OIKOS 101: 91–102, 2003

Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites

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Currie, C. R., Bot, A. N. M. and Boomsma, J. J. 2003. Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. – Oikos 101: 91–102.

Symbioses shape all levels of biological organization. Although symbiotic interactions are typically viewed as bipartite associations, with two organisms interacting largely in isolation from other organisms, the presence and importance of additional symbionts is becoming increasingly more apparent. This study examines the importance of a third mutualist within the ancient symbiosis between leaf-cutting ants and their fungal cultivars. Specifically, we experimentally examine the role of a filamentous bacterium (actinomycete), which is typically carried on the cuticle of fungus-growing ants, in suppressing the growth of a specialized microfungal parasite (*Escovopsis*) of the fungus garden. We conducted two-by-two factorial design experiments crossing the presence/absence of actinomycete with the presence/absence of *Escovopsis* within small sub-colonies of *Acromyrmex octospinosus*. In these experiments, infection by *Escovopsis* became much more extensive within fungus gardens and had a greater impact on the health of gardens in those sub-colonies with the bacterium removed from workers as compared to gardens with the bacterium still present on the ants. We establish that the actinomycete bacterium is most abundant on those major workers tending the garden, providing further support that the bacterium is involved in garden hygiene. We also found a significantly higher abundance of actinomycete on workers in colonies experimentally infected with *Escovopsis* as compared to uninfected control colonies. We suggest that mutualisms between antibiotic-producing microbes and higher organisms may be common associations that are mostly overlooked and that the role of symbionts in reducing the impact of parasites is likely an important aspect in the cost-benefit assessment of mutualisms.

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The importance of mutualism in shaping communities and ecosystems, particularly tropical ones, is widely recognized (Boucher 1985, Margulis and Fester 1991, Douglas 1994, Thompson 1994). However, understanding the evolutionary origins and the long-term stability of mutualisms is a challenge to evolutionary theory. One current theory proposes that mutualistic cooperation evolves from a parasitic association that gradually becomes less exploitative (e.g. commensalism) and may eventually evolve into a form of mutually beneficial symbiosis (Axelrod and Hamilton 1981, Bull and Rice 1991). This theoretical position has been extended to a belief that mutualism is less of a partnership than a mutual exploitation (Nowak et al. 1994, Leigh and Rowell 1995, Maynard Smith and Szathmáry 1995, Herre et al. 1999), indicating that determining the costs

Accepted 1 July 2002

ISSN 0030-1299

and benefits between symbionts and those factors that influence them is fundamental to the study of mutualism (Cushman and Beattie 1991, Bronstein 1994, Herre and West 1997, Herre et al. 1999). Studies of mutualistic interactions typically are founded upon the view that these symbiotic associations are bipartite systems, with two symbionts interacting largely in isolation from other organisms. However, interspecific interactions of mutualists with non-mutualists or even with non-symbionts may alter the costs and benefits operative in the mutualistic association (Gehring and Whitham 1991, Sanders et al. 1993, Gaume et al. 1998, Yu and Pierce 1998, Gastreich 1997). Although the mutualism between fungus-growing ants and their fungi traditionally has been thought to involve only two symbionts associated in near isolation from other organisms (Weber 1966, 1972, Wilson 1971, North et al. 1997), recent work has suggested the presence of both a third mutualist and a highly evolved parasite (Currie et al. 1999a, b, Currie 2001a, b). Here we experimentally explore the importance of this alleged third mutualist and use this model system in the study of symbiosis to illustrate the importance of supernumerary components in mutualisms more generally.

Fungus-growing ants (Attini: Formicidae: Hymenoptera) and their fungi have an ancient and obligate association that originated perhaps as long as 65 million years ago (Wilson 1971, Mueller et al. 2001). The fungi, mostly belonging to the family Lepiotaceae (Agaricales: Basidiomycota) (Chapela et al. 1994, Mueller et al. 1998), are cultivated by the ants and, in exchange, serve as the ants' main source of food. Although the 12 genera of fungus-growing ants use a diversity of substrates for growing their cultivars, the phylogenetically derived sister genera *Acromyrmex* and *Atta* (Schultz and Meier 1995, Wetterer et al. 1998), commonly known as leaf-cutting ants, are specialized to use mostly fresh leaves and flowers. New leaf-cutting ant queens carry an inoculum of fungal material from their parent colony during the mating flight, thereby clonally propagating the fungus from one generation to the next.

The gardens of fungus-growing ants are frequently infected with a specialized and virulent parasitic fungus in the genus *Escovopsis* (Currie et al. 1999a, Currie 2001a). *Escovopsis* is a genus of microfungi that is allied with the Ascomycota order Hypocreales, although no teleomorph has yet been identified (Currie, unpubl.). The only habitat that *Escovopsis* has been isolated from is the gardens and dumps of attine ants (Seifert et al. 1995, Currie et al. 1999a, Bot et al. 2001a). *Escovopsis* has a major impact on the health of the ants' garden; even in the presence of the ants it can overwhelm the garden, killing the colony (Möller 1893, Currie et al. 1999a, Currie 2001b). In addition, *Escovopsis* can maintain a continuous presence within colonies once infection has become established, which results in dramatic decreases in the growth rate of the colony (Currie et al. 1999a, Currie 2001a). Since leaf-cutting ant colonies must reach a minimum size before reproduction is possible, the parasite can have dramatic effects on the fitness of leaf-cutting ants (Currie 2001a).

