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Research article

Fungus garden platforms improve hygiene during nest establishment in *Acromyrmex* ants (Hymenoptera, Formicidae, Attini)

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Abstract. Physically isolating organisms from disease agents should reduce the likelihood of disease transmission and infection, and increase survival and growth, particularly in more vulnerable, early ontogenetic stages. During nest founding in fungus-growing ants, foundresses of most genera use a garden platform to isolate the incipient fungal garden from the soil of the underground chamber. We studied nest founding in *Acromyrmex octospinosus* to test the hypotheses that the use of a platform (rootlets used to suspend the fungus garden): (i) reduces the likelihood that the garden will be contaminated by soil-borne microbial pathogens; (ii) results in more rapid growth of a young colony; and (iii) increases colony survival. We manipulated natural incipient nests to have gardens either in contact with or isolated from soil surrounding the chamber, and nests with and without foundresses present. We found a higher incidence of infection in gardens that were in contact with the chamber soil and without queens, compared with gardens isolated from the chamber soil with and without foundresses. The production of eggs, larvae and pupae, as well as leaf area harvested, were significantly different between nests with and without platforms, but there were no differences in the production of workers nor garden biomass. Likewise, there were no differences between treatments in colony survival rates over 8 weeks. Using smaller incipient gardens, however, gardens with and without platforms differed in survivorship rates after 24 hours. The results indicate that the use of a platform to cultivate an incipient fungal garden is an adaptation to reduce soil-borne diseases and increase colony performance.

Keywords: Acromyrmex, nest founding, hygiene, disease, fungus garden, prophylactic devices.

Introduction

Infectious diseases constitute a substantial source of morbidity and mortality in natural populations (e.g., Ewald, 1994) and therefore they are likely to be important selective factors in social evolution (Alexander, 1974; Hamilton, 1987; Sherman et al., 1988; Gadagkar, 1992; Schmid-Hempel, 1998; Moore, 2002; O'Donnell and Beshers, 2004; Bonds et al., 2005). The aphorism "an ounce of prevention is worth a pound of cure" encapsulates the idea that avoiding contaminants and disease agents is one of the more cost-effective strategies for disease management (Ewald, 1994; Moore, 2002). Growing evidence indicates that animals have evolved behavioral responses to lower the probability of disease transmission, such as decreasing the frequency of contact with sources of contaminants (Hart, 1990; Hart and Ratnieks, 2001; Moore, 2002), including the evacuation of infected areas (Rosengaus et al., 1999).

Fungus-growing ants (Attini), like other social insects (Schmid-Hempel, 1998), confront a diverse array of parasites and pathogens, including those that attack their fungal gardens (reviewed in Mueller et al., 2001; Poulsen and Currie, 2006). To control pathogens in established gardens workers deploy both behavioral and chemical defenses, including weeding and grooming (Bass and Cherrett, 1994; Currie and Stuart, 2001), antimicrobial glandular secretions (do Nascimento et al., 1996; Fernández-Marín et al., 2006) and antibiotic metabolites from bacteria (Currie et al., 1999a, 2003). In contrast to the behavior of workers in established colonies, little is known of the defensive and hygienic behaviors of nest foundresses.

Attine nest founding is unusually complicated, because foundresses transport, establish, care for and grow a fungal symbiont that they will use to nourish their larvae (Weber, 1972; Hölldobler and Wilson, 1990; Mueller et al., 2001; Fernández-Marín et al., 2004). The loss of the symbiont during nest-founding is usually fatal to the new colony (Weber, 1972; Fernández-Marín and Wcislo, 2005), so foundresses have diverse strategies to ensure the survivorship and growth of the fungus garden. Except in the most derived genus *Atta*, attine foundresses are semi-claustral and search for substrate to nourish the fungus gardens (Weber, 1972; Fernández-Marín et al., 2004). These semi-claustral attines use "platforms" during nest founding to physically isolate their gardens from the surrounding soil of the nest chamber (Fernández-Marín et al., 2003, 2004). The architectural details vary among taxa, but we define platforms generally as found objects that support (e.g., rocks, or detached forewings placed on soil pellets) or suspend (e.g., rootlets, or detached forewings inserted into the garden chamber ceiling) a fungal garden away from the soil. Fernández-Marín et al. (2004) showed that the young gardens are isolated from the soil of the surrounding chamber, and they hypothesized that use of a platform may be a prophylactic behavior that reduces the risk of fungal and brood infections from soil-born pathogens. Here we test this hypothesis using a derived leaf-cutter ant, *Acromyrmex octospinosus*.

