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The infrabuccal pellet piles of fungus-growing ants

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Abstract Fungus-growing ants (Attini) live in an obligate mutualism with the fungi they cultivate for food. Because of the obligate nature of this relationship, the success of the ants is directly dependent on their ability to grow healthy fungus gardens. Attine ants have evolved complex disease management strategies to reduce their garden's exposure to potential parasitic microbes, to prevent the establishment of infection in their gardens, and to remove infected garden sections. The infrabuccal pocket, a filtering device located in the oral cavity of all ants, is an integral part of the mechanisms that leaf-cutter ants use to prevent the invasion and spread of general microbial parasites and the specific fungal-garden parasite *Escovopsis*. Fungus-growing ants carefully groom their garden, collecting general debris and pathogenic spores of *Escovopsis* in their infrabuccal pocket, the contents of which are later expelled in dump chambers inside the nest or externally. In this study we examined how a phylogenetically diverse collection of attine ants treat their infrabuccal pellets. Unlike leaf-cutters that deposit their infrabuccal pellets directly in refuse piles, ants of the more basal attine lineages stack their infrabuccal pellets in piles located close to their gardens, and a separate caste of workers is devoted to the construction, management, and eventual disposal of these piles.

Introduction

Fungus-growers (Attini) are distinguished within the ants (Formicidae) by their unique ability to cultivate fungus for food. The ants provide their cultivar with substrate for growth, and, in return, the fungus serves as the ants' primary food source. The mutualism between attine ants and their fungus garden is obligate; one symbiont cannot survive without the other. Because of this, the success of attine ants is directly dependent on the success of the fungal cultivar. Most attine ants maintain their fungus garden in the soil amidst a myriad of highly competitive and potentially parasitic microbes. The fungal cultivar is also exposed to bacteria and fungi present on the nutrient substrate added to the garden.

A major threat to the health of fungus gardens is the potential invasion of general microbial competitors and parasites. In addition, attine-ant nests are attacked by specialized fungal parasites in the genus *Escovopsis* (Ascomycete: Hypocreales) (Currie et al. 1999, 2003). Infections of *Escovopsis*, which are lethal to the garden if not controlled, result in a significant decrease in the growth rate of gardens and a reduction in the production of workers (Currie et al. 1999; Currie 2001). Attine ants protect the fungus garden from the risk of invading microbes by reducing its exposure to microbial contaminants, preventing inoculated microbes from establishing infection within the garden, and suppressing or removing established infections.

The infrabuccal pocket, a structure in the oral cavity of ants, appears to be a fundamental component of the behaviors that attine ants exhibit to defend their garden against microbes. The debris ants gather while cleaning themselves, their nest mates, or the garden is deposited in the pocket, which functions to filter solid materials (Eisner and Happ 1962; Quinlan and Cherrett 1978). Once the pocket is full of compressed debris, the material is regurgitated in the form of a pellet. To keep microbes from re-invading or re-establishing infection in the garden, leaf-cutter ants deposit their infrabuccal pellets

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in refuse piles a safe distance from their garden (Febvay and Kermarrac 1981; Currie and Stuart 2001).

Through the formation and disposal of infrabuccal pellets and a highly evolved social organization, leaf-cutter ants remove microbes from their gardens and minimize their reintroduction. How non-leaf cutting attine genera utilize their infrabuccal pocket and its contents has not been examined. Here we present a detailed analysis of the formation and treatment of infrabuccal pellets in a phylogenetically representative collection of attine ants.

Methods

Study organisms

We examined the behavioral ecology associated with infrabuccal pellet deposition of six species of fungus-growing ants including four higher attines: *Atta colombica* and *Acromyrmex octospinosus* (leaf-cutters), *Trachymyrmex cf. zeteki*, and *Sericomyrmex amabilis*; and two lower attines: *Apterostigma dentigerum* and *Cyphomyrmex costatus*. *Apterostigma* is one of the most basal genera of attine ants, while *Cyphomyrmex* is more derived and likely the sister group to the higher attines, of which the leaf-cutter ants are the most derived (Schultz and Meier 1995). Colonies were collected in the Canal Zone of Panama in 2001, 2002, and 2003 and were maintained in the laboratory at the University of Kansas. Laboratory colonies were kept in dual chambers (one housing the nest and one for foraging and dumping) (7.5×7.5×3 cm for lower and intermediate attines, and 17.5×12×6.5 cm for leaf-cutters) connected by plastic tubes, placed on islands in mineral oil. Leaf-cutter colonies were watered and fed local foliage three times per week, while the rest of the ants were fed a mixture of dried oats and oak catkins and watered once a week.

