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Control of the behaviour of leaf-cutting ants by their 'symbiotic' fungus

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Abstract. There is an obligatory relationship between leaf-cutting ants of the genera *Atta* and *Acromyrmex* (Hymenoptera; Attini) and the fungus, *Attamyces bromatificus* Kreisel, for which they provide a substrate of cut plant material. We show that the ants learn to reject plant material that contains chemicals injurious to the fungus. After an initial period of acceptance, ants from laboratory nests stopped harvesting granular bait containing a fungicidal agent (cycloheximide) and orange peel. This rejection was maintained for many weeks. These colonies also rejected control bait containing no cycloheximide. Some generalisation of the response was observed; colonies rejecting orange granules also rejected grapefruit granules, although they still accepted blackcurrant granules. Rejection of fungicidal bait by colonies in the field was restricted to ants on foraging trails exposed to experimental bait. We conclude that a semiochemical from the fungus, circulated by trophallaxis and grooming, regulates the selection of plant material by foragers. The fungus gains more advantages from the symbiotic relationship than is often realised. The ants provide the fungus with housing, sanitation, defence against diseases and predators, pre-selected food, and a means of dissemination, at the cost to itself of providing food for the ant brood.

Key words. Atta; symbiosis; semiochemical; leaf-cutting ants; sociobiology; fungus.

Leaf-cutting ants forage on living plant material which they transport back to the nest where it is processed to form a substrate on which they cultivate an anamorphic fungus, *Attamyces bromatificus* Kreisel (? = *Leucocoprinus gongylophorus* (A. Moeller Singer), which is the sole food source for the larvae[1]. The adult ants obtain a large proportion of their energy requirements during foraging from the sap of cut plant material[2]. This being so, is the choice of plant material influenced by the requirements of the fungus? It has been suggested that foraging ants respond to the presence of fungicidal compounds in harvested plant material and subsequently cease foraging on these plants[3–5], but no experimental evidence has yet been adduced for this. Other work has indicated that leaf-cutting ants are unable to detect and respond to the growth success of the fungus on various substrates[6].

A variety of factors has been implicated in controlling plant choice, including leaf toughness, moisture content, presence of inimical ants, etc.[7–9]. However, recent evidence[10,11] has suggested that the avoidance of certain plant secondary chemicals is perhaps the most important single factor. Chemicals repellent to foraging ants have been identified in plants that are not attacked[12–14], but it has not been possible to determine the precise role that such chemicals may play in rejection, for example, whether they are injurious to the fungi or the insects, or both[15], Four different categories of rejection responses to unacceptable leaf material based upon chemical cues have been described for *Acromyrmex ostospinosus*[16]: 1) plants are inspected but not cut, suggesting some form of odour-mediated rejection; 2) cutting begins but is not completed, indicating the involvement of aversive gustatory stimuli; 3) on initial presentation the leaves are cut and harvested, but in all subsequent assays the foragers reject the plant material; 4) recruitment ceases with time, and this pattern is repeated on subsequent presentations to the nest.

These observations suggest that learnt responses may affect plant choice during foraging.

Materials and methods

Experiments were carried out on leaf-cutting ant colonies (*Atta* and *Acromyrmex* spp.) imported from Brazil and Guadeloupe (FWI) and maintained in the insectary at 25 °C on an LD 12:12 light cycle. Observations were carried out on colonies of *Atta cephalotes* in the field in lowland rain forest in Trinidad (WI).

Experiment 1. Ants were offered orange bait granules in an open foraging arena connected to a laboratory nest. The granules were produced using a basic micro-encapsulation technique[17]. 2 g of freeze-dried orange peel, 0.3 g of sodium alginate, and (in experimental granules) 0.025 g of cycloheximide were mixed in 25 ml of water. The mixture was introduced dropwise into a solution of calcium gluconate (0.1 M). The resultant beads were removed and allowed to dry at room temperature to form granules. Experimental granules included cycloheximide (100 μg/ml water). Five granules were offered

