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Evolution of Substrate Preparation Behaviors for Cultivation of Symbiotic Fungus in Attine Ants (Hymenoptera: Formicidae)

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Abstract Despite their importance for the evolution of the symbiosis between Attine ants and their fungal cultivar, substrate preparation behaviors have been the focus of few studies. This study aimed to comparatively examining these behaviors in *Acromyrmex disciger*, *Apterostigma pilosum*, *Mycetarotes parallelus*, *Myrmicocrypta* sp., *Trachymyrmex fuscus* and *Trachymyrmex* sp. Nov. to describe the patterns of their evolution. Behavioral observations were carried out with a set of micro cameras and the behavioral frequencies were analyzed by principal components. Our findings revealed that the process can be divided into three parts: physical treatment, chemical treatment, and incorporation. Two behavioral patterns were revealed. The first is exhibited by basal species (*Myrmicocrypta* sp, *A. pilosum* and *M. parallelus*) and is characterized by the absence or low frequency of chemical treatment behaviors, while the second pattern is exhibited by derived species (*Trachymyrmex* sp. Nov., *T. fuscus* and *A. disciger*) and is characterized by great fragmentation of the substrate and deposit of fecal fluid. This suggests that the evolution of the process is marked by an increase in the importance of the chemical treatment, leading to the adaptations observed in leaf-cutting ants.

Keywords *Acromyrmex* · *Apterostigma* · Attini · evolution · *Mycetarotes* · substrate · symbiosis · *Trachymyrmex*

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Introduction

The most significant evolutionary novelty presented by ants of the tribe Attini is their ability to cultivate basidiomycete fungi, mainly of the family Agaricaceae, as food for their colonies. The appearance of this relationship, approximately 50 million years ago, allowed these ants to change their lifestyle from predators/ collectors to "farmers", capable of cultivating their own food. This symbiosis placed them in a privileged position in the habitats they occur, exclusively in the New World. The most extreme condition is presented by leaf-cutting ants of the genera *Atta* and *Acromyrmex*, important herbivores and agricultural pests of the Americas (Holldobler and Wilson 1990; Mueller et al. 2001; Schultz and Brady 2008).

Fungus-growing ants collect different materials and prepare them within the nest to be used as substrate to cultivate their fungus by means of group of behaviors with the main role of initially decomposing those materials before they are put in contact within the symbiotic fungus garden (Holldobler and Wilson 1990; Mueller et al. 2001, 2005; Andrade et al. 2002; Mangone and Currie 2007).

Most of the knowledge on this process is focused on leaf-cutting ants. Workers of the genera *Atta* and *Acromyrmex* lick leaf fragments to remove impurities and the layer of epicuticular wax that protects the leaves, shred them into smaller pieces of approximately 1 mm, and treat them with fecal fluid consisted of several digestive enzymes capable of decomposing polysaccharides. Then they masticate and incorporate fragments into the fungus garden (Mueller et al. 2001; Andrade et al. 2002; Mangone and Currie 2007).

The development of this process by leaf-cutting ants included the rise of a complex system of division of labor. Each task is performed by workers of different sizes, thus a high polymorphism is observed. In addition, within each physical caste, roles are divided based on age; and six castes of workers are estimated to participate in the substrate preparation in leaf-cutting ants. There is also a portioning of the tasks that are performed by different workers so that the plant material is passed from one worker to another until being incorporated. Thus, the process of substrate preparation resembles an assembly line, where each worker is responsible for a part of the process (Holldobler and Wilson 1990; Hart et al. 2002).

The tribe Attini consists of approximately 240 species and most of them do not use fresh plant matter as substrate, but rather, mainly decomposing vegetative material, flowers, fruits, and seeds, and may also use, in smaller quantities, feces and insect carcasses (Holldobler and Wilson 1990; Leal and Oliveira 2000; Mueller et al. 2001; Agosti and Johnson 2005).

In the tribe Attini the leaf-cutting ants are the most derived of this group, and thus their biological characteristics represent a derived pattern. Basal species, in general, are known to prepare the substrate similarly to leaf-cutting ants, although in a much simpler manner. The development of the ability of cultivating fungus with fresh material is believed to be the most important evolutionary innovation of the group (Kusnezov 1963; Schultz and Meier 1995; Wetterer et al. 1998; Mangone and Currie 2007; Schultz and Brady 2008).

This innovation has allowed the ants of tribe Attini to evolve from species of cryptic habits, with small nests and monomorphic workers to species with large nests, highly polymorphic workers and dominant behavior (Brandao and Mayhe-

Nunes 2007; Mangone and Currie 2007). One of the reasons for this transition was certainly the development of the process of substrate preparation. In this way, this study aimed at describing and assessing the behaviors associated with substrate preparation by some species of the tribe Attini, in order to establish a framework for comparison to understand its evolution.

