Waters Co., Milford, MA, USA) was used. The conditions of the mass spectrometer were optimized  
535 for small peptides, using the following settings: (+)-ESI in sensitivity mode; capillary voltage: 3,3 kV,

536 sample cone voltage: 40; source offset: 60; source temperature: 90 °C; desolvation temperature: 250  
537 °C; cone gas flow: 1 L/h; desolvation gas flow: 500 L/h; nebulizer gas flow: 6 bar. Throughout analysis,  
538 UPLC grade solvents by Biosolve BV, Valkenswaard, Netherlands. The UHPLC conditions were  
539 optimized to an injection volume of 7 µL; flow rate: 0,25 mL/min; eluents: A: water + 0,1 % formic acid;  
540 B: acetonitrile + 0,1 % formic acid; column thermostat: 40 °C; gradient: 0 min: 25 % B, 10 min: 40 % B,  
541 13 min: 40 % B, 15 min: 95 % B, 20 min: 95 % B, 21 min: 25 % B, 25 min: 25 % B. This method was  
542 adopted following Taibon<sup>63</sup>. Comparison with literature (retention times and fragmentation pattern)  
543 confirmed the retention times of the destruxins as noted in the chromatograms<sup>62,63</sup>.

## 544 **Statistical analysis**

### 545 **Survival of termites kept individually**

546 Statistical analyses were carried out in R version 4.2.2 and RStudio version 2022.07.02.

### 547 **Grooming**

548 The amount of grooming (defined as the number of grooming states/total observed states) in each  
549 treatment was analysed by fitting a generalised linear mixed model (GLMM) to the data using the  
550 *glmer* function in the R package *lme4*<sup>90</sup>. A binomial error distribution was used to model proportion  
551 data<sup>91</sup>. Models for each incubation time (2, 8, 12, 15 h) were composed of treatment and amount of  
552 cannibalism (defined as the number of cannibalism states/total observed states) as fixed effects. Petri  
553 dish nest ID and colony were used as nested random effects (Plate within colony). The blastospore  
554 filtrate models were constructed for each timepoint (12, 15, 18, 21 h) and contained treatment,  
555 amount of cannibalism and colony as fixed effects and Petri dish nest ID as random effect. All models  
556 were compared using Akaike information criterion (*AIC*-function in R), which revealed the full model  
557 to be the best fit. Model assumptions were checked using the functions *simulateResiduals*,  
558 *testDispersion* and *testZeroInflation* contained within the *DHARMA* package<sup>92</sup>. We performed post  
559 hoc pairwise comparisons using the *glht* function from the *multcomp* package v1.4-10 with Tukey tests  
560 using a Bonferroni correction<sup>93,94</sup>.

### 561 **Cannibalism**

562 The *survfit*-function from the *survival* package<sup>95</sup> was used to model the onset of cannibalistic behaviour  
563 and the *ggsurvplot*-function from the *survminer* package<sup>96</sup> was used to plot the data. Survival curves  
564 were compared using a mixed effects Cox model (*coxme* function from the *coxme* package<sup>97</sup>). For the  
565 blastospore experiment, models for each incubation time (2, 8, 12, 15 h) included treatment as a fixed  
566 effect, and Petri dish nest ID and colony as random effects. For the blastospore filtrate experiment, a  
567 single model included treatment and colony as fixed effects and Petri dish nest ID as a random effect.  
568 In the survival curve analysis, all control data was initially right-censored; in order to fit a mixed effects

569 Cox model to the data, it was necessary to uncensor one arbitrarily-selected control replicate from  
570 each incubation time<sup>17,24</sup>. The *glht*-function was used to perform post-hoc pairwise comparisons using  
571 Tukey tests with Bonferroni corrections.

#### 572 **Mortality of focal termites at 24 hours after introduction into experimental colonies**

573 Following the main blastospore experiment, survival of focal termites was assessed 24 hours after  
574 introduction into experimental colonies (corresponding to 26, 32, 36 and 39 hours after injection for  
575 each incubation time point, respectively). Mortality rates were calculated for each treatment and each  
576 timepoint. A Wilcoxon rank sum test was used to perform pairwise comparisons of mortality rate ranks  
577 between treatments, using a Bonferroni-correction to account for multiple comparisons.