Fungus-growing ants have a fourth symbiont, a filamentous bacterium (actinomycete) currently classified in the genus *Streptomyces* (Currie et al. 1999b). The actinomycete is associated with all species of fungus-growing ants examined; in some taxa it is carried upon modified regions of the ants' integument (Fig. 1), and is localized on genus-specific areas of the ants' cuticle (Currie et al. 1999b). Gynes (female reproductive ants) transfer the bacterium from parent to offspring colony (Currie et al. 1999b), thus transmission of this symbiont appears to be primarily vertical. The bacterium is also transferred between workers within colonies (Poulsen et al. 2003). Although it appears that this bacterium has the capacity to promote the growth of the cultivated fungi among at least some lower attines, the main benefit that the bacterium provides to fungus-growing ants and their fungi is likely the production of antibiotics that suppress the virulent parasite *Escovopsis* (Currie et al. 1999b). Extensive bioassays have established that the actinomycetes produce secondary metabolites that have potent inhibitory properties against *Escovopsis* spp. but apparently do not inhibit other fungi (Currie et al. 1999b). Since these findings were obtained in vitro, experimental evidence indicating that the antibiotics produced by the attine ant–associated actinomycete suppress the growth of *Escovopsis* within gardens is still needed. In this study, we experimentally investigate the role of the actinomycete in dealing with *Escovopsis* infection in gardens of the leaf-cutting ant *Acromyrmex octospinosus*. In addition, we test whether the actinomycete bloom is more prevalent on individual workers specialized on tending the garden, as would be expected if the bacterium is important for maintaining the health of the fungus gardens. Finally, the possibility of pathogen-induced actinomycete growth promotion is investigated by determining whether experimentally infecting previously uninfected colonies with *Escovopsis* influences the abundance of the bacterium on garden-tending individuals. The adaptive significance of symbionts ameliorating the impact of parasites on their hosts is discussed.

Methods

Study organisms

Interspecific interactions among fungus-growing ants, their fungus gardens, the symbiotic actinomycetes, and the garden parasite *Escovopsis* were examined using colonies of the leaf-cutting ant *A. octospinosus*. This species forages on the flower and leaf material of a

variety of different plant species and uses this material to grow its fungus gardens. *Acromyrmex octospinosus* is abundant and widely distributed in the Neotropics, occurring throughout most of South and Central America (Weber 1972). Mature colonies are composed of thousands of workers, with perhaps as many as 35 000 individuals (Wetterer 1999) and a single queen. *Acromyrmex* workers have a size polymorphism that is bimodal with minor and major workers (Bot and Boomsma 1996, Wetterer 1999). The minor workers are specialized in tending the garden while the major workers engage in both garden-tending tasks and foraging for vegetative material. In addition, *Acromyrmex* species appear to have a strong age-based division of labor, especially in the major-worker caste, with young major workers tending the garden, older ones foraging for substrate, and the oldest workers managing dump material (Bot, pers. obs.). In the leaf-cutting ant genus *Acromyrmex*, the actinomycete is most concentrated on the laterocervical plates, which are collar-like structures immediately posterior to the mouthparts on the ventral surface of the ants' thorax. However, the bacterial growth frequently completely covers the ants' exoskeleton as a grayish-white bloom, giving the surface a white appearance that contrasts with the typical reddish to dark-brown color of the ants (Fig. 1).

Experimental colonies were collected in Gamboa, Panama, in 1996 and 1998 and were maintained in the

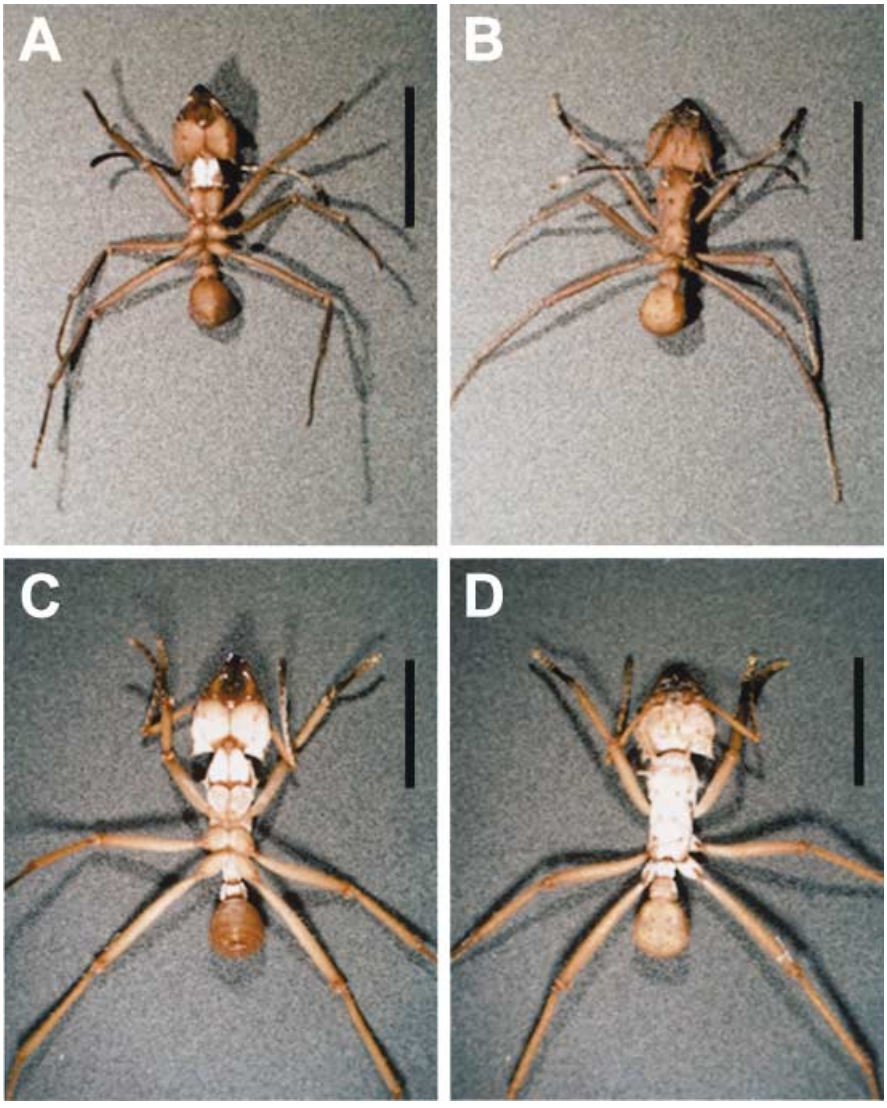

Fig. 1. Location and abundance of actinomycete on major workers of *A. octospinosus*. A and B, ventral and dorsal view, respectively, of a worker representing individuals with none to a small amount of actinomycete present. C and D, ventral and dorsal view, respectively, of worker with thick coverage of actinomycete on the laterocervical plates and other locations on the body. Scale bars represent 4 mm.

