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Within-colony transmission and the cost of a mutualistic bacterium in the leaf-cutting ant *Acromyrmex octospinosus*

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Summary

1. Stable mutualistic interactions require the long-term alignment of fitness interests of participating species. This condition is fulfilled when the benefits of the relationship exceed the costs for all partners.

2. One apparent stabilizing factor in mutualisms is the vertical (parent to offspring) transmission of symbionts, as this tends to reduce the expression of virulent traits and reproductive conflicts. This study examines the cost and mode of transmission of a mutualistic *Streptomyces* bacterium that grows on the cuticle of leaf-cutting ants and produces antibiotics against a specialized fungal parasite of the ant fungus gardens.

3. It is shown that ant respiration rates are elevated by 10–20% when the bacterium is present on their cuticle. This increase is due to direct respiration of the bacterium and possible excess respiration by the ants. Although these two factors cannot be separated, it is clear that the total increase gives a reasonable quantification of the metabolic costs incurred by the *Streptomyces* symbiont.

4. Ants that actively maintain *Streptomyces* cultures on their cuticle tend to consume more of their mutualistic fungus garden than controls and this excess consumption increases with the amount of *Streptomyces* bacteria present.

5. Scanning electron microscopy showed that the mutualistic bacterium is not present on major workers immediately following eclosion, indicating that the bacterium is not transferred to callow workers until later.

6. The results of an experiment simulating within-colony transmission to callow workers suggest that the bacterium is predominantly transmitted from older to newly eclosed major workers, but that transmission may also occur via the fungus garden. The presence of *Streptomyces* bacteria in the fungus garden implies that rare events of horizontal transmission of the fungal cultivar of attine ants may also imply horizontal transmission of strains of the mutualistic bacterium.

Key-words: *Acromyrmex*, mutualism, respiration, *Streptomyces*

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Introduction

Mutualistic relationships are considered to be reciprocal exploitations that provide net benefits for the partners involved (Herre *et al.* 1999). The maintenance of a mutualistic interaction therefore requires that the interests of the partners remain sufficiently aligned to reduce or prevent conflicts among and within each of the partner species (Frank 1996; Herre *et al.* 1999; Bot, Rehner & Boomsma 2001). Several factors are believed to be important for this alignment of interests, such as genotypic uniformity of symbionts within hosts, restricted options for partners outside the mutualism, repeated interactions between potential mutualists, and the mode of symbiont transmission

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(Herre *et al*. 1999). Vertical (parent to offspring) transmission is generally expected to have a stabilizing effect, since it makes the fitness of the symbiont dependent on that of the host (Herre 1993). In contrast, horizontal transmission (between unrelated hosts) is expected to be destabilizing and to lead to an increased expression of conflicts (Frank 1996; Herre *et al*. 1999; Bot *et al*. 2001).

Leaf-cutting ants of the genera *Atta* and *Acromyrmex* (Formicidae: Attini) culture a mutualistic fungus in the family *Lepiotaceae* (Agaricales: Basidiomycota) (Weber 1966, 1972; Chapela *et al*. 1994) for food (Möller 1893). The fungus benefits from the relationship by being manured with suitable plant material and by gaining protection from competitors and pathogens (Bass & Cherrett 1994; North, Jackson & Howse 1997; Currie 2001a; Currie & Stuart 2001). The fungus is vertically transmitted by the colony-founding gynes (winged prospective queens) who carry a fragment of the fungus in their infrabuccal pocket when leaving their maternal colony (Autuori 1956; Hölldobler & Wilson 1990; after Ihering 1898). However, horizontal fungus exchange may occasionally take place during colony founding, either because of colony initiation by multiple gynes (Rissing *et al*. 1989; Mintzer 1990; Bekkevold, Frydenberg & Boomsma 1999), because of several colonies being initiated in close vicinity to each others (Rissing *et al*. 1989), and/or because of garden stealing by colonies that have experienced garden loss (Adams *et al*. 2000: a study on *Cyphomyrmex*). Phylogenetic studies indicate that such exchanges have indeed repeatedly occurred on an evolutionary and long-term ecological time scale (Mueller, Rehner & Schultz 1998; Bot *et al*. 2001; Green, Mueller & Adams 2002). However, as mixing of symbionts is generally against the interest of both host ants and symbionts (Frank 1996), direct or indirect incompatibility reactions (Hansen, Stenlid & Johansson 1993) will probably cause a single lineage of symbiont to prevail within each colony (Bot *et al*. 2001).