Methods

Colonies

Three nests of *Apterostigma pilosum*, *Myrmicocrypta* sp., *Mycetarotes parallelus*, *Trachymyrmex fuscus*, and *Trachymyrmex* sp. Nov., located around the Centro de Estudos de Insetos Sociais, UNESP Rio Claro, Brazil (22° 23′ 44,30″S, 47° 32′ 27,73″W) and three of *Acromyrmex disciger*, located in an urban area of the municipality of Ubatuba, São Paulo State (23° 27′ 24,61″S, 45° 04′ 14,13″W), were dug up and the fungus garden, brood, queen and workers were collected. Colonies were initially kept in the laboratory in nests consisted of plastic containers lined with plaster to allow the ants to clean the fungus garden and recover from the collection. Colonies were then transferred to a plaster nest with one chamber for the fungus garden and one for refuse material and an exit for foraging, covered with a glass lid. During behavioral observations, the nests of *Myrmicocrypta* sp., *Apterostigma pilosum* and *Mycetarotes parallelus* were fed grass seeds, those of *Trachymyrmex fuscus* and *Trachymyrmex* sp. were fed dry flowers of several ornamental plants, and those of *Acromyrmex disciger*, with fresh leaves of Copperleaf (*Acalypha wilkesiana*) and rose flowers and leaves (*Rosa* sp.).

Behavioral Observations

Substrate preparation behaviors were observed with a set of micro video camera system connected to a VCR and a television. Behaviors were recorded in VHS tapes from the moment the substrate was transported to the nest until it was incorporated to the fungus garden by workers. Behaviors were first characterized and later quantified by category. The frequency of each behavior was determined by dividing the number of times it was observed by the total number of behavioral acts observed for each species.

Since *Acromyrmex disciger* is a polymorphic species, workers were visually divided into four castes based on size and the frequency of behaviors were determined for each caste separately and combined. Castes were termed 1–4 in an increasing order of size. The frequency of each behavior for each caste was determined by dividing the number of times each behavior was observed by the total number of behavioral acts of the species, thus determining the participation of castes in the process.

Statistical Analysis

A principal component analysis of the behaviors for each species was performed with the software Bioestat 4.0 to characterize the different patterns of substrate preparation. The components of the analysis were significant when represented at least 10% of the observed variation. Behaviors were considered significant for the determination of each component when values were equal or higher then 0.33 or equal or lower than −0.33. Also, the values obtained in the first two principal components for each behavioral repertoire were graphically represented to determine how each species is positioned in the total variation of behaviors. Since *Acromyrmex disciger* is a polymorphic ant, unlike the other five species examined, the behavior repertoire of each caste was analyzed separately in order to determine the influence of polymorphism in the process of substrate preparation.

Results

In a total of 240 h of observation, seven behaviors were identified and 7,237 behavioral acts were recorded. A description of the behaviors observed is summarized in Table 1. The behaviors, depositing fecal fluid, pressing, and shredding the substrate, are repeated in this order by workers on the resulting fragments.

In *Myrmicocrypta* sp. and *Apterostigma pilosum* the most frequent behavior was licking and the behaviors of depositing fecal fluid, pressing, and shredding the substrate (Table 2) were not observed. In *Mycetarotes parallelus*, the most frequent behavior was licking, the behavior of depositing fecal fluid was observed in lower frequencies, pressing and shredding the substrate were not observed (Table 2).

In *Trachymyrmex fuscus*, *Trachymyrmex* sp. Nov. and *Acromyrmex disciger* all the behaviors were observed, the most frequent was licking, followed by pressing. The least frequent were inserting substrate in the fungus garden and incorporating (Table 2).

Considering the castes of *A. disciger* separately, in caste 1, small-sized workers, the most frequent behavior was inserting the substrate in the fungus garden. Holding,

Table 1 Description of Behaviors Observed for the Study Species During Substrate Preparation Behaviors

Behaviors	Description
Holding	Worker holds the leaf fragment between its mandibles over the fungus garden
Licking	Worker licks the entire surface of the fragment with back and forth movements of its glossa
Depositing fecal fluid	Worker bends gaster forward, touching the tip of the fragment and depositing a drop of fecal fluid
Pressing	Worker presses the fragment with its mandibles in the same area where the fecal fluid was deposited
Shredding	After pressing the fragment borders, a worker shreds the fragment, similar to the cutting behavior
Inserting in the garden	Worker inserts the fragment into the fungus garden and presses the fragment borders with its anterior legs until the fragment is attached
Incorporating	Worker inoculates a small amount of fungus on the recently inserted substrate and performs the same procedures to attach the fragment to the fungus garden

