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Prevalence and impact of a virulent parasite on a tripartite mutualism

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Abstract The prevalence and impact of a specialized microfungus parasite (*Escovopsis*) that infects the fungus gardens of leaf-cutting ants was examined in the laboratory and in the field in Panama. *Escovopsis* is a common parasite of leaf-cutting ant colonies and is apparently more frequent in *Acromyrmex* spp. gardens than in gardens of the more phylogenetically derived genus *Atta* spp. In addition, larger colonies of *Atta* spp. appear to be less frequently infected with the parasite. In this study, the parasite *Escovopsis* had a major impact on the success of this mutualism among ants, fungi, and bacteria. Infected colonies had a significantly lower rate of fungus garden accumulation and produced substantially fewer workers. In addition, the extent of the reduction in colony growth rate depended on the isolate, with one isolate having a significantly larger impact than two others, suggesting that *Escovopsis* has different levels of virulence. *Escovopsis* is also spatially concentrated within parts of ant fungus gardens, with the younger regions having significantly lower rates of infection as compared to the older regions. The discovery that gardens of fungus-growing ants are host to a virulent pathogen that is not related to any of the three mutualists suggests that unrelated organisms may be important but primarily overlooked components of other mutualistic associations.

Keywords *Escovopsis* · Leaf-cutting ants · Mutualism · Parasitism · Symbiosis

Introduction

The importance of parasites, especially disease-producing organisms, in shaping all levels of biological organization is widely recognized (Price et al. 1986; Anderson and May 1991; Herre 1995). Parasites even influence

other interspecific interactions, such as competition and predation (Freeland 1983; Price et al. 1986; Schall 1992; Hudson and Greenman 1998; Yan et al. 1998). Within mutualistic associations, most of the research on parasites has focused on ‘cheaters’: taxa that are closely related to one of the mutualists but do not co-operate, obtaining a reward without providing a benefit in return (Boucher et al. 1982; Mainero and Martinez del Rio 1985). The interest in ‘cheaters’ within mutualisms is at least partially based on the long-term stability of co-operation being a challenge to evolutionary theory (e.g., Addicott 1996; Morris 1996; Pellmyr et al. 1996; Bao and Addicott 1998). However, few studies have examined the importance of unrelated taxa parasitizing mutualisms. In this study, I examine the impact of the microfungus parasite *Escovopsis* on the tripartite mutualism occurring among leaf-cutting ants (Formicidae: Attini), their fungal cultivars, and symbiotic filamentous bacteria (actinomycetes).

This tripartite mutualism likely originated around 50–60 million years ago (Wilson 1971; Mueller et al. 2001). The fungi, mostly belonging to the family Lepiotaceae (Basidiomycota: Agaricales) (Chapela et al. 1994; Mueller et al. 1998), serve as the primary food source for the ants. The filamentous bacteria help protect the fungus gardens from specialized parasites, and may promote the growth of the fungus in some taxa (Currie et al. 1999a; Currie et al., unpublished data). The ants forage for new substrate with which to manure the garden (including plants, insect frass, or seeds), engage in elaborate garden-tending behaviors, and foundress queens disperse both the fungus and bacterium to new colonies (von Ihering 1898 Huber 1905; Currie et al. 1999a; Currie and Stuart 2001).

The ant tribe Attini includes ca. 210 species and 12 genera (Schultz and Meier 1995; Weber 1966), but only the two most derived genera (*Acromyrmex* and *Atta*) use fresh plant material for manuring their gardens. The latter genera are commonly referred to as leaf-cutting ants. Species in the leaf-cutting ant genus *Acromyrmex* have mature colonies with populations of thousands of

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workers, typically with only one or two large fungus gardens (Weber 1966; Wetterer 1999). Leaf-cutting ants in the genus *Atta* have the most complex fungicultural system, which is so successful that mature colonies can support millions of workers and hundreds of fungus gardens (Autuori 1941; Weber 1966). The combined foraging activity of leaf-cutters causes more defoliation than almost any other herbivore group in the Neotropics (Hölldobler and Wilson 1990).