Recently, a third mutualist has been discovered in the ant–fungus symbiosis (Currie *et al*. 1999b). This is a filamentous bacterium (an actinomycete) currently placed in the genus *Streptomyces* (Currie *et al*. 1999b), which produces antibiotics that significantly inhibit a fungal parasite in the genus *Escovopsis* (Ascomycota: anamorphic Hypocreales) (Currie, Mueller & Malloch 1999a). In the absence of the bacterium, this parasite can have a devastating effect on the fungus gardens of leaf-cutting ants (Currie *et al*. 1999a; Currie, Bot & Boomsma 2003), significantly reducing colony fitness and in some cases resulting in colony death (Currie 2001b). The presence and maintenance of the *Streptomyces* bacterium thus seems of prime importance as the bacterium appears to be the primary defence against *Escovopsis* that the ants possess (Currie *et al*. 1999b; Currie *et al*. 2003). Only the major workers of *Acromyrmex* carry a substantial amount of the mutualistic bacterium (Currie *et al*. 1999b; Currie *et al*. 2003; Poulsen *et al*. 2002a). The typical growth pattern is that major workers gain the bacterium a few days after eclosion and that bacterial cover subsequently increases exponentially until the entire ant cuticle is covered after 10–15 days (M. Poulsen, A.N.M. Bot & J.J. Boomsma, personal observations; this study). About 25 days after eclosion, when major workers mature and commence foraging, the *Streptomyces* cover starts to decrease again (M. Poulsen, A.N.M. Bot & J.J. Boomsma, personal observations). The *Streptomyces* bacterium is known to be vertically transmitted on the cuticle of gynes (prospective queens that leave for their mating flights to afterwards found new colonies) (Currie *et al*. 1999b), so that the reproductive fitness of the bacterium depends on the number of gynes that a colony produces. However, it is essential to know whether the mutualistic bacterium is both present on the ant cuticle and in the fungus garden. The latter would indicate that horizontal transfer of the bacterium between colonies may occur in the rare cases that horizontal transfer of the fungal symbiont is successful.

For the relationship between the ants and the bacterium to be truly mutualistic, there must be direct benefits for both partners that exceed the costs of maintaining the partnership. After Currie *et al*. (2003) unambiguously established the advantage of the mutualism to the host, the present study quantifies the cost that the mutualistic bacterium imposes on the host, as any increased ant metabolism, either due to bacterial respiration or due to excess respiration rates of ants carrying the bacterium, will represent costs to the ants. In addition, we investigate the likelihood of horizontal transfer of the bacterium directly via nest-mate workers or indirectly via the fungus garden.

Materials and methods

BASAL METABOLIC RATE OF ANTS WITH AND WITHOUT THE *STREPTOMYCES* BACTERIUM

To measure the cost of carrying the mutualistic bacterium, and thereby to establish whether the bacterium benefits directly from the association, ant respiration was measured in three laboratory colonies of *Acromyrmex octospinosus*. The colonies were collected in Gamboa, Panama, in 1994 (colonies 18 and 19) and 1998 (colony 86), and had subsequently been kept in a climate room at Åarhus University at 60–70% RH and 25 °C under standardized conditions as described in Bot & Boomsma (1996). All colonies were continuously accumulating fungus garden mass, indicating that they were free of infections by the fungal parasite *Escovopsis* when the experiment was conducted (Currie 2001b). Seventy-two callow major workers (head widths: 2·0–2·4 mm; Wetterer 1999), all with a *Streptomyces* coating (a cover of 12; see scale applied in the following paragraph), were sampled from each colony. In the

experimental treatment, 24 of these workers had their bacterial cover removed with a dry brush, another 24 with a wet (water only) brush, and the last 24 with a brush containing an antibacterial solution (penicillin G and streptomycin sulphate, at concentrations of 622 mg l^{-1} and 1230 mg l^{-1}, respectively). All brushing treatments lasted about 10 s. Ants of different treatments and colonies-of-origin were kept isolated in different subcolonies. The subcolonies were established in nestboxes of $10 \times 15 \times 5$ cm^3 and further contained 10 minor workers who only carry a very small amount of the *Streptomyces* bacterium on their cuticle (Poulsen *et al*. 2002a) and 0·5 g of fresh fungus from their colony of origin placed on a (5 cm diameter) Petri dish inside the box. The subcolonies were fed with leaves of bramble (*Rubus fruticosus*).

Ant respiration rates were measured the following day. To obtain an unbiased estimate of the basal metabolic rate, the ants were anaesthetized for 5 min with a 3% v/v solution of enflurane (efrane) in light paraffin oil. This anaesthetic is not metabolized by the ants, does not cause any muscular excitation, and ants have been shown to recover after exposure to 4% v/v enflurane for up to 20 h (Holm-Jensen, Jensen & Nielsen 1980). In the experiments reported here, all anaesthetized ants recovered within 2 h of exposure to enflurane. Respiration rates were measured using a flow-through LiCor 6251 CO_2 analyser with an air flow of 150 ml min^{-1}, allowing sensitive measurements of the production of CO_2 (Nielsen, Elmes & Kipyatkov 1999). The analyser was connected to a data acquisition and analysis system (Sable Systems, Henderson, NV, using Datacan V software) (Nielsen *et al*. 1999). Measurements lasted for 5·33 min and were conducted on single ants placed in a respiration chamber (length = 60 mm; diameter = 13 mm) with rubber plugs in both ends. This chamber was placed in a 25 °C temperature-regulated water bath. Background control measurements monotonously decreased over time, so that it was necessary to adjust ant measurements for background noise by the insertion of two background measurements (after every sixth ant measurement) in the linear regression equation: background measurement = $\beta * \text{time (min)} + \alpha$. The adjusted CO_2 production was subsequently converted to ml CO_2 per hour per gram ant wet mass.