Table 2 Relative Frequency of Substrate Preparation Behaviors for the Cultivation of the Symbiotic Fungus for Each Study Species and it's Castes

	Myrmicocrypta sp. (*N*=440)	*Apterostigma pilosum*. (*N*=506)	*Mycetarotes parallelus* (*N*=1,180)	*Trachymyrmex fuscus* (*N*=912)	*Trachymyrmex* sp. nov. (*N*=515)	*Acromyrmex disciger* 1 (*N*=110)	*A. disciger* 2 (*N*= 1,268)	*A. disciger* 3 (*N*= 2,247)	*A. disciger* 4 (*N*=59)	*A. disciger* Total (*N*=3,684)
Holding	0.319 (140)	0.222 (112)	0.102 (120)	0.033 (35)	0.048 (25)	0.000	0.029 (57)	0.035 (91)	0.071 (4)	0.033 (152)
Licking	0.342 (151)	0.331 (168)	0.476 (562)	0.532 (489)	0.522 (269)	0.100 (12)	0.506 (562)	0.427 (969)	0.643 (38)	0.455 (1,581)
Depositing fecal fluid	0.000	0.000	0.011 (13)	0.048 (49)	0.058 (30)	0.000	0.131 (186)	0.057 (138)	0.000	0.081 (324)
Pressing	0.000	0.000	0.000	0.210 (196)	0.223 (115)	0.000	0.142 (200)	0.273 (625)	0.071 (4)	0.222 (829)
Shredding	0.000	0.000	0.000	0.073 (73)	0.066 (34)	0.000	0.084 (127)	0.167 (383)	0.214 (13)	0.137 (523)
Inserting in the garden	0.111 (49)	0.121 (61)	0.138 (163)	0.034 (35)	0.038 (20)	0.467 (51)	0.058 (74)	0.006 (24)	0.000	0.038 (149)
Incorporating	0.228 (102)	0.326 (165)	0.273 (322)	0.034 (35)	0.049 (22)	0.433 (47)	0.049 (62)	0.003 (17)	0.000	0.034 (126)

N total number of behavioral acts observed for each species, *Acromyrmex disciger* 1 to 4 physical castes visually determined for this species, the number in parentheses represent the number of times each behavior was observed for each species depositing fecal fluid, pressing, and shredding the substrate were not observed for this caste (Table 2).

For caste 2, medium/small-sized workers, and caste 3, medium/large sized-workers, all behaviors were observed and licking was the most frequent. The lower frequent behavior was holding the substrate and incorporating (Table 2). Finally, for caste 4, large-sized workers, the most frequent behavior was licking and the least frequent behaviors were holding and pressing. Depositing fecal fluid, inserting the substrate in the fungus garden, and incorporating were not observed in this caste (Table 2). In general, a strong cooperation among workers was observed during substrate processing in *A. disciger*. This cooperation, however, occurred only in early stages of the process in species of *Trachymyrmex* and is non-existent in the other three study species, whose workers perform solitary tasks, explaining the low number of behavioral acts observed in these species when compared to the others (Table 2).

The principal component analysis revealed that 91% of the variation observed among behavioral repertories may be explained by two components. The first principal component consists of the behaviors: depositing fecal fluid, pressing the substrate, shredding the substrate, holding, inserting the substrate into the garden, and incorporating. This component is positively associated with the three first behaviors and negatively with the remaining three. The second component is strongly determined by the licking behavior and is positively associated to it (Table 3).

The graphic representation of the values of the two principal components for each behavioral repertoire (Fig. 1) shows three groups: the first consists of the repertoires of *Myrmicocrypta* sp., *Apterostigma pilosum*, and *Mycetarotes parallelus*, and is negatively associated with the first component and positively associated with the second. Thus, it is strongly influenced by the frequencies of holding, licking, inserting the substrate into the garden and incorporating; the second group is composed of the repertoires of *Trachymyrmex fuscus*, *Trachymyrmex* sp. Nov. and caste 3 of *Acromyrmex disciger*, and is positively associated with the two components. Thus it is strongly influenced by the frequencies of licking, depositing fecal fluid, pressing the substrate, and shredding the substrate. The third group

Table 3 Values of the Two Principal Components for Each Behavior

Behaviors	PC1 (57,89%)	PC2 (33,80%)
Holding	−0.38	−0.30
Licking	0.04	0.64
Depositing fecal fluid	0.41	0.30
Pressing	0.41	0.34
Shredding	0.42	0.25
Inserting in the garden	−0.40	0.37
Incorporating	−0.42	0.31

