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**Sensitivity of colonies and individuals of *Cephalotes* ants to antibiotics
imply a feeding symbiosis with gut microorganisms.**

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Sensitivity of colonies and individuals of *Cephalotes* ants to antibiotics imply a feeding symbiosis with gut microorganisms.

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ABSTRACT

Ants in the tribe Cephalotini are exceptional in that they keep microorganisms living in their digestive tract. In order to understand what these microorganisms mean to the ants, we observed the feeding habits of *Cephalotes pusillus* and *C. atratus* ants, finding that in nature they feed on extrafloral nectars, homopteran secretions and bird droppings. Feeding the antibiotic kanamycin to colonies of *C. pusillus* in the laboratory kills the ants. Ants desiccate or starve rather than feed on liquids to which the antibiotic gentamicin or netromycin has been added, but feed and survive on liquids with nistatin, penicillin and ampicillin. We identified over 10 microorganisms from the intestine of *C. pusillus* with different antibiotic resistance patterns. The bacteria are from the genera *Corynebacterium*, *Brevibacterium*, *Sphingobacterium*, *Ochrobactrum*, *Myroides*, *Brevundimonas*, *Alcaligenes*, *Stenotrophomonas*, *Moraxella* and *Pseudomonas*. We hypothesize that the microorganisms provide nutrients to the ants by synthesizing amino acids from carbohydrates and nitrates. The ants possibly collect the bacteria from the environment and/or transmit them to their young. They culture them in their digestive tract, eventually feeding on them.

INTRODUCTION

Microorganisms are frequently associated with the digestive tract of vertebrates and insects, but in the case of ants, no such association has been clearly defined. Ants of the tribe Cephalotini are taxonomically, ecologically and behaviorally a distinctive group of Myrmicinae (Kempf 1951). Little is known about their life history and behavior. They are arboreal, inhabiting cavities they carve in dead trunks and branches of trees, and in some cases (i.e. *Cephalotes atratus*) may form large colonies of thousands of individuals. They have been reported to feed on extrafloral nectar (Oliveira and Brandao 1991), on homopteran secretions (Del-Claro and Oliveira 1999), and on wind-transported pollen (Baroni-Urbani and Andrade 1997 and included references). For example, *Cephalotes varians* and *C. maculatus* are highly specialized in collecting extrafloral nectar (Wilson 1976; Adams 1996). Workers have digestive tracts which are specialized for ingestion of liquid food (Caetano 1994) and pollen (Baroni-Urbani and Andrade 1997). They are known to engage in interspecific oral trophallaxis (Del-Claro and Oliveira 1999) and intraspecific oral and oral-anal trophallaxis (Wilson 1976; Wheeler 1984). Oral-anal trophallaxis has been suggested to facilitate the transfer of internal microorganisms (Wheeler 1984); as newly hatched callow (non pigmented) workers have the dilatation of the ileum well developed but completely free of microorganisms (Brandao et al. 1987).

Studies by Caetano (1989) and Caetano and Landim (1985), showed that ants in the tribe Cephalotini differ from other ants in that they have specialized structures in their digestive tract that harbor bacteria and yeast. Although other ant species may have microorganisms in their digestive tract (Caetano et al. 1989a,b; Schroeder et al. 1996 for example), the cephalotines have unique structural adaptations of their digestive tract (Caetano 1989). Yurman and Dominquez (1993) were able to culture anaerobically digestive bacteria from the digestive tract of *C. atratus* and *C. pusillus*, thereby showing that the bacteria were facultative anaerobes.

We explored the feeding habits of *C. atratus* and *C. pusillus* in the field and studied, in the laboratory, the relevance of the associated bacteria in *C. pusillus* by using antibiotics and observing the survival rates of the bacteria and the ants.

MATERIALS AND METHODS

Nests of *C. pusillus* (10 colonies) and *C. atratus* (5 colonies) were observed for at least 120 hours in the field at Chaguaramas (Estado Monagas, Venezuela) and Surumoni (Estado Amazonas, Venezuela). For laboratory studies, *C. pusillus* (30 nests) were collected at Chaguaramas (Estado Monagas); Santa Teresa and Sartenejas (Estado Miranda, Venezuela); and Villa de Cura (Estado Aragua, Venezuela). Colonies were located in dry tree branches. The part of the branch containing the colony was separated, cutting the branch with a chain saw, and placed in a plastic bag for transportation to the laboratory. The branches containing the colonies were placed on a metal grid placed in a plastic container with its walls smeared with glycerin to stop ants from escaping. Humidity, temperature and light exposure were not

controlled as the colonies were placed next to an open window. Voucher specimen of ants were deposited at the Museo de Ciencias Naturales, Universidad Simón Bolívar.