The process of colony reproduction begins with the production of reproductive alates (winged queens and males), which synchronously mate during nuptial flights that typically occur over a few days at the beginning of the rainy season. Foundress queens of the leaf-cutting ants dig subterranean chambers and begin the cultivation of their new gardens from a fungal pellet from their natal nests. Initially these incipient colonies remain sealed within the claustral chamber, where the queens use fecal fluids to maintain the growth of the garden. Foraging for leaf material outside the initial chamber does not occur until the first brood of workers is reared. Colonies continue to accumulate larger biomass of both ants and garden material through the continuous addition of leaf-material and growth of the fungus. The production of new reproductives only occurs when colonies reach sufficient size. For example, sexual maturity requires hundreds of thousands of workers and dozens of individual fungus gardens for *Atta* spp. (Weber 1966, 1972). Leaf-cutting ant colonies are capable of living for 5–10 years or more after initial nest founding (Weber 1972).

Reproductively mature colonies of the leaf-cutting ants can produce thousands of alates annually, yet only an extremely small proportion of queens successfully establish new mature colonies (Weber 1966, 1972). The etiology of this extremely high rate of alate and incipient colony mortality has only partially been established. Predation of gynes (female reproductives) by birds and bats during nuptial flights is extremely high and accounts for a portion of this mortality (Autuori 1950; Water et al. 1962; Mariconi 1970; Fowler et al. 1986). Those gynes that survive to begin digging chambers in the soil are also subject to heavy predation by birds, mammals, and ants (Mariconi and Zamith 1963; Mariconi 1970; Weber 1972). In addition, unsuccessful establishment of fungus gardens by foundress queens who have dug chambers may be a frequent event (Autuori 1941). Once the garden is established, incipient colonies are still vulnerable to being raided by army ants and larger leaf-cutting ant colonies (Autuori 1950; Cherrett 1968; Rockwood 1973; Fowler 1982; Fowler et al. 1984). Leaf-cutting ant colonies need to grow quickly in order to fend off attacks by predators, including other conspecific ant colonies, in order to reach reproductive maturity and potentially to compete successfully for leaf material with other *Atta* colonies in the population (Weber 1972). This strong selection for fast colony growth apparently has led to the evolution of rapid growth rates in leaf-cutting ant colonies, especially in the genus *Atta*, which can reach reproductive maturity ca. 3 years after the mating flight and

reach a colony size in the millions of workers (Weber 1966, 1972; Hölldobler and Wilson 1990).

New research has identified that fungus gardens of leaf-cutting ants are host to a potentially virulent pathogen in the microfungus genus *Escovopsis* (Currie et al. 1999b). This pathogen is frequently present in ant gardens, can be prolific within infected colonies, leads to colony death under some conditions, and is able to persist within the fungus garden for extended periods of time (Currie et al. 1999b). Although it is clear that *Escovopsis* has an impact on the survivorship of leaf-cutting ants, the effect of the parasite on the growth rate of colonies has not been investigated. In this study, I examined the prevalence and impact of *Escovopsis* on leaf-cutting ants in central Panama. Specifically, I studied the following questions:

1. Is the frequency of *Escovopsis* infections different among sympatric species of leaf-cutting ants and between sites across the Isthmus of Panama?
2. Do colonies of different ages have different frequencies of infection?
3. Do persistent infections of *Escovopsis* reduce the growth rate of colonies?
4. Are infections within colonies concentrated in different parts of the fungus gardens?

Materials and methods

Prevalence of *Escovopsis*

First, I examined the prevalence of *Escovopsis* within the fungus gardens of leaf-cutting ants in Gamboa, Panama. Gamboa is located in the canal region of the Panamanian Isthmus, approximately 30 km north of Panama City. This site is composed of secondary tropical moist forest. Sampling was conducted on four common species of leaf-cutting ants in Gamboa: *Acromyrmex octospinosus* ($n=19$), *Acro. echinator* ($n=8$), *Atta colombica* ($n=33$), and *A. cephalotes* ($n=14$) (Weber 1969; Schultz et al. 1998). The two *Acromyrmex* spp. are apparently recently derived sister species (Schultz et al. 1998).

The presence/absence of *Escovopsis* within colonies was determined through the collection of an equivalent amount of garden material (approximate volume of 200–250 ml) from each colony. Since incipient colonies typically are not infected with the parasite because *Escovopsis* is not vertically (from parent to offspring) transmitted (Currie et al. 1999b), they were not included in this study. Garden material was collected by carefully excavating the soil and extracting a single garden chamber, including all the workers tending it. Care was taken to cause minimal disruption to the fungus garden. These sub-colonies were maintained in the laboratory for 3–5 days prior to sampling for *Escovopsis*. Sampling was conducted by placing small individual pieces of garden material (ca. 3 mm³) on nutrient agar. Initially, ten pieces per garden were isolated. If no *Escovopsis* was obtained, absence of infection was confirmed with an additional sampling of ten pieces. Pieces were isolated from younger and older parts of the garden and monitored daily, as outlined in Currie (1999b). All isolations were done on potato dextrose agar (PDA) medium (Difco, Detroit, Mich.) with antibacterial antibiotics (ca. 50 mg/l of penicillin-G and streptomycin sulfate) under aseptic conditions.