ALTERNATIVE TRANSMISSION MODES OF THE MUTUALISTIC BACTERIUM

Two of the three laboratory colonies (18 and 19) of *Acromyrmex octospinosus* were used to study the transmission of the mutualistic bacterium within colonies. For each of the two colonies, eight subcolonies were made in nestboxes of $10 \times 15 \times 5$ cm^3, each containing 1·5 g fresh fungus from the original laboratory colonies placed in a Petri dish with a diameter of 5 cm partly covered with a lid. Twice a week the fungus gardens of the subcolonies were weighed and replaced with fresh fungus from the original colony. Replacement was done to establish a maximally constant and stable environment across subcolonies and to ensure that the possible transmission pathway of the bacterium via the fungus garden was not affected by the treatments applied. The effect of the presence of the bacterium on fungus mass was examined by estimating the daily subcolony fungus mass change. Given that the specific function of the bacterium is to suppress the specialized fungal parasite *Escovopsis*, the removal of the bacterium was expected to have a neutral or positive effect on fungus mass in the absence of the parasite, as energy otherwise allocated to the maintenance of the bacterium would be saved and potentially used for garden growth-enhancing purposes.

Each subcolony contained 50 minor, 20 media and 10 major workers (see Wetterer 1999 for typical caste-especific body sizes in *Acromyrmex* species). The category 'media workers' has been used previously (e.g. Bot & Boomsma 1996), but has recently been shown to be merely the right tail of the skewed size distribution of minor workers (Wetterer 1999), so that our present references to these ants concern only their intermediate size and do not imply that they belong to a distinct caste. All workers of half of the subcolonies were washed for 10 s with the antibacterial solution (penicillin G and streptomycin sulphate, concentrations as above) and any sign of the bacterium still visible after this treatment was scraped off with sharp forceps. In combination, the antibiotic treatment and the scraping almost completely removed the bacterium from the ant cuticle and thus severely inhibited the transmission pathway from nest-mate workers to newly eclosed callows. Directly after this treatment the ants were washed with demineralized water to avoid the spread of antibiotics to other ants or to the fungus garden. Ants from control subcolonies were washed twice with demineralized water and scraped at the back of the mesosoma, where the bacterium was absent. In the rare (< 0·1%) cases that a subcolony worker died it was replaced by an individual of similar size and treatment. Ten major pupae were given to each subcolony to be nursed.

The callow major workers that eclosed in the subcolonies were marked with nail polish between the pronotal spines as soon as they were observed during daily checks. Marked workers were followed for 14 days and the extent of the cover and growth of the mutualistic bacterium on their cuticle was recorded daily using the following approximately linear semiquantitative scale: 0, bacterium absent; 1–3, bacterium present on the laterocervical plates with respective intensities low, intermediate and high; 4–6, bacterium present both on the laterocervical plates and on neighbouring areas with respective intensities low, intermediate and high; 7–9, bacterium covering most of the cuticle with respective intensities low, intermediate and high; 10–12, entire cuticle covered with the bacterium, with respective intensities low, intermediate and high (Fig. 1). This scale was developed on the basis of earlier studies that