Description of infrabuccal pellet piles

Five *A. colombica* (approx. 6,000 workers), five *A. octospinosus* (approx. 4,500 workers), 20 *T. cf. zeteki* (\bar{x} =116.4 workers, $SD\pm 38.84$), five *S. amabilis* (\bar{x} =75.6 workers, $SD\pm 7.16$), ten *C. costatus* (\bar{x} =128.6 workers, $SD\pm 21.45$), and 12 colonies of *A. dentigerum* (\bar{x} =22.4 workers, $SD\pm 6.8$) were observed twice weekly for six weeks using a Nikon SMZ 1500 stereomicroscope. A grid (0.5 cm²) was used to systematically assess the presence or absence of a pellet pile, defined as a cluster of pellets found in the garden chamber that does not contain typical garden refuse such as cultivar substrate, or pieces of unhealthy cultivar. When new piles were present, we recorded the number of piles per colony, location, and other visible constituents of the pile such as invertebrates or fungal contaminants. The number of pellets per pile was counted in all colonies.

Formation, maintenance and disposal of piles

To determine the general behaviors of the ants and their interactions with piles, behavioral scan sampling (Altmann 1974) was done biweekly for 4 weeks with a stereomicroscope and by direct observation. Further observations were made on two model ant systems to monitor the temporal changes and pellet turnover at infrabuccal pellet piles. Five colonies of *T. cf. zeteki* and *C. costatus* were monitored for 20 min of each hour, 9 h a day for 10 days by direct observation and with a Sony video-camera (DCR-VX2000 Digital Handicam) to determine how pellets were deposited on piles, how piles were formed, behaviors associated with pile maintenance, the length of time piles were maintained, whether pellet fusion occurred, and when "dumping" occurred (the movement of pellets from the pellet pile to the refuse pile).

Division of labor and task partitioning

To determine whether pellet piles are tended by a specific worker caste, the dorsal abdominal surface of all *T. cf. zeteki* workers were marked with permanent enamel paints (Testor DS Model Paints) for individual recognition during the 10-day focal behavioral observations. Observations were made to ascertain whether tasks are partitioned. Task partitioning is recognized when two or more workers contribute sequentially to a piece of work (Jeanne 1986). The behaviors of marked individuals were tallied to identify the presence or absence of a division of labor.

Results

Description of infrabuccal pellet piles

Of the six attine species observed, the four non leaf-cutter species constructed infrabuccal pellet piles (Table 1). Pellet piles were not found in either species of leaf-cutter ant, although small clusters of refuse that contained a few pellets were found scattered within their nesting chambers. These were not considered as infrabuccal pellet piles due to the large amount of refuse material they contained. The size and number of piles built per colony varied depending on the species of fungus-growing ant (Table 1). The location of piles varied significantly with species ($\chi^2=163.68$, $df=9$, $P<0.00001$); piles were located most often between the garden and the exit in *T. cf. zeteki* (62%) and *A. dentigerum* (37%), near the garden chamber wall in *A. dentigerum* (37%) and *S. amabilis* (40%), and within the periphery of the fungus garden nearest to the exit in *C. costatus* (48%) (Fig. 1). New piles were observed in the same location in the nesting chamber 86–

Table 1 Description of infrabuccal pellet piles constructed by four species of attine ants

	<i>A. dentigerum</i> n=12	<i>C. costatus</i> n=10	<i>S. amabilis</i> n=5	<i>T. cf. zeteki</i> n=20
Proportion of colonies with infrabuccal pellet piles	0.583	0.900	0.800	0.850
No. of pellets/pile (\bar{x}) ($\pm SD$)	284.21 (69.3)	61.50 (21.04)	412.63 (4.14)	756.86 (182.31)
No. of piles/colony (\bar{x}) ($\pm SD$)	2.92 (0.42)	1.80 (0.23)	1.25 (0.75)	1.42 (0.48)
Proportion of time new piles are built in same location	0.47	0.92	1.0	0.86
Other pile constituents	–	–	Dead ants	Dead ants; whole and in pieces

Fig. 1 Infrabuccal pellet pile of *T. cf. zeteki* (a) located between the garden (left) and exit (top right) and pellet pile workers. Infrabuccal pellet pile of *C. costatus* (b) located within the fungus garden