To examine the possibility that the infection frequency of *Escovopsis* differs between sites across the Panamanian isthmus, I conducted additional sampling of colonies in Fort Sherman Military Reservation (FSMR) and on Barro Colorado Island

(BCI). No colonies of *Acromyrmex* were found at either site, and therefore no representatives of this genus were included in this part of the study. At FSMR, 30 leaf-cutting ant colonies (*A. cephalotes* $n=11$ and *A. colombica* $n=19$) were sampled for *Escovopsis* using the methods outlined above. BCI is a Republic of Panama Nature Monument, so destructive sampling is prohibited. However, *A. colombica* is an abundant leaf-cutting ant on BCI, and is unique among *Atta* spp. in Panama in that the workers remove the refuse (dump) material from the colony, depositing it on the forest floor. Sampling for *Escovopsis* in colonies of *A. colombica* was therefore possible on BCI by isolating from refuse material (composed of old garden material) as it was carried to the dump. In fact, this may be a more reliable method for assessing infection of colonies since workers transport refuse material from all gardens throughout the colony, allowing a representative sampling of the whole biomass of garden (see Bot et al. 2001). Small pieces of refuse material were sampled by aseptically removing refuse (ca. 3–4 mm³) from workers immediately after they emerged from the colonies. In total, 24 pieces of dump material were sampled from each colony ($n=53$ colonies) and placed on PDA medium in the field. As with garden material, refuse pieces were monitored daily in petri plates for the growth of non-mutualistic filamentous fungi; if arising from the inocula, these were then isolated into pure culture, and confirmed to be *Escovopsis*.

Age of colony and presence of *Escovopsis*

The relationship between age of colony and presence of *Escovopsis* was examined in 1997 in Gamboa and FSMR. Since it is difficult to age colonies of *Acromyrmex*, only *Atta colombica* and *A. cephalotes* were included in this part of the study. In total, the age and presence of *Escovopsis* was estimated for 42 colonies of *Atta* spp. from FSMR ($n=30$) and Gamboa ($n=12$). Estimate of colony age was based on the number of openings, the surface area occupied by the colony, and/or (in the case of distinguishing incipient from 1- to 2-year-old colonies) total size of the garden. A logistic growth rate in *Atta* spp. colonies has been established, suggesting that all of the above characteristics increase with colony age (Bitancourt 1941). Further, new colonies are only established following regionally synchronized nuptial flights, so all newly founded colonies within the same season are approximately the same number of days old. Leaf-cutting ant colonies were grouped into four age classes, 1–2, 3–4, 5–6, and >6 years old. Again, incipient colonies were excluded from this study since they are not typically infected with *Escovopsis* (Currie et al. 1999b).

Impact of *Escovopsis* on colony growth rate

To determine if the parasite *Escovopsis* impacts the growth rate of leaf-cutting ant colonies, incipient colonies of *A. colombica* were collected 8–10 weeks after their mating flight in Gamboa. Collected colonies were confirmed to be free of *Escovopsis* infection and subsequently maintained in sterile plastic containers. An inner chamber was used to house the garden, and an outer chamber was provided for foraging and disposing of refuse material. Colonies were maintained in the laboratory for 2 weeks prior to treatment, allowing them to stabilize. It is difficult to determine the initial garden mass or the number of workers within colonies without causing a major disturbance and subsequent loss of significant amounts of garden material, therefore, a comparison was made of the final garden mass and numbers of ants rather than of the growth rates. Since all incipient colonies are approximately the same size and treatments were randomly allocated, differences in initial garden mass would not affect the results obtained in this study.

Colonies were randomly assigned to one of four treatments. Three groups of five colonies were infected with the garden parasite, each group being treated with a different isolate of *Escovopsis*. Prior to the experiment, the three isolates of *Escovopsis* were obtained from three different *A. colombica* colonies from the same population of leaf-cutting ants as those used in this experiment.

