

Planting of fungus onto hibernating workers of the fungus-growing ant *Mycetosoritis clorindae* (Attini, Formicidae)

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Abstract We describe a peculiar fungus-coating behavior of the attine ant *Mycetosoritis clorindae*, where workers plant fungal mycelium on hibernating nestmates. Hibernating nestmates become ultimately enveloped in a live mycelial coat, remain motionless in this coated state, and essentially become integrated into the garden matrix. The shallow nest architecture of *M. clorindae* (depth of main garden is 15–30 cm) in southern Brazil forces the ants to overwinter at relatively low temperatures in the topmost soil layer. Fungal coating may help the ants to survive the prolonged periods of immobility during winter. Fungus-planting on attine adults is so far unknown from other

attine species, but the behavior parallels the planting of mycelium on larvae and pupae occurring in many attine species. Planting of mycelium on adult nestmates may have been overlooked so far in attine ants because this behavior may occur only in dormant nests, which are least frequently collected. The possible adaptive functions of fungus coatings of hibernating adults and developing brood are likely similar, including for example physical protection, prevention of desiccation, shielding against parasites and predators (e.g., army ants), or defense against diseases.

Keywords Hibernation · Leucocoprini · Insect–fungus mutualism · Fungus-coat · Symbiosis

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Introduction

Among the more than 230 fungus-growing ant species in the tribe Attini (Schultz and Brady, 2008), basal attine species are the most arduous to study because of their inconspicuous behaviors, cryptic nests, low-nest abundances, and generally small nest sizes (Weber, 1972; Price et al., 2003; Mueller, 2002; Mueller et al., 2005). A series of recent natural-history studies markedly enriched our understanding of the life-histories of basal attine ants (Weber, 1956; Murakami and Higashi, 1997; Mueller and Wcislo, 1998; Leal and Oliveira, 2000; Schultz et al., 2002; Solomon et al., 2004; Fernández-Marín et al., 2005; Klingenberg et al., 2007; Diehl-Fleig and Diehl, 2007; Lopes, 2007; Pitts-Singer and Espelie, 2007; Rabeling et al., 2007a, 2009; Sanhudo et al., 2008; Vasconcelos et al., 2008; Solomon et al., 2010), but the number of studies on basal attine ants is dwarfed by the dozens of studies reported annually for more derived attine lineages, such as the leafcutter ants (reviewed in Wirth et al., 2003; Solomon et al., 2008).

During a collecting trip to Rio Grande do Sul, the southernmost state of Brazil, we discovered a nest aggregation of the fungus-growing ant *Mycetosoritis clorindae*, a member of a genus for which behavior and nest architecture are almost completely unknown (but see Wheeler, 1907). We report here the first record of the nesting biology of *M. clorindae*, and describe a peculiar behavior of workers that plant live fungus onto hibernating nestmates. Many attine ant species plant fungal mycelium onto their eggs, larvae, and pupa (Weber, 1972; Schultz and Meier, 1995; Adams et al., 2000; Lopes et al., 2005; Camargo et al., 2006), but fungus-coating of adults has not been described yet for any attine species.

Murakami and Higashi (1997) and Lopes et al. (2005) provide the most detailed description to date of the behavior of fungus-planting on attine brood, noting that this behavior is indistinguishable from the fungus-planting behavior on garden substrate during normal gardening. In essence, workers treat the developing brood somewhat like garden substrate (Takahiro Murakami, pers. comm.), inserting the brood into the garden matrix, then planting mycelial tufts onto the brood with the same kind of tamping motions of the front legs that typify normal fungiculture (Weber, 1972; Murakami and Higashi, 1997; Lopes et al., 2005). Despite the larger size of male larvae, planting of fungal tufts occurs more frequently onto worker larvae than onto male larvae of *Acromyrmex subterraneus* (Camargo et al., 2006). In *Acromyrmex* ants, larvae are first thoroughly licked by workers before they plant mycelium onto the larval integument, but workers cease licking after planting (Lopes et al., 2005). Mycelium planted on gardening substrate is fertilized initially with fecal droplets of workers (Weber, 1972), but mycelium planted on larvae is not tended in any comparable way by workers (Lopes et al., 2005), suggesting that the mycelium growing on larvae may subsist in part on secretions derived from the larval integument.