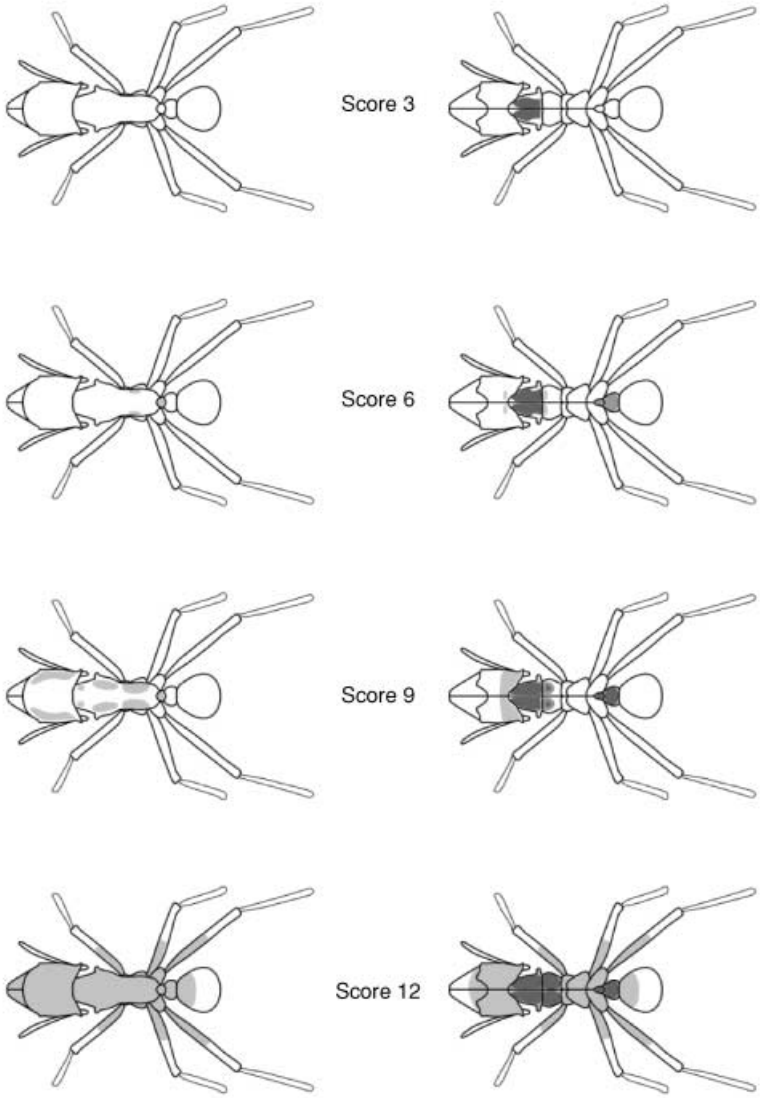

Fig. 1. Schematic drawings representing the scale of age-dependent abundances of the mutualistic bacterium on the cuticle of major workers (left: dorsal view; right: ventral view) of *Acromyrmex octospinosus*. The rows from top to bottom represent the scores 3, 6, 9 and 12. The thickness of the bacterial cover is indicated by the intensity of the gray-scale. See text for further details.

have addressed the abundance of the *Streptomyces* bacterium on the cuticle of major workers of *Acromyrmex* sp. and is believed to represent an optimal compromise between accuracy and feasibility (Currie *et al*. 1999b; Currie *et al*. 2003; Poulsen *et al*. 2002a).

SEM OF *STREPTOMYCES* ON THE CUTICLE OF NEWLY ECLOSED MAJOR WORKERS

The presence of the mutualistic bacterium on newly eclosed major workers was further examined by scanning electron microscopy (SEM). A subcolony originating from colony 86 was constructed in a nestbox of $10 \times 15 \times 5$ cm^3 containing approximately 1·5 g fungus in a Petri dish (diameter = 5 cm), 30 minor workers and 14 pupae of major workers that were allowed to eclose. These callows were marked with nail polish between the pronotal spines on the day of eclosion, except for two callows that were taken out as controls as soon as they eclosed (day 0). The remaining callows were randomly taken out in groups of two to three on days 1, 2, 3, 4 and 5 after eclosion. All callows were subsequently frozen and later examined for the presence of the mutualistic bacterium using SEM.

TESTING FOR DIRECT EFFECTS OF *STREPTOMYCES* ON ANT METABOLIC RATE AND FUNGUS GARDEN MASS

When applying antibiotics to kill the mutualistic *Streptomyces* symbiont (as described above) we implicitly assumed that this treatment did not directly influence ant respiration rates or fungus mass. We tested these assumptions with two additional experiments. The results will be presented here, instead of in the results section, because they concern only a technical component of our study and were done only to justify the experimental approach used in the main experiments.

To ensure that reduced respiration rates of ants were not an artefact of the antibiotic treatment itself, we took 48 additional respiration measurements of single, medium-sized workers from colony 86. None of these workers carried visible amounts of the bacterium on their cuticle. Half of them were brushed for 10 s with antibiotics (penicillin G and streptomycin sulphate, concentrations as above) and the remaining half were left untreated. All were kept in a subcolony containing 1·5 g fungus garden material and approximately 50 minor workers. The following day, ant respiration rates were obtained using the procedure already described. The results showed that respiration rates of workers treated with antibiotics were essentially the same as the respiration rates of untreated controls (mean ± SE: 0·3286 ± 0·0342 and 0·3218 ± 0·0278 for controls and treated ants, respectively; $F_{1,48} = 0{\cdot}026$; $P = 0{\cdot}8729$; ANOVA (JMP)).

A second control experiment was done to determine whether there was any direct effect of the antibiotic treatment on fungus garden mass. Eight subcolonies originating from colony 86 were set up in $10 \times 15 \times 5$ cm^3 nestboxes containing 1 g fungus, 50 minor and 20 media workers. None of these workers carried visible amounts of the *Streptomyces* bacterium. In four of these subcolonies, all workers were washed for 10 s in the solution of penicillin G and streptomycin sulphate (concentrations as above) and in the remaining four subcolonies the ants were left untreated. Every third day (for a total of 12 days) subcolony fungus gardens were weighed and the daily change in fungus mass was calculated. Since the ants initially carried very few or no bacteria, the antibiotic treatment could not alter the amount of bacteria present on ant cuticles, so that the experiment directly evaluated whether there was any direct effect of the antibiotics on fungus garden masses. Mean fungus garden mass was slightly reduced (2·35 mg per day, equal to 0·21%) in subcolonies with antibiotics-treated workers compared with controls, but this effect was not significant ($F_{1,6} = 2{\cdot}664$; $P = 0{\cdot}1538$; repeated measures ANOVA (JMP)) and was 43 and 117 times lower than the differences reported in the results section for colonies 18 and 19, respectively. The test did show a significant effect of time ($F_{3,4} = 10{\cdot}50$; $P = 0{\cdot}0229$), indicating that fungus mass gradually increased during the experiment, similar to what will be reported in the focal experiment.