laboratory for between six months to two years before being used in the experiments reported here. Prior to the experiment, colonies were kept in plastic boxes (32×22 cm), with one fungus garden (1 l volume) under a plastic beaker and covered to exclude light. The experiments were conducted in a climate room at the University of Aarhus in Denmark (25°C, RH = 70%), and, unless otherwise noted, colonies were fed bramble leaves (*Rubus* sp.) three times a week. To prevent ants from escaping, fluon (Northern Products, Inc., Rhode Island) was applied to the interior sides of the colony chambers. No colonies were used in more than one experiment. Colonies were sampled to ensure absence of *Escovopsis* infection prior to being used in the experiment. The strain of *Escovopsis* used in the experiment was obtained from a colony of *Acromyrmex octospinosus* collected in Gamboa in 1998.

Experimental tests of actinomycete suppression of *Escovopsis*

Two possible benefits of the actinomycete for fungus-growing ants and their fungi are examined. We explore the role of the bacterium in: i) reducing the extent of infection of *Escovopsis* within gardens and ii) decreasing the negative impact of *Escovopsis*, once established, on the health of *A. octospinosus* fungus gardens (measured as garden size). It was necessary to address these questions through two separate experiments because of the difficulty of studying both factors within the same experimental setup. Both experiments involved two-by-two factorial designs, crossing the presence/absence of *Escovopsis* (garden experimentally infected versus not infected) and the presence/absence of the actinomycete (not removed from workers versus removed). However, examining the effect of the bacterium on *Escovopsis* prevalence within individual gardens requires long-term experiments during which the actinomycete has an opportunity to influence the growth of *Escovopsis*. This design requires the use of sub-colonies with sufficient garden biomass to sustain relatively stable gardens over sufficient periods of time and to allow the sampling of many garden pieces for the presence of the pathogen. In contrast, investigating the direct effect of the interaction between the bacterium and *Escovopsis* on garden biomass requires a reduction of the variance in this variable, which can be obtained by reducing confounding factors and increasing the number of replicates. We accomplished this by using smaller sub-colonies. While addition of leaf material sometimes occurs in larger sub-colonies, this does not occur in small sub-colonies (Bot, unpubl.). The use of small sub-colonies in the second experiment allowed for greater replication than was possible in the first experiment, however, small sub-colonies are stable for only a limited amount of time (usually 7 days, and well beyond the duration of our experiment).

In both experiments, infections of the parasite *Escovopsis* were achieved by spraying sub-colonies with ca. 50 000–100 000 spores suspended in sterile, distilled water. To disperse the spores evenly within the water, 1–2 µl/ml of the wetting agent Tween 20 was added. As a control, colonies not receiving *Escovopsis* treatment were sham-sprayed with sterile, distilled water with 1–2 µl/ml of Tween 20. Gardens were sprayed using a mist inoculator. To remove the actinomycete from the exoskeleton of workers, each individual was washed in an antibacterial solution (Penicillin and Streptomycin sulfate at 311 mg/l and 641.5 mg/l, respectively) for 30 seconds. In addition, any location with a concentration of the actinomycete, such as the laterocervical plates, was scraped with stiff forceps to ensure maximum removal of the bacterium from the ants. Workers from sub-colonies that were to retain the actinomycete were washed in sterile water, and a location without a concentration of actinomycete was sham-scraped with stiff forceps.

In the first experiment, large sub-colonies were created by dividing five intact colonies each into four parts of approximately 4.0 g garden mass and 25–30 workers. Sub-colonies were placed in individual chambers with an inner circular container (height 3 cm, diameter 7.8 cm) for the garden and an outer chamber ($6 \times 17 \times 11$ cm) for foraging and dumping of refuse material. Each sub-colony was randomly assigned to one of the four treatment combinations, with five replicates (across colonies) of each treatment combination.

After receiving treatments, colonies were maintained in the climate room for 14 days and fresh leaves were provided every other day. At the end of the experimental period, 18 small individual pieces of garden material (ca 4 mm^3) were isolated onto nutrient agar. Aseptic isolations were done on potato dextrose agar medium (Difco Lab., Detroit, Michigan) with antibacterial antibiotics (Penicillin and Streptomycin sulfate at 50 mg/l each). Each piece was monitored daily, and presence of *Escovopsis* growth was noted.

The prevalence of *Escovopsis* within gardens was statistically compared using a logistic analysis (JMP). After ascertaining that the two treatments not receiving *Escovopsis* were still free of infection, they were excluded from the analysis, being structural zeros. The final analysis therefore consists of a single-factor test of the effect of the actinomycete bacteria on sub-colonies that were treated with *Escovopsis*. The garden mass in these large sub-colonies was compared across all four treatments, using a two-factor ANOVA, with the initial and final mass as a repeated measure (SYSTAT).

In the second experiment, the effect of *Escovopsis* and actinomycete bacterium on garden mass was tested according to a procedure based on the use of small sub-colonies (Bot et al. 2001b). Each sub-colony consisted of four minor and four major workers. A single worker pupa was added to each sub-colony to promote

normal behavior in workers. The initial wet-garden mass was ca 80 mg. Moist tissue paper was placed at the bottom of containers (diameter 2.7 cm, height 4 cm) with a small bramble leaf on top. The garden material and workers were placed on the top of the leaf, preventing the direct contact of the fungus with the moist paper. After two days, the fungus was weighed, and both the garden and ants were placed in clean vials with a fresh leaf. Final fungus mass was measured four days after the beginning of the experiment, thus measures were obtained on experimental days zero, two, and four. The instability of these small colonies is caused not by the shriveling of fungus but either by workers in sub-colonies beginning to dismantle the garden or by worker mortality after day four. *Escovopsis* and actinomycete treatments were applied in the same fashion as described above for the first experiment. The treatment with water or *Escovopsis* spores was performed both on day zero and on day two. The use of small sub-colonies allowed each treatment to be replicated 12 times within a single mother colony. We performed the experiment on three colonies, in order to ascertain repeatability of the results obtained. It was impossible to test all colonies at the same time, due to the labour intensive nature of the experiments. Therefore, our experimental design does not allow us to make firm statements about differences between colonies. However, the intention of this experiment is strictly to test the effect of actinomycete presence and *Escovopsis* infection. Statistical comparisons were preformed using a three-way ANOVA (SYSTAT), with 'colony/setup date', 'presence/absence of *Escovopsis*' and 'presence/absence of actinomycetes' as factors. Because we were not interested in the development of fungus weight over time, we used the difference between fungus weight at the start and the end of the experiment as the dependent variable.