Fungus-coating of brood varies between attine species. For example, brood of species in the *Apterostigma pilosum* species group (Lattke, 1997; Villesen et al., 2004) are covered with a thick coat, and the fungus-covered brood is integrated semi-permanently into the garden matrix. In contrast, fungus-coating of brood appears completely absent in the genus *Atta* and in several, phylogenetically disparate, lower attine species (e.g., *Mycetosoritis hartmanni*; UGM, pers. obs.). The mycelial coat of brood is also present in the yeast-cultivating *Cyphomyrmex* species that grow their cultivars in a non-mycelial, yeast state (Wheeler, 1907; Mueller et al., 1998; Wang et al., 1999), but the same fungus is thought to grow as a mycelium on the integument of brood of the yeast-cultivating *Cyphomyrmex* species (Schultz and Meier, 1995).

With the exception of the aforementioned extreme coating in some *Apterostigma* species and the absence of

brood coats in *Atta*, there are no obvious phylogenetic patterns or known ecological correlates that could provide clues to explain the variation in fungus-coating across the attine genera. LaPolla et al. (2002) discuss several untested hypotheses for the adaptive function of the brood coats, including physical protection, protection against parasites or predators (e.g., army ants), and protection from entomopathogen infection. Mueller et al. (2008) were able to identify actinomycete bacteria from a mycelium-covered larva of *Trachymyrmex arizonensis*, suggesting that the fungal coat may protect the immobile brood in conjunction with microbial biofilms embedded in the coat matrix. Fungus-planting on hibernating workers, reported here for the first time for any attine ant, may serve the same kind of functions that have been hypothesized for brood by LaPolla et al. (2002).

Materials and methods

During a survey of leafcutter ants in the state of Rio Grande do Sul, Brazil, we located a nest aggregation of the basal fungus-growing ant *M. clorindae* on 9 September 2004 in an unshaded area of a pasture surrounding a residential house (39 km, Taquara-Porto Alegre highway, Rio Grande do Sul, Brazil; GPS coordinates: S 29°42'55.7", W 50°50'21.5"). At the nearest weather station in Taquara (about 20 km distant from the collection site), average temperatures during winter (July–August) measure 13–15 °C, with average minimum temperatures of 6–8 °C, and occasional temperatures below freezing. September marks the end of winter in southern Brazil, and ants were expected to be in the process of reactivating their nests after a period of reduced activity or winter hibernation. Observations of workers at nest entrances prior to excavations revealed significant ongoing digging activity, indicated also indirectly by the substantial amount of loosely accumulated soil particles at the nest entrance. Such excavation behavior is typical for attine ants reactivating or expanding a nest after quiescence during winter (Weber, 1972; UGM, pers. obs.). Counting the visible mounds of excavated soil at nest entrances, the *M. clorindae* population consisted of at least 40 nests, spread over an area of about 15 m × 7 m in the pasture. Nests of other fungus-growing ant species were interspersed in this nest aggregation (*Acromyrmex landolti* and an unidentified *Trachymyrmex* species).

We excavated two adjacent nests (55-cm apart) following the methods of Schultz (1993) and Mueller et al. (1996). A hole was first dug at 30-cm distance to the entrance mound, and the soil toward the nest was then carefully removed with a pocket knife. The garden

chambers therefore were breached from the side, rather than from above. Such lateral breaching of garden chambers results in minimal soil fallout onto the garden and increases the success of transferring complete, live colonies with healthy gardens to the laboratory. The vertical tunnel of each nest was traced in its entirety, guided by a thin blade of grass inserted into each tunnel. Tunnels emanating below a chamber were similarly traced until they terminated in a dead end. A soil volume of about 40-cm height and 25-cm diameter was then excavated carefully below the apparent dead end of the tunnel, to search for any deeper parts of the nest that may have been missed, but no further tunnels or chambers were found. Nest features were carefully measured.