Colonies were infected with *Escovopsis* by using a mist inoculator with a fungal spore suspension in sterile distilled water. Each colony was sprayed with ca. 40,000–60,000 spores. The fourth group of five consisted of control colonies, which were sprayed with sterile distilled water. Approximately 1–2 $\mu\text{l/ml}$ of a wetting agent (tween 20, Fisher Scientific, Pittsburgh, Pa.) was added to the fungal spore suspensions in order to disperse the spores evenly and was added to the water in the fourth treatment as a control.

Significant precautions were taken to prevent the cross-contamination of *Escovopsis* between colonies. *Escovopsis* produces wet spores, so airborne contamination is extremely unlikely; however, vectoring by either mites moving between colonies or human contact during the maintenance of the colonies is possible. To prevent the movement of mites between colonies, uninfected and infected colonies were maintained on different, spatially separated tables. In addition, the outer surfaces of each colony's outer chamber were covered in fluon (Northern Products, Rhode Island), and a small film of heavy mineral oil was applied to the upper edge of the inner surface of each chamber. To prevent human vectoring of *Escovopsis*, uninfected colonies were always handled first. In addition, sterile techniques (i.e. washing hands, sterilizing forceps, etc.) were employed rigorously during maintenance of colonies. Colonies were provided *ad libitum* access to fresh vegetation for foraging, and water was added onto cotton-plugs in the outer chamber once per week. The colonies were maintained in natural climatic conditions in an open air laboratory in Panama for 7 weeks.

To determine the impact of *Escovopsis* infection on the growth rate of leaf-cutting ant colonies, the garden mass as well as the number of workers, larvae, and pupae were determined at the end of the 7-week experimental period. The 'wet-weight' of the garden was measured because it is strongly correlated to 'dry-weight' (Currie, unpublished data) and allows sampling of the garden material for the presence of *Escovopsis* following weighing. The presence or absence of *Escovopsis* was confirmed by isolating 28 garden pieces from each infected and control colony onto PDA (as described above). The effect of treatment on these variables was compared with a one-way ANOVA using JMP (version 3.2.2, SAS 1997). The effect of different isolates of *Escovopsis* was statistically compared by means comparisons using the Tukey-Kramer HSD test with JMP (version 3.2.2, SAS 1997). Normality of the data was confirmed using the Shapiro-Wilk W test. The variable larvae had a non-normal distribution and therefore was transformed using the natural log.

Location of *Escovopsis* within gardens

To determine whether infection of *Escovopsis* is spatially aggregated within gardens, the prevalence of the parasite in different locations within the infected colonies was examined. This was investigated using the colonies infected with *Escovopsis* in the above mentioned experiment. At the end of the 7-week experimental period, 21 pieces of garden were isolated from each of the 15 infected colonies, with seven pieces isolated from each of the top, middle, and bottom of gardens. A vertical sampling gradient (top to bottom) within gardens was examined because new vegetation is added to the top of the garden while old material is removed from the bottom. The prevalence of infection across these garden areas was statistically compared by means comparisons using the Tukey-Kramer HSD test with JPM (version 3.2.2, SAS 1997).

Results

Prevalence of *Escovopsis*

The proportion of colonies infected with *Escovopsis* was very similar within congeneric leaf-cutting ant species

Table 1 Frequency of *Escovopsis* in colonies of leaf-cutting ant species from different locations in the canal region of Panama (Barro Colorado and Fort Sherman Military Reservation are abbreviated BCI and FSMR, respectively). Sampling of *Atta colombica* colonies

Ant species	Gamboa (garden)	FSMR (garden)	BCI (refuse)
<i>Acromyrmex echinator</i>	75% (n=8)	–	–
<i>Acro. octospinosus</i>	68.4% (n=19)	–	–
<i>Atta cephalotes</i>	50.0% (n=14)	18.2% (n=11)	–
<i>Atta colombica</i>	48.5% (n=33)	47% (n=19)	66.0% (n=53)

occurring in Gamboa (Table 1). However, *Escovopsis* was isolated more frequently from colonies of *Acromyrmex* than *Atta*, with at least 70.4% and 48.9% of the colonies infected, respectively ($X^2=3.79$, $df=1$, $P=0.051$; Table 1).

The frequency of *Escovopsis* in colonies of *Atta* differed between the three locations, suggesting that the parasite is more prevalent in some locations for some species. For example, only 18.2% of *A. cephalotes* colonies were infected in FSMR as compared to 50.0% of colonies of this species in Gamboa ($X^2=2.79$, $df=1$, $P=0.093$; Table 1). In addition, a higher proportion of colonies of *A. colombica* on BCI was infected than in Gamboa and FSMR ($X^2=3.20$, $df=1$, $P=0.074$; Table 1), although this could be a sampling artifact, since isolating from refuse bits is not equivalent to isolating directly from the garden.