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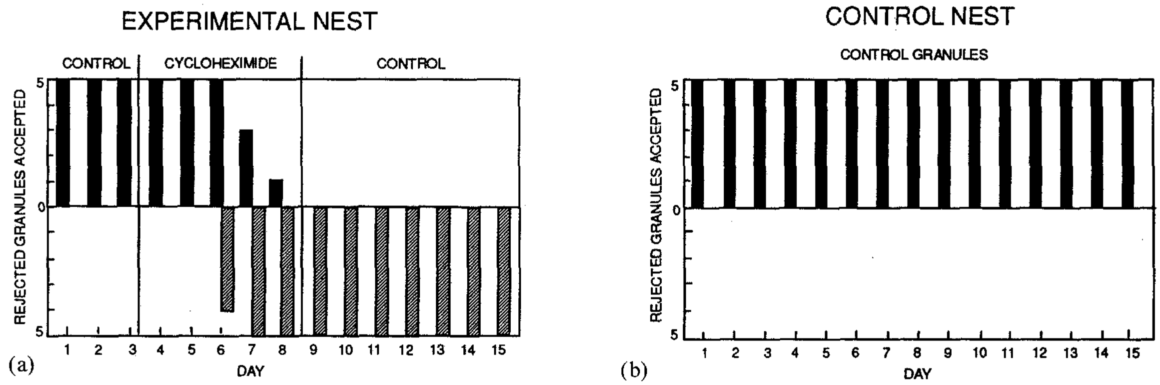

Figure 1. Response of laboratory colonies to a bait containing the fungicide cycloheximide. *a)* Control granules (without cycloheximide) were accepted by the colony on days 1–3. Experimental granules were accepted initially (days 4 and 5) but the number taken into the nest by foragers decreased (days 6–8). Complete rejection of the experimental granules occurred by day 8. Control granules offered on subsequent days were also rejected. *b)* Over the same period the control colony accepted all control granules offered.

each day to laboratory colonies and the number of granules taken into the nest during the following hour was recorded. Granules appearing in the refuse pile and foraging arena after 24 h were recorded as rejected granules and removed. These orange granules constitute a semi-artificial plant material of controlled form and composition which acts solely against the symbiotic fungus. The granules were: a) novel food material (colonies had no previous foraging experience of orange) and b) a highly attractive and preferred food source[18]. Experimental granules were not repellent – there was no difference in the pick-up rate of control and experimental granules.

Separate experiments were carried out to determine if there was any toxic action of cycloheximide on ants, or any delayed conditioning effect. Ants readily drank cycloheximide solution in 0.1 M sucrose at a concentration of 100 μg ml^{-1} and showed no reduction of intake after 48 hours when compared with a control of 0.1 M sucrose solution. Experimental granules were, however, toxic to the fungus; *Attamyces bromatificus* is very sensitive to cycloheximide[19], but the granules were not toxic to the ants. Fluorescence bioassays indicated no ingestion of material by the ants during either foraging or substrate preparation. While it is theoretically possible that cycloheximide could be taken into the insects through a different route, it is clear that direct insecticidal effects were not responsible for rejection of orange granules at the concentrations used here.

Experiment 2. For the second experiment, orange, grapefruit and blackcurrant fruit granules were produced as described above; grapefruit granules contained freeze-dried grapefruit peel and blackcurrent granules contained Ribena™ (4 ml in 30 ml of water) with 4 g of flour added to form the matrix. Prior to conditioning, all nests were offered five control orange granules and the time taken for all granules to be picked-up and transported to the nest entrance was recorded. All the granules were removed before they reached the colony entrance. This was repeated ten times for all types of fruit granule. Nests were then conditioned to reject orange granules as described in experiment 1. Following this conditioning procedure, the acceptance or rejection of all granule types were recorded. The fate of all granule types in control nests was also recorded.

Field experiments. Fifteen colonies of *Atta cephalotes* were located and mapped; the nest area, and the number and size of permanent foraging trails were measured. The most active trail of each nest was designated the 'experimental trail'. A plastic vial containing 1 g of orange granules + cycloheximide (prepared as explained above) was placed on the experimental trail of each colony at approximately 1 meter from the nest entrance. 1 g of these experimental orange granules was offered each day for 10 days. On the 11th day, 1 g of experimental orange granules was offered to all trails, 'naive' (no previous exposure to orange granules) and experimental, and the number of granules taken by each trail recorded after 24 h.