Abundance of actinomycete on different castes

To investigate the association of leaf-cutting ants with the actinomycete bacterium we examined the abundance of the bacterium on different castes within three colonies of *A. octospinosus*. To allow a comparison of foraging workers with garden-tending workers, the intact 1 l fungus gardens of each colony were broken down into two sections: the inside core (predominately workers involved in garden maintenance) and the outside foraging area (predominately foraging workers). Abundance of actinomycete on individuals working in the dump was examined separately for six other colonies in the laboratory in February 2001. Using a dissecting microscope, the abundance of actinomycete was categorized into two very distinct and conservative groupings: i) none to a small amount present, but restricted to the laterocervical plates of the propleura (Fig. 1a, b), or ii) a thick growth on the laterocervical plates and on other locations on the body, which frequently included complete coverage of most of the body of the ant (including a majority of the dorsal and ventral surfaces of the head, thorax, and abdomen: Fig. 1c, d). First, we examined the differences in actinomycete abundance between the distinct size classes of the ants, comparing minor versus major workers. In addition, the abundance of actinomycete on gynes (female reproductives) was examined using individuals collected during a nuptial flight of *A. octospinosus* on May 9, 1997, in Gamboa, Panama. Second, the abundance of actinomycete on major workers inside the garden chamber (i.e. young individuals that are involved in garden-tending tasks) was compared with major workers outside the garden chamber (i.e. older individuals involved in foraging for vegetative substrate). Since minor workers do not forage, a comparison between nest locations was not made for this size caste. The two abundance-classes of actinomycete biomass on workers were used as the dependent variable in a logistical analysis comparing position of workers (inside or outside the garden chamber, JMP).

Pathogen-induced actinomycete growth promotion

We conducted a final experiment on *A. octospinosus* workers to examine the potential for pathogen-induced growth promotion of actinomycete. The abundance of actinomycete on major workers was assessed for three colonies of *A. octospinosus*. The amount of actinomycete, using the same two broad categories outlined above, was determined for each major worker. Two sub-colonies were created from each parent colony of *A. octospinosus*. Each sub-colony consisted of ca 4 g of garden and half of the workers from the parent colony. Queens were not included in any of the sub-colonies. As in the first experiment, the sub-colonies were maintained in a two-chamber system with the garden in a circular container (height 3 cm, diameter 7.8 cm) situated within an outer chamber for foraging ($6 \times 22 \times 17$ cm). The sub-colonies were allowed to stabilize their gardens for three days in the laboratory while being fed bramble leaves. Half of the colonies (one randomly selected sub-colony of each pair from the parent colonies) were stressed by spraying the garden with ca 50 000–100 000 spores of *Escovopsis* dissolved in sterile, distilled water using a mist inoculator (see details of the spraying methods above). Control colonies were sprayed with sterile water. The experimental colonies were maintained in the laboratory for eight days before being broken down to determine the abundance of actinomycete. The abundance of actinomycete on workers between infected and uninfected colonies was compared using a logistic analysis (JMP).

Results

Experimental tests of actinomycete suppression of *Escovopsis*

In the first experiment, the prevalence of *Escovopsis* was significantly lower in sub-colonies with the actinomycete present as compared to sub-colonies with the bacterium removed (Fig. 2a; Likelihood-ratio test on sub-colonies treated with *Esocovopsis*: $X^2 = 30.24$, df = 1, $P < 0.0001$). An insignificant part of the variation in garden mass was explained by either the presence/absence of *Escovopsis* or the presence/absence of actinomycete (Fig. 2b; repeated measure ANOVA: $F_{1, 16} = 0.0025$, $P = 0.96$; $F_{1, 16} = 1.4157$, $P = 0.25$ respectively). Also the interaction between these factors was not significant ($F_{1, 16} = 0.0005$, $P = 0.98$).

In the second experiment, a marginally significant three-way interaction between colony/setup date, the presence/absence of actinomycete and presence/absence of *Escovopsis* was obtained (Fig. 3, Table 1, repeated measures analysis, three-way interaction; $F_{2, 156} = 3.1711$, $P = 0.0447$). More specifically, of those sub-colonies experimentally infected with the parasite, those with actinomycete-covered workers had significantly larger gardens at the end of the experimental period than did those with workers without actinomycete. The intensity of this effect differed slightly between the three times the experiment was performed for different colonies. The highly significant two-way interaction between presence/absence of *Escovopsis* and presence/absence of actinomycetes (Table 1; $F_{1, 156} = 8.0647$, $P = 0.0051$) confirms the importance of the effect of actinomycetes on the impact of *Escovopsis*. However, the two-way and three-way interactions contribute little to the total variance that is explained. The bulk of the variance is explained by treatment with *Escovopsis* ($F_{1, 163} = 238.31$; $P < 0.0001$) and by differences between colonies/day of setup ($F_{2, 163} = 18.95$; $P < 0.0001$). The main effect explains 63% of the variance, while the full model explains 69% of the total variance (Table 1). There is no overall effect of the presence of actinomycetes ($F_{1, 163} = 0.98$; $P = 0.32$), which may be explained by the slightly negative effect of these bacteria on fungus weight when *Escovopsis* is absent (Fig. 3), a fact that may reduce the overall influence of the positive effect that they have when *Escovopsis* is present.