Age of colony and presence of *Escovopsis*

There is a trend towards *Escovopsis* being less prevalent in older colonies of *A. cephalotes* and *A. colombica* (Fig. 1). More than 45% of 1- to 2-year-old *Atta* spp. colonies were infected with this parasite as compared to only ca. 20% of colonies that are at least 5 years old; however, this difference is not statistically significant ($X^2=1.62$, $df=3$, $P=0.116$).

Impact of *Escovopsis* on colony growth rate

There was a significant effect of treatment on garden mass and number of adults, pupae, and larvae in this experiment (one-way ANOVA, $F_{3,16}=7.983$, $P=0.0018$; $F_{3,16}=5.604$, $P=0.008$; $F_{3,16}=6.753$, $P=0.0037$; $F_{3,16}=10.895$, $P=0.0004$, respectively). Treatment of colonies with *Escovopsis* resulted in a negative effect on the growth and health of colonies. After 7 weeks, colonies of *A. colombica* infected with *Escovopsis* had significantly smaller final garden mass compared to uninfected colonies (t -test=4.38, $P=0.0004$; Fig. 2a). The garden size of uninfected colonies was on average twice as large as infected colonies. Infections of *Escovopsis* within *A. colombica* gardens also had a significant impact on the production of new workers within colonies. Specifically, infected colonies had significantly fewer larvae, pupae, and

on BCI was conducted by collecting refuse material as it was removed from the colony. Sampling for the remaining species and locations was done by collecting garden material and plating pieces on nutrient agar. (n represents the number of colonies sampled)

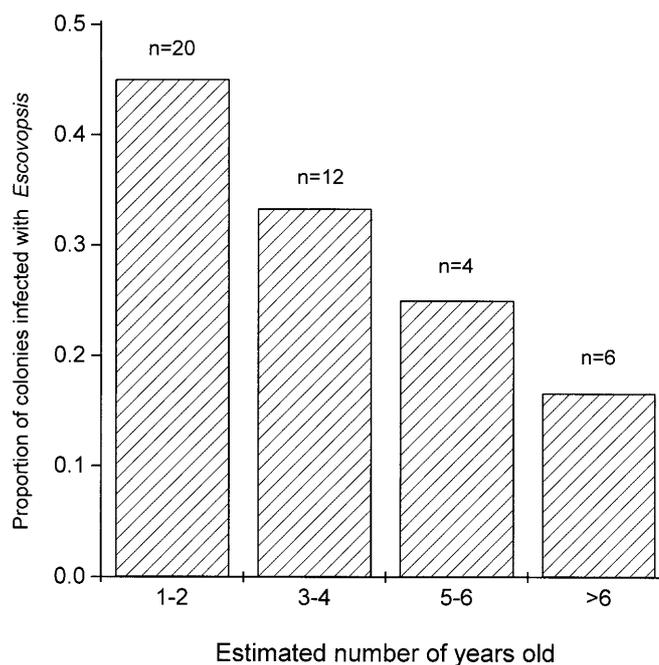


Fig. 1 The relationship between colony age and presence of the parasite *Escovopsis* in *Atta* spp. colonies. The age of each colony was estimated based on the number of openings and surface area (see Materials and methods)

adult ants than the uninfected control colonies at the end of the experimental period (t -test=5.20, $P<0.0001$; t -test=4.74, $P<0.0001$; t -test=4.34, $P=0.0004$, Fig. 2b–d).

The impact of infection on the health of the colony was also affected by the isolate of *Escovopsis* used in the study. No significant difference in effect on garden mass was observed between the three isolates used in the experiment (Fig. 2a). However, a significant effect of *Escovopsis* isolate was obtained for the final number of larvae, pupae, and adult ants within colonies (Fig. 2b–d).