Results

Experiment 1. Fruit granules (orange, grapefruit and blackcurrant) provided a highly attractive and acceptable food source to all nests prior to the conditioning experiment. When we incorporated a fungicide into a highly attractive and acceptable (orange) granular bait, foraging worker ants of a colony of *Atta laevigata* rejected this bait for at least ten days, after an initial period of acceptance lasting approximately 48 h (fig. 1). Granules taken into the nest and subsequently rejected then appeared either in the refuse pile or in the foraging arena. Complete rejection occurred by day 7, and control granules offered on subsequent days were also rejected. Control orange granules were accepted throughout the experimental period (fig. 1b). Similar

Table 1. Uptake of fruit granules by laboratory colonies before and after conditioning to orange granules, and time taken to pick-up (5 granules presented in 10 replicate tests).

Granule Type	Number of granules taken pre-conditioning	Average pick-up time (seconds) pre-conditioning	Number of granules taken post-conditioning
Orange control	all	194 (SD = 138)	none
Orange experimental	all	220 (SD = 156)	none
Grapefruit control	all	199 (SD = 103)	none
Grapefruit experimental	all	188 (SD = 48)	none
Blackcurrant control	all	204 (SD = 98)	all
Blackcurrant experimental	all	203 (SD = 67)	all

There was no significant difference in the pre-conditioning pick-up rate between the different fruit granules (whether experimental or control).

laboratory trials were carried out on individual colonies of *Atta sexdens rubropilosa*, *Acromyrmex octospinosus*, and *Ac. subterraneus*. In *Atta sexdens* complete rejection first occurred on day 7, in *Acromyrmex octospinosus* on day 6, and in *Ac. subterraneous* on day 6. The same rejection phenomenon was shown also in the 10 replicates of each treatment in experiment 2.

Experiment 2. In this experiment there was no significant difference in preconditioning pick-up rate between the different fruit granules, either experimental or control (table 1). Nests rejecting experimental granules also rejected control granules without cycloheximide, suggesting that the rejection mechanism involves negative conditioning of the ants to some chemical aspect of

