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Facultative mushroom feeding by common woodland ants (Formicidae, *Aphaenogaster* spp.)

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ABSTRACT

Despite the remarkable diversity of food resources exploited by ants, fungi are rarely known to be part of their diet. The notable exception is the fungal-feeding specialists in the tribe Attini, which cultivate fungi inside their nests in what is one of the earliest forms of agriculture. Previous accounts of fungivory in ants outside these fungal-feeding specialists have been questioned due to whether or not ants consume fungal tissue or prey on mycophagous insect larvae present in or on mushrooms. Here we show that ants in the widespread genus *Aphaenogaster* recruit to mushroom baits in the field regardless of whether or not mushrooms contained insects upon which ants might prey. When dye-stained mushrooms were provided to colonies in the lab, ants fed on mushroom tissue and dye was visible throughout their digestive tract. Evidence of mushroom feeding in *Aphaenogaster* suggests that facultative fungal feeding is more common in ants than previously reported, including within the myrmecine clade that contains the attines. Previous accounts of fungivory in ants have been limited to fungal-feeding specialists, but mushroom feeding by *Aphaenogaster* shows that fungi can also be part of a generalist ant diet. By feeding on mushrooms and transporting mushroom tissues back to the nest, *Aphaenogaster* workers may also serve as dispersers of fungal spores.

1. Introduction

Ants are among the most ecologically successful groups on earth, both in terms of their abundance and diversity (Folgarait, 1998; Lach et al., 2010; Wilson, 1987). In addition to their complex social structure, one factor that may have contributed to the success of ants is their ability to exploit diverse dietary niches (Blüthgen and Feldhaar, 2010). Ants feed on a wide variety of resources ranging from carrion, dung, and invertebrate prey to nectar, seeds, and other assorted plant materials (Lanan, 2014). Despite the remarkable diversity of plant- and animal-based food sources exploited by ants, fungi are rarely reported to be part of their diet. The notable exception to this is the fungus-growing ants in the tribe Attini, a highly successful group of New World ants that practice one of the earliest forms of agriculture (Mueller et al., 1998). Higher attines feed on special structures, called gongylidia, that are produced by their fungal cultivars (Henrik et al., 2014; Quinlan and Cherrett, 1979), while lower attines are thought to feed on hyphal tissue directly. Outside of the attines and their social parasites (Adams et al., 2000), clear examples of fungivory in ants have only been recognized in a single genus of Old World formicine ants (*Euprenolepis*) (von Beeren et al., 2014; Witte and Maschwitz, 2008) and all of these

groups are thought to be fungal-feeding specialists. Ants with a generalist diet are rarely known to consume fungi, and there remains debate over the evolutionary origins of fungal farming (reviewed in Mueller et al., 2001).

Fungivory is common across diverse groups of arthropods having arisen repeatedly within the Coleoptera, Diptera, Collembola, Orthoptera, Acari, and Lepidoptera (Bruns, 1984; Courtney et al., 1990; Epps and Arnold, 2010; Fogel, 1973; O'Connell and Bolger, 1997; Powell, 1980). Mushrooms and other fungal fruiting bodies, although ephemeral and patchy in their distribution (Hanski, 1989), offer a supply of easily harvestable material containing minerals, vitamins, fats, and amino acids (Chang and Miles, 2004; Wallis et al., 2012). The apparent absence of fungi from the diet of most ants is therefore surprising. Although ants of diverse genera have long been observed as common visitors to mushrooms (Lewis and Worthen, 1992; Orr and Charles, 1994), such ants are thought to visit mushrooms strictly as predators, where they feed on the many mycophagous arthropods and their larvae that aggregate in fungal tissues (Mueller et al., 2001; Worthen et al., 1994). Indeed, these ants are often observed collecting mycophagous larvae from mushrooms (Courtney et al., 1990; Lewis and Worthen, 1992; Worthen et al., 1994). Whether mushroom-visiting ants

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also feed on fungal tissue itself is not clear (Mueller et al., 2001).

