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Research Article

Keywords: Behavioral parasitism, geographic mosaic theory, symbioses, fungus farming ants

Posted Date: June 29th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9888518/v1

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Insectes Sociaux on October 30th, 2026.
See the published version at https://doi.org/10.1007/s00040-026-01145-2.

Regulation of a symbiotic species network by a *Megalomyrmex* guest ant parasite: Establishment, maintenance, and threat of breakdown

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Abstract

The establishment of social interactions between distantly related species is rare and requires effective communication across different sensory and signaling systems. In social insects, interspecies cohesion is particularly striking given the importance of nestmate recognition and colony defense. *Megalomyrmex symmetochus* guest ants are central to an emerging symbiotic species network model that encompasses ants, bacteria, and fungi. These guest ants are obligate social parasites of the fungus-growing ant *Sericomyrmex amabilis*, residing within the host nest, exploiting resources, but also protecting against lethal invaders (i.e., predatory ants). However, how the guest ant locates and maintains community balance in collaboration with their host is not well understood. We show that young, mated *M. symmetochus* queens eavesdrop on host trail pheromones to locate a nest, and once inside, eavesdrop on host alarm signals. In addition to communicative function, we found that *M. symmetochus* alkaloid venom is also antimicrobial and may influence the microbial community makeup. Finally, we discuss the timing of alate production and dispersal ability of young queens. This work illustrates how guest ant parasites infiltrate and may stabilize host networks through multi-functional chemical tools. Framing symbioses within interactive communication processes contributes to the understanding of how complex multispecies symbioses emerge and persist in social insect systems over ecological and evolutionary time.

Keywords: Behavioral parasitism, geographic mosaic theory, symbioses, fungus farming ants

Introduction

Social insects host some of the most complex networks of organisms in the animal kingdom. Ants create stable microhabitats that support communities of predators, parasitoids, parasites, mutualists, and scavengers (Box 1; Adams et al., 2020; Combes, 2005; Hughes et al., 2008; Suarez & Goodisman, 2021). These associations are shaped by environmental context, but also co-evolutionary dynamics (Collin et al., 2026; Macit et al., 2025; Thompson, 1994, 2005). For example, antagonistic interactions between ants and parasites may result in coevolutionary arms races where both species continuously adapt and diversify in response to one another (Dawkins & Krebs, 1979; Foitzik et al., 2003). Host and parasite distributions are crucial because population size, density, dispersal capacities, and gene flow also influence evolutionary outcomes. According to the geographic mosaic theory of coevolution (Thompson, 1994, 1999, 2005) arms races occur at the population level, with environmental conditions and community compositions leading to divergent local adaptation.

Observations of a species in the natural world that highlight linkages between the environment or other organisms that is inherently cross-disciplinary and multiscaled is the study of *natural history* (Tewksbury et al., 2014). Despite decades of research documenting ant associations, the natural history of many specialized symbionts remains poorly described, limiting our ability to interpret behavioral strategies in an evolutionary context. Moreover, social parasitism (one social species living permanently, temporarily, or at least regularly in dependence on another social species for survival; Forel, 1898) is remarkably common (Buschinger, 2009; Gray & Rabeling, 2023; Rabeling, 2020). Understanding the natural history of these interactions is therefore critical, not only for interpreting chemical and behavioral strategies that parasites and hosts have evolved, but also for broader conservation efforts (Nanglu et al., 2023; Tewksbury et al., 2014). The obligate dependence of social parasites makes them ecologically vulnerable; the loss of a host population may lead to parasite extinction (Casacci et al., 2019; Thomas et al., 2009). Preservation of the host and its habitat is essential for maintaining diversity of interconnected associates.

The genus *Megalomyrmex* includes 46 described species, many are free-living predators and one-fourth are social parasites on fungus-growing ant species (Boudinot et al., 2013; Brandão, 2003; Sozanski et al., 2020). While not all natural history is known, three parasitic strategies have been well described. “Thief ants” (e.g., *M. silvestrii, M. mondabora,* and *M. mondaboroides*) exhibit lestobiotic parasitism, where the parasites live near the host nest periodically raiding it for resources, consuming a small amount of garden and brood (Adams et al., 2015). Agro-predators, as described in *M. wettereri, M. incisus* and distantly related *Gnamptogenys hartmani* (Box 1A & 2D), are parasites that displace and/or kill host workers in raids ultimately usurping the nest structure with the garden, consuming host larvae and fungal cultivar (Adams et al., 2000; Cardoso et al., 2016; Dijkstra & Boomsma,