Location of *Escovopsis* within gardens

The presence of *Escovopsis* within infected colonies is spatially aggregated. *Escovopsis* was most prevalent in the bottom and middle portions of the garden and least prevalent at the top, or youngest part of the garden (Fig. 3, $P<0.05$, Tukey-Kramer HSD). This result sug-

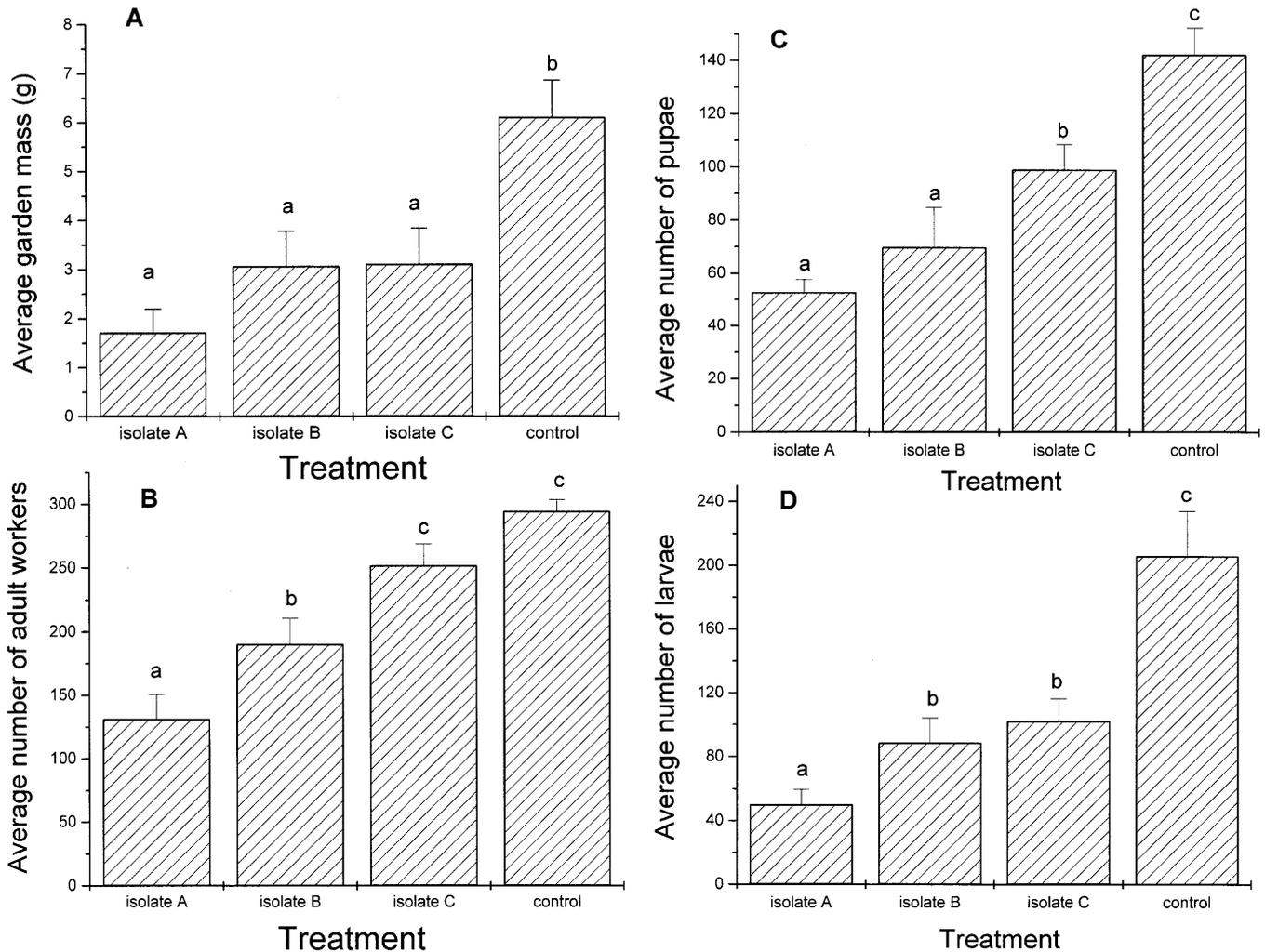


Fig. 2A–D Impact of *Escovopsis* on the growth rate of *A. colombica* colonies. Three different isolates of *Escovopsis* obtained from *A. colombica* colonies within the same population were used to explore the possibility of differences in virulence. Each treatment was replicated 5 times using complete incipient (approximately 3 months old) *A. colombica* colonies, and differences were compared using the Tukey-Kramer HSD test (*a*, *b*, and *c* represent significant differences in treatments). **A** Difference in garden mass. Gardens infected with *Escovopsis* had significantly lower mass compared to uninfected colonies. **B** Difference in number of workers. Infected gardens had significantly fewer workers than uninfected colonies. **C** Difference in number of pupae. *Escovopsis*-infected colonies had significantly fewer pupae than uninfected colonies. **D** Difference in number of larvae. Colonies that were infected had significantly fewer larvae than uninfected colonies

gests that infections persist in the lower or older regions, spreading upward as new leaf material is added. No significant difference in the prevalence of *Escovopsis* between the middle and bottom of garden was observed.