We used a combination of field and laboratory methods to test whether ants visiting mushrooms forage on fungal fruiting bodies not only as predators of insects but also as facultative fungivores. We focused on the ant *Aphaenogaster rudis*, which is among the most common ants in eastern forests of North America (King et al., 2013). There is strong observational evidence that ants in the genus *Aphaenogaster* forage for mushrooms and consume mushroom tissue rather than exclusively prey on mycophagous insects (Carroll et al., 1981). Here we used an experimental approach to test whether ants were attracted to mushrooms themselves rather than to the presence of potential prey insects, predicting (1) that *A. rudis* workers would be attracted to mushroom baits in the field, and (2) that they would recruit equally to insect-infested mushrooms and insect-free mushrooms. Next, we tested whether *A. rudis* consumed mushroom tissue in the lab by feeding colonies mushrooms stained with fluorescent dye. We predicted that dye would be present throughout the gut of dissected workers if they fed on dye-stained mushrooms. Based on our findings for *Aphaenogaster*, we compiled evidence from other ant species that have been previously reported to feed on mushrooms to evaluate whether fungivory may be more common in ants with generalist diets than previously expected.

2. Methods

2.1. Field baiting trials

We used food baits as a behavioral assay to test whether ants were attracted to mushrooms in the field and whether they would recruit to mushrooms that were free of potential prey insects. We conducted mushroom baiting trials at Duke Forest, NC (36.0366°N, 79.0772°W) on 3 July 2014. We positioned 13 experimental arrays haphazardly over a 0.25 ha area of mature forest dominated by a mix of oak (*Quercus* spp.) and pine (*Pinus* spp.). Experimental baits consisted of three types: (1) insect-infested mushrooms, (2) insect-free mushrooms, and (3) plaster casts of mushrooms moistened with deionized water to serve as a control. For mushroom baits, we raised fresh oyster mushrooms from a single strain of *Pleurotus ostreatus*, a species native to our study site, on commercially inoculated straw blocks in the lab until fruiting bodies were just emerging. To manipulate the presence of mycophagous insect prey, half of the straw blocks were placed in a shaded yard at the edge of a forested lot in Raleigh, NC until mushrooms were fully matured, during which time they were naturally and abundantly colonized by mycophagous insects and their larvae (including both flies and beetles). The insect-free straw blocks were kept in the lab where their mushrooms matured at a similar rate but free of insect visitors.

Within each array, baits were placed 0.5 m apart on individual pieces of waxed paper to facilitate the observation of visiting ants. Baits were placed at 9:30 AM, when colonies were observed foraging. After this time, we censused ants on or under each bait twice at 1 h intervals beginning at 10:30 AM. We collected all ants present on baits at the end of 2 h to confirm species identifications in the lab. In addition to our primary bait arrays, we distributed 13 cookie baits (pecan sandies) around the perimeter of our study area to compare visitation of ants at mushroom baits to a more common bait used for ant surveys (Bestelmeyer et al., 2000).

2.2. Laboratory feeding and observation

We provided oyster mushrooms to colonies of two *Aphaenogaster* species in the lab (*A. rudis* and *A. picea*) to observe how colonies interacted with mushrooms and to determine whether or not workers ingested mushroom tissue. We collected whole colonies of *A. rudis* from Albemarle County, VA (38.0936°N, 78.4219°W) and Orange County, NC (36.0366°N, 79.0772°W) in August and September of 2014, and we collected whole colonies of *A. picea* from Floyd County, VA (36.8586°N, –80.4075°W) in July of 2014. Colonies were maintained in artificial

nest boxes (19 × 27 cm) with a plaster floor and glass-covered nest chamber (12 × 15 cm). The plaster in each nest was moistened bi-weekly to maintain humidity. Colonies were kept without food for one week to encourage foraging activity, and we then placed fresh, laboratory-grown oyster mushrooms in the foraging arena of 10 *A. rudis* colonies and eight *A. picea* colonies. We checked colonies daily over one week to record behaviors associated with mushroom foraging and potential consumption.