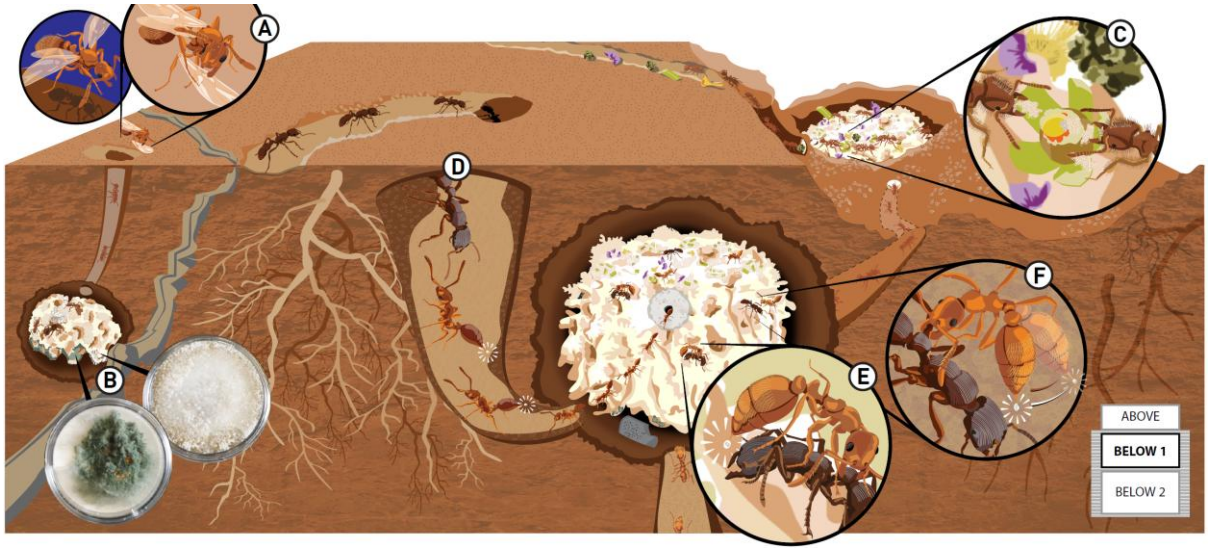


Box 1: Above ground view of daily activity and threats. *Sericomyrmex amabilis* maintains inconspicuous nests with active trails passing under the leaflitter. A large colony may have several nest entrances, connected by tunnels beneath the leaflitter leading down to as many as 16 chambers with fungus gardens. Threats from social parasites that invade the nest are common: (A) *Gnamptogenys hartmani*, (B) *Megalomyrmex symmetochus*. Other threats include (C) parasitoids (e.g., Phorid fly) that attack (D) foraging *S. amabilis* workers.

2003; Neupert et al., 2018). The last strategy exhibits the most sophisticated host nest integration and cohabitation in *Megalomyrmex* parasites. The "guest ants" (*M. adamsae, M. symmetochus,* and *M. miri*) are obligately dependent on their host (Boudinot et al., 2013; Neupert et al., 2018; Weber, 1940; Wheeler, 1901, 1925), associate with distantly-related ant species, and exploit host resources such as nest structure and food while maintaining a worker caste and caring for their own brood (Huang & Dornhaus, 2008; Oberski et al., 2025; Wheeler, 1901). To cohabit with another ant species successfully, guest ant parasites use fine-tuned chemosensory systems to mediate interactions with their host. We have been studying *Megalomyrmex symmetochus*, a guest ant parasite that not only lives within the nest of their fungus-growing ant *Sericomyrmex amabilis* host but builds small fungus garden cavities where the parasite queen(s) and brood reside, fully integrating into the host nest environment (Box 2&3).

We provide a comprehensive account of the guest parasite's natural history, by combining field observations, colony surveys, behavioral data, and microbiological culturing and assays. Furthermore, we evaluate this complex symbiotic species network from the lens of an ongoing exchange of communication processes that have evolutionary consequences. By placing *M. symmetochus* within a broader evolutionary, ecological and behavioral

framework, we aim to strengthen comparative understandings of host–parasite relationships and underscore the value of natural history as a foundation for all studies.



Box 2: Below ground activities of microbial and parasite threats. (A) Following a mating flight a young *Megalomyrmex symmetochus* queen removes her wings and begins a search for a young host colony. (B) A young *Sericomyrmex amabilis* colony that is not yet parasitized. Workers manage microbial threats (e.g., *Trichoderma* (green); *Escovopsis* (white) that are maintained at low levels throughout the garden. (C) *Sericomyrmex amabilis* workers forage for and process substrate (e.g., cut flower petals and leaves, insect frass) that they feed the garden. (D) *Gnamptogenys hartmani* raider parasite attempt to usurp a mature *S. amabilis* colony. (E) The raiders are attacked by the *Megalomyrmex symmetochus* guest ants that occupy the mature *S. amabilis* nest. The guest ants wipe their venom using a gaster-tuck sting. (F) The guest ants can also deliver their toxic alkaloid venom using a side-swipe sting. Regardless of the delivery method, the guest ant venom causes confusion (e.g., raiders attack one another) and mortality, preventing further recruitment of the raiding parasites (Adams et al., 2013).