A. octospinosus foundresses use living rootlets as a platform to which they attach their gardens, and forage for fresh vegetable materials to nourish the fungus during the nest-foundation stages (Fernández-Marín et al., 2003). We experimentally evaluated whether the use of a garden platform reduces the probability that the fungal garden becomes infected, and we then ascertained whether or not use of a platform has consequences for colony performance and survival.

Materials and methods

The role of platforms in controlling microbial infections of gardens

Sixty incipient nests of *A. octopinosus* were marked in the field near Gamboa, central Panama (Colon Province), during the early part of the wet season (May-June, 2002), approximately 4 weeks after foundation. Each nest contained a fungus garden (~ 1 cm in diameter) suspended from living rootlets, a single foundress, and no workers. To manipulate garden platforms we excavated a small amount of soil and then carefully opened each nest on one side using a knife; care was taken to avoid harming the foundress and touching the garden during the manipulation of the platform (rootlet). To re-suspend the rootlet platform, we used a sterile toothpick that at one end was intertwined with the rootlets and the other end was inserted into the surrounding soil. Nests were randomly assigned to one of the following treatments:

(1) *control:* nests with a foundress and a platform (i.e., a garden isolated from the soil-chamber). A toothpick was placed in contact with the rootlets but the orientation and position of the garden was unchanged.

(2) *platform without foundress:* the foundress was removed from the nest, and a sterile toothpick was used as above to suspend the garden.

(3) *no platform and no foundress:* the foundress was removed from the nest, and the toothpick was intertwined with the rootlets so as to change the orientation of the garden and bring it into contact with the soil of the surrounding chamber.

The entrance of each nest was then re-built using moist soil collected at the site that was compacted to seal the chamber, except for a small entrance as in a natural nest. Thirty-six hours later we re-opened each chamber. We used sterile forceps to grab the rootlets and cut them; we then transferred individual gardens to sterile Petri dishes. The knife used during excavations was cleaned with ethanol before manipulating nests, and manipulations were made in a way to avoid contact with the fungus gardens. To assay the degree of infection of each fungus garden, 20 fungal pieces (~ 3–5 mm diameter) were taken from each nest and placed in one of two sterile Petri dishes (10 pieces/dish) with potato dextrose agar (Difco) with antibiotics against bacteria (50 mg/l each of penicillin and streptomycin). We observed the dishes twice per day, and we removed any fungal pieces that were contaminated with an overgrowth of mycelium or yeast-like forms. After ten days, we recorded the total number of contaminated and healthy fungus pieces growing on the Petri dishes.

Colony growth with and without garden platforms

To determine if the use of a fungal platform enhances fungal symbiont growth and brood production, we maintained incipient nests in the laboratory using methods from Fernández-Marín et al. (2003), in which each artificial nest was housed in a soil-filled plastic box with a small sapling of *Bactris* sp. (Arecaceae) growing in it. We could not use natural nests for a "no rootlet" (no platform) treatment because in nature *Acromyrmex* foundresses dig for new rootlets or dig a new chamber if rootlets are not available or are substantially modified (HFM personal observation). We established three treatments using the artificial nests:

(1) *root platform* (Root): ants were able to use the rootlets (~ 1.0 mm diameter) of *Bactris* sp. saplings that passed through the chamber area.

(2) *plastic platform* (Pl-P): a nylon thread (~ 1.0 mm diameter) passed through the chamber area, which the foundress could use as a platform.

(3) *no platform* (No-P): the chamber area lacked any materials suitable for use as a platform to isolate the garden from the soil.

