insects

MDPI

Article

Hygiene defense behaviors used by a fungus-growing ant depend of the fungal pathogen stages

Ernesto Bonadies[1, 2, 3], William T. Wcislo[3], Dumas Gálvez[2], William O.H. Hughes[4], Hermógenes Fernández-Marín[1*]

[1] Centro de Biodiversidad y Descubrimiento de Drogas, Instituto de Investigaciones Científicas y Servicios de Alta Tecnología (INDICASAT AIP), Panamá, Apartado 0843-01103.
[2] Programa Centroamericano de Maestría en Entomología, Universidad de Panamá.
[3] Smithsonian Tropical Research Institute, Balboa, Ancon, Panama, Republic of Panama
[4] School of Life Sciences, University of Sussex, Brighton BN1 9QG, UK
* Corresponding: hermogenes_f@hotmail.com

Received: date; Accepted: date; Published: date

Abstract: Parasites and their hosts use different strategies to overcome the defenses of the other, often resulting in an evolutionary arms race. Limited animal studies have explored the differential responses of host when challenged by differential parasite loads and different developmental stages of a parasite. The fungus-growing ant *Trachymyrmex* sp. 10 employs three different hygienic strategies to control fungal pathogens: grooming of the antibiotic-producing metapleural glands (MG), and planting or weeding of their mutualistic fungal crop. By inoculating *Trachymyrmex* colonies with different fungal parasite (*Metarhizium* or *Escovopsis*), different parasite concentrations or stage (germinated conidia or ungermianted conidia), we tested whether ants modulate and change hygienic strategies depending on the nature of the parasite challenge. There was no effect of the concentration of parasite on the frequencies of the defensive behaviors, indicating that the ants did not change defensive strategy according to the level of the threat. When challenged with conidia of *Escovopsis* sp. and *M. brunneum* that were germinated or not-germinated, however, the ants adjusted hygienic behavior to fungal planting and MG grooming behaviors using one strategy or the other depending on the conidia germination status. Our study suggests that fungus-growing ants can adjust the use of hygienic strategies based on the nature of the parasites.

1. INTRODUCTION

Living in societies may increases the transmission of parasites within groups and this can be exacerbated by high genetic relatedness among group members [1,2]. Natural selection on social organisms has driven the evolution of strategies for disease prevention and control, including rapid detection of pathogens, increased immune responses, waste and corpse disposal, social grooming, prophylactic- and self-medication [3-7]. The availability of different hygienic strategies could enable individuals or groups to deploy them differentially, according to the nature of the parasite threat and the efficiency of each defense strategy [8]. However, little is known about how individuals determine which particular hygienic strategies to deploy, and what triggers the switch between them [9].

Fungus-growing ants (Formicidae:Myrmicinae) such as *Trachymyrmex* form an obligatory mutualism with a fungi crop (Basidiomycota: Lepiotaceae and Pterulaceae; [12]), and both ant and crop are challenged by diverse parasites [10,11]. These ants use several different behavioral strategies to deal with parasites [13,14], relying primarily on grooming with their antibiotic-producing metapleural gland (MG), or planting or weeding the fungal mutualist [15]. Some species also utilise antibiotic-producing actinomycete bacteria, while others, such as *Trachymyrmex* sp. 10 lack this symbiont and instead make more use of MG grooming or fungal planting [15]. Here we use *Trachymyrmex* sp. 10 to test the hypothesis that hosts will alter their behavioural defense strategy either quantitatively or qualitatively, depending on the dose or nature of parasite challenge.

2. METHODS

We collected colonies (ants and mutualist fungus garden) of *Trachymyrmex* sp. 10 on the campus of the University of Panama in Panama City, Panama. Colonies were kept in plastic boxes under laboratory conditions at the Smithsonian Tropical Research Institute in Gamboa. The colonies had wet paper towels placed in the boxes to maintain humidity and were fed twice a week with corn flour, oatmeal and occasionally leaves of *Lagerstroemia speciosa*. We used two parasites in our experiments: i) the entomopathogenic fungus, *M. brunneum* and ii) a pathogen of the mutualist fungus garden, *Escovopsis* sp. We isolated both parasites from a local study site in Gamboa and cultured them on agar plates. Isolates of both parasites had >99% conidia viability when used in the experiments.

