

Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens

Ainslie E. F. Little^{1,2,3,*}, Takahiro Murakami^{2,4}, Ulrich G. Mueller^{3,5} and Cameron R. Currie^{1,2,3}

¹Department of Bacteriology, University of Wisconsin-Madison, Madison, WI 53706, USA

²Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, KS 66045, USA

³Smithsonian Tropical Research Institute, Apartado Box 2072 Balboa, Ancon, Republic de Panama

⁴Chromosome Research Unit, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

⁵Section of Integrative Biology, Patterson Laboratories, University of Texas at Austin, Austin, TX 78712, USA

*Author for correspondence (alittle@wisc.edu).

Parasites influence host biology and population structure, and thus shape the evolution of their hosts. Parasites often accelerate the evolution of host defences, including direct defences such as evasion and sanitation and indirect defences such as the management of beneficial microbes that aid in the suppression or removal of pathogens. Fungus-growing ants are doubly burdened by parasites, needing to protect their crops as well as themselves from infection. We show that parasite removal from fungus gardens is more complex than previously realized. In response to infection of their fungal gardens by a specialized virulent parasite, ants gather and compress parasitic spores and hyphae in their infrabuccal pockets, then deposit the resulting pellet in piles near their gardens. We reveal that the ants' infrabuccal pocket functions as a specialized sterilization device, killing spores of the garden parasite *Escovopsis*. This is apparently achieved through a symbiotic association with actinomycetous bacteria in the infrabuccal pocket that produce antibiotics which inhibit *Escovopsis*. The use of the infrabuccal pocket as a receptacle to sequester *Escovopsis*, and as a location for antibiotic administration by the ants' bacterial mutualist, illustrates how the combination of behaviour and microbial symbionts can be a successful defence strategy for hosts.

Keywords: actinomycete; behavioural ecology; *Escovopsis*; host–parasite interaction; mutualism; pathogen

1. INTRODUCTION

Parasites are direct agents of natural selection; they regulate host populations and influence community and ecosystem dynamics (Poulin & Morand 2004). Social insects have a high risk of parasitism due to the high density and genetic homogeneity of

individuals in colonies, thus they have developed a range of defensive mechanisms. Termites (*Coptotermes*) wall off nestmates infected by nematodes (Fujii 1975), honey bees remove larvae infected by foulbrood (Spivak & Reuter 2001) and ants have glands (metapleural) with antibiotic properties active against general microbial infections (Bot *et al.* 2002; Poulsen *et al.* 2002).

Fungus-growing ants (New World tribe Attini) provide their fungal cultivar with substrate for growth and, in return, the fungus serves as the ants' main food source (Weber 1972). The obligate nature of this symbiosis requires the ants to protect both themselves and their fungal mutualist from parasites. Fungus garden health is constantly threatened by microbes inhabiting the soil and substrates collected to manure the cultivar. To separate new gardens from soil, queens use hygienic strategies during nest construction (Fernandez-Marin *et al.* 2004). The fungus garden is also threatened by parasitic fungi in the genus *Escovopsis* (Ascomycota, Hypocreales), infections of which can be lethal if not controlled (Currie *et al.* 1999a). Leaf-cutter ants (the two most derived genera of fungus-growing ants) use specialized hygienic behaviours called fungus grooming and weeding to remove *Escovopsis* spores and infected garden material (Currie & Stuart 2001), and waste-management tasks are partitioned to prevent the spread of potentially harmful microbes from the refuse into the garden (Hart & Ratnieks 2001). To further defend against *Escovopsis* infection, fungus-growing ants have a mutualistic association with filamentous bacteria (actinomycete) housed on the ants' cuticle that produce antibiotics that specifically inhibit *Escovopsis* (Currie *et al.* 1999b).

The infrabuccal pocket, a filtering structure within the oral cavity of ants, is a key component in leaf-cutter hygienic behaviours (Quinlan & Cherrett 1978). Detritus and potentially hazardous debris that ants gather while cleaning themselves, the nest area, or the fungus garden is accumulated in the pocket (Bailey 1920). Once full, the compressed material is expelled from the pocket as a pellet. To prevent microbes from re-establishing infection in the garden, leaf-cutter ants deposit their infrabuccal pellets in refuse piles segregated from their nest (Febvay & Kermarrec 1981). In contrast, ants in the more phylogenetically basal fungus-growing ant lineages stack and maintain their pellets in piles near their gardens (Little *et al.* 2003). It is currently unclear why most genera of fungus-growing ants build piles of infrabuccal pellets. In this study, we experimentally examine whether the piling of infrabuccal pellets is a response to garden infection by microbial parasites and we explore the microbial ecology of the infrabuccal pocket to further understand its role in fungus-growing ant nest hygiene.