The values in parentheses represent the percentage of participation of each principal component in the total variation among species

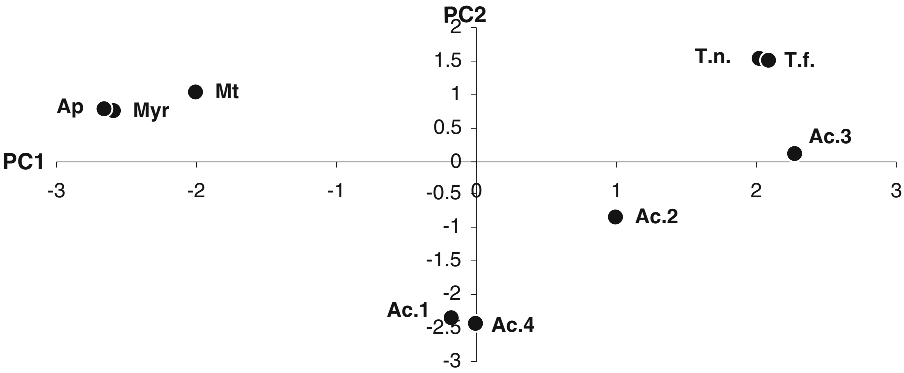

Fig. 1 Graphic representation of the values of each behavioral repertoire for the first two principal components. Ap.—*Apterostigma pilosum*; Myr—*Myrmicocrypta* sp.; Mt.—*Mycetarotes parallelus*; T.f.—*Trachymyrmex fuscus*; T.n.—*Trachymyrmex* sp. Nov.; Ac. 1–4—physical castes of *Acromyrmex disciger.*

consists of repertoires of castes 1 and 4 of *A. disciger*, which do not seem to participate efficiently in the preparation process. Caste 2 of *A. disciger* seems to be intermediary between the second and third groups.

Discussion

The process of substrate preparation can be divided into three stages, determined in the same way than those of *Atta sexdens*, *A. laevigata* and *A. bisphaerica* by Diniz and Bueno (2009). Each stage consists of different behaviors that are performed in sequence by workers. The first stage is the physical treatment in which workers lick and hold the substrate. Our findings revealed that the behavior of licking the substrate observed in this stage is in general the most frequent, similarly to those described in the literature for leaf-cutting ants and also species of *Trachymyrmex*, *Cyphomyrmex*, and *Myrmicocrypta* (Beshers and Traniello 1996; Murakami and Higashi 1997; Andrade et al. 2002; Mangone and Currie 2007).

The physical treatment is carried out when the substrate is brought into the nest. Thus the high frequency of these behaviors, mainly licking, indicates that their main role is to remove impurities from the substrate, preventing the contamination of the fungus garden. In this phase, workers of leaf cutting ants remove the layer of epicuticular wax present in the leaf fragments that could prevent the growth of the symbiotic fungus. Considering the species examined in this study, it is more likely that the role of removing the epicuticular applies only to *Acromyrmex disciger* (Andrade et al. 2002; Mangone and Currie 2007; Diniz and Bueno 2009). The second stage is the chemical treatment, when workers deposit fecal fluid, press, and shred the substrate. This sequence of behaviors is repeated by workers in the resulting fragments, so that at the end of this stage, the original substrate is reduced to a large number of small fragments.

The frequencies of these behaviors varied among the study species. *Myrmicocrypta* sp. and *Apterostigma pilosum* did not exhibit these behaviors, and only depositing fecal fluid was observed for *Mycetarotes parallelus*. This contradicts the report in the literature for *Myrmicocrypta ednaella* and *Cyphomyrmex* spp., that exhibit high frequencies of these behaviors (Murakami and Higashi 1997), but agrees with Mangone and Currie (2007).

In *Trachymyrmex fuscus* and *Trachymyrmex* sp. Nov. the behaviors associated with this stage were more frequent and pressing was the most frequent among the three, similarly to *T. septentrionalis* (Beshers and Traniello 1996). In general, *Acromyrmex disciger* was similar to these two species. However, if its physical castes are considered separately, castes 2 and 3 (medium-sized) were the most active in substrate preparation, while castes 1 and 4 participated little, similarly to the leaf-cutting ants of the genera *Atta* and *Acromyrmex* (Wilson 1980; Andrade et al. 2002; Camargo et al. 2007; Mangone and Currie 2007).