2.1. Experimental groups and study behaviors

From each collected colony, we created groups of 20 workers, randomly chosen and kept in Petri dishes with wet paper strips and 0.5 g of the fungus garden placed in the center of the dish. We quantified four different behaviors to determine the response of ants to pathogens, following Fernández-Marín et al. [15]: i) *fungal grooming* with ants licking the surface of the mutualist fungus to remove contaminated particles which are stored and treated in the infrabuccal pocket and later discarded as pellets; ii) *metapleural gland (MG) grooming*, with ants rubbing their forelegs over the MG opening to transfer antibiotic MG secretions to a site of contamination; iii) *fungal cultivar planting*, with ants placing healthy pieces of the fungus garden on a contaminated area of the garden; and iv) *weeding*, with workers removing a piece of the garden that is then placed in a garbage dump.

2.2. Hygienic responses to different pathogen concentrations

We performed inoculations with conidia of the entomopathogenic fungus *Metarhizium brunneum*. We tested three different concentrations, high (~2.3 $x10^8$ conidia), medium (~6.0 $x10^7$ conidia), and low (~2.1 $x10^7$ conidia). We inoculated conidia by placing them on a piece of Parafilm, which was then rubbed on the surface of the fungal gardens (methods from [11]). The high, medium and low concentrations correspond to areas in the Petri dish of 25 mm^2, 9 mm^2 and 4 mm^2 respectively. We used 10 experimental groups for each pathogen concentration.

2.3 Hygienic responses to different stages of the pathogen

For this experiment, we performed inoculations using an entomopathogen fungus, *M. brunneum* and a pathogen of the fungal cultivar, *Escovopsis* sp. For both pathogens, we investigated the effect of the developmental stage of the pathogen by using non-germinated and germinated conidia. We germinated 4 mm^2 of conidia on a piece of fungus garden for 30 hours to obtain germinated conidia. As a control for conidia we used 4 mm^2 of talcum powder on the fungus garden. As a control for germinated conidia we inoculated 4 mm^2 of talcum powder on a piece of fungal garden and left for 30 h. We applied the germinated conidia, ungerminated conidia or control treatments by gently rubbing on to the fungus garden in the experimental groups. We used 10 experimental groups for each treatment (N = 60). Data from both experiments were analysed with PERMANOVA [16].

Voucher specimens from the study are deposited in the collections of the Museo de Invertebrado de la Universidad de Panamá and in the Smithsonian Tropical Research Institute.

3. RESULTS

Overall, the hygienic responses of workers did not vary with parasite concentration (PERMANOVA: $F_{2,29} = 1.0$, $p = 0.4$). We did not detect significant correlations between MG grooming and weeding or planting (respectively, $r = -0.19$, $t = -1.01$, $df = 28$, $p = 0.32$; $r = -0.34$, $t = -1.94$, $df = 28$, $p = 0.06$), nor between planting and weeding ($r = 0.15$, $t = 0.80$, $df = 28$, $p = 0.43$).

The developmental stage of the parasite triggered different responses by workers (PERMANOVA: $F_{1,39} = 13.4$, $p < 0.0001$). Against both parasites, workers used MG grooming more frequently than planting when they encountered germinated conidia of the pathogens (One-way

Permutation Test: *Escovopsis*, Z = -2.3, p = 0.006 *Metahizium*, Z = - 2.5, p = 0.008), and planted more frequently than MG grooming when they encountered ungerminated conidia (*Escovopsis*, Z = 2.3, p = 0.006 *Metarhizium*, Z = 2.3, p = 0.04; Figure 2). There were no differences in frequencies of behaviours in control treatments, when they simulated germinated or ungerminated conidia (One-way Permutation Test, MG grooming: Z = 0.51, p > 0.05, Planting: Z = -0.46, p > 0.05; Figure 2). There was also no effect of parasite on the hygienic behaviours comparing *Metarhizium* and *Escovopsis* ($F_{1,39}$ = 2.7, p= 0.06, Figure 2).