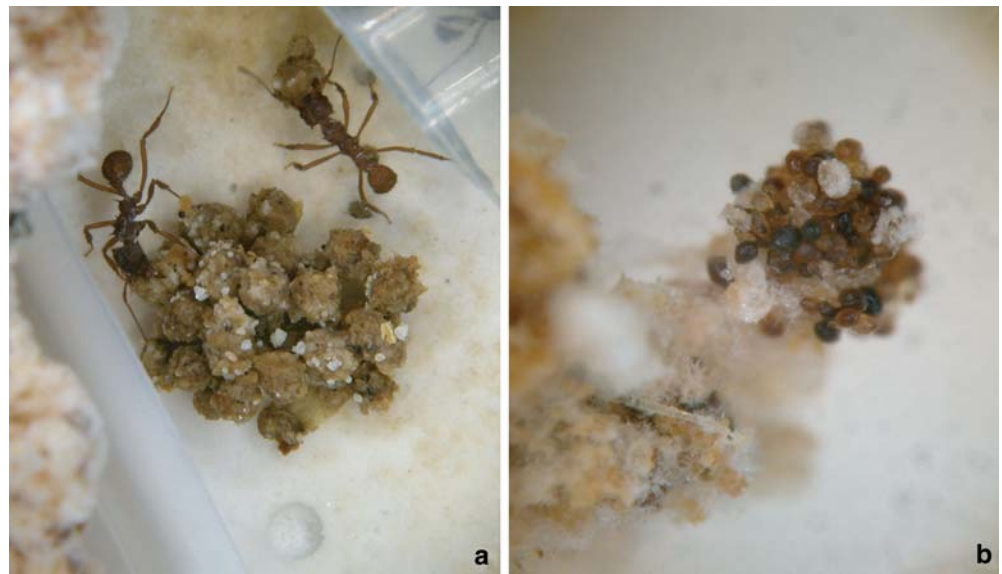


Table 2 Behaviors of *T. cf. zeteki* and *C. costatus* infrabuccal pellet pile workers. The number of observations corresponds to the average number of times the task was completed during ninety 20-min observation periods in each colony (150 h)

Task	Description	No. of observations			
		<i>T. cf. zeteki</i> (n=5)		<i>C. costatus</i> (n=5)	
		Mean	SD	Mean	SD
Deposition (direct)	Placing a newly formed pellet directly on the pile	80.20	11.45	15.50	8.10
Deposition (indirect)	Placing a pellet on the pile that was taken from another location in the chamber	1.60	3.57	2.20	1.60
Clustering	Gathering numerous pellets together into a sphere-shaped pile small enough to carry	93.60	16.29	4.20	2.42
Fusion	Further clustering of pellets using mouth parts, resulting in a fused mass of pellets	15.60	3.36	0.00	0.00
Rearrangement	Picking up pellets, or clusters of pellets, with mandibles and replacing them in a different location in the pile	105.0	19.33	1.00	0.70
Wandering	Picking up a pellet, or cluster of pellets, and carrying it around the chamber, ultimately returning it to the pile	56.80	3.56	0.03	0.07
Addition of dead ants	Adding dead ants or ant parts to the pile	16.80	2.77	0.00	0.00
Fungus grooming	Fungus grooming around pile area	0.00	0.00	13.10	16.40
Planting fungi	Placing fungal cultivar on the pile	0.00	0.00	1.50	0.84
Dumping	Removal of pile, transport to the dump	3.80	0.45	0.00	0.00

100% of the time in all species except *Apterostigma*, in which location varied week to week (Table 1). We found that the most common constituent of pellet piles built by healthy colonies, other than pellets, is dead ants; either whole or in pieces (legs, wings, antennae) (Table 1).

Construction, maintenance and disposal of infrabuccal pellet piles

In most instances, *T. cf. zeteki* and *C. costatus* deposited pellets directly on the infrabuccal pellet pile (Table 2). Ants stood near the pile, made one or several lateral movements with their mandibles, then expelled a small

pellet which they caught between their mandibles and placed on the pile. Once about 15 pellets had been deposited, the ants began to cluster them. *T. cf. zeteki* did this by rotating the pellets against the underside of their thorax using their forelegs and mouthparts, while *C. costatus* stood upright beside the pile and clustered the pellets together using their forelegs.

After the initial clustering by *T. cf. zeteki*, we observed a further compaction of the pellets into a fused mass (Table 2), done primarily by rotating the cluster repeatedly with the mouth parts. The fused pellet cluster became progressively darker in color, making individual pellets indistinguishable. The pile workers apply a brown secretion in the area immediately surrounding pellet piles.

