

Delayed rejection in a leaf-cutting ant after foraging on plants unsuitable for the symbiotic fungus

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Leaf-cutting ants culture a mutualistic fungus for which they collect and process a great diversity of fresh plant material as substrate. It has previously been observed that workers show “delayed rejection” behavior toward substrate that is harmful for the fungus but not for the ants: workers initially accept such materials but thereafter avoid its collection. In this study, we investigated delayed rejection behavior toward natural leaves in several 2-choice experiments in laboratory subcolonies of *Acromyrmex lundii*. We experimentally manipulated leaf suitability for the fungus by infiltrating them with a fungicide (cycloheximide) not detectable to the ants. The ants’ delayed rejection behavior was specific toward the respective fungicide-treated plant species. Delayed rejection was also observed in naive ants after contact with the fungus garden containing treated leaves, confirming previous results with artificial bait. The onset of delayed rejection occurred 10 h after incorporation of treated leaves into the fungus garden. Rejection behavior was maintained for at least 9 weeks when incorporation of the previously unsuitable plant species was precluded. However, acceptance resumed after 3 weeks when ants were “forced” to feed on untreated leaves of the previously treated plant species. The observed species-specific, rapidly expressed, and flexible rejection of unsuitable substrate may be a mechanism to successfully avoid the provisioning of the fungus garden with plants containing harmful compounds as they occur in the highly diverse natural habitat of the colonies. *Key words:* *Acromyrmex lundii*, avoidance learning, behavior, foraging decisions, host plant selection, symbiosis. [*Behav Ecol*]

Leaf-cutting ants (genera *Atta* and *Acromyrmex*, Formicidae) culture a mutualistic fungus (*Leucocoprinus gongylophorus*, Lepiotaceae, Basidiomycota), for which they harvest fresh plant material. In the nest, this material is further processed and incorporated into the fungus garden, which is consumed by the ants and their brood (Quinlan and Cherrett 1979; Bass and Cherrett 1995). Leaf-cutting ants are extremely polyphagous. Up to 50–80% of the available species in often highly diverse plant communities are used, and typically several plant species are harvested and incorporated into the fungus garden simultaneously (Cherrett 1989; Wirth et al. 2003). Nevertheless, foragers show marked preferences for leaves of certain plant species, individuals and even within individual plants (Hubbell and Wiemer 1983; Howard 1990; Meyer et al. 2006), which result in a ranked host plant use from high acceptance to complete rejection.

Research on the underlying causes of host plant selection in leaf-cutting ants has focused on those plant characteristics used by foraging ants to make their decisions at the cutting site. Leaf toughness and nutrient content, as well as the presence of attracting and/or deterring secondary compounds, are regarded the main factors influencing such decisions (e.g., Cherrett 1972; Howard 1987, 1988; Cherrett 1989; Nichols-Orians and Schultz 1990; Camargo et al. 2004; Meyer et al. 2006). Furthermore, learning of the odor of the food items carried by nest mates along the trail or inside the nest may

influence foragers’ decisions toward particular food sources (Roces 1990, 1994; Howard et al. 1996).

In addition to the selection of suitable plants by foragers at the cutting site, a second step of “quality control” occurs once the material has been carried into the nest. Materials unsuitable as fungal substrate may be postselected by gardening workers and as a consequence carried out and dropped around the nest entrances. This was the case for inert material initially carried and then discarded by workers of the leaf-cutting ant *Acromyrmex subterraneus brunneus* in experiments (Camargo et al. 2003) and can also occasionally be observed in natural leaf-cutting ant colonies (Herz H, personal observation).

Plasticity in decision making during host plant selection by leaf-cutting ants has been documented in laboratory colonies: when fed repeatedly with certain plant species, foraging workers initially accepted them but showed delayed rejection over the next days, that is, a declining interest in harvesting those leaves (Knapp et al. 1990). Such delayed rejection implies avoidance learning by the foragers, for which Knapp et al. (1990) proposed 2 possible mechanisms: first, it may occur because some leaf compounds are harmful to the ants that ingest them (e.g., noxious plant sap), thus leading to rejection, a phenomenon known for other insect species (Bernays and Chapman 1994). Second, it may arise because the harvested substrate is deleterious to the fungus yet harmless for the ants, so that workers react to changes in fungus performance by discontinuing the harvest of such material. Ridley et al. (1996) and North et al. (1999) demonstrated that in fact, workers stopped harvesting fungicide-containing baits that were initially incorporated into the fungus garden in response to the detrimental effects of this material, even though it was harmless for the workers.

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The flora in the tropical and subtropical habitats of leaf-cutting ants is chemically highly diverse, and secondary compounds are frequently fungicidal (Rosenthal and Berenbaum 1991). A feedback mechanism via the fungus that enables foraging workers to reject unsuitable food plants after their incorporation into the fungus garden, through avoidance learning, could be important to help ants maintaining a healthy culture. Avoidance learning by foragers is expected to be fast and plant (stimulus) specific, so that harvesting of alternative, suitable host plants continues. In addition, specific avoidance of unsuitable substrates should be long lasting in order to avoid further intake of harmful material. However, recurrent assessment of previously unsuitable plants and reversal learning should be advantageous, thus allowing renewed harvesting of plants when favorable changes in their quality occur, for example, through phenology (Coley and Kursar 1996).

Ridley et al. (1996) and North et al. (1999) showed that rejection behavior toward baits supplemented with fungicide was maintained for more than a week. Interestingly, treated colonies even sustained rejection toward bait without the fungicide, indicating that workers may have associated the toxic effects of the incorporated bait with its characteristics (odor and potentially other chemical or physical features), thus allowing its recognition at the foraging site on subsequent days, and its avoidance. Delayed rejection is based on a postforaging mechanism via effects of the incorporated material on the fungus garden. This was demonstrated by removing experienced workers (those that incorporated the fungicide-containing bait) from the "treated" garden and introducing naive workers. After exposure to the garden, they were shown to reject the bait at the foraging arena on subsequent days, even though they never contacted the bait previously (North et al. 1999).

In their studies, Ridley et al. (1996) and North et al. (1999) employed an artificial bait laced with a fungicide undetectable by the ants. In the present study, we extend such investigations and go beyond by studying 1) the occurrence of delayed rejection of natural plants (leaves) laced with this fungicide and its species specificity; 2) the time interval elapsing from the incorporation of the plant until the occurrence of rejection; 3) the time span during which changes in the fungus garden are detectable by the ants, thus inducing rejection, after a single incorporation of fungicide-containing leaves; 4) the duration of memory for the plant species inducing rejection after being incorporated; and 5) the time course of reversal learning when a plant species initially harmful for the fungus becomes suitable.