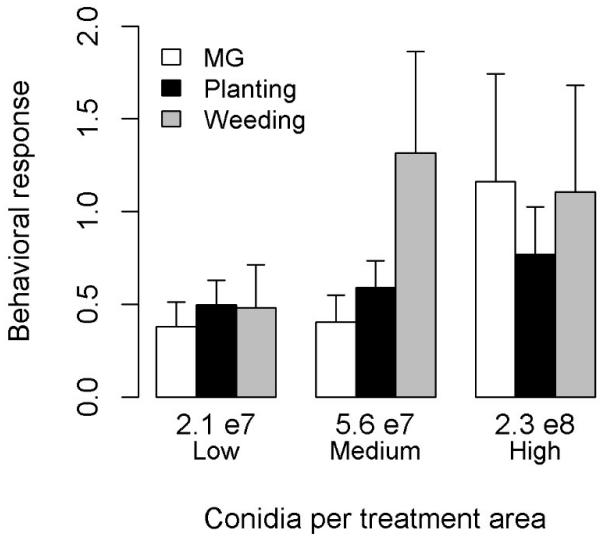

Figure 1. Ant workers behavioral response exposed to three different *Metarhizium brunneum* conidia amounts. Error bars represent standard errors.

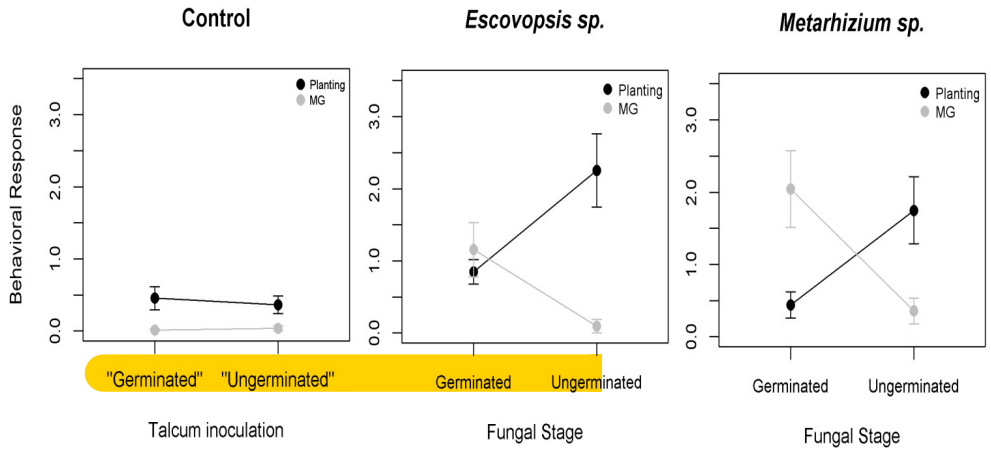

Figure 2. Mean ± standard errors frequencies of the hygienic behaviors metapleural gland grooming (MG; grey dots) and fungal mutualist planting (black dots) exhibited by *Trachymyrmex* sp. 10 fungus-growing ants in response to the germinated conidia or ungermianted conidia of the *Escovopis* and *Metarhizium* fungal parasites. Control treatments were talcum powder (for ungermianted conidia) or talcum powder contaminated fungal mutualist (for germinated conidia).

4. DISCUSSION

Fungus growing ants are able to detect the presence of parasitic fungi ~~presence~~, and have previously been shown to increase cleaning rates depending on the parasite concentration [11]. In this study, we found the ants adjust which behavior is used as the main hygienic strategy depending on the nature of the parasite threat, using primarily metapleural gland (MG) grooming against germinated conidia from both *Metarhizium* and *Escovopsis* fungi, and planting their mutualistic fungus against ungerminated conidia.

Trachymyrmex sp. 10 lack visible antibiotic producing bacteria on their cuticle, and the MG secretions are consequently the main source of antimicrobial compounds to fight parasites, as is also the case for *Atta* leafcutter ants [17, 18], and derived attine ants in general employ the MG more than lower attine ants [20]. The synthesis and deployment of MG grooming secretions entails a high metabolic cost, approximately 13-20% of basal metabolism [19], so adjusting its use according to the threat will be beneficial. The energetic cost of planting behavior is not known, but is likely significant as the ants have to collect substrate for, and clean, the fungal mutualist in order to grow it. More studies are needed to understand planting costs and function [15].