STATISTICAL ANALYSIS

All numerical data were tested for homogeneity of variances using Bartlett's tests (JMP) and for normality using Shapiro-Wilk *W*-tests (JMP). Variances of respiration rate measurements were homogeneous and normally distributed, so that an ANOVA (SYSTAT) could be conducted. The abundance of bacterial growth on the cuticle also appeared to be normally distributed and variances were not significantly different between groups, so that a repeated measures ANOVA (SYSTAT) with subcolonies nested within treatments and colonies was used to test whether the abundance of the bacterium on the cuticle of nest-mates had an effect upon the development of the bacterium on newly eclosed ants. The observations on days 1 and 2 were not included in this analysis since no ants carried the mutualistic bacterium directly after eclosion. The daily decrease in fungus mass of subcolonies was normally distributed and had homogeneous variances after raising them to the second power, so that an ANOVA could be conducted with subcolonies nested within treatments and colonies.

Results

The respiration rates of major workers carrying the mutualistic bacterium were elevated on average by 15·4%, 21·5% and 13·6% in colonies 18, 19 and 86, respectively, in comparison with workers treated with antibiotics (Fig. 2). This treatment effect is highly

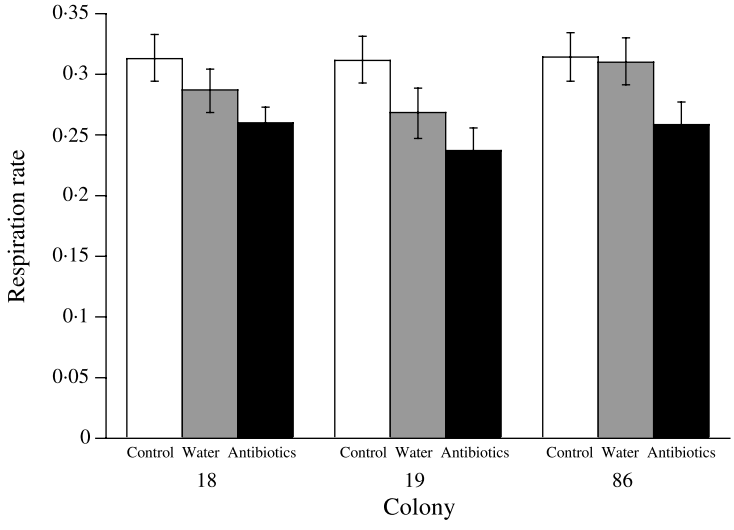

Fig. 2. Respiration rate (ml CO_2 g^{-1} h^{-1} (wet mass); mean ± SE; $n = 24$) of major workers from three colonies (18, 19 and 86) after two treatments (water and antibiotics) and controls.

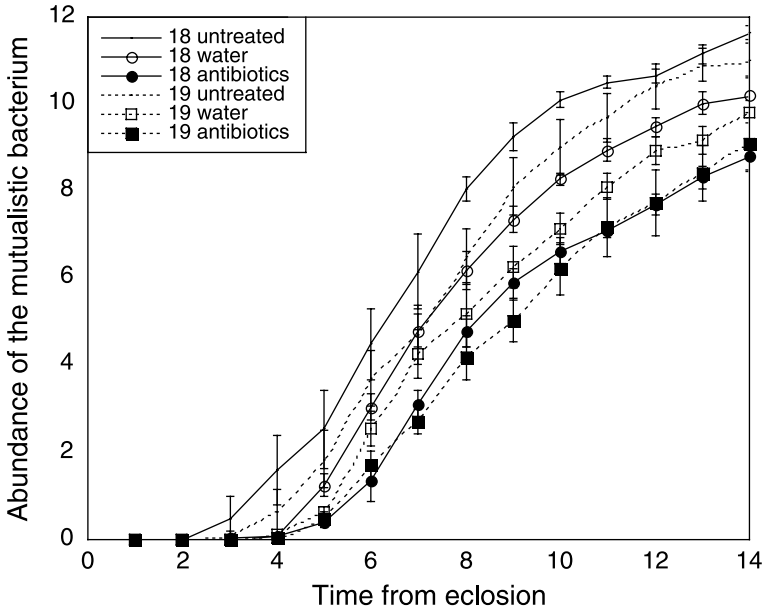

Fig. 3. The cover with mutualistic bacterium on the cuticle of newly eclosed major workers as a function of time from eclosion (days) for the colonies 18 and 19 after the different treatments (water, antibiotics) (mean ± SE; $n = 10$). See Fig. 1 and text for details on the cover scale applied. The curves of the development of the bacterium on the cuticle of untreated ants are taken from a similar experiment by M. Poulsen, A.N.M. Bot & J.J. Boomsma unpublished observations) and are plotted here to facilitate comparison.

significant ($F_{2,207} = 8{\cdot}230$; $P = 0{\cdot}0004$), but there is no significant difference between the three colonies ($F_{2,207} = 1{\cdot}090$; $P = 0{\cdot}3382$), nor a significant interaction term ($F_{4,207} = 0{\cdot}3612$; $P = 0{\cdot}8361$). The total model ($F_{8,207} = 9{\cdot}683$) was highly significant ($P < 0{\cdot}0001$) and explained 27·9% (R^2) of the total variance in the dependent variable. In colonies 18 and 19 the workers treated with water had reduced respiration rates, indicating that the water treatment can also remove a substantial portion of the bacterial cover.