Ants rearranged the contents of the pile by picking up a pellet or pellet cluster and replacing it in a different spot on the pile (Table 2). In addition, *T. cf. zeteki* frequently lifted a pellet cluster and wandered about the chamber with no apparent destination, never making contact with the garden, never laying the cluster down, and ultimately returning it to a different location in the pile (Table 2). Wandering was sometimes prolonged: at four times, wandering occurred for the entire 20 min observation period. Garden and pile workers of *T. cf. zeteki* were observed depositing dead ants or ant parts on pellet piles (Table 2). Wandering and the addition of dead ants to pellet piles were not observed in *C. costatus*. However, fungus grooming and the addition of cultivar to the area of the infrabuccal pellet pile were observed in *C. costatus* (Table 2).

T. cf. zeteki piles were maintained for an average of 58.9 h, although they were observed to last as long as 198.5 h. Disposal of *T. cf. zeteki* infrabuccal pellet piles occurred all at once (Table 2). Pellet clusters were picked up by several workers and taken directly to the dump. Pile workers then cleaned the pellet pile site. Partial dumping of a pellet pile was not observed. *C. costatus* were not observed disposing of their infrabuccal pellets in the refuse pile, and thus the duration of the time they spend maintaining their piles is unknown (Table 2).

Division of labor and task partitioning

Observations revealed that *T. cf. zeteki* garden workers are responsible for pellet deposition. Pellet deposition most often occurred shortly after garden workers had tended the garden (98%). A specific caste of ants tends the infrabuccal pellet piles. Pellet pile workers were observed in association with the pile the majority of the time (91.3%), and spent less time in the garden (4.5%), in which they were observed wandering, not performing specialized tasks. Pile-tending workers are also responsible for pile removal. The addition of dead ants and ant parts to piles does not appear to be a task that is segregated to one group of ants. Tasks associated with pile maintenance are not partitioned.

Discussion

In our study we found that colonies of a diverse group of fungus-growing ants constructed piles of their infrabuccal pellets. The following evidence suggests that piling is a complex behavior exhibited by many genera of attine ants, and is not a laboratory artifact. First, pellet piles are present in most colonies and exist within individual nests for long periods of time. Second, piles are located in specific locations within or near the fungus garden. Third, workers actively tend pellet piles and some deposit a secretion in the vicinity of the pile that is apparently not deposited elsewhere in the nesting chamber. Fourth, in *T. cf. zeteki* there is a division of labor associated with

tending pellet piles. Pellet piles built in situ have likely been overlooked for two reasons: attine infrabuccal pellets are extremely small, and their close proximity to nests would make it difficult to observe them during nest excavation.

Infrabuccal pellets of leaf-cutting ants contain spores of the garden pathogen *Escovopsis* and other invasive microbes (Quinlan and Cherrett 1978; Currie and Stuart 2001; T. Murakami and C.R. Currie, unpublished data). Thus, our findings that a phylogenetically diverse group of fungus-growing ants pile their pellets, maintain them in close proximity to their garden, actively tend the piles, and do not frequently dispose of the material are surprising. Although it is possible that pellet piles serve simply as a half-way point for refuse material, this is unlikely because pellet piles do not exhibit the same characteristics as refuse piles. Most attine dumps harbor mites and fungal contaminants (Bot et al. 2001), and garden workers unrelated to waste management actively avoid physical contact with rubbish piles (Zeh et al. 1999). Conversely, garden workers do not actively avoid pellet piles (A.E.F. Little, personal observation), whereas they are repelled by dump material. Moreover, healthy *T. cf. zeteki* colonies have a specialized caste of workers devoted to the construction and maintenance of piles. This suggests that infrabuccal pellet piles serve an important, and yet unknown, function within the attine ant–microbe mutualism. Given that leaf-cutter ants utilize their infrabuccal pocket to remove the virulent parasite *Escovopsis* from their garden, it is possible that infrabuccal pellet piles are constructed to help defend the garden from *Escovopsis* or general microbial contaminants.

The piling of pellets within gardens of *C. costatus* is particularly intriguing, and runs counter to the supposition that pellet piles are composed of pathogenic waste material. *C. costatus* adds the fungal cultivar to their within-garden pellet piles, and they were not observed moving the contents of their piles to the refuse heap. This suggests the ants may be using pellets as a substrate for cultivar growth. The function of infrabuccal pellet piles remains elusive; however, experimental manipulation of colonies and pellet piles may shed light on their role in the disease ecology of the fungus-growing ant–microbe mutualism.

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