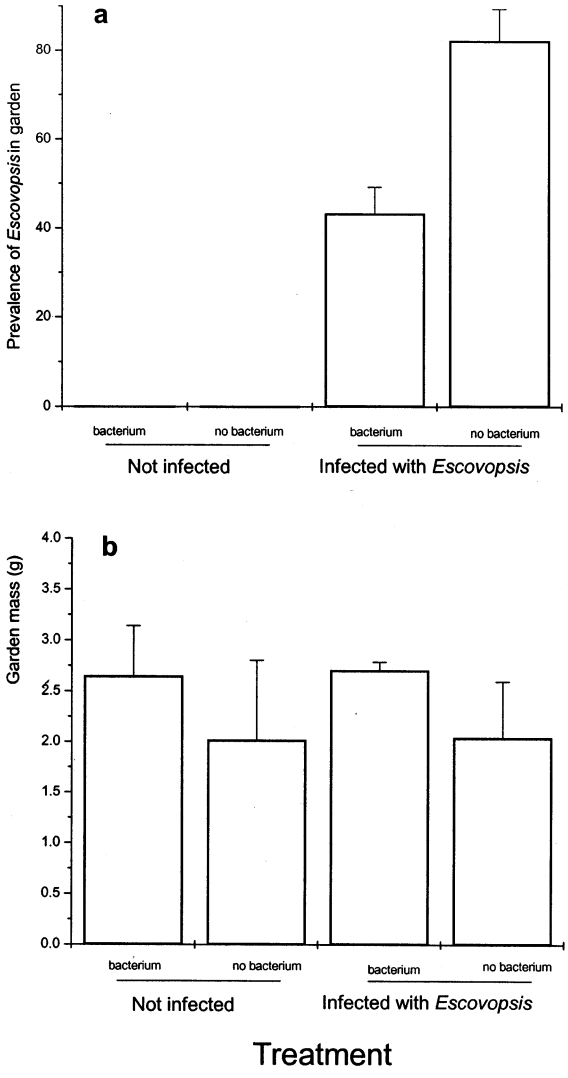

Fig. 2. Examination of the role of actinomycete in suppressing the growth of the parasite *Escovopsis* within *A. octospinosus* fungus gardens using a two-by-two factorial design crossing infection of the parasite with the presence/absence of the actinomycete on workers ($n = 5$ sub-colonies per treatment, mean $\pm$ SE). a. Mean prevalence of the parasite *Escovopsis* within gardens ($X^2 = 30.24$, df = 1, $P < 0.0001$). b. Mean final garden mass of sub-colonies (infection treatment $F_{1, 16} = 0.0$, $P < 0.99$; bacterial removal treatment $F_{1, 16} = 0.1587$, $P < 0.6956$; respectively).

Abundance of actinomycete on different castes

We found that different worker castes have different abundance of actinomycete on their integument. The minor workers typically had actinomycete only on the laterocervical plates of the propleura, while major worker and female reproductive alates are frequently completely covered with the bacterium (Table 2). Major workers within the garden chamber have significantly more actinomycete on their integument compared to major workers foraging for leaf material (Fig. 4; Likelihood ratio chi square, $X^2 = 82.00$, df = 1, $P < 0.0001$). More specifically, major workers tending the garden were often completely covered in actinomycete, while foragers usually had no actinomycete or just a small amount on the laterocervical plates. Workers tending the dump have the smallest abundance of actinomycete on their cuticle (Table 2).

Pathogen-induced actinomycete growth promotion

An increase in abundance of actinomycete was observed on major workers when colonies were stressed by infecting them with *Escovopsis* (Fig. 5). At the end of the experimental period, more workers in the infected colonies had a heavy covering of actinomycete on their integument as compared to sub-colonies not sprayed with *Escovopsis* (Likelihood ratio chisquare, $X^2 = 8.24$, df $= 1$, $P = 0.0043$).

Discussion

The results of this study in combination with those of Currie et al. (1999b) clearly show that the symbiotic filamentous bacterium present on attine ants is a third mutualist modulating the mutualism between leaf-cutting ants and their fungal cultivars. Currie et al. (1999b) showed that the actinomycete is vertically transmitted between parent and offspring colonies, a typical mode of transmission in mutualistic symbioses (Bull et al. 1991), and that the actinomycete produces antibiotics in vitro that specifically target the pathogen *Escovopsis*. In addition, the bacterium is associated with fungus-growing ants representative of the generic diversity of the tribe, is most concentrated in specialized locations on the integument of workers, and is localized in different areas of the ants across attine genera (Currie et al. 1999b). Our study provides experimental evidence within colonies that the bacterium helps to suppress the parasite *Escovopsis*. Specifically, we found both the prevalence of *Escovopsis* infection and the rate of garden loss to be significantly lower in sub-colonies with the actinomycete present as compared to sub-colonies with the actinomycete experimentally removed.

Despite the removal of the actinomycete from ants, gardens were not completely devastated by *Escovopsis* in our study. However, this does not detract from the importance of the actinomycete in helping maintain the health of the garden. It is possible that a small biomass of the bacterium was still present after our actino-