To experimentally address these questions, we took advantage of the method developed by Ridley et al. (1996), using a fungicide (cycloheximide) undetectable by and harmless to the ants, and combined it with an infiltration method that enables the internal air space of leaves to be filled with fluids (e.g., Beyschlag and Pfanz 1990). Using this novel approach, we were able to infiltrate leaves with the fungicide and therefore to manipulate the quality of the leaves for the symbiotic fungus, but not for the ants, because the leaf characteristics (e.g., toughness, specific surface, and/or odor characteristics) that are expected to influence foraging decisions remained unchanged.

MATERIAL AND METHODS

Ant subcolonies

We used 3 large laboratory colonies of *Acromyrmex lundii* originating from Buenos Aires, Argentina, reared at 25 °C. Experiments were performed with subcolonies, established by isolating approximately 100 cm³ of fresh fungus garden and

approximately 500 workers from a large laboratory colony. We placed the fungus and ants in experimental nests, consisting of 3 plastic boxes (400 cm³ each) connected by plastic tubes (10 mm diameter, 6 cm length). The first box served as the feeding arena, the second central box contained the fungus garden, and the third box was used by the ants as refuse chamber. All subcolonies were set up at least 1 week prior to experiments to allow for garden reestablishment and growth. We provided subcolonies daily with fresh leaves *ad libitum* and fresh water in small plastic cups. To ensure high foraging activity, we did not feed the subcolonies 1 day prior to the experiment and removed any remaining leaves from the feeding arena. Subcolonies exhibited regular foraging and gardening behavior and were viable for many weeks.

Plant material and treatment of leaves

We maintained subcolonies on a mixed diet of leaves of privet (*Ligustrum vulgare*, Oleaceae), rose (*Rosa canina*, Rosaceae), and bramble (*Rubus fruticosus*, Rosaceae) collected at the university campus. We used the same plant species in the experiments.

The rationale of the experiments to be described below was to make leaves unsuitable for the fungus, but not for the ants, by infiltrating them with a fungicide, and to investigate the occurrence and dynamics of plant rejection. We manipulated leaf quality for the fungus by lacing the leaves with the fungicide cycloheximide (Sigma-Aldrich, Deisenhofen, Germany), which, as indicated above, has previously been shown not to be detectable by the ants (Ridley et al. 1996). When manipulating leaf quality, we aimed to maintain leaf features such as odor, surface characteristics, and toughness, which may influence decision making of foraging ants and fungal culturing in gardening ants. To fill the internal air space of leaves with an aqueous solution (0.02%, w/w) of cycloheximide, we adopted a pressure-vacuum infiltration method from plant physiological studies (e.g., Beyschlag and Pfanz 1990). Pressure-vacuum infiltration of leaves was performed as follows: freshly cut leaves were placed in a 60-cm³ syringe, which was half filled with the cycloheximide solution. After insertion of the piston, all remaining air in the syringe was removed through the outlet. The outlet was then closed, and a negative pressure was generated by forcefully pulling the piston outward. The suction drags the air from the intercellular space in the leaves through open stomata and the petiole. After removing any air bubbles from the leaf surface by shaking, the piston was vigorously pushed back into the closed syringe. The positive pressure forced the solution into the leaf. Suction and pressure were alternated several times to achieve complete infiltration. Successful infiltration was visible by a darkening of the leaf. On average, 3.4 ± 0.4 μl (mean ± standard deviation), 6.9 ± 1.1 μl, and 4.5 ± 0.5 μl of solution per square centimeter of leaf area could be pressed into a leaf for rose, privet, and bramble, respectively (determined by weighing, *N* = 25 leaves each). Infiltrated leaves were briefly rinsed with water and blotted dry with paper tissue.

Because a high dose of fungicide is expected to be lethal for the fungus, we established in preliminary tests that the incorporation of leaves infiltrated with a 0.02% solution of cycloheximide causes no visible effects on the fungus garden (i.e., no signs of browning or decay) and does not affect worker survival. This fungicide concentration was used throughout the experiments. When presented the first time with both infiltrated and noninfiltrated leaves of the same species, workers showed no preference for any of them, indicating that fungicide infiltration itself did not influence decision making by foragers. Thus, the leaves treated with a fungicide acted analogously to "Trojan horses," carrying the harmful compound into the fungus

garden without being recognized by the ants, which displayed normal foraging behavior and leaf processing.

Experimental procedure for quantifying workers' preferences

To evaluate workers' preferences, we offered leaves as disks (6 mm in diameter) punched with a paper clipper. To avoid possible contamination of disks, punchers were thoroughly cleaned when a different leaf type was punched and disks were only handled with forceps. Two-choice preference tests were conducted with the subcolonies. In "treatment" preference tests, leaf disks of one plant species infiltrated with the fungicide cycloheximide (hereafter "treated leaves") and untreated leaves from a second plant species were simultaneously presented at the foraging arena of a subcolony. For logistic reasons, we opted to compare fungicide-treated leaves with completely untreated leaves in the preference tests, after preliminary trials had indicated that infiltration with water but no fungicide had no effects on foraging decision (data not shown). Disks of the 2 leaf types (20 each, if not indicated otherwise) were presented simultaneously in the foraging arena in 2 small plastic dishes set side by side. During preference tests, the subcolonies were observed continuously, and for each leaf disk collected and carried back to the fungus garden, its identity ("leaf type") and the time elapsed from the beginning of the test was noted. The tests were stopped when all disks were collected or 2 h had elapsed. The pickup rate for each of the 2 leaf types was calculated as the slope of the cumulative number of disks taken over time.

The collected leaf disks were readily processed and incorporated into the fungus garden. During this process, gardening ants shredded the leaf disks into minute fragments of 1–2 mm² in size. They softened the fragments by chewing and then placed the particles on the surface of the fungus garden. At this stage, the particles received fecal droplets and one to several fungal tufts to enhance the colonization of the vegetative material by the fungus (Herz H, personal observation; as described by Weber 1956, 1972; de Andrade et al. 2002). In subcolonies that readily picked up all 40 leaf disks, the material was usually processed within 2 h after the start of the preference test.