For each of the two excavated nests, we collected all gardens and all ants with a sterile forceps, then transferred these to a laboratory nest chamber (see description in Schultz, 1993). We accumulated descriptive observations of worker behavior in these two nests within the 36 h following excavation, more sporadically during the following 5 days of the collecting trip through Rio Grande do Sul, and again more carefully afterwards in the Bacci Laboratory at UNESP Rio Claro, São Paulo, Brazil. All collected material (ants, gardens) is vouchered in the Bacci Ant Collection at UNESP Rio Claro. Ant vouchers are also deposited at the Museo de Zoología, Universidade da São Paulo.

The cultivated fungus of one nest (UGM040909-01) was sequenced at UNESP Rio Claro and identified via the BLAST at Genbank of the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), following the methods of Mueller et al. (1998) and Solomon et al. (2004) for DNA extraction and sequencing of the ITS-gene region. The ITS-sequence for this fungus is deposited as Genbank accession GU301643.

Results and discussion

Both of the excavated colonies were queenright and monogynous, with colony sizes of 97 workers (Nest A, UGM040909-01) and 44 workers (Nest B, UGM040909-02). None of the workers appeared callow or recently enclosed. Alates were absent. No brood was found (not even eggs) during careful searches through gardens under a microscope (45× magnification) later on the day of excavation. The absence of brood was consistent with the expectation (see Sect. “Methods”) that the nests were in the process of reactivation after a period of quiescence or hibernation during winter. Most workers were collected hidden in the garden. About 10–15 workers per nest were collected with forceps when they moved about the garden chamber.

Fungus-coating of workers and queens

While surveying gardens for the presence of any brood or alate ants a few hours after collection, we noticed that the queens and most workers remained immobile in the garden, had garden fragments attached to their integument, or were completely encased in mycelium (Fig. 1). Ants with mycelial attachments remained motionless in a curled-up position that is typical for many attine species during cryptic defense: head and metasoma pulled downward, and all legs folded under the mesosoma, with the femoral–tibial joints extending somewhat laterally, but the tibial–tarsal joints protected under the mesosoma and the adducted metasoma (Fig. 1).

Mycelium-covered workers were initially believed to have become accidentally entangled in tacky garden fragments, and the adhering fragments were therefore carefully removed with forceps to free these ants from the putative, mycelial encumbrances. When inspecting these nests again a day later under the microscope, the majority of workers and the queens were immobilized again in curled-up positions in the gardens and were variably encased or covered with mycelial tufts. The queens showed the thickest covering, with about 60 mycelial tufts and about 5 small grass fragments adhering to each queen. Workers showed different degrees of mycelial coating, with some