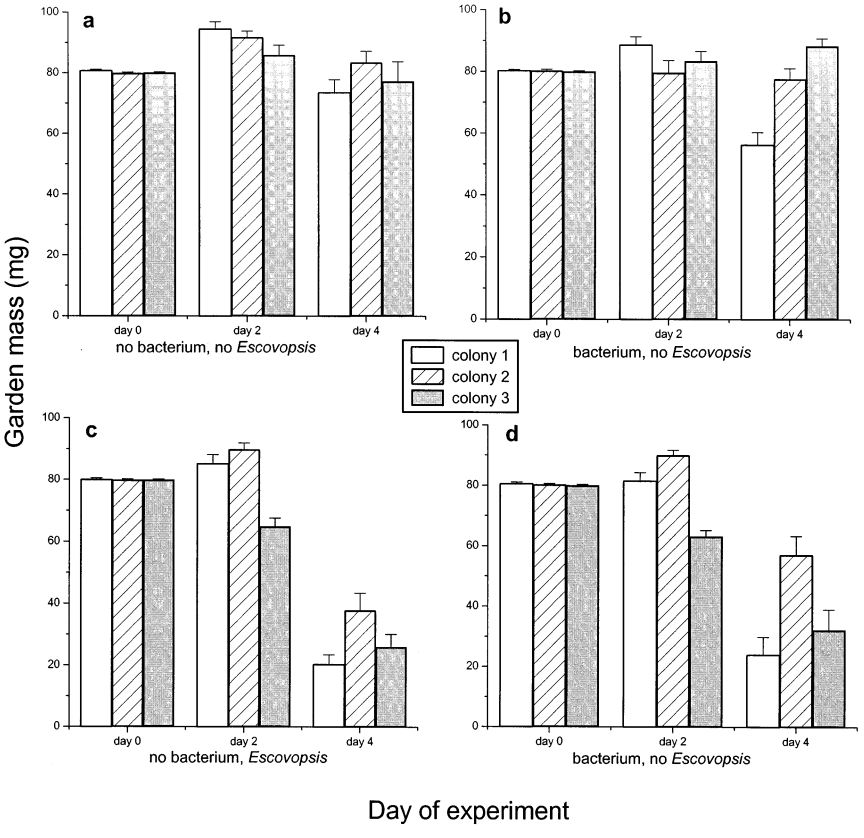

Fig. 3. Examination of the role of actinomycete in preventing garden loss due to the parasite *Escovopsis* within *A. octospinosus* fungus gardens using a second two-by-two factorial design experiment crossing infection of the parasite with the presence/absence of the actinomycete on workers. Mean garden mass is plotted for each of the three dates measured during the experiment for each set of sub-colonies from the three parent colonies ($n = 12$, 12 sub-colonies from 3 different parent colonies, mean $\pm$ SE). a. Average garden mass for sub-colonies with bacterium removed from workers and receiving no experimental infection of the garden with the parasite *Escovopsis*. b. Average garden mass for sub-colonies with bacterium present on workers and no experimental infection of the garden with the parasite *Escovopsis*. c. Average garden mass for sub-colonies with bacterium removed from workers and experimental infection of garden with the parasite *Escovopsis*. d. Average garden mass for sub-colonies with bacterium present on workers and experimental infection of the garden with the parasite *Escovopsis*. A statistically significant interaction between presence/absence of the bacterium and the parasite *Escovopsis* was observed (Table 1; $F_{1,\ 156} = 8.0647$, $P = 0.0051$).

Table 1. Summary of the statistical analyses of data presented in Fig. 3. The experiment tests the effect of actinomycetes on *Escovopsis* infection. The first column gives the predictor variables and their interaction terms. The following columns give the results for the reduced models and the full model (F-values with df's as subscripts and P-values in brackets) of the weight change of the fungal fragments between day 0 and day 4. The fit of the entire model and the percentage of the total variance of the dependent variable explained by the model (R^2) are given at the bottom.

Source	Main effects only	Plus first order interactions	Full model
Colony/setup date	$18.95_{2,\ 163}$ (<0.0001)	$21.05_{2,\ 158}$ (<0.0001)	$21.64_{2,\ 156}$ (<0.0001)
Escovopsis	$238.31_{1,\ 163}$ (<0.0001)	$264.83_{1,\ 158}$ (<0.0001)	$272.17_{1,\ 156}$ (<0.0001)
actinomycete	$0.98_{1,\ 163}$ (0.3231)	$1.09_{1,\ 158}$ (0.2977)	$1.12_{1,\ 156}$ (0.2911)
colony × *Escovopsis*		$5.07_{2,\ 158}$ (0.0073)	$5.21_{2,\ 156}$ (0.0064)
colony × actinomycete		$3.29_{2,\ 158}$ (0.0398)	$3.38_{2,\ 156}$ (0.0365)
Escovopsis × actomycete		$6.41_{1,\ 158}$ (0.0123)	$6.59_{1,\ 156}$ (0.0112)
colony × *Escovopsis* × actomycete			$3.19_{2,\ 156}$ (0.0439)
Fit of model F	$69.30_{4,\ 163}$ (<0.0001)	$36.80_{9,\ 158}$ (<0.0001)	$31.52_{11,\ 156}$ (<0.0001)
R^2	0.6297	0.6770	0.6897

mycete removal treatment, either in the garden or on workers, at an abundance below detectable levels. In addition, secondary metabolites secreted by the bacterium onto the garden prior to the experimental setup would likely still be present in the actinomycete removal treatments, at least early in the experimental period, and thus provide some temporary inhibition of *Escovopsis*. Finally, greater devastation of the garden in the absence of the actinomycete was likely diverted by the other defense mechanisms that are employed by the ants. For example, the ants physically remove the fungus *Escovopsis* through weeding and fungus grooming behaviors (Currie and Stuart 2001). Nevertheless, we obtained a significant effect even over a short time period. Leaf-cutting ant colonies must survive for several years and grow to large sizes to reach sexual maturity, so any immediate marginal benefit would likely result in a significant advantage over the long life span of a colony (which may be more than 10 years). Although it would be valuable to conduct these experiments over a longer period, it would be extremely difficult. Only large colonies with queens are sufficiently stable for long-term experiments, and in such set-ups it would be difficult to remove a significant proportion of the actinomycete as well as ensure that it does not grow back on cleaned or newly emerging workers.