Discussion

The fungal pathogen *Escovopsis* has a significant negative impact on the success of the tripartite mutualism

among leaf-cutting ants, their fungal cultivars, and filamentous bacteria. Leaf-cutting ant colonies can be completely devastated by *Escovopsis*, with the fungus overgrowing the ants' fungus garden (Möller 1893; Currie et al. 1999b). Also, as seen in this study, persistent infections of the garden have a dramatic negative impact on the growth rate of colonies, both in terms of fungus garden mass and number of workers. This finding is also supported by unpublished laboratory observations that many *Escovopsis* infected colonies of *A. colombica* maintained in the laboratory for over 2 years did not expand their fungus garden much beyond the initial size, while uninfected colonies grew quickly (Currie, personal observation). In addition, some colonies infected with *Escovopsis* appeared to have decreased in both mass of garden and numbers of workers during the experimental period.

The significant reduction in fungus garden growth rate in *Escovopsis* infected colonies suggests this parasite has a major negative effect on the fitness of leaf-cutting ant colonies. Colonies must accumulate a vast amount of fungus garden (i.e., dozens of individual garden chambers in *Atta* spp.) to support the production of reproductive alates (Weber 1966, 1972); therefore any

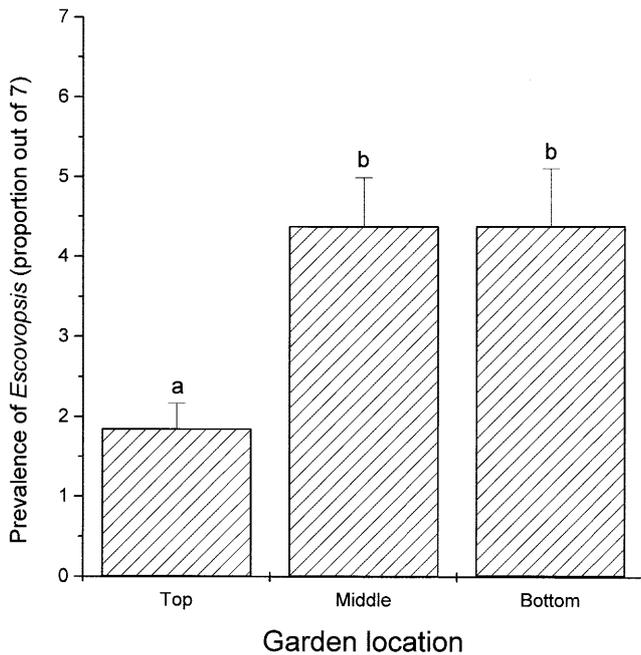


Fig. 3 Prevalence of *Escovopsis* within different locations of the infected gardens of *A. colombica*. The upper, or younger, region of the garden had significantly lower prevalence of *Escovopsis* than the older regions ($n=15$, a and c represent significant differences based on Tukey-Kramer HSD)

decrease in growth rate will result in a delay in reproduction. *Escovopsis* may even prevent some colonies from ever reaching reproductive maturity by severely limiting the accumulation of fungus garden. In addition, this decrease in growth rate of the garden could have serious indirect effects on the success of the mutualism. For example, small colonies are more susceptible to raids by army ants or larger leaf-cutting ant colonies (Autuori 1950) and are likely competitively inferior.

My finding of fewer brood and workers in infected colonies compared to uninfected colonies is likely due to several factors. There is probably a decrease in the rate of egg laying by queens when the garden growth rate is reduced. However, it appears that a higher mortality rate in infected colonies is at least partially responsible for the observed differences in number of brood and workers in the infected versus uninfected experiment. It can take even longer than 7 weeks, the duration of this experiment, for eggs to completely develop into adult ants (Autuori 1950; Weber 1966, 1972). Therefore, it is unlikely that the large differences in number of workers between *Escovopsis* infected and uninfected colonies could be completely related to a decrease in egg laying by queens. Instead, it is possible that eggs, and even larvae, may have been consumed by workers and the queen, as occurs in incipient colonies (Weber 1966, 1972). This would not only decrease the demands on the fungal mutualist by reducing the amount of brood that must be supported by the garden, but also recycles some nutrients back to the colony. Other ant species are known to consume brood under nutrient stress (Hölldobler and

Wilson 1990). Another possibility is that survivorship of workers and even brood decreased under the nutrient stress present in infected colonies.