Subcolonies were not fed between consecutive preference tests and thus received only the leaf material offered in the tests. Within each of the 4 experimental series (see below), all subcolonies stemmed from only 1 of the 3 available laboratory colonies. Subcolonies that received treated leaves were used only once in the experiments and were not merged again with the laboratory colony to avoid the introduction of experienced workers and treated fungus garden material.

We performed 4 different experimental series, as described below, aimed at answering different questions.

Experimental series 1: delayed rejection of cycloheximide-treated leaf disks

To examine whether ants show delayed rejection toward leaves treated with the fungicide and whether the behavior is specific toward the treated species, we conducted preference tests with treated and untreated leaf disks presented on 2 consecutive days, using 2 different pairs of plant species, either rose and privet or bramble and privet.

In a first set of 3 choice tests, we presented to "treatment" (experimental) subcolonies disks of 1) treated rose leaves and untreated privet leaves (on both days) and 2) the reciprocal, that is, disks of treated privet leaves and untreated rose leaves. "Control" subcolonies were presented with 3) disks of both untreated rose and privet leaves (6 subcolonies each). A second set of 3 choice tests was performed comparing 4) treated bramble and untreated privet disks (again on both days), 5) the

reciprocal (3 subcolonies each), and 6) untreated bramble and privet leaf disks as control (4 subcolonies).

Experimental series 2: onset of delayed rejection

In order to determine the onset of rejection behavior, preferences for either treated or untreated leaf disks were tested repeatedly at 2-h intervals for either 16 h (6 subcolonies) or 24 h (for a random subset of 3 of the subcolonies). Two parallel choice tests were conducted, comparing 1) "treatment" subcolonies, which were presented disks of treated privet leaves and untreated rose leaves throughout the series (6 subcolonies) with 2) "control" subcolonies that were presented untreated disks of both species (6 subcolonies). For the first preference test of the series, colonies received 20 disks of each leaf type, whereas in the consecutive tests they received 10 disks per leaf type.

Experimental series 3: delayed rejection in naive ants transferred to treated fungus gardens—how long are changes detectable?

These experiments were conducted to assess how long the fungus garden induces rejection behavior, that is, how long are changes in the fungus detectable for the ants after treated leaves have been incorporated into the garden. To control for potential effects of the experience of foraging ants with the treated material, "naive" ants, which had never been exposed to treated leaf material or fungus garden containing treated leaves, were transferred to "treated" fungus gardens, into which cycloheximide-treated leaves had been incorporated (by different ants). The transfer of naive ants into the treated fungus garden was conducted, in various experiments, after different intervals since the incorporation of the treated leaves; however, in all experiments the naive ants remained in the treated garden for 24 h before their preferences were tested at the foraging box. In these experiments, any rejection behavior of naive ants toward leaves treated prior to incorporation would be brought about by the treated fungus, and not by the handling of treated leaf material, because those ants neither collected nor incorporated the treated leaves. This induction of delayed rejection in naive workers mediated by the fungus was already demonstrated for the leaf-cutting ant *Atta sexdens rubropilosa* (North et al. 1999). In the present experiments, we took advantage of this phenomenon to investigate for how long changes in the fungus garden that induced delayed rejection are detectable for the ants in the subcolony.

In the reciprocal experiment, "experienced" ants, that is, those that collected and processed the treated leaf material, were transferred after different time intervals to fungus gardens that only contained untreated leaf material. After 24 h in the untreated garden, their preferences were tested at the foraging box. These experiments allowed assessment of the effects of both handling of treated leaf material and variable exposures to treated gardens on the expression of rejection behavior.

In all subcolonies used in these 2 experiments, we conducted an initial preference test at the very beginning (see below). Thereafter, the total worker force (ca. 500 workers) was transferred about 2 h or 1, 2, or 3 days after the initial preference test was finished (4 pairs of subcolonies each time). As indicated, the second preference test was performed after the ants had remained for 24 h on their "new" garden.

Worker forces of naive ants and untreated gardens were obtained by presenting 20 disks of untreated privet leaves to each of 16 subcolonies and allowing the ants to process and incorporate the leaf material. For experienced ants and treated fungus gardens, 20 disks of treated privet leaves were presented to each of 16 subcolonies. For initial preference tests, 20 untreated rose disks were presented in parallel with the untreated or treated privet disks, respectively. In the second test, both leaf

types were offered untreated. Subcolonies were randomly assigned to pairs of treated and untreated subcolonies, and after different time intervals (see above), all ants were removed from their nest-box and fungus garden and transferred to the new ant-free garden: workers from treated gardens to untreated ones and vice versa. Workers were carefully removed with soft forceps, and care was taken to avoid damage to the fungus garden. To minimize garden desiccation, the fungus box was not completely uncovered during ant collection, but a lid with a hole was used to reach in with the forceps. The collected ants were kept in a box with damp tissue paper before they were transferred to the ant-free garden of their paired subcolony.

Two additional pairs of subcolonies were used as controls to test for handling effects. For these controls, naive workers were exchanged between untreated gardens about 2 h after the initial preference test with untreated privet and rose disks, and a second preference test was carried out 1 day after the transfer. Foraging behavior did not significantly change between consecutive tests (rejection index [RI], see below, $N = 4$, paired t -test, $t = 0.38$, degrees of freedom [df] = 3, $P = 0.73$), indicating that there were no handling effects.

Experimental series 4: long-term rejection and resumption of acceptance

To examine if, and how long, rejection behavior persists in subcolonies, delayed rejection was initiated on the first 2 days with treated leaves, and preference tests with nontreated leaves of the same species were repeatedly performed for several weeks. Additionally, one experiment was conducted to investigate whether reversal learning occurs, that is, whether ants, after having rejected a leaf type harmful to the fungus, resume their foraging when those leaves become suitable.

In the first experiment, subcolonies were initially presented with treated privet leaves and delayed rejection was observed to occur, as expected, at the subsequent day. From that day, subcolonies were offered exclusively rose leaves (fed 3 times/week ad libitum) and workers' preferences between rose and untreated privet leaves were investigated. In the second experiment, subcolonies were similarly presented first with treated privet leaves and delayed rejection occurred. After that, they were offered exclusively *untreated* privet leaves (fed 3 times/week ad libitum), that is, they were "forced" to accept the leaves they have rejected on the second day or to remain deprived of forage. Again, workers' preferences between untreated privet and rose leaves were investigated.