Finding and entering the fortress

For social parasites to be successful, they must have specialized sensory systems that allow them to detect and locate their host (Adams et al., 2020; Chaisson & Hallem, 2012; Combes, 2005). Guest ants face the particular challenge of locating and entering the well-defended fortress of another ant colony, but achieve this by exploiting host communication. For example, some species eavesdrop on host trail pheromones to locate nests (Lenoir et al., 1992; Morgan, 2009), while others use cuticular hydrocarbon profiles to confirm host identity (Howard, 1993). Once host identity is confirmed, guest ants may gain entry and maintain social harmony by mimicking the host's odor profile – either through biosynthesis, or through CHC acquisition (Akino, 1994; Casacci et al., 2019; Tsuneoka & Akino, 2012). Regardless of strategy, guest ants must first locate their host before breaching the host nest to exploit resources.While we have studied the integration of *M. symmetochus* into their host colony (Neupert et al., 2018), we know little about dispersal timing and early stages of host finding. Like other Solenopsidini, *M. symmetochus* reproductives mature inside their natal colony until they are ready to mate (Lofgren et al., 1975). Mating triggers dramatic morphological and physiological changes in ant queens,

including brain gene expression (Calkins et al., 2018), reproductive organ development (Vander Meer et al., 2021) and immunological changes (Castella et al., 2009). These post-mating changes may also alter host-seeking behavior, transforming virgin gynes into host-searching parasites.

We hypothesized that following mating, young *M. symmetochus* queens eavesdrop on host trail pheromones to locate a host colony. This is supported by our field observations, where we found parasite gynes walking in leaf litter after their nuptial flight (Supplemental Video SV1). Their host, *S. amabilis,* is an accessible chemical target since *S. amabilis* queens are non-claustral foundresses that forage early in colony life (Wheeler, 1925), laying trail pheromones for navigation through nesting sites just below the leaf little (Box 2, far left). To test whether mating triggers trail-following behavior, we used a Y-tube olfactometer assay comparing virgin and mated female alates. Female and male individuals were mated in the lab in a clear, ventilated plastic container in groups of 1:4 respectively (Supplemental Protocols S1). Activity peaked around dusk, when males made small flights and aggregated in corners to copulate. Females were removed if copulation was observed (Supplemental Video SV2), remaining ants were left overnight to ensure all individuals had mating opportunities. By dawn, all activity had ceased and females were moved to individual nestboxes until behavioral trials began.

We noticed a dramatic behavioral difference between unmated and mated gynes (Fig. 1). Each gyne was exposed to a glass Y-tube that was used by a mature foraging *S. amabilis* colony. One arm was plugged, ensuring foragers used only one side. Gynes were placed in a 1.5mL Eppendorf acclimation chamber before exposure to the Y-tube. All unmated gynes (20/20) avoided the *Sericomyrmex* trail, antennating it briefly before backing away (exact binomial test: $p < 0.001$; Fig. 1a). In contrast, mated gynes antennated the trail entrance and entered, with 95% (19/20) following host trails past the bifurcation on their first trial (exact binomial test: $p < 0.001$; Fig. 1a). To assess choice consistency across repeated exposure, we repeated this experiment two additional times with the same mated gynes. Preference remained high across attempts two and three, with no significant difference between trials. However, latency to enter increased significantly with each subsequent assay, while total time spent investigating the *S. amabilis* trails remained constant once gynes entered the y-tube (Supplemental Results S2).

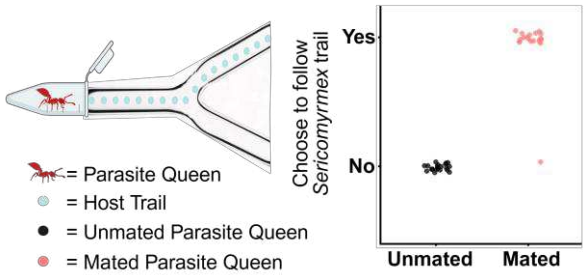


Figure 1. **Y-tube assays determined if unmated and mated parasite queens follow host-laid trails**. Unmated queens never entered the assay within the 40-minute observation window ($n = 20$; $p < 0.001$, Exact Binomial Test), while mated queens overwhelmingly choose to enter the assay and follow the host's trail ($n = 20$, $p < 0.001$, Exact Binomial Test) Created in BioRender https://BioRender.com/c3gmj1m