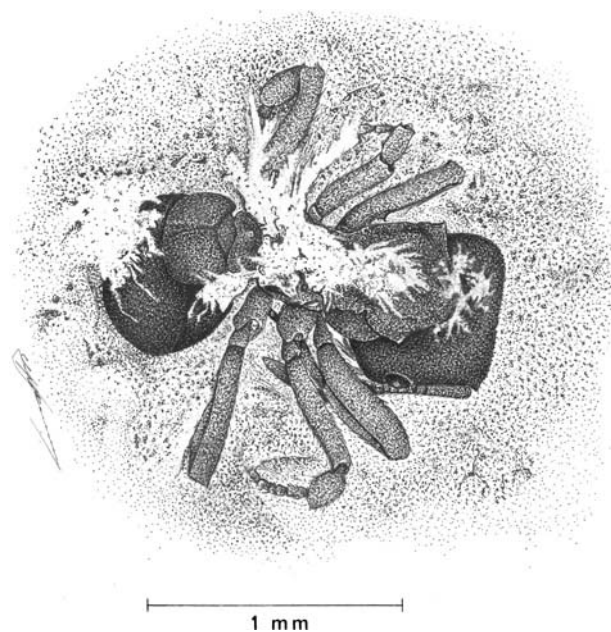


Fig. 1 Overwintering worker of *Mycetosoritis clorindae* with mycelial tufts planted by other workers onto its integument. The tufts are planted here primarily in the crevices of the neck and petiolar regions, but the mycelial coat can also completely envelop a worker. The worker exhibits the typical immobile posture of a hibernating worker, with head and metasoma curled downward, and legs folded underneath. Drawing by Jaime Roberto Somera

workers completely covered like the queens, while other workers bore only a few tufts (Fig. 1). The tufts adhered to the adults like they adhere to garden substrate when planted by workers during normal fungiculture. About 10–15 workers were active in each nest, moving about the artificial nest chamber and performing gardening tasks; these workers were free of visible mycelial tufts.

The mycelial tufts on workers were located frequently in the neck region (crevice between the mesosoma and the appressed head; Fig. 1), the crevices between the legs pulled up under the mesosoma, and the petiolar region. The frequent placement of the tufts tucked in body crevices of the curled-up ants, as well as the immobility of the ants, suggested that the tufts did not adhere accidentally, but that the tufts had been placed there by other workers.

Because a comparable number of workers (about 10–15) were active in field nests at the time of collection and in lab nests shortly after collection, it appeared that workers were comprised of two cohorts, one cohort of active, mycelium-free workers and a second cohort of immobile, mycelium-coated workers. Moreover, removal of the mycelial coat a few hours after collection did not immediately stimulate typical intranidal activities in the coated workers. Although workers were not marked for individual recognition, this interpretation of behavioral specialization is further supported by the following observations. Workers from which the mycelial coat was carefully removed with forceps, and which were then placed into the garden chamber at some distance to the garden, exhibited sluggish movements, but walked back to the garden and inserted themselves into the garden by assuming the curled-up, immobile posture. The sluggish movement back to the garden suggest some physiological un-preparedness for walking, which in turn suggests a state of hibernation rather than simple resting. Five of these returned workers were observed for half an hour each (4 workers from Nest A, 1 worker from Nest B), and in three of these cases some other, active worker arrived, inspected the curled-up worker, then planted a mycelial tuft onto the body of the immobile worker. In one case, one active worker planted two mycelial tufts and one grass fragment onto an immobile worker during the half an hour of observation. Workers onto which mycelium was planted remained motionless (except for small antennal movements) and therefore seemed to cooperate with the workers planting the mycelium on their bodies.

Workers attach mycelial tufts to the integument of nestmates with tamping motions of the front legs. Specifically, workers gather a mycelial tuft from mature garden, carry it with their mandibles to a motionless nestmate, then pack the tuft into place with quick, alternating tappings of the front legs. The fungus-planting behavior on nestmates, therefore involves the same kind of motions performed by

workers during normal fungal planting on gardening substrate (Murakami and Higashi, 1997; Lopes et al., 2005). We did not notice the kind of licking that precedes mycelial planting on larvae in *Acromyrmex* ants (Lopes et al., 2005), but such behavior could have been missed in our observations. More detailed observations are needed to discern possible differences between fungal planting on adult nestmates, brood, or garden substrate.

The process of fungus-coating of the queen by workers cannot easily be observed, because the queen invariably hides under or inside the garden, but it can be inferred indirectly when removing the coat experimentally and inspecting the queen after some time. The extensive fungus covering of queens within a day after experimental removal of the fungus-coat (see above) suggests that fungus-planting workers pay particular attention to the queen, such that mycelial coating of the queen may progress faster than the coating of the average worker.