Our study shows that the actinomycete occurs in different abundances on different castes of *A. octospinosus* workers, with foundress queens and young major workers who tend the fungus gardens typically being covered completely in the actinomycete, while the older major workers which forage for vegetation or tend the dump have a very small abundance of bacterium on their exoskeletons. These differences also are found in field colonies of both *A. octospinosus* and *A. echinatior* colonies (Currie, pers. obs., more than 20 colonies). Since it appears that the maintenance of actinomycete is energetically costly to the ant colonies (Poulsen et al., unpubl.), and that the primary benefit provided by the actinomycete is helping to protect the health of the fungus garden, the distribution of actinomycete being highest on garden tending workers suggests this symbiotic association is energetically optimized. Expending energy to maintain actinomycete blooms on foraging workers and/or dump tenders would provide little or no benefit to the colony since these workers do not come in contact with *Escovopsis*, but would represent a energetic cost. In addition, since the actinomycete depends on the successful production of new queens for dispersal to new colonies (i.e. the bacterium is vertically transmitted between generations, Currie et al. 1999b), the bacterium itself would not likely obtain a benefit from being abundant on these worker castes. Leaf-cutting ants have one of the most complex caste systems among social insects, with tasks performed by workers being correlated to the individuals' age and physical size, and this fine-grained worker division of labor is believed to have evolved to optimize ergonomic efficiencies (Wilson 1980a, b). Thus the evolution of energetic optimization of actinomycete distribution on workers would not be surprising.

Our finding of lower abundance of actinomycete on minor workers as compared to major workers is unexpected, since minor workers are specialized in tending the garden. Two possible explanations for this are: i) because of the small body size (i.e. surface area) of minor workers, the cuticle and plate are not a good location for the growth of the bacterium, or ii) due to

Table 2. Abundance of actinomycete on minor workers, major workers, gynes (incipient queens), and dump workers in *A. octospinosus*. The proportion of individuals for each group was compared by assigning the amounts of actinomycete on each ant to one of two distinct groupings: i) none visible or just small coverage on the laterocervical plates (LP) (Fig. 1a, b), or ii) thick growth on the LP and present on other location on the body (Fig. 1c, d). Gynes were collected during a nuptial flight on May 9, 1997 in Gamboa, Panama.

Ant caste	None present or on the LP only	Thick covering of LP and other location on ants
Minor workers	80.3	19.7
Major workers	45.3	54.7
Gynes	4.2	95.8
Dump workers	100	0

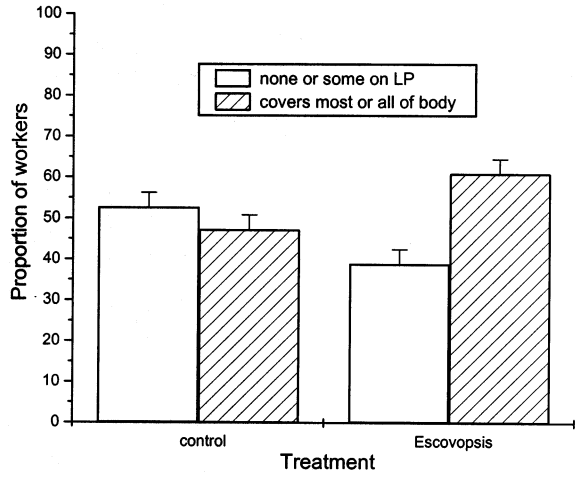

Fig. 4. A comparison of the abundance of actinomycete on major workers tending the fungus garden versus that on foragers. Abundance of the bacterium on individual workers was placed into two very distinct groupings: i) none visible or present only on the laterocervical plates of the propleura (LP) (Fig. 1a, b), or ii) thick growth on the LP and other locations on the body (Fig. 1c, d). A significantly higher abundance of bacterium is present on workers inside the garden as compared to foragers ($n = 3$, mean $\pm$ SE, $X^2 = 82.00$, df $= 1$, $P < 0.0001$).

the garden-tending tasks performed by minor workers, presence of the actinomycete is only needed on the laterocervical plates. A third, but not mutually exclusive, possibility is that the actinomycete not only benefits the health of the garden but protects the ants from entomopathogenic microbes. Although it appears that the actinomycete does not produce secondary metabolites with general anti-fungal properties that would help protect workers from ubiquitous entomopathogenic fungi (Currie et al. 1999b), the possibility remains that specialized antibiotic activity is present that protects the ants from an as-yet-unknown parasite(s) of the ants. In addition, the thick layer of actinomycete (Fig. 1c, d and Currie et al. 1999b Fig. 2a) would prevent any fungal spores from coming in contact with the exoskeleton of the ants, a common mode of entry for entomopathogenic fungi (Charnley 1984, St. Leger 1991); thus the bacterium may also serve as a physical barrier. If the actinomycete does help protect the ants themselves from microparasites, this may explain the pattern of abundance on different castes of *A. octospinosus*. Production of the large major workers is obviously more energetically costly to colonies than small minors. In addition, as previously noted, major workers that tend the garden are younger than those that engage in activities outside the garden, and since younger workers have a longer life span remaining to serve the needs of the colony, they are more valuable. Colonies may only receive a net energetic benefit from the heavy coverage of actinomycete when protecting the most valuable workers (e.g. young major workers). In fact, female reproductives alates (gynes) are energetically the most costly individuals for colonies to produce, and they typically have the highest abundance of actinomycete on their integument (Table 2). Obviously, this heavy abundance of actinomycete is important for the establishment and maintenance of the vulnerable new fungus gardens that foundress queens attempt to establish. Future research on this tripartite mutualism should both attempt to determine why different castes have different amounts of actinomycete and examine the potential role of the bacterium in protecting workers from entomopathogenic microbes.

Fig. 5. A comparison of the abundance of actinomycete on major workers from sub-colonies stressed with the parasite *Escovopsis* and unstressed colonies. The abundance of the bacterium on individual workers was placed into two distinct groupings: i) none visible or present only on the laterocervical plates of the propleura (LP) (Fig. 1a, b), or ii) thick coverage of the LP and other location on the body (Fig. 1c, d). A significantly higher abundance of bacterium is present on workers from *Escovopsis*-infected colonies compared to uninfected colonies ($n = 3$, mean $\pm$ SE, $X^2 = 8.24$, df $= 1$, $P < 0.0043$).