When major workers eclose, the mutualistic bacterium is absent on their cuticle. This is documented both by the subcolony experiment (Fig. 3) and by the SEM pictures of the cuticle of newly eclosed major workers (Fig. 4), which showed that the bacterium was present at the earliest 3 days after eclosion. When nurtured in subcolonies with workers treated with antibiotics, callow major workers experience a delay in the initial establishment of the bacterium and in the time required to develop a full coating (score 10–12) of the mutualistic bacterium ($F_{1,144} = 16{\cdot}38$; $P < 0{\cdot}0001$) (Fig. 3; Table 1). Comparing the results of this experiment with an experiment in which the development of the bacterium on the cuticle of untreated workers was examined (M. Poulsen, A.N.M. Bot & J.J. Boomsma personal observations), it is clear that the water treatment removes a substantial amount of the bacterium on the cuticle of subcolony workers (Fig. 3). There was a significant effect of the colony-of-origin on the development of the mutualistic bacterium ($F_{1,144} = 5{\cdot}869$; $P = 0{\cdot}0167$; see Fig. 3 and Table 1) and, more importantly, there was a statistically highly significant interaction effect of time and treatment ($F_{11,1584} = 5{\cdot}11$; $P < 0{\cdot}0001$), indicating that the effect of treatment increased over time (Fig. 3). In addition, there were less pronounced, but significant, interaction terms of time and colony ($F_{11,1584} = 2{\cdot}87$; $P = 0{\cdot}001$) and time and subcolony ($F_{132,1584} = 1{\cdot}39$; $P = 0{\cdot}003$). The overall analysis shows that the rate of increase towards being fully covered with the bacterium is significantly reduced by the treatment and that this effect increases over time, although not entirely consistent across colonies and subcolonies (Table 1).

Colonies deprived of the *Streptomyces* bacterium tend to be more efficient in maintaining their subcolony fungus garden. On average 9·02% and 24·4% more fungus was consumed when bacteria were present for the colonies 18 and 19, respectively. This effect was not statistically significant, but we did find that subcolony fungus gardens mass losses were 42·9% higher in colony 18 than in colony 19. This difference between colonies was significant ($F_{1,147} = 4{\cdot}05$; $P = 0{\cdot}0459$) and corresponded to a similar difference in the average colony-specific bacterial cover scores of large workers in these colonies during the 14 days of experiment (means ± SE of the sums of *Streptomyces* scores were 69·7 ± 3·32 and 49·7 ± 2·09 for colonies 18 and 19, respectively).

Discussion

THE COSTS OF MAINTAINING THE *STREPTOMYCES* SYMBIONT

Although we do not know how much of the elevated respiration rate is due to bacterial respiration or to excess respiration rates of ants carrying the bacterium, it is clear that the metabolic costs of either metabolic activity have to be paid by the ant. The differences in