Incipient *A. octospinosus* nests were collected in late May (2004) (~ 4 weeks after foundation). Each nest contained a small garden, eggs, larvae and a single queen. Within each artificial nest, a fungus garden (~ 0.05 g wet weight ≈ 0.015 g, dry weight) without brood was placed on the soil floor of the chamber, along with the foundress, and the entrance was closed. Individual nests were placed in an arena (35×25×15 cm) with non-sterile soil. After 24 hours, the entrance was opened to permit the foundress to forage. We used 30 artificial nests per treatment as described above. If a nest failed between 1 and 3 days after it was established, then it was replaced with a new one. Every second day for 8 weeks, 2 pieces (each 2×3.5 cm) of young, fresh leaves (*Lagerstroemia speciosa*, Lythraceae) were added to the arena. The nests and foraging arenas were kept moist by spraying them with non-sterile water. For each nest, the leaf area that queens harvested was recorded. After 8 weeks, the fungus gardens were collected from surviving nests, and we recorded the number of workers, pupae, larvae, eggs, and staphylae. Each fungus garden was dried and subsequently weighed.

Table 1. One-Way ANOVA with post hoc Tukey's HSD tests for differences in brood production and foraging activity of *Acromyrmex octospinosus* during nest founding among treatments involving root platforms (Root), plastic platforms (Pl-P) and no platforms (No-P).

Variables	One-Way ANOVA		Post hoc Tukey's (HSD) test comparison among treatments (*p* value)		
	$F_{2, 52}$	*p* value	Root vs. Pl-P	Root vs. No-P	Pl-P vs. No-P
Eggs	3.25	0.04	0.93	0.050	0.17
Larvae	3.23	0.04	0.94	0.10	0.07
Pupae	6.69	0.002	0.96	0.004	0.01
Workers	0.52	0.59	–	–	–
Staphylae	1.43	0.24	–	–	–
Dry garden biomass	2.52	0.14	–	–	–
Leaf area cut	3.51	0.03	0.88	0.03	0.16

Incipient garden survivorship

Incipient nests of *A. octospinosus* were collected in May 2006. Artificial nests with and without root platforms were made as described above. Each nest contained a foundress and a small piece of garden (wet weight ≈ 0.0025 g). We recorded the survivorship of the garden after 24 hours. The fungus garden was considered living if it had white hyphae, and dead if the garden was discarded, cut in pieces, or brown in color.

Colony survivorship

We monitored colonies with and without garden platforms to determine whether foundresses and colonies (fungus garden) survived to 24 hours, as well as 8 weeks from the start of the experiment.

Data analyses

The fungal garden infection data were transformed (natural log, ln) to meet assumptions of parametric statistical tests. Other data were normally distributed and were analyzed with one-way ANOVA and post-hoc comparisons, using Statistica Statsoft software, and tests from Zar (1999) as indicated in the text.

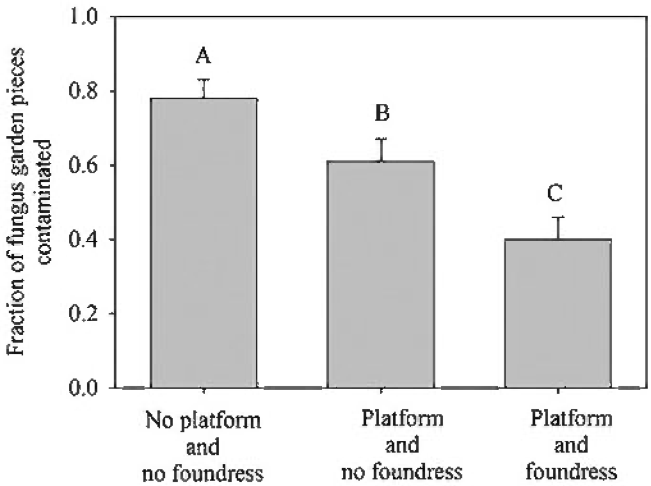

Figure 1. Proportion of contaminated sub-samples per fungus garden from incipient nests of *Acromyrmex octospinosus*, comparing treatments where gardens lacked a platform and foundress or lacked only a foundress, *versus* controls with both platforms and foundresses. Mean values are given with their standard error. Levels of contamination are significantly different among treatments, and significant pairwise comparisons are indicated by different letters (one-way ANOVA, Least Significant Difference LSD, post hoc analysis, $p < 0.05$).