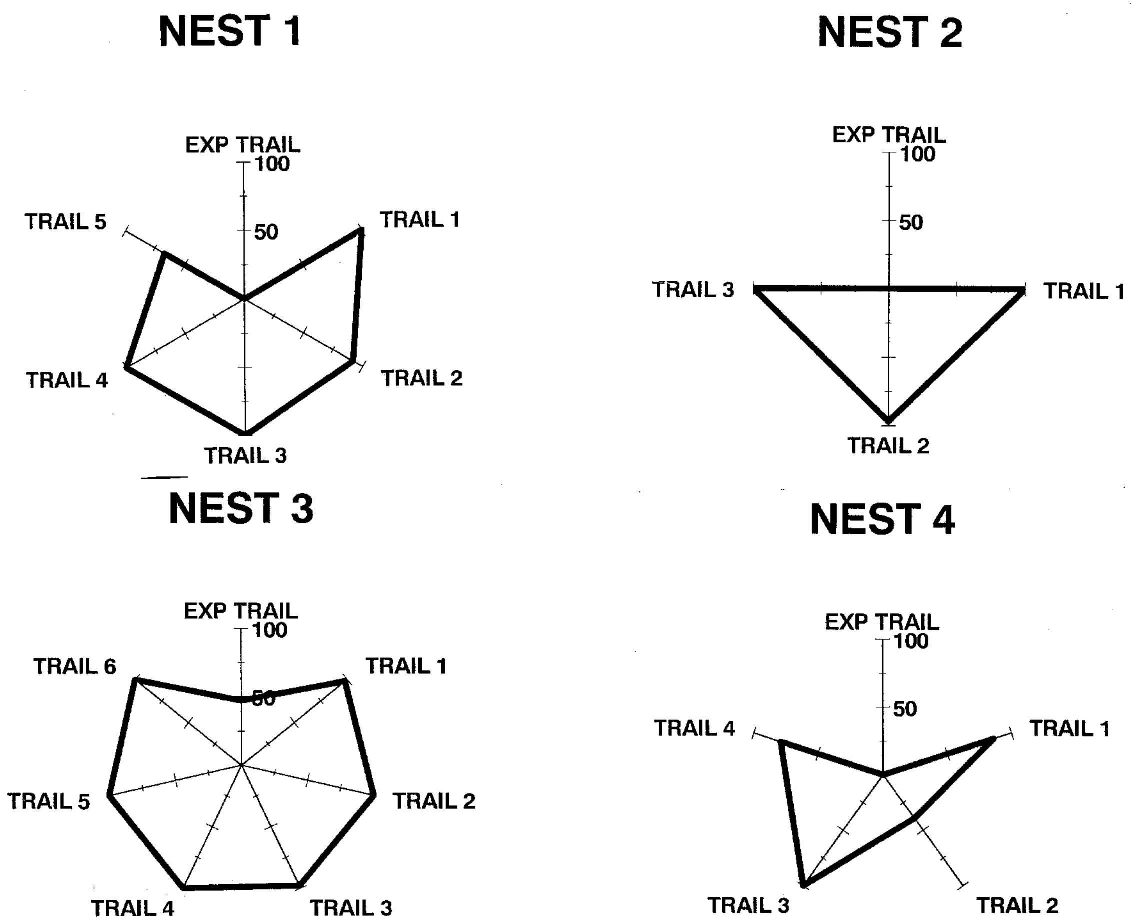

Figure 2. Activity of ants on foraging trails leading from four *Atta cephalotes* nests in Trinidad, W.I. Each axis on the four nest plans represents a permanent foraging trail. A vial of ~100 orange granules was put on each trail about 1 metre from the nest entrance, and was replaced daily for five days prior to the test. Subsequently, the vials were replaced, but one vial, placed on the experimental trail marked 'EXP', contained the fungicide cycloheximide. 24 hours later, the percentage of approximately 100 granules taken by foragers on each trail from each plastic vial was recorded. The percentage is plotted on the nest plan.

orange peel rather than to the cycloheximide. Furthermore, foraging ants that did not take orange granules generalised their response by rejecting granules containing freeze-dried grapefruit peel. However, they continued to accept experimental and control blackcurrant granules. Foragers from control nests continued to accept all granule types for the duration of the experiment. The granules were neither toxic nor repellent to the ants kept in isolation from their fungal symbiont, and therefore this change in foraging behaviour can only be accounted for by ants responding to the injurious effect of this food source on the symbiotic fungus. The ability of foragers to generalise their response to odours with common components could help to protect the colony. Following the rejection of a plant containing fungitoxic chemicals, foragers that generalise to a similar set of odours or gustatory stimuli associated with those toxins can inhibit the colony from harvesting closely related plants with similar chemical defences.

The field studies on *Atta cephalotes* colonies provide a clearer picture of the effect of fungicidal material on foraging behaviour (fig. 2). Mature colonies had a number of permanent foraging trails radiating out from the nest in various directions. Ants on trails leading to a bait of orange granules plus fungicide rejected these orange granules to a greater degree than foragers on 'naive' trails (trails not previously exposed to granules) of the same nest (Mann-Whitney U test-Statgraphics: $Z = 3.90$, two tailed probability of equalling or exceeding Z, $P < 1.0 \times 10^{-4}$. 15 nests, 15 experimental trails, 48 naive trails). However, foragers from the same nest, but on 'naive' trails which had not previously been baited, accepted the fungicidal bait. These results demonstrate trail fidelity by foragers and indicate a lack of communication within the nest between ants exploiting differently located food sources. This may be due in part to foragers on a given trail servicing particular fungus gardens (*Atta* nests consist of numerous small well-spaced subterranean fungus gardens connected by galleries).

Discussion

We conclude that, in a mature nest, *Atta* workers are relatively faithful to a particular trail. While they may reject certain plant material on the basis of physical characteristics or aversive chemical stimuli, other material is removed to the nest and incorporated into the fungus garden substrate. If the forage material has toxic effects on the fungus, an allomone is produced by the fungus which acts as negative reinforcement to the ants servicing that particular fungus garden. We do not know how this signal reaches the incoming foragers, and it may be necessary to postulate that it is transmitted by the minims ('gardeners') which tend the fungus gardens. The rejection is then a conditional response to the conditional stimulus of odour associated with the forage material. It is long-lasting: we have found that it persists for up to 30 weeks in laboratory colonies, and its loss may therefore be due to turnover of the population of foragers.