Following observations on the day of collection, the two colonies were transported by car to the Bacci Laboratory at UNESP Rio Claro (S 22°23'43.9", W 47°32'32.3"), arriving 7 days after collection. The colonies were kept in a cooler during transport, and occasional readings of a thermometer kept in the cooler indicated temperatures between 20 and 26°C. Once stabilized in the laboratory, the two colonies were carefully searched again under the microscope and found to contain, respectively, only one and two hibernating workers with some mycelium coating. The rest of the workers were coat-free and appeared engaged in typical worker tasks (e.g., gardening, foraging). Maintenance at higher temperatures for several days or the general disturbance during transport appeared to have triggered the colonies to complete the transition from winter hibernation to spring activities. It may be possible to induce the reverse process experimentally and stimulate fungus-coating behavior in lab colonies by subjecting them to gradually decreasing temperatures. It is also possible that similar fungus-coating of hibernating workers may occur in other attine species from temperate regions or from higher altitudes.

Identification of the cultivated fungus

The fungus cultivated by nest UGM040909-01 (Genbank accession GU301643) of *M. clorindae* falls within the Clade-2 fungi of lower attine ants, one of the two dominant fungal groups associated with lower-attine ants (Mueller et al., 1998; Vo et al., 2009). This extends the known range of Clade-2 cultivars of lower-attine ants to Southern Brazil (previously known only from Northern South America, Central America, and North America). Because Clade-2 cultivars were first imported into cultivation by lower-attine ants from free-living populations an estimated

11 mya (Mikheyev et al., 2010), much more recent than the origin of the lower attine ants (Schultz and Brady, 2008), Clade-2 fungiculture appears to have spread since that time through essentially the entire range of lower attine ants.

Nest architecture

One and two gardens were found, respectively, in Nests A and B of *M. clorindae*. A single, unbranched tunnel (1.0–1.5 mm diameter) leads vertically downward from the entrance mound, ending in the ceiling of the first garden chamber at depths of 26.7 cm (Nest A) and 18.3 cm (Nest B) (measuring depth from the ground surface to the bottom of a chamber). The top nest chamber measured 3.2 cm × 4.5 cm widths and 2.4 cm height in Nest A, and 2.3 cm × 2.4 cm widths and 1.9 cm height in Nest B. This chamber design (lateral dimensions exceeding the chamber height) is characteristic for many ground-nesting attine ants (e.g., Weber, 1972; Rabeling et al., 2007a; UGM, unpubl. obs.). Nest A also had a small, empty chamber of 9-mm diameter at depth 5.1 cm, which may have been the original chamber constructed by the foundress queen at nest initiation. No such foundress chamber was found in Nest B; however, Nest B had a second, small garden at depth 29.5 cm in a chamber measuring 2.1 cm width and 1.6 cm height.

Three additional nests of *M. clorindae* that we accidentally broke into during excavation of *Acromyrmex* nests at this site had one chamber (21 cm and 29 cm depths in Nests C and D, respectively) or two chambers (vertically arranged at depths of 17 and 21 cm in Nest E). The accumulated evidence from all five nests therefore indicates that *M. clorindae* builds nests with either one or two chambers at depths of between 15 and 30 cm. The overall nest architecture of *M. clorindae* (a long, thin, vertical, unbranched tunnel leading straight down to a first nest chamber at about 20-cm depth, then continuing to lower chambers arranged in a vertical series; sessile, spherical gardens) resembles most closely the one known for the congener *Mycetosoritis hartmanni* (Wheeler, 1907; Himler, 2007; Himler and Mueller, in prep.).