Results

The role of platforms in controlling microbial infections of gardens

Fungal platforms reduced the extent of infection of the fungus garden compared to nests without platforms, and the lowest infection rate was observed in nests with both platform and foundress. After 36 hours, fungal gardens from control nests (i.e., isolated garden and foundresses) had an average of 40% fungal pieces contaminated, whereas 61% were infected in nests with platforms and without foundresses, and 78% were infected when neither foundresses nor platforms were present ($F_{2,55} = 10.76$, $p = 0.0001$) (Fig. 1).

Colony growth with and without garden platforms

The three treatment groups differed with respect to some measures of performance. Nests with a root platform differed from those without platforms in the production of eggs, pupae and leaf area harvested, but did not differ from those with plastic platforms (Table 1, Fig. 2). Pairwise comparisons showed that root and plastic platform nests differed from those without platforms in the production of larvae and pupae, and there were overall treatment effects on production of both larvae and pupae. Worker production, fungal staphylae number, and garden dry weights did not differ among treatments (Table 1, Fig. 2).

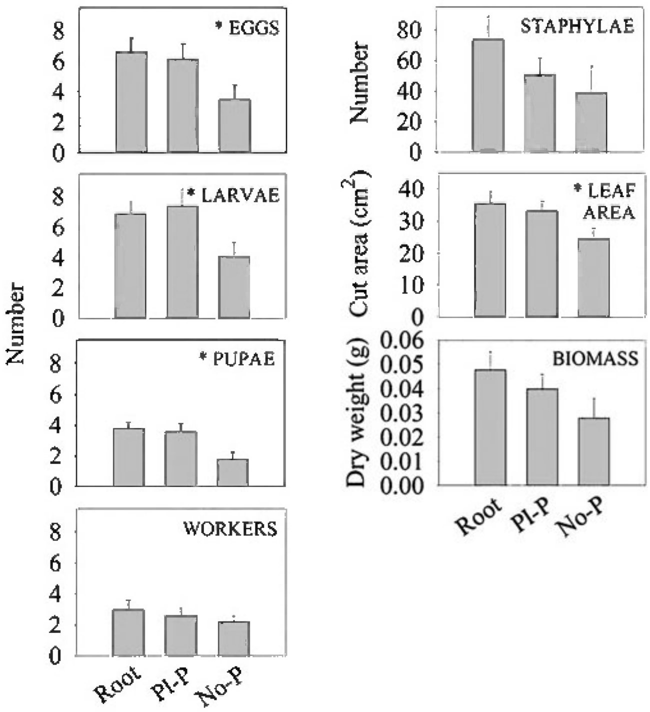

Figure 2. The effect of using a natural fungal platform (Root), a plastic platform (Pl-P), or no platform (No-P) on colony performance in artificial foundress nests of *Acromyrmex octospinosus*, as measured by production of eggs, larvae, pupae, and workers, as well as the number of staphylae, leaf area harvested, and fungal garden dry weight. Mean values are given with their standard errors. Significant differences in overall performance measures are indicated by an asterisk (*). For pairwise significant differences see Table 1.

Fungus garden and colony survivorship

Foundress nests of *A. octospinosus* with very small pieces of garden differed in survivorship rates after 24 hours, comparing nests with root platforms (43% of n = 30 nests survived) and those without platforms (13% of n = 39) (Chi-Square, $df = 1$; $X^2 = 6.68$, $p < 0.01$). In contrast, when artificial nests were started with larger fungal gardens (0.05 g) the presence or absence of platforms had no effect on short-term survivorship because all nests survived for 24 hours. Eight weeks after artificial *A. octospinosus* nests were established, 20 out of 30 colonies survived in nests with root (natural) platforms and 20 of 30 colonies survived without platforms, whereas 15 out of 30 colonies with plastic platforms survived (G test = 5.36, $df = 2$; $p > 0.05$).