In those subcolonies fed exclusively with rose leaves, after the initial 2 tests, no privet disks picked up during the preference tests were allowed to reach the garden in order to avoid changes in the experience with this plant type initially harmful to the fungus. To that end, an additional box was inserted between the feeding arena and the fungus garden, in which the privet leaf disks were carefully taken away from the ants with forceps before they entered the fungus chamber. In this experiment, only 5 treated colonies were available after week 6, and after week 9 the overall vitality of the subcolonies declined, so that the test series was stopped.

A total of 22 subcolonies were used in both "treatment" series (6 and 5 subcolonies in first and second experiment, respectively) and "control" series (6 and 5 subcolonies in first and second experiment, respectively). "Control" subcolonies received both leaf types untreated in the initial preference tests.

Analyses

Based on the pickup rates from the preference tests, an RI was calculated as the difference of the pickup rates of untreated (r_u) and treated (r_t) leaf disks relative to the total pickup rate:

$$RI = (r_u - r_t) / (r_u + r_t).$$

Thus, an RI of 0 indicates equal pickup rates for both leaf types offered. An RI toward 1 (>0) corresponds to a rejection of the treated disk and a preference for untreated leaf disks, whereas an RI toward -1 (<0) indicates a preference for the treated leaf disks and rejection of the untreated disk.

It is important to note that differences in acceptance of different species are likely, so that an RI of 0 is not necessarily expected when comparing leaves of different species. Instead, if the treatment of the leaves with fungicide has an effect, a difference in preference and therefore RI between tests involving a treated leaf type and the equivalent tests with untreated leaves is expected. Alternatively, a treatment effect can manifest itself in a change in preference behavior when colonies are tested repeatedly with treated leaves. Therefore, treatment effects were assessed by comparing the RIs of the "treatment" subcolonies with the RIs of "control" subcolonies with the same leaf species untreated or by following RIs over time.

Overall tests for effects of treatment, time, and their interaction on RI were conducted using analyses of variance (ANOVAs) for repeated measurements (STATISTICA, version 7, StatSoft, Inc., Tulsa, OK). Differences between RIs from "treatment" and "control" subcolonies on single days were analyzed by t -tests. Paired t -tests were used to assess differences of RIs between repeated preference tests on the same subcolonies. To comply with requirements of normality and homogeneous variances, all RI values were transformed [$\arcsin(RI \times 0.5 + 0.5)$] prior to statistical analyses.

RESULTS

Experimental series 1: delayed rejection of cycloheximide-treated leaf disks

Initially, ants picked up leaf disks treated with the fungicide cycloheximide and untreated disks of another species both at very similar rates, resulting in an RI around 0. On the second day, the RI was significantly higher (Figure 1). This was independent of the plant species involved, indicating that the ants strongly and species specifically rejected the disks of the treated leaf type 24 h after the incorporation of the material into the fungus garden (Table 1). In contrast, RI did not change in experiments where 2 types of untreated leaf disks were presented (Table 1).

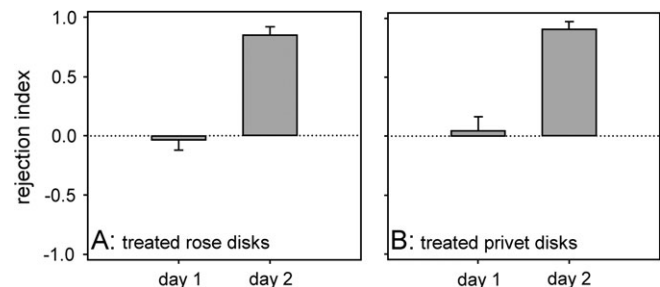


Figure 1 RIs of *Acromyrmex lundii* subcolonies on 2 consecutive days for leaf disks treated with cycloheximide. Two types of leaf disks (rose and privet) were presented and the pickup rate of treated disks relative to the overall pickup rate compared between 2 consecutive days. The dotted line marks the RI of 0 at which both disks types are picked up at the same rate. (A) Rose leaves were treated with cycloheximide, and privet leaves remained untreated ($N = 6$ subcolonies); (B) the reciprocal was tested: privet treated and rose untreated ($N = 6$ subcolonies). Data are mean \pm standard error. For statistical results, see Table 1.

Table 1
Results of 2-choice preference tests with various combinations of leaf disks treated with the fungicide cycloheximide and untreated disks

Combination of leaf disks		RI _{day1}	RI _{day2}	N ^a	t	P
Treated	Untreated					
Rose	Privet	-0.04 ± 0.20	0.85 ± 0.17	6	8.88	<0.001
Privet	Rose	0.05 ± 0.28	0.91 ± 0.15	6	9.99	<0.001
	Privet-rose	-0.02 ± 0.34	-0.17 ± 0.16	6	1.47	>0.20
Bramble	Privet	-0.03 ± 0.17	0.78 ± 0.11	3	4.70	<0.05
Privet	Bramble	-0.08 ± 0.14	0.81 ± 0.16	3	9.09	<0.02
	Privet-bramble	0.12 ± 0.27	0.01 ± 0.21	4	0.95	>0.4

Data are the mean RI ± standard deviation of subcolonies on day 1 (first exposure) and day 2 and the results of paired *t*-tests. The same combination of leaf disks was presented on both days.

^a Number of subcolonies tested.

Experimental series 2: onset of delayed rejection

The short-term course of the RI at 2-h intervals for both “treatment” and “control” colonies is shown in Figure 2. In subcolonies that were offered treated leaves, RI strongly increased over time, indicating an increasing strength of rejection of the treated leaf type. In “control” subcolonies that were offered untreated leaves, in contrast, RI stayed rather constant for the 24 h of the experiment, with the RI not significantly deviating from 0 ($N = 6$, *t*-tests, *t* values ranging from 0.09 to 0.91, *df* = 5, $P > 0.40$), except in the first test at hour 0 ($N = 6$, *t*-tests, $t = 2.81$, *df* = 5, $P < 0.04$). Pairwise comparisons of RIs of “treatment” and “control” colonies indicated a significant rejection of the treated leaf type 10 h after incorporation of the treated substrate. The overall ANOVA results for treatment and time effects and their interaction are given in the caption of Figure 2.

Experimental series 3: delayed rejection in naive ants transferred to treated fungus gardens—how long are changes detectable?