Feeding preferences were assessed in the laboratory by leaving colonies without food but with water for 5 days, and then offering them different types of food. Colonies were filmed using a video camera during their active hours (4 hours around noon) and their feeding preferences estimated. Each of at least 6 different colonies was tested at least 5 times for each food, by observing if they fed on it during their active hours the day after 5 days of starvation.

In order to test the effect of antibiotics on the ants, 0.5 ml of the commercially available antibiotic was diluted in 3 ml of 30% sugar solution and an additional sample of 0.25 ml of antibiotic in 3 ml of water. Antibiotics used were commercially available kanamycin (0.25 mg/ml Kantrex, Bristol), nistatin (100,000 units/ml Micostatin, Squibb), gentamicin (20 mg/ml Gentalyn, Schering-Plough), netilmicin (33 mg/ml Netromicin Schering-Plough), penicillin (1,000,000 u.i., Pfizer) and ampicillin (1 mg/ml MK, Laboratorios Calox, Caracas). They were chosen so as to cover both gram positive and gram negative bacteria and a range of fungi. Two different experiments were made.

Experiment 1 evaluated ant survival when feeding them continuously with antibiotics. Thirty-six groups of 10 workers each were kept in plastic chambers, isolated from their colony. Six groups of ants were left with no food nor water (No food in Table I) and six groups with food (sugar water) and water *ad libitum* but no antibiotics (Control). A single antibiotic diluted in the water and food was offered to groups of ants (six for each antibiotic) during the duration of the experiment. This experiment was performed with *C. pusillus* and for comparison, with six groups of 10 *Camponotus rufipes* workers collected at the university campus (Sartenejas, Caracas). *C. rufipes* is not known to have microorganisms in its digestive tract but is known to have intracellular endosymbiotic bacteria (Shroeder et al. 1996) and thus may serve as an interesting comparison to *C. pusillus* with its extracellular endosymbionts.

Experiment 2 was designed to overcome the effects of desiccation and starvation observed in experiment 1 (see results). Each antibiotic was offered to five different colonies of *C. pusillus*, which had been transferred previously from their nest in the trunks or branches to clean glass tubes by crushing their nests and letting the ants establish their new nest in the glass tubes. Each of the 30 colonies used were left to establish its nest and was fed with sugar and water for 15 days. We then offered each colony the specific antibiotic for 24 h. Six control colonies were offered water and sugar solution only. Then, the antibiotics were removed and colonies were offered water and sugar solution *ad libitum*. Twice a week, the foraging area of the colonies were examined and corpses of dead ants found were collected during 30 days. After the experiment, all surviving ants were counted. We calculated the percentage of ants which died using the cumulative number of dead ants collected, the number of ants present at the beginning of the experiment and the number surviving ants at the end of the experiment (See Table I).

For the study of the bacteria, ants were washed with a 3% NaClO solution and then their digestive tracts were dissected under a stereoscopic microscope, by submerging the ants in distilled water and performing the dissection in a sterile laminar flux chamber. The digestive tracts were dissected under totally aseptic conditions. The dissected intestine were incubated for 3 weeks in Luria-Bertani broth (10

g Bacto tryptone, 5 g Bacto yeast extract, 10 g NaCl, see also Smibert and Krieg 1981) under anaerobic and aerobic conditions. The bacteria present in the cultures were isolated at the Venezuelan Center for Culture Collections (CVCM), and identified using API Coryne, I32 GN and API 20NE strips and the semi-automated ATB-PLUS system (bio-Mérieux S.A. Marcy l'Etoile, France). Complementary tests for identification were also made. Susceptibility to antibiotics was determined by disk diffusion test according to the NCCL (1984) guidelines. The following disk were selected: BBL penicillin (10 IU/IE7UI), BBL ampicillin (10 Ug) BBL kanamicin (30Ug) BBL gentamicin (10Ug) and Shaering Plough netromicin (30Ug). All bacteria identified are preserved at the Venezuelan Center for Culture Collections, and registered as indicated in Table II.

RESULTS

General observations: Workers of the two cephalotine species studied are relatively slow-moving and are strictly diurnal. Their maximum activity coincides with maximum luminosity, except during very hot days, when they stop foraging from noon to early afternoon. They are often seen immobile or walking very slowly on sunny spots of tree branches or around their nest entrance. They do forage in columns to water sources, extrafloral nectaries and/or homopteran colonies, but we never observed them collecting pollen from flowers, although they are often seen licking leaf and branch surfaces. Observations of workers of both, *C. atratus* and *C. pusillus*, when returning to their nests in the field, revealed that the only recognizable material they transported was bird droppings. No insects or other solid materials could be detected as carried by ants when screening over 200 workers observed at 3 different sites in Venezuela. However, when presented in the field with dead insects or with human urine, they collected it. Food preferences in the laboratory revealed that ants foraged mainly on sugars (sucrose and honey), bird droppings, and licked cooked meat and food preserved with nitric salts, but never fed on meat, cricket eggs, seed flour (manioc, maize, barley), freshly collected flower pollen from flowers nor artificial diets (peanut butter, dog food).