Discussion

A constellation of factors plays a role in how attine ants prevent and manage diseases, including hygienic behavior, disease-suppressing microbes, the antibiotic properties of the cultivated fungi, antimicrobial glandular secretions, and immune responses (Currie and Stuart, 2001; Baer et al., 2005; Fernández-Marín et al., 2006; Poulsen et al., 2006; Poulsen and Currie, 2006). The relative importance of any one factor is not well understood. For example, the absence of a queen tending the fungus garden slightly increases the levels of contamination, as expected based on the fact that grooming and weeding reduces contamination levels (Weber, 1972; Bass and Cherrett, 1994; Currie and Stuart, 2001). Attine foundresses, except *Atta* spp., physically isolate their fungus gardens by using platforms (Fernández-Marín et al., 2004). Our results suggest that the use of platforms constitutes another adaptation to reduce the impact of soil-borne microbial diseases that attack the ants' symbiotic fungus. Given that the use of antimicrobial compounds is metabolically expensive (e.g., Poulsen et al., 2002), and their use is limited to actively combating diseases (e.g., Fernández-Marín et al., 2006), we hypothesize that prophylactic behaviors such as the use of platforms (Fernández-Marín et al., 2004), or weeding and grooming (Bass and Cherrett, 1994; Currie and Stuart, 2001), are important components of an integrative strategy that attine ants use to manage diseases at a lower cost. The cost of constructing platforms is unknown, but in *Acromyrmex*, for example, foundresses clean the rootlets only one time in the early stages of nest foundation, and do not continually tend the platform like they do the fungus garden with grooming and weeding behavior (e.g., Currie and Stuart, 2001). Likewise, the relative costs of disease prevention versus treatment of established diseases are unknown for attines. By analogy, it is vastly less expensive to prevent disease than to treat it in human health care systems (e.g., Kahn and Muennig, 2001).

Incipient nests are potentially contaminated both by substrate material brought into the nest (i.e., fungi and bacteria on the surfaces of fresh leaves), and by contaminants that the ants acquire while foraging, which they then inadvertently transfer to the garden. Aseptic fungus gardens were rare in our study, such that only 60% of the fungi pieces per nest were not contaminated in incipient nests with platforms. Comparative data suggest that this rate of contamination is unlikely to have been an artifact of the experimental manipulation. Currie et al. (1999b), for examples, showed that gardens from diverse attines had contamination rates over 40%, while Geraldo et al. (2004) showed that 12 to 60% of *Cyphomyrmex* nests were infected with *Escovopsis.* Low levels (~zero) of infection of the parasitic fungus *Escovopsis* were reported for foundress nests of *A. colombica* (Currie et al., 1999b), but this species is claustral, foundresses do not forage, and they do not use platforms (Fernández-Marín et al., 2004).

Our results also indicate that use of a platform is associated with a modest increase in some measures of brood production during nest founding. Root platforms are associated with enhanced production of some brood classes (but not workers), as well as increased rates of foraging relative to nests without platforms. Nest survivorship, however, did not differ between nests with and without platforms, which suggests that in nests without platforms, queens allocated energy to colony survival instead of to growth, though this hypothesis remains to be tested. The higher mortality of small fungus gardens in comparison with larger gardens implies that competitors quickly overgrow a smaller garden. Larger fungus gardens may better resist diseases and contaminants. This conjecture rests on untested assumptions that i) larger gardens have a higher diversity of substrate material than smaller gardens, and ii) the diversity of substrata limits the dispersion of a pathogen throughout the garden (i.e., cultivar and competitors have differential growth rates on different substrata).

Finally, we also showed that *A. octospinosus* are able to grow their garden and produce brood even in the absence of a platform. This observation suggests that foundresses could behaviorally compensate for its absence (e.g., by increased rates of weeding and grooming, increased use of antimicrobial products, or reducing foraging trips), although behavioral observations to test this hypothesis are lacking. Observations on rates of colony development when platforms are lacking are relevant to an understanding of the evolutionary divergence between *Acromyrmex* and *Atta,* because the use of a garden platform is evolutionarily lost in the latter (Fernández-Marín et al., 2004). The fact that *Atta* foundresses are claustral and do not forage may be related to the loss of a platform, as a means to minimize the probability that contaminants are introduced into the garden chamber. Detailed behavioral studies are needed now to measure the relative costs of different strategies for disease management, and to assess the trade-offs between low- and high-cost antimicrobial strategies and the consequences for colony growth and performance.

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