At first glance, finding a trend towards lower frequency of *Escovopsis* in older leaf-cutting ant colonies suggests that infected colonies are more likely to die off before reaching large, sexually mature size as compared to uninfected colonies in the same age cohort. However, this explanation is based upon several incorrect assumptions. First, the size of the colony was assumed to reflect the age of the colony. However, in this study I show that colonies infected with the parasite *Escovopsis* have a significantly slower rate of colony growth. Therefore, colonies might have been small not because they were young, but because of a reduction in growth rate from being infected with *Escovopsis*. The second invalid assumption is that colonies only become infected at an early age, not continuously, as would be expected for *Escovopsis*. Third, it assumes that the sampling effort to determine the presence/absence of *Escovopsis* was equivalent for large and small colonies. Only a single chamber from each colony was examined for the presence of *Escovopsis*, however, larger colonies are composed of dozens of chambers. It is currently not clear whether the presence of *Escovopsis* within one chamber means that all chambers within colonies are infected, or whether the ants are able to localize infections and prevent their spread to other chambers. If, in fact, *Escovopsis* is less prevalent in larger colonies, I propose two independent, but not mutually exclusive, explanations for this pattern. First, it is possible that only colonies with an inherent resistance to *Escovopsis* reach large sizes. If some colonies are more resistant to *Escovopsis*, perhaps because of differences in their symbiotic actinomycete (Currie et al. 1999a, Currie et al., unpublished data), then this would suggest a fascinating evolutionary arms race between the tripartite mutualists and the specialized parasite. Second, large colonies may be more resistant to becoming infected by the parasite, or perhaps are able to suppress or even eliminate it once infections are established.

All three isolates of *Escovopsis* were collected from the same population from which the experimental colonies were obtained, but there was a significant difference in their impact on the number of adults, pupae, and larvae in the colonies (Fig. 2). Large *A. colombica* colonies may be composed of up to hundreds of individual chambers of fungus gardens (Weber 1972), and since *Escovopsis* is horizontally transmitted (between colonies within the same generation, see Currie et al. 1999b), it is likely that within these large colonies different strains of pathogens are present in different garden chambers, or even within different regions of the same garden. This would result in competition occurring among *Escovopsis* strains within populations, colonies, and even individual gardens. Studies on the evolution of virulence in *Escovopsis* could provide insight into the evolution of parasites and pathogens in general.

Workers continuously add new leaf material to the top of the garden, while the used-up vegetative material and

old fungus is removed from the bottom and placed into the dump. My finding that *Escovopsis* is less prevalent in the top of gardens indicates that this pathogen persists in the older regions, and therefore is not present in the vegetative substrate added to the garden. This is further evidence that *Escovopsis* is a specialized pathogen of the gardens of fungus-growing ants, maintaining a presence within gardens by circumventing the defenses of this tripartite mutualism. In turn, the ants attempt to suppress or eliminate *Escovopsis* through a mutualistic association with antibiotic-producing bacteria (Currie et al. 1999a, Currie et al, unpublished data) and by the physical removal of the parasite by workers (Currie and Stuart 2001). This suggests an interesting host-parasite battle within individual gardens, with *Escovopsis* spreading upward towards the fresh, and likely more nutrient-rich, regions of the garden and the ants attempting to suppress and eliminate it. The physical removal of *Escovopsis* by workers (Currie and Stuart 2001) may help explain the finding of a decrease in garden accumulation in infected colonies. Specifically, ants in infected colonies may remove older garden material at a faster rate due to the infection, resulting in a slower rate of biomass accumulation in these colonies.

The symbiosis among fungus-growing ants, their fungi, and filamentous bacteria is exploited by a specialized and virulent parasite not related to any of the mutualists. The discovery of an unrelated parasite within this ancient association suggests that other mutualisms may be exploited by organisms that are not 'cheaters', but are instead whole new taxa. For example, it is well established that lichens are colonized by a diverse assemblage of non-lichen forming (lichenicolous) fungi (Hawksworth 1979, 1981, 1982; Hawksworth and Hill 1984; Santesson 1989; Petrini et al. 1990). The importance of these fungi within these ancient mutualisms has received little attention from ecologists, however, it is likely that some are highly specialized and virulent parasites. Studies examining the presence and importance of unrelated taxa (especially microbes) in other highly evolved mutualisms should be rewarding.

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