To determine whether ants actually ingested mushroom tissue, we fed colonies oyster mushrooms soaked in an aqueous solution of 0.5% rhodamine B fluorescent dye as per methods of Gayahan and Tschinkel (2008). Two *A. rudis* colonies were supplied dyed oyster mushrooms, two colonies were supplied dyed mealworms (*Zophobas morio* larvae), and two colonies were supplied dyed sugar water (20% sucrose solution). We included mealworms and sugar water as positive controls since both are known to be part of *Aphaenogaster*'s diet and represent common sources of proteins/lipids (mealworms) and carbohydrates (sugar water). Again, colonies were kept without food for one week before feeding trials to encourage foraging activity. We let colonies feed for ~48 h after food was provided, and we then removed and dissected 51 adults and 49 larvae under a microscope to examine whether dye was present in the gut. For adults, we additionally noted whether dye was present in the crop, midgut, and/or hindgut. Dissections were conducted blind to treatment.

2.3. Statistical analyses

For baiting trials, we focused our analyses on *A. rudis* because they composed the majority of species on baits of all types. Since ant abundance data were not normally distributed, we tested for differences in recruitment between bait types using the Friedman test in GraphPad Prism 6 (GraphPad Software, Inc. 2015). We then tested for differences in ant abundance between bait types using the Wilcoxon test without a Bonferroni correction to avoid type II error.

3. Results

3.1. Attraction to mushroom baits

We predicted that workers of *Aphaenogaster rudis* would be attracted to mushroom baits compared with plaster controls, and this prediction was supported (Fig. 1). The abundance of *A. rudis* workers at baits differed significantly among bait types (Friedman test, $N = 13$, $Q = 15.17$, $p = 0.0005$), and workers were over twelve times more abundant on mushroom baits compared to plaster controls (Wilcoxon test, $N = 13$, insect-infested mushrooms vs. control: $p = 0.001$; insect-free mushrooms vs. control: $p = 0.0029$). We next predicted that *A.*



Fig. 1. Abundance of *A. rudis* ants on mushroom baits (insect-ridden and insect-free) and plaster decoys. Box plots show median, 25–75%, and non-outlier range of ants present at baits pooled across two censuses. Letters indicate significant differences ($p < 0.05$).

rudis workers would be attracted to mushrooms whether or not they contained potential prey insects, and this prediction was also supported; there was no significant difference in the number of workers present at insect-infested and insect-free mushrooms (Wilcoxon test, $N = 13$, insect-infested mushrooms vs. insect-free mushrooms: $p = 0.21$). Furthermore, we observed *A. rudis* workers removing pieces of mushroom baits and carrying them back to their nests.

In total, four ant species visited experimental baits of all types, including *A. rudis*, *Camponotus pennsylvanicus*, *Temnothorax curvispinosus*, and *Crematogaster lineolata*. We counted 455 individuals over both censuses, of which 98% were *A. rudis*. In addition to mushroom baits and plaster controls, we placed cookie baits around the perimeter of our study area to compare how ants responded to a more common bait type. Cookie baits had three times more visiting workers than mushroom baits; across our experiment, ants recruited to cookies the most, followed by mushrooms, and plaster the least. Consequently, mushrooms were not as attractive as a sugar- and lipid-rich bait (cookies), but mushrooms were more attractive to ants than plaster controls.

3.2. Mushroom consumption by laboratory colonies

To test whether ants consumed mushroom tissue inside their nests, we provided mushrooms to *Aphaenogaster* colonies in the lab and observed their behavior. Workers of all colonies antennated mushrooms presented in their foraging arenas within the first 5 min of their placement. Seven out of eight colonies of *A. picea* were already heavily recruiting foragers to mushrooms within this period. After four days, all *A. picea* colonies showed evidence of mushroom feeding as indicated by cutting mushroom fragments and transporting pieces into the nest where they were chewed in piles by nestmates. Colonies of *A. rudis* behaved similarly (Video 1), with all ten colonies exhibiting distinct signs of mushroom feeding, and six colonies showing unambiguous signs of piling chewed mushroom tissue inside their nests.