Our results demonstrate that mating triggers a dramatic shift in host-seeking behavior. Unmated gynes actively avoid *Sericomyrmex* trails, while 95% of newly mated gynes followed them (Fig. 1). The progressively longer latencies across repeated trials, suggests either aging effects (Boulay & Lenoir, 2001; Giraldo & Traniello, 2014) or learned avoidance from repeated unrewarded encounters (Piqueret et al., 2019). To our knowledge, this is the second report of a distantly-related guest ant exploiting host trail pheromones (Lenoir et al., 1992; Morgan, 2009), suggesting convergence of eavesdropping on host communication signals.

Guest ant queen acceptance

Once a mated queen locates a host nest, she must gain entry. As documented in other guest ant systems, queens often enter their host's nest with minimal resistance (Adams et al., 2012; Martin et al., 2007). We suspect *M. symmetochus'* infiltration proceeds similarly. While queen introductions were not tested, Neupert et al., (2018) conducted introduction experiments with workers: where *S. amabilis* workers from non-parasitized colonies snapped at parasites but also showed a submissive response to parasite venom exposure, with no lethal consequences for either species. A follow-up study revealed that when aggressed, *M. symmetochus* workers performed a gaster-flag action that volatilizes their venom instead of a sting (Boot et al., in review). The venom appears to function as a signal that suppresses aggressive behaviors and warns hosts of a potential threat. Unlike other guest parasites (Casacci et al., 2019; Iwai et al., 2022; Tsuneoka & Akino, 2012), *M. symmetochus* does not use chemical acquisition to maintain peace within their host colony; instead they maintain a muted CHC profile (Neupert et al., 2018). Despite this, they do not remain hidden from their hosts.

To test if workers from parasitized nests would accept a recently mated *M. symmetochus* queen from a different colony, we moved the 20 mated queens used in the trail-following trials to individual Petri dishes. Each dish had five host workers from parasitized colonies and a garden fragment (~1 cm^3). Cohabitation behaviors were observed for six weeks. We observed little to no aggression toward the mated parasite queens from the host workers, similar to what was observed in the guest ant parasite *M. adamsae* (Adams et al., 2012). Queens removed their wings between 0.5 and 7 days following mating (Supplemental Video SV3). Although our observations were limited and the duration of the experiment was short (6 weeks), parasite queens and host workers did not die or have visible signs of conflict (clipped appendages) nor were larvae detected.

Our results suggest that newly mated *M. symmetochus* queens encounter little resistance when integrating into incipient *S. amabilis* colonies if the host queen has experience living with parasites. In other words, host queens originating from a parasitized host nest are more likely to tolerate a parasite invasion. While our study used workers, we suspect that all host colony members, including gynes, habituate similarly to a parasite-influenced nestmate recognition profile. Just as in Neupert et al., (2018), *S. amabilis* workers that have

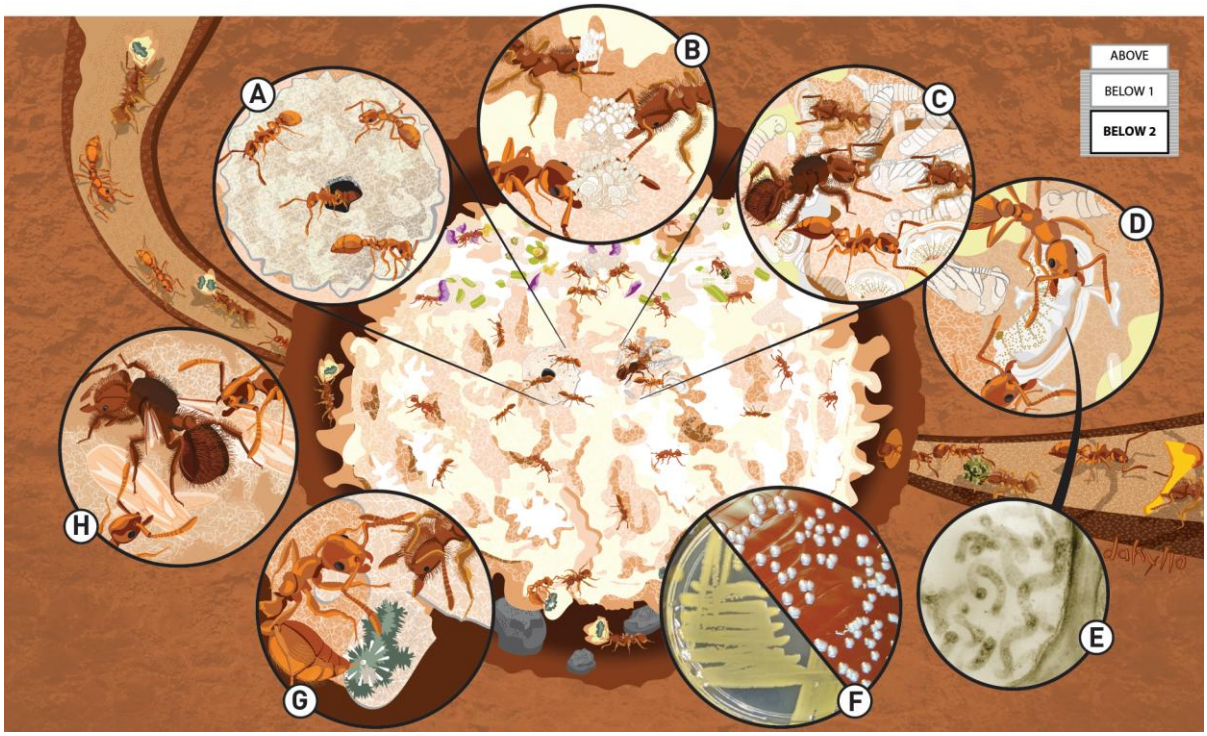
experience with parasites are not likely to aggress the guest ant parasite, whether worker or young queen.