The occurrence and strength of rejection behavior in naive workers 24 h after exposure to treated fungus gardens strongly

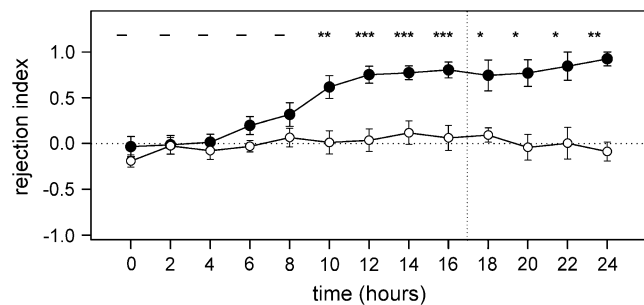


Figure 2
 Short-term course of RIs. In order to determine the onset of rejection behavior, repeated preference tests were made with *Acromyrmex lundii* subcolonies presenting treated privet leaf disks and untreated rose leaf disks (filled circles). Control colonies (open circles) were offered both leaf types untreated (each group $N = 6$ subcolonies). Tests were conducted at 2-h intervals for 24 h. After hour 16 (vertical dotted line), only 3 subcolonies in each group were tested. Data are mean ± standard error. Significance of *t*-tests between the 2 groups for each time of measurement are given as –, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Overall, there was a significant effect of leaf treatment (ANOVA, $F_{1,10} = 15.9$, $P < 0.003$), time ($F_{8,80} = 16.4$, $P < 0.001$), and a significant time × treatment interaction ($F_{8,80} = 8.4$, $P < 0.001$).

depended on the time elapsed after the incorporation of the treated leaves (Figure 3). The RI decreased significantly with the length of the time interval before transfer of workers ($R = 0.99$, $N = 4$, $F_{1,2} = 108.4$, $P < 0.01$). When naive ants were transferred

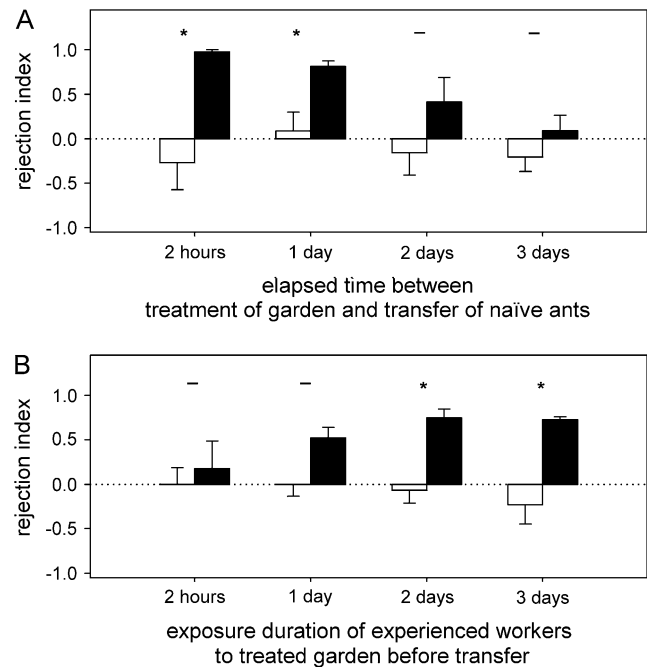


Figure 3
 RIs of “naive” and “experienced” *Acromyrmex lundii* subcolonies before (open bars) and after (filled bars) experimental transfer of workers to “treated” or “untreated” ant-free fungus gardens. (A) Naive ants that had never been in contact with treated leaf material were transferred, after different time intervals, to ant-free gardens in which privet leaves treated with cycloheximide had previously been incorporated. After transfer, they remained 24 h and were tested for the occurrence of rejection. (B) Workers from subcolonies treated with cycloheximide were transferred to untreated, ant-free fungus gardens after having spent different times after the incorporation of treated leaves in the garden. After transfer, they remained 24 h and were tested for the occurrence of rejection. Rejection responses were compared with the initial preference tests (open bars) conducted at the beginning of the experiment. All preference tests were conducted with untreated privet and rose leaves, except for the initial test in B when treated privet leaves were used to generate “experienced ants” before the transfer. Data are mean ± standard error, and $N = 4$ subcolonies for each group. Significance of paired *t*-tests of RI within each group before and after the transfer of the ants is given as –, not significant and * $P < 0.05$.

to “treated” fungus gardens within 2 h after the incorporation of treated privet leaves, they strongly rejected untreated privet leaves after 24 h in the garden (Figure 3A, on the left). Their RI after the transfer to the treated garden was significantly higher than the initial RI before the transfer ($N = 4$, paired t -test, $t = 4.05$, $df = 3$, $P = 0.03$). When naive ants were transferred one day after treated leaves were incorporated, they still rejected the previously treated leaf type 24 h later ($N = 4$, paired t -test, $t = 3.41$, $df = 3$, $P = 0.04$). However, when naive ants were transferred 2 or 3 days after incorporation of treated leaves, they accepted the previously treated leaf type (paired t -tests, nonsignificant [NS]), thus indicating that fungicide-induced changes in the fungus were no longer detectable.

In the reciprocal experiment, experienced ants that had interacted with both treated leaves and treated gardens for different time intervals were transferred to untreated gardens and their rejection behavior was recorded. Experienced ants removed from their treated garden after 2 h and transferred to an untreated garden did not reject the previously treated privet disks after 24 h (paired t -tests, NS, Figure 3B, on the left). Experienced ants remaining one day on the treated garden and then transferred to an untreated one showed a higher RI, although not statistically different, compared with the initial tests before the transfer ($N = 4$, paired t -test, $t = 2.41$, $df = 3$, $P = 0.09$). Finally, experienced workers transferred to an untreated garden after 2 or 3 days in the treated one showed rejection behavior toward the previously treated leaf type (Figure 3B, on the right, day 2: $N = 4$, paired t -test, $t = 3.96$, $df = 3$, $P = 0.03$; day 3: $N = 4$, paired t -test, $t = 3.84$, $df = 3$, $P = 0.03$).

Experimental series 4: long-term rejection and resumption of acceptance

After the initial exposure to treated privet leaves, ants showed rejection of these previously treated leaves as expected, and this response was observed to persist for 9 weeks when no subsequent incorporation of untreated leaves of this same species into the fungus garden was allowed (Figure 4A). In control subcolonies presented only untreated leaves, workers also developed moderate rejection behavior toward privet leaves over time for unknown reasons. Despite this phenomenon, “treatment” subcolonies consistently had a significantly higher RI than “control” subcolonies, except in week 4 and 7 (Figure 4A, overall ANOVA results presented in the figure caption).