Another reason why ants could change between behaviors may be related to the specificity or efficacy of the hygienic behavior. The fungal mutualist produces a wide variety of compounds with antimicrobial activity [21], and there is evidence they inhibit endophytes [22], mycoparasites such as *Escovopsis* sp. [23], and also slow down *Metarhizium* growth [24,25]. Additionally, planting behavior covers the pathogenic conidia that may be beneficial. For example, *Metarhizium* sp. requires oxygen during germination [26], so planting behavior could potentially create a microenvironment unfavorable to the germination or growth of the pathogen [27]. Fungal symbionts in *Acromyrmex* ants can inhibit *Metarhizium* fungal germination and growth [25], but both *Metarhizium* sopres and hyphae are sensitive to MG compounds, as *Escovopsis* spores, but not *Escovopsis* hyphae [8]. On other hand, *Escovopsis germination and growth is sensitive to* MG compounds but not *Metharizium* (15). Those studies permit suggest that ungerminated conidia are susceptible to fungal planting strategy, while germinated conidia could to be susceptible to MG compounds. Using the same compound to also control a parasite could lead to the faster evolution of resistance, as has been abundantly illustrated by human use of pesticides and antibiotics [28]. While, that using different compounds and hygienic strategies with different modes of action has been proposed to avoid pesticide resistance in agricultural ecosystems, and antibiotic resistance in medicine [28]. Fungus growing ants arose ~60-50 million years ago, and utilising different strategies according to the nature of the parasite threat may have helped them to avoid resistance to their antimicrobial compounds.

ACKNOWLEDGEMENTS: Thanks to Ministerio de Ambiente for the permits. Funds coming from SENACYT Fellowhip to EB, and INDICASAT AIP and STRI provided supplemental support to EB. HF-M was supported by SNI and SENACYT grants. Additional support came from research funds from STRI to WTW.

REFERENCES

1. Hamilton WD. 1987 Kinship, recognition, disease, and intelligence: constraints of social evolution. In *Animal Societies: Theories and Facts*, ed. Itô Y, Brown JL, Kikkawa J. pp. 81-102. Tokyo: Japan Science Society Press.
2. Godfrey SS, Bull CM, Murray K, Gardner MG. 2006 Transmission mode and distribution of parasites among groups of the social lizard *Egernia stokesii*. *Parasitol Res*, **99**: 223-230.
3. Bot AN, Currie CR, Hart AG, Boomsma JJ. 2001 Waste management in leaf-cutting ants. *Ethol Ecol & Evol* **13**: 225-237.
4. Diez L, Lejeune P, Detrain C. 2014 Keep the nest clean: survival advantages of corpse removal in ants. *Biol lett* **10**: 20140306. https://doi.org/10.1098/rsbl.2014.0306 PMID: 25009241
5. Yanagawa A, Shimizu S. 2007 Resistance of the termite, *Coptotermes formosanus* Shiraki to *Metarhizium anisopliae* due to grooming. *BioControl* **52**: 75-85.