Host alarm substance recognition

Obligately dependent social parasites coevolve with their hosts, driving the convergence and divergence of communication processes (Brandt, Foitzik, et al., 2005; Brandt, Heinze, et al., 2005). Host-parasite cohabitation is maintained for many years with a single host colony, managing over a dozen garden chambers (Box 2). When colonies are excavated, guest parasites are found throughout, though they are in higher numbers in small chambers within the garden cavities (Shik et al., 2018) housing the parasite queen(s) and brood (Box 3A). The overall nest structure is similar to a diminutive *Atta* colony (Wheeler, 1925), with many chambers (4 to 65 cm deep; Geisse Pers. Obs.) and multiple entrances connected to surface tunnels in the leaflitter (Box 1). Because of the life-long dependence of the guest ants, they must navigate a complex chemical landscape created by their host and likely exploit host signals to avoid conflict. Ant parasites have been documented eavesdropping on diverse host communication channels (Adams et al., 2020), however, eavesdropping of host alarm pheromones (chemicals that coordinate colony defense) by social parasites, has been unexplored.

For parasites cohabitating with their hosts, the ability to detect alarm signals could contribute to long-term colony survival. *Megalomyrmex symmetochus* not only lives alongside *S. amabilis*, but will defend it against *G. hartmani* raider ants (Box 2; Adams et al., 2013). *Megalomyrmex symmetochus* workers deploy aggressive venom weaponry either via direct stings or as aerosolized droplets by gaster-flagging (Box 2) while the stingless *S. amabilis* retreat and take shelter inside the garden (https://www.pnas.org/doi/10.1073/pnas.1311654110#supplementary-materials). Functioning as a soldier caste, *M. symmetochus* may recognize when host colonies are under threat, potentially through eavesdropping on host alarm communication.

Fungus-growing ants produce volatile alarm mixtures from their mandibular glands that signal immediate danger to nestmates, triggering rapid defensive recruitment and coordinated responses (Norman et al., 2017). These compounds differ across species, with shared behaviorally active pheromones like 3-octanol and 4-methyl-3-heptanone (Norman et al., 2017). Alarm compounds are released when workers gape their mandibles (Blum, 1969; Crewe & Blum, 1972). If *M. symmetochus* can eavesdrop on these signals, it would provide a mechanism for parasites to assess colony threat levels and coordinate their defensive strategy.



Box 3: Activities within the queens' chambers, and the potential for microbial symbiont sharing. (A) Once integrated, the *Megalomyrmex symmetochus* guest ants construct a chamber within the fungus garden, with a small opening only they can pass through. (B) Both ant species harvest fungal hyphae for nutrition. (C-D) *Megalomyrmex symmetochus* guest ants periodically take host brood for consumption. This diet may explain the overlap in symbionts Entomoplasmatales (E; found in hosts) and *Burkholderia* and *Pantoea* (F; found in gardens). (G) *Megalomyrmex symmetochus* guest ants may help their host by recognizing garden pathogens and applying venom as a functional antimicrobial. (H) *Megalomyrmex symmetochus* workers will castrate host female reproductives by physically cutting their wings, ultimately rendering them as part of the host-worker caste and reducing host reproductive potential.