In support of our prediction that *Aphaenogaster* workers consume and digest mushroom tissue as food, we observed rhodamine-dye in the guts of all 20 *A. rudis* workers fed dye-stained mushrooms (Fig. 2A). Similarly, we observed rhodamine-dye in the guts of all workers in our positive control groups that were fed dye-stained mealworms ($N = 20$) or sugar water ($N = 11$), both of which are known foods of *A. rudis* colonies. For workers fed dye-stained mushrooms, dye was visible in the crop and the hindgut but was rarely visible in the midgut (Fig. 2B). Dye was visible in all regions of the gut in workers fed dye-stained mealworms, while dye was only visible in the crop of workers fed dye-stained sugar water. Because foraged foods are also consumed by larvae—especially proteinaceous foods, such as insect prey—we examined whether dye was present in the guts of larvae. Dye was not visible in the gut of larvae fed dye-stained mushrooms ($N = 20$) nor sugar water ($N = 9$) but was visible in 90% of larvae fed dye-stained mealworms ($N = 20$).

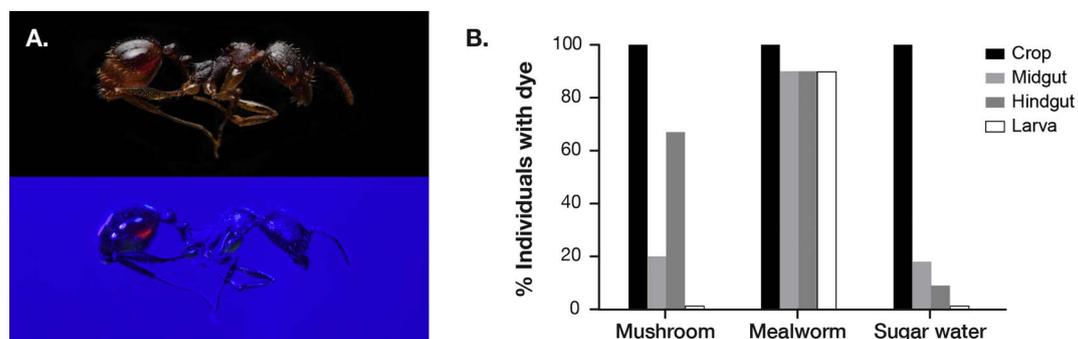


Fig. 2. (A) *A. rudis* worker imaged under normal and UV light after feeding on mushrooms stained with fluorescent dye. Fluorescence is visible in the abdomen. (B) Bar graph showing the percent of adult workers and larvae with dye present in the gut after feeding on dye-stained mushrooms, mealworms, or sugar water. For adult workers, the location of dye was noted as being in the crop, midgut, and/or hindgut.

4. Discussion

Ants are well known dietary generalists (Blüthgen et al., 2003; Lanan, 2014; Penick et al., 2015), but until now, fungal feeding was thought to be limited to a few lineages of mycophagous specialists (Mueller et al., 2001; Witte and Maschwitz, 2008). Here we show that ant species with a generalist diet also feed on fungi. Ants from the widespread genus *Aphaenogaster* recruited to mushroom baits in the field, and they did so whether or not mycophagous insect prey were present in mushroom tissue. When mushrooms were presented to colonies in the lab, workers chewed off mushroom pieces and brought them back to their nests (Video 1). Once inside, workers continued to chew mushroom pieces and ingested fungal tissue as revealed through dissection of workers fed mushrooms stained with fluorescent dye. Ants in the genus *Aphaenogaster* are known scavengers of insect prey, myrmecochorous seeds, and liquid food sources (Lubertazzi, 2012), and here we show that *Aphaenogaster* colonies also feed on mushrooms when present. Evidence of mushroom feeding in a generalist consumer such as *Aphaenogaster* suggests that fungal feeding may be more common in ants than previously thought.