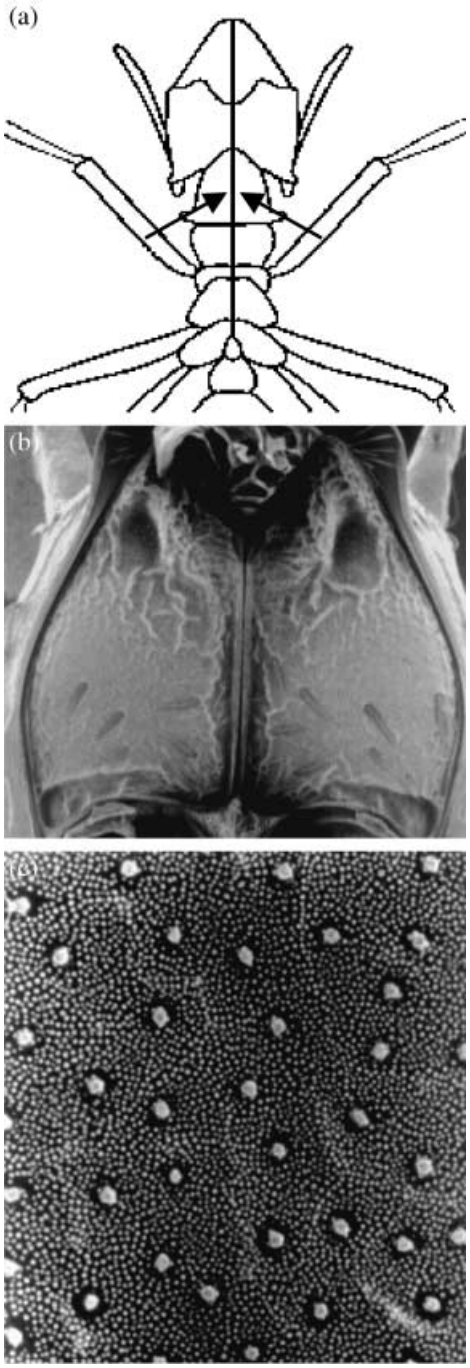

Fig. 4. (a) Schematic diagram of an ant showing the location of the laterocervical plates (arrows) and scanning electron microscope (SEM) micrograph pictures of (b) the laterocervical plate of a newly eclosed major *A. octospinosus* worker. (c) A close-up of the same laterocervical plate. For a comparison with workers carrying the *Streptomyces* bacterium on the laterocervical plate, see Fig. 1 (this study) and Fig. 2(a) in Currie *et al*. (1999b).

respiration rates between treated ants and controls (Fig. 2) are in the order of 10–20% and these differences cannot be explained by the antibiotic treatment itself (see control experiments in Materials and methods). This implies that the costs of maintaining the specialized defence mechanism of the *Streptomyces* bacterium are of the same magnitude as the costs incurred by the general defence mechanism of active metapleural gland secretion, which we have recently found to be equivalent to 15–20% of the basic metabolic rates of major *A. octospinosus* workers (Poulsen *et al*. 2002b). It is as yet unclear what nutrient source supports the growth of the bacterium on newly eclosed ants and where they come from. In another study we have shown that metapleural gland secretions are of some importance for the bacterium in the later phase (starting 15–25 days after eclosion) of declining *Streptomyces* cover, but not in the initial phase of exponential growth of the bacterium on newly eclosed major workers (M. Poulsen, A.N.M. Bot & J. J. Boomsma personal observations). Further work to identify the source and identity of the resources that allow the *Streptomyces* bacterium to grow on the ant cuticle is obviously needed.

The elevated respiration rates in workers with the bacterium present compared with workers with the bacterium removed suggest that the relationship between the ants and the *Streptomyces* bacterium is truly mutually beneficial. However, we cannot quantify the benefits and costs for the *Streptomyces* bacterium, as it remains unknown whether the bacterium still has alternative substrates independent of ants and what its metabolic efficiency on such hypothetical substrates would be. It seems almost unavoidable that the domestication of the bacterium by the ants has reduced the bacterial opportunities for horizontal transmission and has increased selection pressure towards specialization in the evolutionary arms-race with *Escovopsis*. It may therefore be that the bacterium no longer has alternative substrates and is exploited rather than mutualistically serviced by the ants at its present evolutionary advanced stage of symbiosis with the ants.

The investment by the ants in the maintenance of their *Streptomyces* mutualist is remarkably high and may have a direct link to the consumption rate of fungus garden material. Previous studies investigating the role of the fungus in the diet of leaf-cutting ants have found that the fungus garden is of little importance for the energetic requirements of adult ants, although it was shown that fungus is ingested both directly (Möller 1893; Weber 1972; Quinlan & Cherrett 1978, 1979) and indirectly via anal trophallaxis after larval processing of fungus material (Schneider 2000). Our results suggest that specific situations (as imposed by our experimental treatment where alternative feeding resources are absent) may force the ants to use their fungus garden as a backup resource for feeding at the expense of larval development.

TRANSMISSION OF THE *STREPTOMYCES* SYMBIONT

The results obtained by SEM show that newly eclosed major callow workers do not carry the bacterium on their cuticle, corroborating the negative visual scores during the first days of the transmission experiment.

Table 1. Results of a repeated measurements ANOVA testing for effects of the presence of the mutualistic bacterium on nest-mate workers (treatment), colony and subcolony (nested within colony and treatment) on the development of the *Streptomyces* bacterium on newly eclosed major workers of *A. octospinosus*

Source	d.f.	SS	*F*	*P*
Within days				
Treatment	1	373·7	16·38	< 0·0001
Colony	1	133·9	5·869	0·0167
Subcolony{Treatment, Colony}	12	433·8	1·585	0·1021
Treatment*Colony	1	4·901	0·2148	0·6437
Error	144	3 285		
Between days				
Time	11	22 318	1140	< 0·0001
Time*Treatment	11	99·66	5·107	< 0·0001
Time*Colony	11	56·05	2·872	0·001
Time*Subcolony{Treatment, Colony}	132	325·1	1·388	0·003
Time*Treatment*Colony	11	18·16	0·9304	0·5098
Error	1584	2 810		

However, the development of the bacterium cover is only delayed, not prevented, in colonies where adult workers have severely reduced abundances of the bacterium. The results obtained suggest that the normal and most efficient transmission of the bacterium within colonies is a direct one via other ants that already have established *Streptomyces* cultures on their cuticle. This would explain why older major workers still carry minor quantities of the mutualistic bacterium (score 1–3) (Poulsen *et al*. 2002a), because it guarantees transmission of the *Streptomyces* symbiont to the next cohort of major workers. However, three observations suggest that callow major workers may also acquire the mutualistic bacterium indirectly via the fungus garden:

1. The *Streptomyces* bacterium can be isolated from the fungus garden (C.R. Currie personal observation).
2. Newly eclosed callow workers are relatively immobile and keep their ventral sides in close contact with the fungus garden (M. Poulsen personal observations).
3. The pattern of initial growth of the bacterium ventrally on the coxae and/or the laterocervical plates of the ants (M. Poulsen personal observations) would make this indirect transfer relatively efficient. However, if only the ants are able to produce the specific resources needed for the bacterium to grow, this would imply that the bacterium may only be present in the fungus garden as inocula, which could reduce the efficiency and speed of this mode of transfer significantly.