To test whether *M. symmetochus* detects host alarm pheromones, we applied choice assays using a still air pitfall olfactometer to determine if parasite workers, males, and gynes respond to three crushed *S. amabilis* heads (Supplemental Protocols S1). One vial contained stimulus, and the other vial was empty and served as a control. Host alarm recognition is determined if the parasite workers entered the control (avoidance) or treatment (attractant) vials more frequently than 50% of the time. One ant was used per assay, and ant choices were counted after 12 hrs. We found that all castes of parasites were more likely to enter the empty vial than the vial containing crushed heads (gynes: 46/65, $p = 0.002$, males: 18/24, $p = 0.023$, workers: 29/36, $p = 0.001$), suggesting avoidance of the host's alarm compounds. General linear mixed models determined no differences in choice behaviors among castes, suggesting they react and respond similarly (likelihood ratio test: $\chi 2 = 1.69$, $df = 2$, $p = 0.43$).

Our results show that all *M. symmetochus* castes avoid entering a vial with crushed heads. This suggests that parasites can detect their host's defensive substance, 4-methyl-3-

heptanone, which makes up 70% of the host ants mandibular chemistry (Norman et al., 2017). It has been established in- and outside of fungus-growing ant systems that ants avoid higher concentrations of 4-methyl-3-heptanone, and are attracted at lower concentrations (Lopes et al., 2023; Moser et al., 1968; Wilson, 1958) suggesting this pheromone elicits dose-dependent defensive behaviors. Additional studies are needed to determine what host alarm compounds and concentrations trigger avoidance and attraction of *M. symmetochus,* and if there is observed cooperation between the host and parasite colonies mediated by chemical communication.

Colony regulation: Host colony growth and size

Obligate social parasites regulate the host colony in order to maximize fitness gains (Adams et al., 2020; Buschinger, 2009; Cini et al., 2019; Schmid-Hempel, 1998) beginning in the early stages of colony life, when guest ant queen expands her worker force alongside the host queen (Box 3; Adams et al., 2012)**.** Finding incipient colonies is rare but we have been observing the development of a parasitized *S. amabilis* colony collected in 2023. In the early stages of the colonies' growth, the host and parasite queens were observed side-by-side inside a crevice within the fungus garden. Brood was present along with five host and three parasite workers. With stable laboratory conditions and unlimited foraging material for the garden, it was only after 8 months that the guest ant queen built her chamber (Adams et al., 2012). As the garden and host numbers grew, the *M. symmetochus* queen maintained 3-5 workers. Once the garden reached approximately 7 cm the queens were rarely seen together. By 12 months, the *Sericomyrmex* queen's colony was estimated to have 100-200 workers, whereas the *Megalomyrmex* queen produced about 10 workers. Like the *M. adamsae* guest ant, it is likely that the parasite queen withholds colony growth until the host colony is large enough to sustain both species (Adams et al., 2012).

Based on 25 years of laboratory and field observations, we believe that parasitized colonies maintain a careful balance. While conflict is frequently observed in laboratory colonies and in some field colonies (Adams et al 2013), the parasite venom appears dose-dependent and not very toxic to host workers. We also have seen that the microbe infused fungus garden suffers following a host/parasite conflict and guest ant alkaloid levels increase (Goes Pers. Obs.). However, it is unclear if host and parasite conflict alters the microbial community or damages the fungal garden.

Bacterial associates and transmission

The mode of transmission is a key determinant of symbiont evolution. Vertically transmitted symbionts are inherited across generations, aligning host and symbiont fitness and favoring cooperation, specialization, and genome reduction (Bright & Bulgheresi, 2010; Sachs et al., 2011). In contrast, horizontally acquired symbionts increase ecological flexibility and genetic diversity but weaken partner fidelity and can favor more opportunistic interactions (Ebert, 2013; Moran et al., 2008). Many systems exhibit mixed transmission, balancing stability with flexibility (Ebert, 2013; Russell et al., 2017).

The fungus garden bacterial community is heavily influenced by horizontally acquired environmental bacteria (Barcoto et al., 2025; Bringhurst et al., 2023), but some taxa are consistently transmitted vertically in a host species-specific manner (Bringhurst et al., 2023; Sadowski et al., 2025). Regardless of transmission mode, attine-associated bacteria provide important nutritional and immunological functions. Vertically transmitted Enterobacteriaceae (e.g., *Klebsiella* and *Pantoea*: Box 3F) fix nitrogen in leaf-cutter ant gardens (Pinto-Tomás et al., 2009), while horizontally acquired bacteria (i.e., *Bacillus* and *Niallia*) aid in plant substrate breakdown (Martiarena et al., 2023). Several bacteria produce antifungals that inhibit microfungal pathogen growth (e.g., *Escovopsis*), and can be horizontally (i.e., *Burkholderia*: Box 3F) or vertically (i.e., *Pseudonocardia* and *Amycolatopsis*) acquired (Francoeur et al., 2021; Sen et al., 2009). Together, these microbial partners form an integrated network supporting nutritional recycling, substrate processing, and pathogen defense within the attine system.