6. Bos N, Sundström L, Fuchs S, Freitak D. 2015 Ants medicate to fight disease. *Evolution* **69**: 2979-2984.
7. Castella G, Chapuisat M, Christe P. 2008 Prophylaxis with resin in wood ants. *Anim Behav* **75**: 1591-1596.
8. Bot ANM, Ortius-Lechner D, Finster K, Maile R, Boomsma JJ. 2002 Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants. *Insect Soc* **49**: 363-370.
9. Cremer S, Armitage SA, Schmid-Hempel P. 2007 Social immunity. *Curr Biol* **17**: R693-R702.
10. Hughes WOH, Eilenberg J, Boomsma JJ. 2002 Trade-offs in group living: transmission and disease resistance in leaf-cutting ants. *Proc R Soc Lond B* **269**: 1811-1819.
11. Fernández-Marín H, Zimmerman JK, Rehner SA, Wcislo WT. 2006 Active use of the metapleural glands by ants in controlling fungal infection. *Proc R Soc Lond B* **273**: 1689-1695.
12. Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR. 2005 The evolution of agriculture in insects. *Annu Rev Ecol Evol Syst* **36**: 563-595.
13. Currie CR, Scott JA, Summerbell RC, Malloch D. 1999 Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* **398**: 701-704.
14. Fernández-Marín H, Zimmerman JK, Nash DR, Boomsma JJ, Wcislo WT. 2009 Reduced biological control and enhanced chemical pest management in the evolution of fungus farming in ants. *Proc R Soc Lond B* **276**: 2263-2269.
15. Fernández-Marín H, Bruner G, Gomez EB, Nash DR, Boomsma JJ, Wcislo WT 2013 Dynamic disease management in *Trachymyrmex* fungus-growing ants (Attini: Formicidae). *Am Nat* **181**: 571-582.
16. Anderson MJ. 2017 Permutational Multivariate Analysis of Variance (PERMANOVA). In Wiley StatsRef: Statistics Reference Online (eds Balakrishnan N, Colton T, Everitt B, Piegorsch W, Ruggeri F,Teugels JL). doi:10.1002/9781118445112.stat07841.
17. De Lima Mendonça A, Da Silva CE, de Mesquita FLT, da Silva Campos R, Do Nascimento RR, de Azevedo Ximenes ECP, Sant'Ana AEG. 2009 Antimicrobial activities of components of the glandular secretions of leaf cutting ants of the genus *Atta*. *Antonie Van Leeuwenhoek* **95**: 295-303.
18. Fernández-Marín H, Nash DR, Higginbotham S, Estrada C, van Zweden J S, d'Ettorre P, Wcislo WT, & Boomsma JJ. 2015 Functional role of phenylacetic acid from metapleural gland secretions in controlling fungal pathogens in evolutionarily derived leaf-cutting ants. *Proc R Soc Lond B* **282**: 20150212.
19. Poulsen M, Bot ANM, Nielsen MG, Boomsma JJ. 2002 Experimental evidence for the costs and hygienic significance of the antibiotic metapleural gland secretion in leaf-cutting ants. *Behav Ecol and Sociobiol* **52**: 151-157.
20. Hughes WOH, Pagliarini R, Madsen HB, Dijkstra MB, Boomsma JJ. 2008 Antimicrobial defense shows an abrupt evolutionary transition in the fungus-growing ants. *Evolution* **62**: 1252-1257.
21. Richard FJ, Poulsen M, Drijfhout F, Jones G, Boomsma JJ. 2007 Specificity in chemical profiles of workers, brood and mutualistic fungi in *Atta*, *Acromyrmex*, and *Sericomyrmex* fungus-growing ants. *J Chem Ecol* **33**: 2281-2292.
22. Van Bael SA, Fernández-Marín H, Valencia MC, Rojas EI, Wcislo WT, Herre EA. 2009 Two fungal symbioses collide: endophytic fungi are not welcome in leaf-cutting ant gardens. *Proc R Soc Lond B* **276**: 2419-2426.
23. Gerardo NM, Jacobs SR, Currie CR, Mueller UG. 2006 Ancient host–pathogen associations maintained by specificity of chemotaxis and antibiosis. *PLoS Biology* **4**: e235.
24. Armitage SA, Fernández-Marín H, Wcislo WT, Boomsma JJ. 2012 An evaluation of the possible adaptive function of fungal brood covering by attine ants. *Evolution* **66**: 1966-1975.
25. Armitage SA, Fernández-Marín H, Boomsma JJ, Wcislo WT. 2016 Slowing them down will make them lose: a role for attine ant crop fungus in defending pupae against infections? *J Anim Ecol* **85**: 1210-1221.
26. Braga GU, Destéfano RH, Messias CL. 1999 Oxygen consumption by *Metarhizium anisopliae* during germination and growth on different carbon sources. *J Inverteb Pathol* **4**: 112-119.

210 27. Axelrod R, Axelrod DE, Pienta KJ. 2006 Evolution of cooperation among tumor cells. *PNAS* **103**:
211 13474-13479.
212 28. Palumbi SR. 2001. Humans as the world's greatest evolutionary force. *Science* **293**: 1786-1790.