It has been long known that the fecal fluid contains several enzymes, mainly pectinases, xylanases, amylases, and proteases. These enzymes have several origins; some are from the symbiotic fungus and cross the digestive tract of ants without being digested. Some authors believe that during chemical treatment, workers masticate the substrate depositing enzymes produced by the symbiotic fungus, and fragment it by pressing and shredding, thus promoting an initial break down and facilitating the development of the symbiotic fungus (Erthal et al. 2004; Ronhede et al. 2004; Silva et al. 2003).

This system of biochemical interaction was considered for a long time the basis for the symbiosis between these two organisms. However, since the behaviors associated with chemical treatment were not observed in *A. pilosum* and *Myrmicocrypta* sp., and only depositing fecal fluid was observed in *M. parallelus*, this indicates that the importance of this biochemical system to symbiosis does not apply to all species of this group, and may be absent in some species (Mueller 2002; Ronhede et al. 2004).

The last stage is incorporation, in which ants insert the substrate in the fungus garden and inoculate the symbiotic fungus. These behaviors are very similar among all study species as well as other Attine ants. This is the only stage that had more participation of caste 1 in *A. disciger*, showing the specialization of this caste in these tasks (Beshers and Traniello 1996; Murakami and Higashi 1997; Andrade et al. 2002; Mangone and Currie 2007).

In addition to the stages of the preparation process, species differed in the way they partitioned this task. *Myrmicocrypta* sp., *A. pilosum*, and *M. Parallelus* carried out the entire process in a solitary manner, in which the same worker performs all tasks. *T. fuscus* and *Trachymyrmex* sp. Nov. performed the physical treatment in groups of workers. In *A. disciger*, all stages were performed with the cooperation of several workers resembling an assembly line, with different workers responsible for different roles (Anderson et al. 2001; Hart et al. 2002; Burd and Howard 2005).

The most accepted phylogeny of the tribe divides it into two lineages: Paleoattini and Neoattini. The first is formed by the genera *Myrmicocrypta*, *Apterostigma*, and *Mycocepurus*, and is considered basal. The second consists of the genera *Mycetarotes*, *Mycetosoritis*, *Mycetophylax*, *Paramycetophylax*, *Kalathomyrmex*, *Cyphomyrmex*, *Mycetagroicus*, *Sericomyrmex*, *Trachymyrmex*, *Acromyrmex*, *Pseudoatta* and *Atta*, and is considered derived (Kusnezov 1963; Schultz and Meier 1995; Wetterer et al. 1998; Schultz and Brady 2008; Klingenberg and Brandão 2009). According to the principal component analysis, when ants began to cultivate their fungus, the process of substrate preparation was very simple with total or partial absence of a chemical treatment, as shown by the repertories of *Myrmicocrypta* sp., *A. pilosum*, and *M. parallelus*.

Throughout the evolutionary history of the tribe Attini, these behaviors evolved and differentiated, first depositing fecal fluid, which involved the development of the ability to store fungal enzymes in the digestive tract, and then pressing and shredding the substrate, which involve the ability of fragmenting the substrate. The development of the chemical treatment seems to have been slow during the evolution of the tribe, as species of genera considered derived, such as *Cyphomyrmex*, exhibit a pattern similar to those of basal species (Murakami and Higashi 1997; Mangone and Currie 2007; Schultz and Brady 2008).

The genus *Trachymyrmex* is considered the closest, phylogenetically, to leaf-cutting ants. From an evolutionary point of view regarding substrate preparation, the behavioral pattern of species of this genus may represent the limit of the adaptation of a monomorphic ant to chemical treatment. They are highly skilled in the use fungal enzymes and fragmentation of the substrate, and thus have an elaborated chemical treatment, very similar to that of leaf-cutting ants. They, however, rarely cut plant leaves, clearly indicating that this pattern is not enough to prepare fresh plant matter at the same rate as in the leaf cutting ants. Similarly to a large number of ant species, monomorphic workers of *Trachymyrmex* need to perform, throughout their lives, all tasks of colony maintenance, thus the level of complexity of the substrate preparation process is limited. If they become too specialized in these behaviors, they might not be able to perform several tasks in the colony (Holldobler and Wilson 1990; Leal and Oliveira 2000). The solution for this impasse came with the development of intense polymorphism exhibited by leaf-cutting ants, which allowed the rise of large workers, such as caste 4 of *A. disciger*, specialized in leaf cutting outside the nest; medium workers specialized in the physical and chemical treatments; and small workers that perform incorporation tasks. Thus, they can break down fresh plant material at an increased rate, as they have castes specialized in substrate preparation without affecting other tasks within the colony, performed by other less specialized castes.

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