The microbiota of *Megalomyrmex* remains poorly studied; however, the bacterial communities of socially parasitic species, such as *M. symmetochus,* are closely tied to those of their fungus-growing ant hosts (Liberti et al., 2015). In *M. symmetochus*, bacterial taxa including Entomoplasmatales (likely *Spiroplasma*; Box 3E) and Bartonellaceae are shared with host ants and their fungal gardens (Liberti et al., 2015). These are likely horizontally acquired through intimate interactions such as the consumption of host larvae (Box 3G) and fungal garden (Box 3B). These findings suggest that *M. symmetochus* may rely on at least some host-associated microbes.

Aside from these acquired bacterial symbionts, *Megalomyrmex* parasite venom likely influences the host-associated bacterial community found in the fungus gardens. It has been found that *Megalomyrmex* venom is used during offensive (Adams et al., 2015) and defensive actions (Adams et al., 2013), and also has communicative function (Boot et al., in review) as well as antimicrobial properties (Sozanski et al., 2020). Even when not in conflict, it appears that *M. symmetochus* applies their venom to the fungus garden (Adams et al., 2013), exposing the microbial community to their venom. Despite this, it is unclear whether the parasite presence affects fungal garden bacterial communities.

To test the impact of parasitism in bacterial communities, we conducted a culture-based study growing garden-derived bacteria from recently collected parasitized and non-parasitized *S. amabilis* (Supplemental Protocols S1). Our results found bacterial communities retained putatively important taxa, such as *Pseudomonas* and *Pantoea* (Fig. 2), suggesting they have *M. symmetochus* venom tolerance. However, some taxa, including *Spiroplasma* and *Burkholderia*, were not recovered, likely reflecting culturing limitations (Francoeur et al., 2021; Green et al., 2025). This highlights the need for culture-independent approaches to fully assess how parasitism influences microbial community structure.

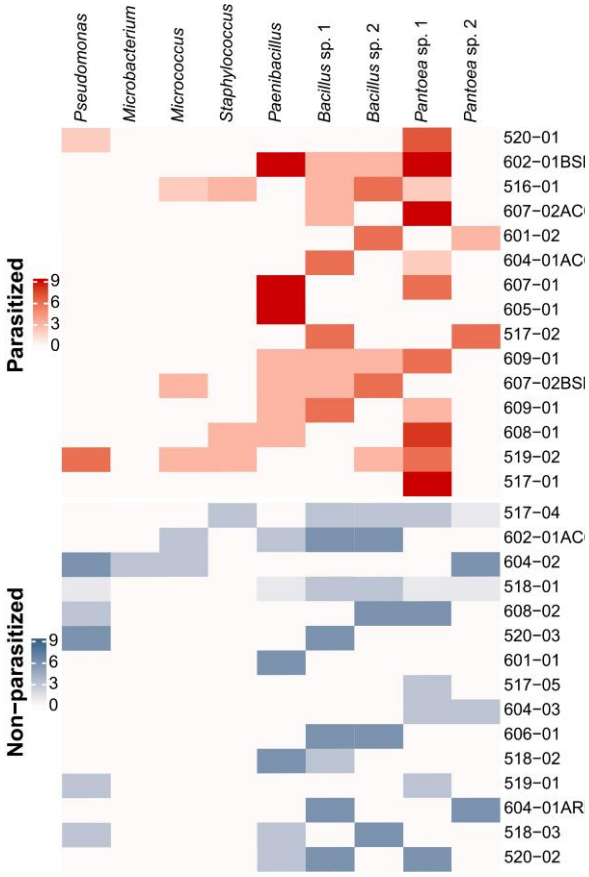


Figure 2. Presence of cultured bacteria from the fungus gardens of parasitized (n = 15) and non-parasitized (n = 15) *Sericomyrmex amabilis* colonies. Each colony had bacteria grown with 9 replicates on LB agar. Shortened collection codes denote the colonies.

Vertical transmission may facilitate associations over evolutionary time, potentially resulting in coevolution between hosts and symbionts (Bright & Bulgheresi, 2010; Krishnan et al., 2024; Wilson & Duncan, 2015). Under such conditions, bacterial strains may adapt to the chemical environment of their hosts, including exposure to alkaloids produced by *M. symmetochus*. The similarity we found between parasitized and non-parasitized gardens may be due to vertically transmitted microbiota with parasite venom tolerance.