Experiments with antibiotics: *C. pusillus* started to die after 24 h and were all dead after 7 days when left without food, or with food containing antibiotics, except nistatin, penicillin, ampicillin and kanamicin (Table I : Isolated ant groups, experiment 1). Ants fed with nistatin, penicillin, ampicillin or with pure sugar water showed statistically lower mortality than all other groups. Isolated ants fed kanamicin started to die later than did ants fed netromicin or gentamicin. Isolated ants fed netromicin or gentamicin had a mortality pattern similar to that of desiccated and starved ants, suggesting that they did not consume the water and sugar contaminated with the antibiotic. Ants fed kanamicin were all dead after 7 days although most workers survived for more than two days. None of the antibiotics tested killed *C. rufipes* workers significantly more than the control *C. rufipes* fed water and sugar. When the antibiotics were fed to whole *C. pusillus* colonies for only one day (Table I: Colonies, experiment 2), worker mortality was much lower. After 2 weeks, ants of colonies fed once with kanamicin started to die significantly more than did controls and those offered one of the other antibiotics.

Identification of microorganisms and susceptibility to antibiotics: We could isolate from the intestine of worker ants of *C. pusillus* at least one species of yeast and 10 different species of bacteria (Table II). The bacteria were identified as *Corynebacterium* sp. and *Brevibacterium* sp. (GRAM+); and as *Sphingobacterium spiritivorum*, *Sphingobacterium multivorum*, *Ochrobactrum anthropi*, *Myroides* sp., *Brevundimonas diminuta*, *Alcaligenes faecalis* 1, *Alcaligenes faecalis* 2, *Stenotrophomonas* sp., *Moraxella* sp., and *Pseudomonas alcaligenes* (non fermenting GRAM - bacteria). The profile of antibiotic resistance was highly variable for the various species (Table II), but clearly, more than one species of bacterium showed a pattern compatible to what happened with ants: That is, *Brevundimonas diminuta*, *Alcaligenes faecalis* (2) and *Pseudomonas alcaligenes* were susceptible to kanamicin but resistant to penicillin and ampicillin.

CONCLUSIONS

The results of experiments in which antibiotics were fed to workers of isolated *C. pusillus* suggest that ants feed on liquids containing penicillin, ampicillin, kanamicin or nistatin but not on liquids that contain netromicin or gentamicin. The mortalities of groups of isolated ants observed after 24 h when offered netromicin and gentamicin are probably from desiccation; workers avoid feeding on liquids contaminated with those antibiotics. Isolated ants offered kanamicin start to die later, suggesting that these ants did not desiccate but fed on liquids contaminated with kanamicin, which then killed them by killing symbiotic bacteria. Ants in isolated groups fed penicillin, ampicillin, nistatin and controls had significantly lower mortality. Mortality was more likely caused by lack of water intake than by failure to feed; laboratory colonies survived weeks without food if water was available.

Experiments with whole colonies confirmed that ants ingesting a one day dose of kanamicin eventually die. *C. rufipes*, which has no known microorganisms living in its digestive tract, was affected by the absence of water and food but not by contamination with antibiotics, suggesting that the antibiotics did not kill *C. rufipes* and that the mortality observed with *C. pusillus* was not the result of a putative insecticidal effect. That is, isolated ant workers fed with these antibiotics died, either because of the effect of the antibiotic, as was probably the case of kanamicin, or by desiccation, as was probably the case of netromicin and gentamicin which seem to inhibit feeding and drinking. Colonies were more resistant than were isolated groups of workers, probably because they could feed and drink antibiotic-free solutions after the first day of the experiment.

The bacteria we found in the intestine of workers of *C. pusillus* are compatible with those reported by Yurman and Dominquez (1993) in the stomach, ileum and rectum of *C. pusillus*. All the species found are known as free living bacteria. None of the bacteria seem to have specific adaptations for living in the digestive tract of *C. pusillus*, but seem to be rather common bacteria which had been ingested by the ants. These bacteria are different from those recently found in the digestive tract of termites and isopods

(Margulis et al 1990; Jorgensen et al. 1997) for example.

The ant mortality caused by feeding on kanamycin was probably the result of the antibiotic killing one, some or all the associated bacteria susceptible to kanamycin. An interpretation of our results is that kanamycin kills the most important of the associated bacteria and thus, the ants. Although possible, it is unlikely that kanamycin has a direct toxic effect on these ants (see above). Other antibiotics kill the ants indirectly as they are so repellent that ants prefer to desiccate or starve rather than ingest liquids contaminated with them (netromycin and gentamicin). All in all, our results suggest that some of the bacteria in the digestive tracts of *C. pusillus* are essential to the ants, probably forming a symbiosis with their host.