2. MATERIAL AND METHODS

(a) Study organisms

To examine the behavioural and microbial ecology associated with infrabuccal pellets, *Trachymyrmex cf. Zeteki*, which frequently build infrabuccal pellet piles (IPPs), was used as a model species (Little *et al.* 2003). In January 2003, 40 queenright colonies with 75–250 workers and a single fungus chamber were collected in Gamboa, Panama. Colonies were maintained at the University of Kansas in plastic dual chambers (one housing the garden and one for feeding,

foraging and dumping of refuse) connected by plastic tubes, and placed on islands surrounded by mineral oil to prevent potential transfer of microbes between colonies via vectors (e.g. mites). Colonies were fed a mixture of dried oats and oak catkins and watered once a week.

(b) Experimental infections

We examined the function of IPPs by experimentally infecting gardens of 40 *T. cf. zeteki* colonies. To determine whether pellet piling is a response to general microbial parasites or to the specialized parasite *Escovopsis*, colonies were randomly assigned to one of four treatments: (i) *Escovopsis*, (ii) *Trichoderma viride*, a generalist mycoparasitic fungus closely related to *Escovopsis* (Currie *et al.* 2003a,b), (iii) non-viable *Escovopsis* (UV irradiated for 1 h to kill spores) or (iv) sterilized, distilled water. Pre-existing IPPs were removed prior to treatment. *Escovopsis* and *T. viride* strains (sub-cultured from single colony-forming units) were isolated in Gamba from a *T. cf. zeteki* colony and the soil, respectively. Isolates were grown on potato dextrose agar (PDA) (Difco, Sparks, MD) with 1000 iu ml⁻¹ of penicillin–streptomycin (MP Biomedicals Inc., Aurora, OH). Spores were added to ddH₂O with Tween 20 [5 × 10⁻⁵] (Fisher Scientific, Pittsburgh, PA) to evenly disperse spores in solution. Each colony received 0.5 ml of solution (ca 40 000 spores) via mist inoculation, then colonies were monitored at regular intervals for 48 h.

(c) Microbial ecology

To further examine the role of IPPs in *T. cf. zeteki* garden hygiene, we isolated microbes from pellets dissected from the infrabuccal pocket and those in IPPs built after experimental treatments. From each pile present 48 h after treatments (15 piles total), 12 randomly selected pellets were removed. Six pellets per pile were particle-plated on PDA (with 1000 iu ml⁻¹ penicillin–streptomycin) and six were particle-plated on actinomycete specific medium (chitin agar with 1000 iu ml⁻¹ Nystatin, Sigma-Aldrich, St. Louis, MO) and incubated at 21 °C for 10 days and four weeks, respectively. Growth was monitored, and the presence of fungal cultivar, *Escovopsis* or actinomycetes was scored (present/absent) and then sub-cultured into pure culture.

Forty-eight hours post treatment, the heads of six ants from each of three (randomly chosen) *Escovopsis*-treated colonies were aseptically dissected and the pellets removed to identify viable microbes in infrabuccal pellets prior to regurgitation from the infrabuccal pocket. Three pellets from each colony were particle-plated on PDA, while the other three were particle-plated on chitin agar. Plates were incubated, and microbes were scored and sub-cultured as above.

To determine whether actinomycetes isolated from the infrabuccal pocket produce *Escovopsis*-inhibiting antibiotics, we challenged one actinomycete isolate (representative of the most common morphotype found during experimental isolation of *T. cf. zeteki* pellets) *in vitro* with *Escovopsis* (Currie *et al.* 1999b). Challenges were scored 10 days after *Escovopsis* inoculation, and growth/inhibition of *Escovopsis* was scored as complete inhibition or a reduction of growth when compared to *Escovopsis* growth in the absence of the actinomycete ($n=15$).