In subcolonies exclusively fed with untreated leaves of the previously treated leaf type, rejection of that leaf type again strongly increased after the initial exposure to treated leaves, as expected (Figure 4B; statistics presented in the figure caption). However, in contrast with the previous experiment, “treatment” colonies only had a significantly higher RI than the “control” colonies in the first and second week (Figure 4B). In the following weeks, RI of “treatment” and “control” colonies did not differ any more and acceptance of the treated leaf type was resumed, indicating that enforced incorporation of previously unsuitable leaves led to resumption of acceptance. Again, a moderate rejection of privet leaves was observed in control subcolonies over time suggesting a decline in their quality.

DISCUSSION

Species-specific delayed rejection and its onset

In this study, we showed that foragers of the leaf-cutting ant *Ac. lundii* exhibit delayed rejection of leaves infiltrated with the fungicide cycloheximide and are able to discriminate between suitable and harmful leaves for the fungus in 2-choice

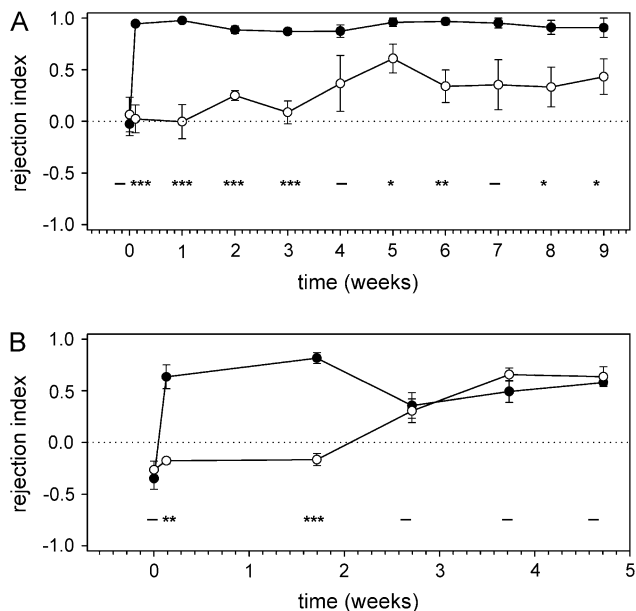


Figure 4

Long-term course of RIs of *Acromyrmex lundii* subcolonies fed (A) with a leaf type that had not been previously treated (rose) or (B) on untreated leaves of the initially treated leaf type, which had induced rejection (privet). “Treatment” colonies (filled circles) received privet leaf disks treated with cycloheximide and untreated rose leaf disks on the first 2 days of the experiment, and “control” colonies (open circles) were offered untreated leaf disks of both types. Note differences in scaling of the x axis between A and B. Significance of t -tests comparing “treatment” and “control” subcolonies for each time of measurement are given as –, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Overall ANOVA results for A, treatment: $F_{1,9} = 137.0$, $P < 0.001$; time: $F_{10,90} = 4.1$, $P < 0.001$; time \times treatment: $F_{10,90} = 1.9$, $P = 0.051$; and for B, treatment: $F_{1,8} = 12.3$, $P < 0.008$; time: $F_{5,40} = 22.1$, $P < 0.001$; time \times treatment: $F_{5,40} = 15.5$, $P < 0.001$.

preference experiments. Delayed rejection of natural plants in leaf-cutting ants was demonstrated for the first time by Rahbe et al. (1988) and now has been shown to occur in several leaf-cutting ant species (*Ac. lundii*, *Acromyrmex octospinosus*, *Ac. subterraneus*, *Atta cephalotes*, *A. leavigata*, *A. sexdens*, *A. colombica*) both in the laboratory (Knapp et al. 1990; Ridley et al. 1996; North et al. 1999; Camargo et al. 2003; this study) and in the field (Ridley et al. 1996; Saverschek 2004; Wagner 2004). It therefore seems to be a decision-making process common among leaf-cutting ants.

Delayed rejection behavior in our experiments was specific toward the leaf type laced with the fungicide, indicating that the ants were able to recognize leaves of different plant species. From feeding trials, it is known that leaf-cutting ants are highly competent in distinguishing among leaves of different species and even among leaves from the same tree (Hubbell and Wiemer 1983; Howard 1990; Meyer et al. 2006). Our data suggest that the ants not only can assess leaf quality on the cutting site but can also learn to associate particular leaf characteristics with the specific reaction of the fungus toward the leaf. These outcomes extend previous findings by Ridley et al. (1996) using artificial bait.

The onset of delayed rejection behavior in the experimental subcolonies occurred within 10 h after foraging on the leaves. This is considerably faster than in previous experiments with artificial bait (Ridley et al. 1996), which was in the range of several days. The time lag between foraging and incorporation of the substrate into the garden and observation of rejection

behavior may depend on the kind and amount of treated material, the ant species, and the size and the motivational state of the colony.

The fast induction of rejection is effective in avoiding further exposure of the fungus to unsuitable, harmful plants and also circumvents the further allocation of workers to their harvesting. Scouts explore the foraging territory for potential new host plants and are observed to return to the nest with a load after a number of trips, so that the buildup of a foraging column might require a relatively long time (Jaffe and Howse 1979; Howard et al. 1996). The observed fast induction of rejection behavior suggests that workers may be able to respond to the unsuitability of a new substrate within a day, that is, before their next foraging cycle, diurnal or nocturnal, begins (e.g., Hodgson 1955; Waller 1986; Wirth et al. 1997). Foragers may thus abandon the newly discovered plant if it is unsuitable for the fungus, or exploitation may continue and be amplified by recruitment of nest mates if it is suitable. The fast induction of rejection behavior may be one of the reasons why this phenomenon has not yet been observed to occur under natural conditions in the field. It may be a rather cryptic episode in a given foraging day, because scouts occur in small numbers and they harvest small quantities. A brief occurrence of a plant species in the harvest of leaf-cutting ants and in very low quantities, as observed in field studies (Wirth et al. 2003), may reflect such initial acceptance that further leads to delayed rejection behavior, although it has so far been attributed to a depletion of the source or to the recent discovery of a new one.