The complexity of this ant–bacterial association is further illustrated by our findings that there is an increase in the abundance of the actinomycete on workers in response to infection by *Escovopsis*. Our findings of increased abundance of the bacterium in *Escovopsis*-stressed colonies is supported by the observation that both *Trachymyrmex* and *Acromyrmex* colonies stressed by sub-optimal gardening conditions and colonies experiencing an actual decline in garden volume tend to exhibit a much higher abundance of the bacterium on workers and/or the queen (Currie, pers. obs., Bot, pers. obs.). This suggests that the ants utilize the actinomycete as an inducible defense, with increased energy going toward growth of the bacterium when colonies are facing high parasite pressure, and less energy when parasite pressure is low. Inducible defenses are well documented in plants, and it is assumed that it is adaptive for the plant to avoid producing costly defenses in the absence of pathogens and/or herbivores (Karban and Baldwin 1997, Karban et al. 1999). In the fungus-growing ant mutualism, the actinomycete could be an energetically costly defense for colonies (see above). In fact, in this study, there appears to be a slight increase in garden mass (although not statistically significant) in the absence of any *Escovopsis* infection in those sub-colonies that have the bacterium removed (Fig. 3). The source of nutrients supporting the growth of the fungus-growing ant–associated actinomycete is currently unknown. The filamentous bacterium does not appear to break down and consume the cuticle of the ants' exoskeleton. Using scanning electron microscopy, extensive observations of older workers with the actinomycete removed revealed that no deterioration or penetration of the ants' cuticle had occurred in the locations where the bacterium had been abundant (Currie, unpubl.). One possibility is that the ants secrete nutrients for the growth of the bacterium at specialized locations of the integument, which could explain the genus-specific location of the bacterium, the age-specific abundance, and the apparent ability of the ants to promote the growth of the bacterium under stressed conditions.

Species can interact in harmful (parasitic), beneficial (mutualistic), and neutral (commensal) ways, but attempts to delineate symbiotic associations within these categories can be difficult (Herre et al. 1999). Therefore, determining the costs and benefits associated with interspecific interactions, especially mutualistic ones, is essential for understanding the stability and evolution of these associations (Cushman and Beattie 1991, Bronstein 1994, Herre and West 1997, Herre et al. 1999). Our finding of a mutualistic bacterium protecting its symbiont from a pathogen might be a common but vastly overlooked benefit within mutualisms in general. Similar benefits have been suggested or shown in a few other mutualistic associations, including some mycorrhizae that suppress pathogens of their plant mutualists (Dehne 1982, Hooker et al. 1994, Kjøller and Rosendahl 1996) and some microbes associated with insects that produce antibiotics that may benefit their hosts (Dillon and Charnley 1986, 1988, Boucias et al. 1996, Mueller, in prep.). In addition, in humans and likely many other animals, the normal bacterial flora of healthy intestinal and vaginal systems are thought to inhibit the establishment of harmful or pathogenic bacteria (Marrie et al. 1980, Cheng et al. 1995, Reid et al. 1995). If a mutualist decreases the parasite pressure of its symbiont, this could result in a net benefit to itself. This may be especially important within mutualisms in which one partner has undergone a transition from sexual to asexual reproduction (e.g. some lichenized algae, dinoflagellates symbiotic with marine invertebrates, and fungi associated with fungus-growing ants and termites). A decrease in sexual reproduction is theoretically predicted to result in an increase in parasite pressure because parasites are believed to be able to adapt quickly to genetically homogenous hosts. This theory, specifically referred to as the Red Queen hypothesis, suggests that parasites are a selective force maintaining sexual reproduction (Van Valen 1973, Jaenike 1978, Hamilton 1980). If, as proposed by Law and Lewis (1983), there is a selective advantage for one symbiont to be asexual, preventing it from escaping from its sexual host, then parasite pressure could be a significant cost to mutualism. This could select for mutualists to help protect their symbiont from parasites, or perhaps even result in establishment of additional mutualists within symbioses, as has occurred in the tripartite mutualism among attine ants, their cultivars, and their filamentous bacteria.

It is clear that clonal propagation of genetically homogenous cultivars for food by both ants and humans results in serious pathogen problems (Lucas 1980, Barrett 1981, Currie et al. 1999a, Currie 2001a). Interestingly, attine ants, like humans, have resorted to chemical warfare with pathogens to maintain the health of their cultivars. It appears that both have failed to successfully develop a 'magic bullet' approach to disease or pest control. This is evident for human populations as illustrated by the extensive evolution of antibiotic and pesticide resistance by human diseases and agricultural pests. The lack of a 'magic bullet' in the attine ant symbiosis is illustrated by the finding that even though the actinomycete improves the ability of the ants to deal with *Escovopsis*, this pathogen still can cause dramatic damage in gardens (Currie 2001a). In addition, despite the apparent selective pressure of the actinomycete-produced antibiotic on *Escovopsis*, the pathogen apparently has not evolved complete resistance over the long evolutionary history of this symbiosis. This suggests that the bacterium and fungal parasite have been engaged in a co-evolutionary 'arms race', perhaps one in which the bacterium evolves more potent metabolites to suppress *Escovopsis* and the parasite

evolves increasingly greater resistance to the compounds. Identification of the secondary metabolite(s) produced by these bacteria is now needed to gain a better understanding of this fascinating tripartite mutualism.

Acknowledgements – We thank the Smithsonian Tropical Research Institute and Autoridad Nacional del Ambiente of the Republic of Panama for logistic help and granting of collecting permits. For maintaining the lab colonies, we are grateful to M. Goldsmith, R. Gadeberg, A. Jensen, B. Jessen, and L. Liboriussen. We thank D. Nash and G. Gage for helping prepare Fig. 1. This work was supported by Smithsonian and NSERC pre-doctoral awards to C. R. C., an EU-Marie Curie fellowship to A.N.M.B., and Carlsberg foundation support to J. J. B. Special thanks to S. Barrett, G. Carroll, G. Currie, J. Eckenwalder, M. Heath, P. Kotanen, D. Malloch, U. Mueller, S. Price and T. Myles for invaluable comments on this study and/or manuscript, and to the Entomological Society of Canada for a graduate student travel award that provided the opportunity for C. R. C. to travel to Aarhus, Denmark, to conduct this research.

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