3. RESULTS

(a) Experimental infections

In response to experimental infections, IPPs are built very quickly. Piles were present within 3 h following treatment in 60% of *Escovopsis*-infected colonies (figure 1). IPP construction differed significantly between experimental treatments (G -test, $G=13.05$, $p<0.005$, d.f.=3; figure 1). After 48 h, 80% of colonies exposed to *Escovopsis* had built IPPs, significantly more than control colonies ($G=16.12$, $p<0.001$, d.f.=1). The proportion of colonies that constructed IPPs was also significantly higher when exposed to viable *Escovopsis* than when treated with non-viable *Escovopsis* ($G=7.72$, $p<0.010$, d.f.=1). Although some piles were built in colonies exposed to *Trichoderma* and non-viable *Escovopsis*, their proportions were not significantly different

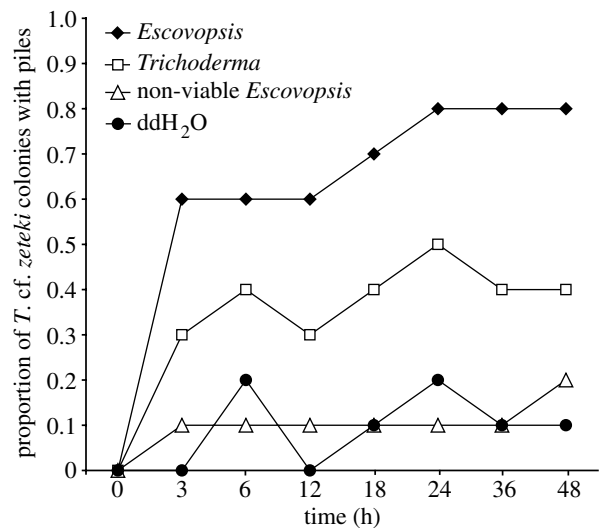


Figure 1. Proportion of *Trachymyrmex cf. zeteki* colonies that built infrabuccal pellet piles after exposure to one of four infection treatments over a 48 h period.

from the control nests ($G=2.776$, $p>0.050$, d.f.=1; $G=0$, $p>0.100$, d.f.=1, respectively).

(b) Microbial ecology

Microbial isolations reveal that pellets in IPPs do not contain viable fungal cultivar tissue and very few contain viable *Escovopsis* (6.25%); however, many contain viable actinomycetes (64.44%) (table 1). Similarly, isolations from pellets dissected from the infrabuccal pocket do not contain viable cultivar or *Escovopsis*, but 88.9% contain actinomycetes (table 1). There were significantly more actinomycetes isolated from pellets in piles and pockets of *Escovopsis*-treated colonies than statistically expected when compared to sham-treated colonies ($G=8.71$, $p<0.005$, d.f.=1 for both non-viable *Escovopsis* and ddH₂O). The proportion of actinomycetes isolated from pellets formed during *Escovopsis* treatment were significantly greater than those formed during *Trichoderma* treatments ($G=3.88$, $p<0.050$, d.f.=1).

Strong inhibition of *Escovopsis* by the infrabuccal actinomycete isolate was found in bioassay challenges as seen in Currie *et al.* (1999b), Germination and growth of *Escovopsis* were completely suppressed in 40% of challenges, while fungal growth in the remaining challenges was inhibited by $85.32 \pm 3.29\%$ (mean \pm s.e.) when compared to *Escovopsis* growth in the absence of actinomycetes.

4. DISCUSSION

The success of agriculture by ants has, in part, been attributed to efficient removal of microbial parasites from fungus gardens by workers (Currie & Stuart 2001). Our study shows that the removal of microbial parasites from fungus gardens is even more complex than previously realized and that the infrabuccal pocket plays a key role (figure 2). Experimental infection of *T. cf. zeteki* gardens resulted in a fivefold increase in IPP abundance compared to baseline pile abundance in sham-treated nests (distilled water and non-viable *Escovopsis*). Examination of pellets

Table 1. Mean numbers of isolates per pellet, given as proportions, in which fungus-growing ant-microbe symbionts, cultivar, *Escovopsis* and actinomycetes were isolated following exposure to one of four infection treatments.