Long-term rejection and resumption of acceptance

After workers from a subcolony learned to reject a specific plant, their memory lasted for at least 2 months, even though no further negative reinforcement had occurred after the initial incorporation of the treated leaves. The subcolonies in the experiment did not survive long enough to determine the extent of the long-term memory, which has been suggested to last between 5 and 30 weeks (Knapp et al. 1990; Ridley et al. 1996). This phenomenon and the subsequent decline of the rejection behavior have been attributed to lifetime memory and the turnover of the foragers in a colony (Knapp et al. 1990; Ridley et al. 1996), but no experimental work addressing this question has been performed so far.

When subcolonies that had learned to reject a plant harmful for the fungus were fed with untreated leaves of the same type without alternatives, they resumed acceptance of the initially rejected type within 3 weeks. Thus, acceptance recommenced considerably faster when the ants were exposed to leaves that were actually suitable, yet previously harmful, indicating the existence of behavioral plasticity and reversal learning, a fact incompatible with the existence of a stable lifetime memory.

Host plants of leaf-cutting ants exhibit phenological changes in the quality and quantity of their defenses and may therefore be suitable only for some periods of the year (Fowler and Stiles 1980; Hubbell et al. 1984; Howard 1987; Coley and Kursar 1996). Because territories of leaf-cutting ant colonies are spatially limited, it may be beneficial for scouts to revisit plants, so that harvesting can be resumed after changes in plant quality occur. Periodic use of individual host plants by single leaf-cutting ant colonies has actually been observed (Rockwood 1976; Fowler and Stiles 1980; Wirth R, personal communication) and may mirror such dynamics of leaf quality and subsequent changes of foraging decisions in the workers.

The role of the fungus garden for delayed rejection

We showed experimentally that contact of the workers with a fungus garden with unsuitable (treated) leaves incorporated

was necessary to induce delayed rejection behavior. An exposure of ants to treated leaf material alone was not sufficient to provoke delayed rejection. This indicates that some unidentified changes in the fungus garden are responsible for the induction of delayed rejection via avoidance learning. Ridley et al. (1996) and North et al. (1999) first showed this phenomenon using fungicide-containing bait that was incorporated as fungal substrate. Our experiments using leaves instead of artificial bait indicate that avoidance learning induced by a fungal stimulus also occurs under more realistic conditions, that is, in response to changes in the suitability of leaves for the fungus. The induction of delayed rejection exclusively through the fungus, as in our study, shows that it was independent of avoidance learning mediated by the toxic effects of compounds directly ingested by the ants (Knapp et al. 1990).

Stimuli from the fungus garden and the leaf material

Little is known about the nature of changes occurring in the fungus after incorporation of harmful substrates that leads to rejection behavior in workers. When patrolling the garden, ants may directly detect these changes, or respond to a semiochemical released by the fungus, as suggested by several authors (Knapp et al. 1990; Ridley et al. 1996; North et al. 1999). Results from experiments with pairs of subcolonies of *A. sexdens* separated by gauze indicated that there are no volatiles involved (North et al. 1999). Although identifying the nature of the fungal changes was not the aim of this study, our results emphasize 2 points. First, the rapid induction of delayed rejection indicates that learning occurs via some direct alteration of the fungus, rather than an effect on the quantity and/or quality of the nutritious gongylidia produced by the fungus. Those are known to be produced in the fungal mass 4–5 days after incorporation of substrate (Bass and Cherrett 1996). Second, the fungal changes inducing delayed rejection are likely to occur at a very small, localized scale, in the range of square millimeters, because workers incorporated particles of treated and untreated leaf types evenly mixed and distributed across the apical region of the single fungus garden, without obvious separation of the different substrates (Herz H, personal observation).

In our experiments, induction of delayed rejection in workers when exposed to a treated garden was possible only for a short time, 1–2 days, which indicates that either the changes in the fungus garden occur only for a brief period or the leaf characteristics used by the ants for plant recognition are no longer detectable. Regarding leaf characteristics, the fungus mycelium almost completely covers an incorporated plant fragment after 2 days (Herz H, personal observation), so that this overgrowth may mask the relevant leaf stimulus for plant identification and learning.

So far, it also remains largely unknown as to how the information on the unsuitability of a substrate is distributed within the colony. Previous experiments aimed at answering the question of whether the foragers need to have direct contact with the fungus or whether gardening workers, which directly process the substrate, obtain the information and then transmit it to foragers suggest that both channels may operate (North et al. 1999).

Ecological implications

The combination of foraging ants capable of coping with mechanical plant defenses and a fungus able to cope with plant chemical defenses is regarded as a main component for the great ecological success of the fungus–leaf-cutting ant mutualism (Cherrett 1989). In this study, we showed that avoidance

learning in the foragers, mediated by the fungus, is 1) fast and 2) specific for the harmful leaf, 3) the specific reaction is memorized for several weeks, and 4) it is plastic and can be modified when previously unsuitable substrate becomes suitable. All these aspects contribute to an efficient protection of the mutualist against loading with harmful compounds. Such protection of the fungus may enhance or ensure its growth and productivity and is ultimately beneficial to both mutualistic partners. In highly diverse, spatially and temporarily variable environments, with many fungicidal plant compounds potentially unknown to the ants, it is advantageous for a colony to be able to avoid plants harmful to the fungus through a mechanism that directly responds to the fungus and not only relies on the effects of harmful plant compounds on those workers that make foraging decisions at the cutting site.