Previous accounts of mushroom-feeding in ants have been questioned due to the possibility that ants were attracted by potential prey insects living in fungal tissue rather than to mushrooms themselves (Mueller et al., 2001; Worthen et al., 1994). Ants are common predators of litter invertebrates, and *Aphaenogaster* spp. are noted predators of mycophagous insect larvae (Lewis and Worthen, 1992; Worthen et al., 1994). Yet we observed no difference in the number of *A. rudis* workers that were attracted to insect-infested mushrooms versus insect-free mushrooms (Fig. 1). Moreover, workers readily consumed insect-free mushrooms in the lab, though we did not observe mushroom feeding in larvae. Unlike other ant species, workers of *A. rudis* do not share food with larvae through trophallaxis (Lőrinczi, 2014). Instead, workers allow larvae to feed on insect prey directly, while other food items are consumed exclusively by workers or supplied indirectly to larvae via trophic eggs (Iwanishi et al., 2003). Mushrooms and other non-prey foods may fall into the latter category.

In addition to the fungus-growing attines, their social parasites (*Megalomyrmex* spp.), and mushroom-feeding specialists in the genus *Euprenolepis*, ants from at least four other genera—*Aphaenogaster*, *Camponotus*, *Iridomyrmex*, and *Pheidole*—have been reported visiting mushrooms (Carroll et al., 1981; Lewis and Worthen, 1992; Orr and Charles, 1994; Worthen et al., 1994). Mushroom feeding appears to be especially common in ants of the genus *Aphaenogaster*, which are globally distributed and common in both temperate and tropical environments (Guénard et al., 2012). We observed workers of three *Aphaenogaster* species (*A. lamellidens*, *A. picea*, and *A. rudis*) feeding on mushrooms in mountain and lowland habitats in the eastern United States, and we observed an unidentified *Aphaenogaster* species feeding on mushrooms in Japan (Fig. A1A). Our results confirm the findings of

Carroll et al. (1981), who first suggested the presence of mycophagy in eight *Aphaenogaster* species (*A. ashmeadi*, *A. flemingi*, *A. floridana*, *A. fulva*, *A. lamellidens*, *A. miamiana*, *A. tennesseensis*, and *A. treatae*). In addition to *Aphaenogaster*, we observed workers of the pavement ant, *Tetramorium* sp. E—one of the most common and widespread ant species in the world (Steiner et al., 2008)—foraging on mushrooms in an urban city center (Raleigh, NC, USA; Fig. A1B). Although it has not been confirmed whether all of these species actually consume fungal tissue, past assumptions that fungal-feeding is rare in ants should be revised.

While it is clear from our experiments that ants collect and ingest mushroom tissue, it is not clear what nutritional value they provide to ant colonies. As described above, it seems that mushrooms are not fed to larvae, which are the primary growth stage in ants (Penick et al., 2017). The diet of adult ants often differs from the diet of larvae, with adults primarily consuming liquid foods, like nectar. *Aphaenogaster* adults are known to collect liquids by dropping small debris into liquid food sources to soak them up and bring back to their nests (Banschbach et al., 2006; Fellers and Fellers, 1976). It is therefore possible that *Aphaenogaster* ants collect mushrooms primarily to consume liquids present in mushroom tissue. Mushrooms are less nutrient-rich than insect prey that is normally consumed by *Aphaenogaster* (Barker et al., 1998; Finke, 2015), but mushrooms contain carbohydrates and protein, including the complete set of essential amino acids for insects (Chang and Miles, 2004; Maák et al., 2017; Mueller et al., 2001). It is possible that mushrooms contain nutrients that are not readily found in *Aphaenogaster*'s typical diet, but because mushrooms are patchy in their distribution and *Aphaenogaster* colonies have relatively small foraging ranges (Lubertazzi, 2012), mushrooms are likely only available to some colonies in a given year. While our experiments focused on consumption of oyster mushrooms (*Pleurotus ostreatus*), we observed foragers of *Aphaenogaster* actively cutting pieces from fruiting bodies of diverse fungi in the field, including species of *Cantherellus*, *Boletus*, *Lactarius*, *Amanita*, and *Laetiporus* (Epps, personal observation).