treatment	pellet location	sample size	proportion of infrabuccal pellets from which isolates were obtained \pm s.d.		
			cultivar	<i>Escovopsis</i>	actinomycetes
<i>Escovopsis</i>	pellet pile	48	0.0	0.06 ± 0.16	0.79 ± 0.17
	infrabuccal pocket	9	0.0	0.0	0.89 ± 0.19
<i>Trichoderma</i>	pellet pile	24	0.0	0.0	0.58 ± 0.22
non-viable <i>Escovopsis</i>	pellet pile	12	0.0	0.0	0.33 ± 0.24
distilled water	pellet pile	6	0.0	0.0	0.33 ± 0.0
total		99			

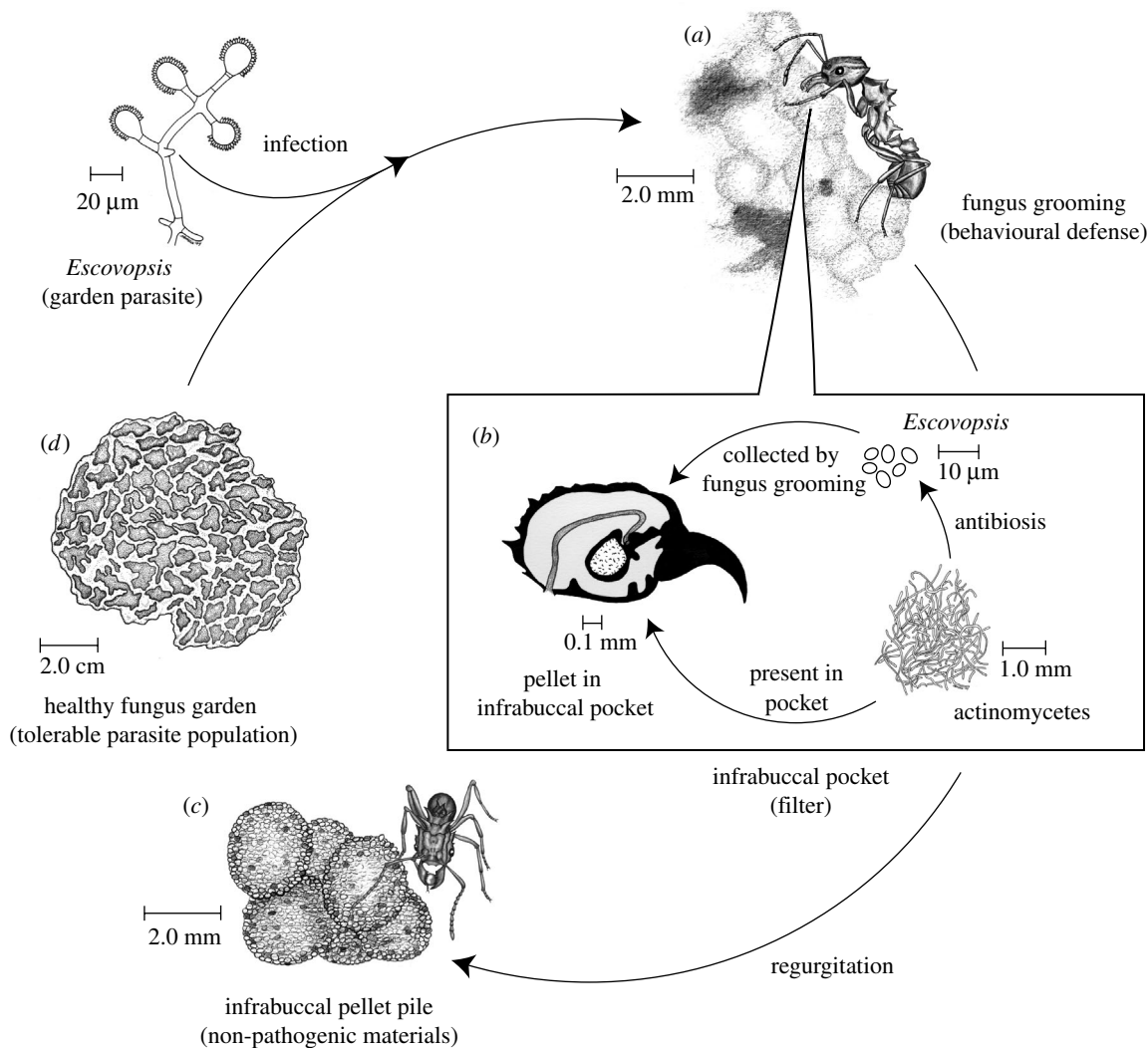


Figure 2. Removal of the fungus garden parasite *Escovopsis* by *Trachymyrmex* cf. *zeteki*. Following infection, ants collect parasitic spores in their infrabuccal pocket via fungus grooming (a). In the pocket, *Escovopsis* spores are exposed to inhibitory antibiotics produced by actinomycetous bacteria (b). Once the pocket is full, the non-pathogenic contents are regurgitated in the form of pellets and stacked together in piles tended by the ants (c), leaving the fungus garden with tolerable levels of the parasite (d).

dissected from the infrabuccal pocket, and of those collected after expulsion, confirms that pellets contain *Escovopsis* spores used to infect the garden (A. Little, personal observation). However, in addition to storing and compacting spores, we found that the infrabuccal pocket also appears to function as a specialized sterilization device, killing spores of the garden parasite *Escovopsis*. Microbial isolations of pellets taken from IPPs and those dissected from the infrabuccal

pocket reveal that pellets rarely contain viable spores of *Escovopsis*.

Our finding of actinomycetes in infrabuccal pellets probably explains the absence of viable *Escovopsis*; antibiotics produced by actinomycetes in the infrabuccal pocket probably kill *Escovopsis* spores. The infrabuccal pocket is a confined space that permits the ants to place parasitic material in quarantine, subjecting it to antibiotics produced by their

mutualistic bacteria, then expelling the resultant non-pathogenic material. Whether actinomycetous bacteria are housed within the infrabuccal pocket regularly or they are acquired from the ants' cuticle in response to infection is unknown. Other factors, such as enzymes in the salivary secretions of fungus-growing ants, may also aid in the inhibition of *Escovopsis* in the oral cavity (Febvay *et al.* 1984). Fungal inhibition in the infrabuccal pocket may be specific to *Escovopsis*; the fungal cultivar is successfully transmitted to new nests via the infrabuccal pocket (Weber 1972). Our data provide the first evidence that fungus-growing ants actively distinguish between viable and non-viable spores of *Escovopsis*, suggesting that the ants may recognize a secondary metabolite produced by *Escovopsis*, perhaps related to germination.