The mutualistic bacterium is dependent upon the production of gynes for vertical transmission to the next generation of leaf-cutting ant colonies (Currie *et al*. 1999b). The fitness of the bacterium is thus tightly linked to the fitness of the ant colony (i.e. to the number of gynes, prospective queens, that are produced), a situation that is not uncommon in vertically transmitted nonvirulent symbionts (Herre 1993). Vertical transmission allows the ant host to control the movement and mixing of symbiont lineages during mating flights, which should lead to a higher within-host relatedness of symbionts and thus a better alignment of interests between symbiont and host (Frank 1996; Herre *et al*. 1999). The presence of inocula in the fungus garden implies that the *Streptomyces* symbiont can be horizontally exchanged between colonies in rare cases where exchange of the other symbiont, the mutualistic fungus, happens.

MULTIPLE SYMBIOTIC INTERACTIONS IN ATTINE ANTS

The fact that attine ants have evolved specialized mutualistic and ectosymbiotic interactions with two different microorganisms, a fungus garden symbiont and a *Streptomyces* symbiont (Mueller *et al*. 2001; Mueller 2002; Currie *et al*. 2003) offers special opportunities to test current theories on the ecological efficiency and evolutionary stability of mutualistic interactions. As already partly discussed above, both the colony-level and the population-wide genetic diversity of the two mutualists and their host ants may differ widely and this may have profound effects on the stability of the interaction. *Acromyrmex* ants are known to be outbred (Boomsma, Fjerdingstad & Frydenberg 1999) and to have very high within-colony genetic diversity owing to queens mating with many males (Boomsma *et al*. 1999; Villesen *et al*. 2002). The fungal mutualists are single clones per colony but have substantial genetic diversity at the population level and are shared by sympatric *Acromyrmex* species (Bot *et al*. 2001). The genetic structure of the *Streptomyces* mutualists is as yet unknown, but hypotheses on this issue can be generated based on recent evolutionary theory predicting that mixing of symbionts may be problematic if it induces competition between symbiont strains and reduces their overall level of service to the host (Frank 1996).

Recent evidence has confirmed this prediction for the mutualistic fungus of the ants, as it could be shown that *Acromyrmex* workers actively kill fungal transplants of a different genetic make-up from their resident fungus (Bot *et al*. 2001). It remains to be seen, however, to what extent the *Streptomyces* bacterium has a similar clonal structure to the fungal symbiont, because the ecological roles of the two symbionts are fundamentally different. The fungus in higher attines has a long history of clonal propagation (Chapela *et al*. 1994; Mueller *et al*. 2001). This implies that it would be rapidly wiped out by sexually propagated parasites, both generalists and specialists such as *Escovopsis*, were it not for the continuous care and effective chemical defences of the ants (cf. Currie 2001a). However, the *Streptomyces* bacteria may lack such a breeding structure and, given the role of this bacterium in the association with the ants, mixing and sex among *Streptomyces* strains may in fact be beneficial for the ants. If a complex of recombining bacterial genotypes would provide better protection against *Escovopsis* than genetically pure cultures, selection might favour processes of bacterial sexual exchange that facilitate control of the presumably fast evolving *Escovopsis* parasites. The exchange of genetic material between free-living *Streptomyces* strains is indeed known to occur under natural conditions (Waksman 1959 and references therein; Ravel, Wellington & Hill 2000; Hu, Hopwood & Khosla 2000). The further evolutionary study of genetic diversity in attine symbionts will thus have great potential to understand general issues concerning the stability of mutualistic interactions.

The attine ant system also provides unique opportunities to further our general understanding of mutualistic interactions. The interactions of the ants with their fungus gardens are known to be obligatory (e.g. Chapela *et al*. 1994) and the same almost certainly holds for the interactions with the *Streptomyces* bacteria (Currie *et al*. 1999b; Currie 2001a). However, this does not mean that the productivity of these mutualistic interactions is constant across species and habitats. As infection rates and strain virulence of *Escovopsis* are known to vary across attine ant species (Currie 2001b), future ecological studies may reveal highly complex mosaics of genus-specific, species-specific and even population-specific interactions. That the two mutualists and *Escovopsis* are all obligate ectosymbionts provides excellent opportunities for field and laboratory studies, which may clarify the ecological cost–benefit trade-offs involved in this tripartite mutualism in much more detail than the pioneering studies by Currie *et al*. (2003) and the present one have achieved.

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