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REFERENCES

- Bass M, Cherrett JM. 1995. Fungal hyphae as a source of nutrients for the leaf-cutting ant *Atta sexdens*. *Physiol Entomol.* 20:1–6.
- Bass M, Cherrett JM. 1996. Fungus garden structure in the leaf-cutting ant *Atta sexdens* (Formicidae, Attini). *Symbiosis.* 21:9–24.
- Bernays EA, Chapman RF. 1994. Host-plant selection by phytophagous insects. New York: Chapman and Hall.
- Beyschlag W, Pfanz H. 1990. A fast method to detect the occurrence of nonhomogeneous distribution of stomatal aperture in heterobaric plant leaves. *Oecologia.* 82:52–55.
- Camargo RS, Forti LC, de Matos CAO, Lopes JF, de Andrade APP, Ramos VM. 2003. Post-selection and return of foraged material by *Acromyrmex subterraneus brunneus* (Hymenoptera: Formicidae). *Sociobiology.* 42:93–102.
- Camargo RS, Forti LC, de Matos CAO, Lopes JF, de Andrade APP. 2004. Physical resistance as a criterion in the selection of foraging material by *Acromyrmex subterraneus brunneus* Forel, 1911 (Hym., Formicidae). *J Appl Entomol.* 128:329–331.
- Cherrett JM. 1972. Chemical aspects of plant attack by leaf-cutting ants. In: Harbourne JB, editor. *Phytochemical ecology*. London: Academic Press. p. 13–24.
- Cherrett JM. 1989. Leaf-cutting ants. In: Lieth H, Werger MJA, editors. *Tropical rain forest ecosystems: biogeographical and ecological studies*. Amsterdam (The Netherlands): Elsevier. p. 473–488 (Ecosystems of the world; vol. 14B).
- Coley P, Kursar T. 1996. Anti-herbivore defenses of young tropical leaves: physiological constraints and ecological trade-offs. In: Mulkey SS, Chazdon RL, Smith AP, editors. *Tropical forest plant ecophysiology*. New York: Chapman and Hall. p. 305–336.
- de Andrade APP, Forti LC, Moreira AA, Boaretto MAC, Ramos VM, de Matos CAO. 2002. Behavior of *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) workers during the preparation of the leaf substrate for the symbiont fungus culture. *Sociobiology.* 40:293–306.
- Fowler HG, Stiles EW. 1980. Conservative resource management by leaf-cutting ants? The role of foraging, territories and trails, and environmental patchiness. *Sociobiology.* 5:25–41.
- Hodgson ES. 1955. An ecological study of the behavior of the leaf-cutting ant *Atta cephalotes*. *Ecology.* 36:293–304.
- Howard JJ, Henneman ML, Cronin G, Fox JA, Hormiga G. 1996. Conditioning of scouts and recruits during foraging by a leaf-cutting ant, *Atta colombica*. *Anim Behav.* 52:299–306.
- Howard JJ. 1987. Leafcutting ant diet selection: the role of nutrients, water, and secondary chemistry. *Ecology.* 68:503–515.
- Howard JJ. 1988. Leafcutting ant diet selection: relative influence of leaf chemistry and physical features. *Ecology.* 69:250–260.
- Howard JJ. 1990. Infidelity of leafcutting ants to host plants: resource heterogeneity or defense induction? *Oecologia.* 82:394–401.
- Hubbell SP, Wiemer DF. 1983. Host plant selection by an attine ant. In: Jaisson P, editor. *Social insects in the tropics*. Paris: Université Paris-Nord. p. 133–154.
- Hubbell SP, Howard JJ, Wiemer DF. 1984. Chemical leaf repellency to an attine ant: seasonal distribution among potential host plant species. *Ecology.* 65:1067–1076.
- Jaffe K, Howse PE. 1979. The mass recruitment system of the leaf cutting ant, *Atta cephalotes* (L.). *Anim Behav.* 27:930–939.
- Knapp JJ, Howse PE, Kermarrec A. 1990. Factors controlling foraging patterns in the leaf-cutting ant *Acromyrmex octospinosus* (Reich). In: Vander Meer RK, Jaffe K, Cedenio A, editors. *Applied myrmecology: a world perspective*. Boulder (CO): Westview Press. p. 382–409.
- Meyer S, Roces F, Wirth R. 2006. Selecting the drought stressed: effects of plant stress on intraspecific and within-plant herbivory patterns of the leaf-cutting ant *Atta colombica*. *Funct Ecol.* 20:973–981.
- Nichols-Orians CM, Schultz JC. 1990. Interactions among leaf toughness, chemistry, and harvesting by attine ants. *Ecol Entomol.* 15:311–320.
- North RD, Jackson CW, Howse PE. 1999. Communication between the fungus garden and workers of the leaf-cutting ant, *Atta sexdens rubropilosa*, regarding choice of substrate choice of substrate for the fungus. *Physiol Entomol.* 24:127–133.
- Quinlan RJ, Cherrett JM. 1979. The role of fungus in the diet of the leaf-cutting ant *Atta cephalotes* (L.). *Ecol Entomol.* 4:151–160.
- Rahbe Y, Febvay G, Kermarrec A. 1988. Foraging activity of the attine ant *Acromyrmex octospinosus* (Reich) (Hymenoptera: Formicidae) on resistant and susceptible yam varieties. *Bull Entomol Res.* 78: 329–337.
- Ridley P, Howse PE, Jackson CW. 1996. Control of the behaviour of leaf-cutting ants by their 'symbiotic' fungus. *Experientia.* 52:631–635.
- Roces F. 1990. Olfactory conditioning during the recruitment process in a leaf-cutting ant. *Oecologia.* 83:261–262.
- Roces F. 1994. Odour learning and decision-making during food collection in the leaf-cutting ant *Acromyrmex lundii*. *Insectes Soc.* 41:235–239.
- Rockwood LL. 1976. Plant selection and foraging patterns in two species of leaf-cutting ants (*Atta*). *Ecology.* 57:48–61.
- Rosenthal GA, Berenbaum MR, editors. 1991. *Herbivores: their interactions with secondary plant metabolites*. Vol. I: The chemical participants. 2nd ed. San Diego (CA): Academic Press.
- Saverschek N. 2004. Host plant selection by the leaf-cutting ant *Atta colombica*—the role of experience in the process of decision making [diploma-thesis]. Göttingen (Germany): University of Göttingen. p. 79.
- Wagner M. 2004. Trailtreue und Ablehnungsverhalten der Blattschneiderameisenart *Atta colombica* [diploma-thesis]. Würzburg (Germany): University of Würzburg. p. 104.
- Waller DA. 1986. The foraging ecology of *Atta texana* in Texas. In: Lofgren CS, Vander Meer RK, editors. *Fire ants and leaf-cutting ants: biology and management*. Boulder (CO): Westview Press. p. 146–158.
- Weber NA. 1956. Treatment of substrate by fungus-growing ants. *Anat Rec.* 125:604–605.
- Weber NA. 1972. Gardening ants: the attines. Philadelphia (PA): The American Philosophical Society.
- Wirth R, Beyschlag W, Ryel RJ, Hölldobler B. 1997. Annual foraging of the leaf-cutting ant *Atta colombica* in a semideciduous rain forest in Panama. *J Trop Ecol.* 13:741–757.
- Wirth R, Herz H, Ryel RJ, Beyschlag W, Hölldobler B. Herbivory of leaf-cutting ants: a case study on *Atta colombica* in the tropical rain-forest of Panama. Berlin (Germany): Springer. p. 230 (Ecological studies; vol. 164).