Gardens were spherical, rested on the chamber bottoms, and appeared to fill almost completely the garden chambers. No rootlets permeated the chambers and gardens, and gardens therefore were not suspended. The garden-filled chambers indicate that *M. clorindae* does not collapse its gardens significantly during the period of relative inactivity during the colder winter months, contrasting with the winter-garden reduction known for other temperate attine species (Wheeler, 1907; Seal and Tschinkel, 2006; Rabeling et al., 2007b; UGM, pers. obs.). Perhaps some foraging activity continues in *M. clorindae* during warmer days in winter, allowing workers to continually add substrate to

gardens, or perhaps the cultivated fungus digests substrate slowly at lower temperatures (i.e., the substrate lasts longer before exhaustion by the fungus). Garden substrate consisted of a mix of short grass leaf-fragments, grass seeds, and other unidentified seeds. Seeds measured about 1.0–1.5 mm in diameter, and grass fragments rarely exceeded 4 mm in length.

A dense, tarry mass of black-brown coloration was found at the bottom and to the side of the chamber in Nest A. The mass (henceforth called “wad”) measured 13 mm × 8 mm widths and 7 mm height, had a relatively dry surface, and appeared not to touch the garden. The wad rested on five little pebbles and was therefore somewhat elevated from the chamber floor. Dissection under the microscope revealed that the wad consisted of densely packed debris, including soil particles, sand grains, seed husks, a few germinating seeds, and small round pellets that may have been expelled infrabuccal pellets of worker ants. Filamentous fungi and a few nematodes inhabited the tarry mass. Similar wads also occur in representatives of several other attine genera (e.g., *Myrmicocrypta*, *Mycetophylax*, *Mycocepurus*, *Mycetosoritis*, *Trachymyrmex relictus*; Rabeling, 2004; C. Rabeling, pers. comm.; UGM, pers. obs.), where such wads can be found year-round (i.e., not only at particular times of the year; UGM, pers. obs.). The wads may represent mere accumulations of compacted, exhausted garden mixed with some other debris; however, the fact that the ants do not discard this debris outside the nest (a behavior typical for many attine species) but keep it compacted next to the garden suggests that the wads may serve an unknown function in garden chambers.

Implications for understanding of attine ant-microbe interactions

In in vitro bioassays, Sen et al. (2009) showed that antimicrobial secretions of actinomycete bacteria growing on the integument of attine ants severely inhibit or kill the fungal cultivars of diverse attine species. The ability of *M. clorindae* to maintain cultivar growth on hibernating adults, therefore suggests that *M. clorindae* does not carry inhibitory actinomycetes on their integument (at least not during periods of hibernation), or that in vitro assays of attine-associated actinomycete bacteria do not reflect their antibiotic properties under natural conditions in the nest, as discussed by Sen et al. (2009). Fungus-planting on attine adults is so far unknown from other attine ants, but in a laboratory study of the behavior of *Trachymyrmex zeteki*, workers were seen twice to place mycelium on queens of the social parasite *Megalomyrmex symmetochus* parasitizing *T. zeteki* nests (Adams, Shah and Mueller, in review). Such planting on adult ants parallels the planting of mycelium on larvae and pupae characteristic for many

attine species (see Sect. [Introduction](#); Weber, 1972; Lopes et al., 2005; Camargo et al., 2006). The adaptive functions of fungus coatings of hibernating adults and developing brood could be similar, possibly including physical protection, prevention of desiccation, shielding against parasites and predators (e.g., army ants), or defense against diseases, as discussed by LaPolla et al. (2002). Planting of mycelium on adult nestmates may have been overlooked so far in attine ants because this behavior may occur only in dormant nests; such nests are least frequently collected because nest entrances are difficult to spot, and collectors tend to avoid periods of ant inactivity (i.e., winter). Even though pilot experiments with the temperate attine ant *Cyphomyrmex wheeleri* from Central Texas have failed so far to induce fungal-coating behavior at lower temperatures during winter (UGM, pers. obs.), similar laboratory studies under temperature-controlled conditions may represent a promising approach to study fungus-coating behavior in *M. clorindae* and possible other attine species adapted to temperate habitats or higher elevations.

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