The lack of previous evidence that ants commonly feed on fungi has left open questions about the evolution of fungal farming in the attines. There are two main models to explain how fungal farming could have evolved: the “consumption first” model and the “transmission first” model (Bailey, 1920; Mueller et al., 2001; Weber, 1958). The consumption first model postulates that ants began consuming free-living fungi first and then started cultivating fungi later, while the transmission first model postulates that specialized fungi used ants to disperse their spores first and that fungal consumption and cultivation evolved later. The fact that fungal-feeding appeared rare or non-existent in ants has been used to lent support to the transmission first model (Mueller et al., 2001), but documentation of fungal-feeding in *Aphaenogaster* and potentially other ant genera suggests that fungi could have been part of

a generalist ant diet. Branstetter et al. (2017) recently hypothesized that attines evolved from a generalist hunter-gatherer during the ‘nuclear winter’ that followed the K–Pg extinction event, which drove organisms to specialize on food that did not immediately depend on photosynthesis, including fungi. It is therefore possible that fungal tissue was already part of the generalist diet of the attine ancestor, which later specialized on fungal consumption as a result of the K–Pg extinction event. Evidence of fungivory in *Aphaenogaster* therefore challenges past critiques of the consumption first model, but it does not rule out the transmission first model.

By feeding on mushrooms, it is likely that *Aphaenogaster* and other mushroom feeding ants serve as spore dispersers. *Aphaenogaster rudis* and *A. picea* forage through leaf-litter and build their nests in decaying wood, both of which provide rich substrate for decomposer fungi. Notably, *Aphaenogaster* spp. are keystone seed dispersers for understory plants (Ness et al., 2009), and it would therefore be interesting if they served a similar role for fungi. Other insects, including bees and wasps, play a critical role in the dispersal of yeasts and other microbes among floral resources (Schaeffer et al., 2014; Stefanini et al., 2012). Most mushroom-forming fungi are not known to be dispersal-limited, but the order of colonization by different fungal species can cause multiplicative differences in community structure and decomposition rates (Fukami et al., 2010). Ants alter the composition of microbial communities inside their nests (Dauber and Wolters, 2000; Laakso and Setälä, 1998; Wagner et al., 1997), and the consequences could favor the establishment of certain fungal species over others. Future research on ant–fungal interactions should provide insight into the role of fungi in ant diets as well as the importance of ants in fungal spore dispersal.

All data generated or analyzed during this study are included in the article or supplement. Supplementary data associated with this article can be found in the online version, at <https://doi.org/10.1016/j.fooweb.2017.12.001>.

Conflict of interest

The authors have no conflicts of interest to declare.

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Appendix 1

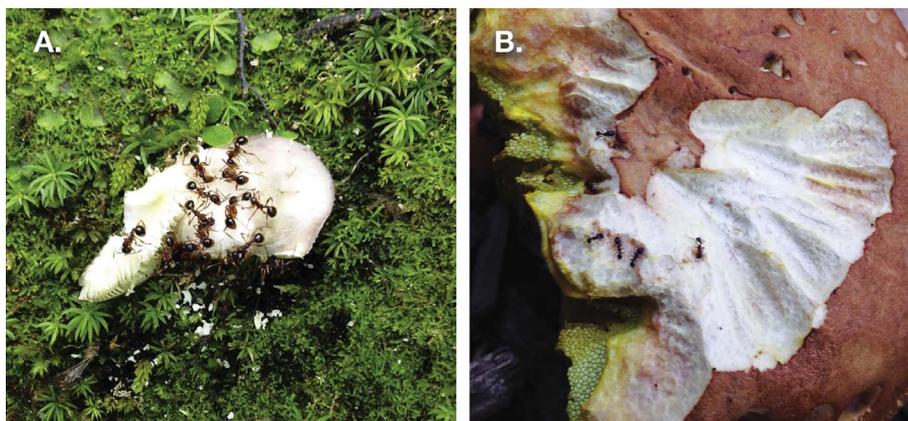


Fig. A1. Observations of ant species feeding on mushrooms. (A) *Aphaenogaster* sp. feeding on unknown mushroom in Kyoto, Japan (35.0267°N, 135.7988°E); (B) *Tetramorium* SpE feeding on Bolete mushroom in Raleigh, USA (35.7816°N, 78.6527°W).

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