While it is theoretically possible that a barren fungus garden will act as negative reinforcement, we can see no alternative to postulating a chemical mediator in the rejection process: it is scarcely conceivable that ants compare an empty fungus garden with a thriving one by non-chemical signals alone. Furthermore, rejection occurs on a timescale much shorter than that needed to eliminate fungal growth over a whole fungus garden.

Our results show that the fungus is not a passive partner in this symbiotic relationship. The fungus subverts a colony of ants into providing it with a healthy diet. In addition, the fungus has the benefits of housing, defence, and sanitation (attine ants are known to control entry of alien fungi by mechanical and chemical means[20–22]). Furthermore, at regular intervals, the fungus is distributed to other sites during the nuptual flight of the ants in the infrabuccal pouch of the foundress queens. The fungus can therefore increase its own fitness by contributing to brood-rearing of the ants and by manipulating the behaviour of foragers so that they harvest food that provides the optimal medium for fungal growth. The evidence for communication between a fungal symbiont and an animal has implications for evolutionary sociobiology and may also help to explain the success of the Macrotermitinae (fungus-growing termites) in the Old World. More immediately, it provides a hypothesis to explain the feeding preferences of leaf-cutting ants and the reasons for their changing foraging patterns.

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1 Quinlan, R. J., and Cherrett, J. M., Ecol. Ent. *4* (1979) 151.
2 Littledyke, M., and Cherrett, J. M., Bull Ent. Res. *66* (1976) 205.
3 Febvay, G., and Kermarrec, A., in: Fire Ants and Leaf-Cutting Ants: Biology and Management, p. 247. Eds C. S. Lofgren and R. K. Vander Meer. Westview Press, Boulder (Colorado) 1986.
4 Nichols-Orions, C. M., and Schultz, J. C., Ecol. Ent. *15* (1990) 311.
5 Bueno, O. C., Hebling-Beraldo, M. J. A., Aulino da Silva, O., Pagnocca, F., Fernandez, J. B., and Vieira, P. C., in: Applied Myrmecology: A World Perspective, p. 420. Eds A. Cedeno, K. Jaffe and R. K. Vander Meer. Westview Press, Boulder (Colorado) 1990.
6 Quinlan, R. J., and Cherrett, J. M., Ecol. Ent. *3* (1978) 221.
7 Nichols-Orions, C. M., and Schuiltz, J. C., Biotropica *21* (1989) 80.
8 Bowers, M. A., and Porter, S. D., Ecology *66* (1981) 861.
9 Cherrett, J. M., and Jutsum, A. R., in: Social Insects in the Tropics, pp. 155–163. Ed. P. Jaisson. Universite Paris Nord, Paris 1983.
10 Howard, J. J., Ecology *68* (1987) 503.

11 Howard, J. J., Ecology *69* (1988) 250.

12 Hubbell, S. P., Wiemer, D. F., and Adejare, A., Oecologia *60* (1983) 3221.

13 Hubbell, S. P., Howard, J. J., and Wiemer, D. F., Ecology *65* (1984) 1067.

14 Hubert, T. D., and Wiemer, D. F., Phytochemistry *24* (1985) 1197.

15 Howard, J. J., Cazin Jr. J., and Wiemer, D. F., J. chem. Ecol. *14* (1988) 59.

16 Knapp, J. J., Howse, P. E., and Kermarrec. A., in: Applied Myrmecology: A World Perspective, p. 382. Eds A. Cedeno, K. Jaffe and R. K. Vander Meer. Westview Press, Boulder (Colorado) 1990.

17 Fravel, D. R., Marois, J. J., Lumsden, R. D., and Connick, W. J., Phytopath. *75* (1985) 774.

18 Cherrett, J. M., and Seaforth, C. E., Bull ent. Res. 59 (1970) 615.

19 Cazin Jr., J., Wiemer, D. F., and Howard, J. J., Appl. environment. Microbiol. *55* (1989) 1346.

20 Quinlan, R. J., and Cherrett, J. M., Ecol. Ent. *2* (1977) 161.

21 Boyd, N. D., and Martin, M. M., Insect. Biochem. *5* (1975) 619–635.

22 Boyd, N. D., and Martin, M. M., J. Insect. Physiol. *21* (1975) 1815.