This study clearly indicates that the piling of infrabuccal pellets by *T. cf. zeteki* is a behavioural response to microbial infection of the fungus garden, particularly to *Escovopsis*. Interestingly, actinomycetes are found significantly more often in infrabuccal pellets formed after exposure to viable *Escovopsis* than in those formed after exposure to *Trichoderma*, non-viable *Escovopsis* or water. This could be the result of increased uptake of actinomycetes into the infrabuccal pocket from an external source, such as the exoskeleton, during times of infection. Alternatively, if actinomycete populations are maintained in the infrabuccal pocket it is possible that *Escovopsis* collected during infection stimulates the growth of actinomycetes in the infrabuccal pocket, as seen on the ants' cuticle during infection (Currie *et al.* 2003a).

Behaviour is an important part of an organism's parasite defence strategy and, in this case, hygienic behaviours are critical. The development of hygienic practices was very important in decreasing the prevalence of human disease (Ewald 1994), and sanitary measures, such as deep ploughing, pruning and burning, have been used for centuries to eradicate or decrease parasitic inoculum in agriculture (Agrios 1997). Apparently, natural selection pressures have led fungus-growing ants to evolve a similar suite of parasite defence strategies (Currie & Stuart 2001; Mueller *et al.* in press; this study). The association between fungus-growing ants, their cultivar and *Escovopsis* is ancient (Currie *et al.* 2003b). It is likely that prior to domestication by fungus-growing ants, the fungi they cultivate were parasitized by *Escovopsis*, thus the ants have had to adapt to the presence of *Escovopsis* for millions of years. Our data suggest that the development of the infrabuccal pocket as a receptacle for parasites and locale for antibiotic administration by the ants' bacterial mutualist may be one of the key evolutionary innovations in fungus-growing ants. This combination of behaviours and microbial symbionts, which constitutes the fungus-growing ants' parasite defence strategy, has undoubtedly allowed for their enduring success.

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