#### 578 **Scanning electron micrographs (SEMs) of blastospores**

579 Representative SEMs of germinating blastospores on PDA media at different time points (0, 2, 4, 8, 12  
580 and 15 h (Supplementary Figure S2)) were taken to determine the *in vitro* germination rate. For SEM  
581 preparations, small and thin pieces of PDA with blastospores were mixed 1:1 with 25% glutaraldehyde  
582 in 0.1 M cacodylate buffer pH 7-7.2 for 60 minutes. After three 5-10 minutes washing steps with 0.1 M  
583 cacodylate buffer, the samples were fixed in 1% OsO<sub>4</sub> in distilled water for 60 minutes and washed  
584 three times in distilled water for 5-10 minutes each. Then the blastospores were transferred into  
585 vessels covered with planktonic gauze (mesh-size 10 µm) and were dehydrated in ascending ethanol  
586 concentrations (30%, 50%, 70%, 90%, 2 x 100%, 2 x 100% absolutely water free) about 10-15 minutes  
587 each. Critical point drying with CO<sub>2</sub> was performed with a Balzer CPD 030. The dried samples were  
588 transferred onto double-sided adhesive tape on specimen stubs before they were sputtered with gold  
589 in a Balzer SCD 040. Images were taken with a FEI Quanta 200 ESEM.

#### 590 **Cuticular Hydrocarbon analysis**

591 Extractions of pools of three individual termites per treatment were performed in 2 ml glass vials  
592 (Agilent Technologies, Santa Clara, California, USA) on an orbital shaker (IKA KS 130 Basic, Staufen,  
593 Germany) for 10 minutes. Extracts were subsequently evaporated under a constant stream of gaseous  
594 carbon dioxide and then resuspended in 5 µL of a hexane solution containing 7.5 ng/µL dodecane (C<sub>12</sub>)  
595 as an internal standard. Following this, 3 µL of the resuspended extracts were injected in splitless mode  
596 with an automatic liquid sampler (ALS) (PAL RSI 120, CTC Analytics AG, Switzerland) into a gas-  
597 chromatograph (GC: 7890B) simultaneously coupled to a flame ionization detector (FID: G3440B) and  
598 a tandem mass spectrometer (MS/MS: 7010B, all provided by Agilent Technologies, Waldbronn,  
599 Germany). The system was equipped with a fused silica column (DB-5MS ultra inert; 30 m x 250 µm x  
600 0.25 µm; Agilent J&W GC columns, Santa Clara, CA, USA) at a temperature of 300 °C with helium used  
601 as a carrier gas under a constant flow of 1.8 ml/min. The FID had a temperature of 300 °C and used

602 nitrogen with a 20 mL/min flow rate as make-up gas and hydrogen with a 30 mL/min flow rate as fuel  
603 gas. The column was split at an auxiliary electronic pressure control (Aux EPC) module into an  
604 additional deactivated fused silica column piece (0.9 m x 250  $\mu$ m x 0.25  $\mu$ m) with a flow rate of 0.8  
605 mL/min leading into the FID detector, and another deactivated fused silica column piece (1.33 m x 250  
606  $\mu$ m x 0.25  $\mu$ m) at a flow rate of 1.33 mL/min into the mass spectrometer. The column temperature  
607 program started at 60 °C and was held for 5 min, increasing 20 °C/min up to 200 °C and then increasing  
608 3 °C/min to the final temperature of 325 °C, held for 5 min.

609 CHC peak detection, integration, quantification and identification were all carried out with  
610 Quantitative Analysis MassHunter Workstation Software (Version B.09.00 / Build 9.0.647.0, Agilent  
611 Technologies, Santa Clara, California, USA). CHCs were identified according to their retention indices,  
612 diagnostic ions, and mass spectra as provided by the total ion count (TIC) chromatograms, whereas  
613 their quantifications were achieved by the simultaneously obtained FID chromatograms, allowing for  
614 the combination of the best-suited method for hydrocarbon quantification (Agilent Technologies,  
615 Waldbronn, Germany, pers. comm.) with reliable compound identifications. Absolute CHC quantities  
616 (in ng) were obtained by standardizing all peak areas of the identified CHC compounds according to  
617 the concentration of the internal C12 standard, and additionally divided by three to obtain CHC amount  
618 equivalents per single individual. A discriminant analysis (DA) was performed with the R package MASS  
619 to test whether CHC profiles statistically differ between the investigated treatment groups and to  
620 visualize the degree of separation between them. Wilk's  $\lambda$  was calculated to measure the quality of the  
621 DA. Furthermore, quantitative differences between the treatments for each individual CHC compound  
622 were accessed with Benjamini-Hochberg corrected Man-Whitney U tests, and visualized with boxplots  
623 where significant differences were detected.

#### 624 **Data Availability Statement**

625 All data generated or analysed during this study are included in this published article and its  
626 supplementary information files.

627

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