Ants in the tribe Cephalotini, and specially *C. atratus* and *C. pusillus*, are known to feed on extrafloral nectar and possibly on wind dispersed pollen but not on pollen in flowers (Baroni-Urbani and Andrade 1997), which seems mysterious. The fact that these ants maintain bacteria in their digestive tracts increases this mystery. We do not know what role the bacteria have, but based on what these ants feed on and based on the nutritional requirement of any multicellular organisms, we might suggest that bacteria provide the ants with aminoacids by incorporating nitrogen from urea and nitrates present in the bird droppings collected by the ants. Alternatively, bacteria may help in degrading pollen or fungal spores and hyphae or other substances collected by workers when scraping leave surfaces. The internal anatomy reported for these ants though, suggest that pollen and bacteria are kept in different parts of their digestive tracts. That is, the filtering bulb in the fore-gut stops pollen from passing to the hind-gut where bacteria are found (Caetano and Cruz-Landim 1985). The bacteria found in the digestive tracts of these ants are common free living soil bacteria, suggesting that *C. pusillus* probably feeds on bacteria found on leaf surfaces or on the soil and allows them to reproduce in its digestive tract. Bacteria may profit from this arrangement by having access to a relatively stable environment with adequate pH, temperature and oxygen concentrations for their growth. Bacteria isolated from these ants grow best at about 40° C (Yurman and Dominguez 1993). Ants profit from this arrangement by being able to sustain themselves on low protein foods. The low level of activity shown by ants also suggests a low metabolic rate. Their feeding habits, their associated metabolism, and in most species also their black color, suggest that they serve as a biochemical reactor, accumulating solar energy to regulate their body temperature (Heinrich 1993), favoring the growth of microorganisms in their digestive tract.

We do not know if all cephalotines possess symbiotic microorganisms, as their characteristic intestinal morphology suggests (Caetano 1984; 1989; Caetano and Cruz-Landim 1985). Our work should suggest further research into possible symbiotic relations between microorganisms and ants among the ants in other tribes, possible those paleotropical ants in the genus *Cataulacus*, which have similar digestive tracts as the cephalotines and also contain microorganisms (Caetano et al. 1994)

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Table I: Cumulative mortality of ants (ants that died as a % of the number of workers at the beginning of the experiment) when fed different liquids. Data are the mean of 6 replicates for experiment 1 and 5 replicates for experiment 2

Experiments	Groups of ants (experiment 1)			Colonies (experiment 2)	
	Day 1	Day 3	Day 7	Day 15	Day 30
<i>C. pusillus</i>					
No food	48 a	66 a	100 a	no data	no data
Kanamycin	8 b	58 a	100 a	36 a	54 a
Netromicin	40 a	70 a	100 a	6 b	18 b
Gentamicin	44 a	81 a	100 a	6 b	25 b
Nistatin	22 b	34 b	52 b	8 b	25 b
Penicillin	2 b	2 b	5 b	1 b	2 b
Ampicillin	2 b	3 b	5 b	2 b	2 b
Control	14 b	19 b	36 b	1 b	9 b
<i>C. rufipes</i>					
No food	15 b	79 a	100 a		
Kanamycin	0 b	2 b	22 b		
Netromicin	2 b	2 b	7 b		
Gentamicin	0 b	9 b	16 b		
Nistatin	0 b	4 b	23 b		
Control	0 b	0 b	5 b		

Percentages in the same column marked with letter a are statistically different from those marked with b ($p < 0.05$, chi-square test on total number of dead workers). Those marked with b are indistinguishable from controls.

Table II: Susceptibility (S) and resistance (R) of bacteria found in the digestive tract of *C. pusillus* workers, to the various antibiotics previously tested on ants.

	Accession number *	Penicillin	Ampicillin	Kanamycin	Netromicin	Gentamicin
<i>acterium</i> sp.	1077	S	S	S	S	S
<i>terium</i> sp.	1078	R	R	S	S	S
<i>bacterium spiritivorum</i>	1079	S	S	R	R	R

<i>Bacterium multivorum</i>	1080	R	R	R	S	S
<i>Centromyces anthropi</i>	1081	R	R	R	S	S
<i>Centromyces</i> sp.	1082	R	R	R	R	R
<i>Centromyces diminuta</i>	1083	R	R	S	S	S
<i>Centromyces faecalis</i> (1)	1084	R	S	S	S	S
<i>Centromyces faecalis</i> (2)	1085	R	R	S	S	S
<i>Centromyces thomomas</i> sp.	1086	R	S	S	S	S
<i>Centromyces</i> sp.	1087	R	R	R	R	R
<i>Centromyces alcaligenes</i>	1088	R	R	S	S	R

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