In the future, we intend to test this with experimental assays. We propose that attine-associated core bacteria may have unique strains that have coevolved within the host-parasite lineages. The same bacterial species are likely found with sympatric congeners (i.e., *Sericomyrmex opacus* Mayr *or Sericomyrmex mayri* Forel) but do not host *Megalomyrmex* social parasites. Such comparisons would help disentangle whether venom tolerance reflects historical coevolution within parasitized systems or generalized *M. symmetochus* venom alkaloid resilience by attine-associated bacteria.

Ant and microfungi threats

The stability of ant symbioses can be disrupted by predators, social parasites, and microbes that can be transient or more persistent over time. There are several natural

enemies of fungus-growing ants, including other ants (e.g., *Neivamyrmex* army ants, *G. hartmani* agro-predators; (Dijkstra & Boomsma, 2003; LaPolla et al., 2002). Although rarely seen, army ants are expected to raid *S. amabilis* nests, as they do in other attine ant systems (e.g., *Apterostigma*, *Mycocepurus*, and *Mycetophylax*; Ješovnik & Schultz, 2017). *Gnamptogenys hartmani* is a more complex interspecific interaction with the host and guest ant. The agro-predators raid *Sericomyrmex* colonies usurping their nest structure and stealing their garden and brood (Dijkstra & Boomsma, 2003).

Sericomyrmex amabilis workers can resist raids, but these encounters are costly (Adams et al., 2013). However, nests parasitized by *M. symmetochus* guest ants show higher survival during raids since the guest ants confront the agro-predators, deploying alkaloid venom to repel and confuse the attackers (Box 2 E&F; Adams et al., 2013). Also, *G. hartmani* appears to avoid raiding colonies that harbor *M. symmetochus*, suggesting that the presence of the guest ant itself acts as a deterrent. Colonies with *M. symmetochus*, should therefore incur a higher lifetime fitness benefit, resulting in strong selection to tolerate guest ant invasions, as is expected in other protective symbiont systems (Hopkins et al., 2017; Jaenike et al., 2010). Selection for host protection by the guest ant likely arose from resource conflicts with other parasites (Gerardo et al., 2020; Mideo, 2009; Weinersmith & Earley, 2016), combined with the life-long dependence of *M. symmetochus* on a single host colony (Adams et al., 2013).

Microbes also threaten both ant species and the symbiotic species network (Box 2B). Given their social lifestyle, *M. symmetochus* and its host face similar pathogenic pressures (May & Anderson, 1979; Schmid-Hempel, 1998). Ants are vulnerable to generalist entomopathogenic fungi and use cleaning behaviors and social organization to control infections, providing colony-level protection (Cremer et al., 2007). Transient microbes are brought to the nest through harvesting plant material (Box 1C; Van Bael et al., 2012) or by the foragers (Augustin et al., 2017). Some may be harmless, while others, opportunistic pathogens to the fungiculture system (e.g., *Escovopsis weberi*; Currie et al., 1999; Montoya et al., 2025; Rodrigues et al., 2011). Since *M. symmetochus* is dependent on the fungus garden for nutrients and shelter, it is reasonable to expect that they will help their host in protecting the fungal garden. *Sericomyrmex amabilis*, like other fungus-growing ants, has grooming and weeding behaviors to remove contaminants from the garden (Currie & Stuart, 2001). Both ant species clean themselves and nestmates with self- and allo-grooming and the *S. amabilis* host grooms the guest ant *M. symmetochus* after fungal pathogen exposure (Bruner et al., 2014; Wheeler, 1925).

From the parasite's perspective, it stands to reason that they would use their alkaloidal venom prophylactically. Ant alkaloids serve diverse functions, such as pheromone signaling, offensive toxins, and antimicrobial activity (Fox & Adams, 2022). Alkaloids disrupt cell membranes by incorporating into lipid bilayers, altering membrane fluidity, and facilitating pore formation ultimately leading to cell death (Efimova et al., 2020; Jubair et al., 2021). Much like alkaloidal fire ant venoms found to inhibit fungal pathogens including *Fusarium* and *Beauveria* (Sharma & Upadhyay, 2021; Storey et al., 1991), the venom of *Megalomyrmex peetersi* can also inhibit bacterial growth (Sozanski et al., 2020). This leads us to expect that *M. symmetochus* venom also has antimicrobial properties, since this natural product has been well-known for its antimicrobial potential (Cushnie et al., 2014). To test this, we performed disk-diffusion assays to assess whether its alkaloidal venom inhibits pathogenic fungi isolated from *S. amabilis* colonies. We evaluated fresh *M. symmetochus* venom (with biomolecules), its synthetic alkaloid (without biomolecules), methanol as a solvent control, and Amphotericin B (an antifungal) as a positive control for fungal inhibition.

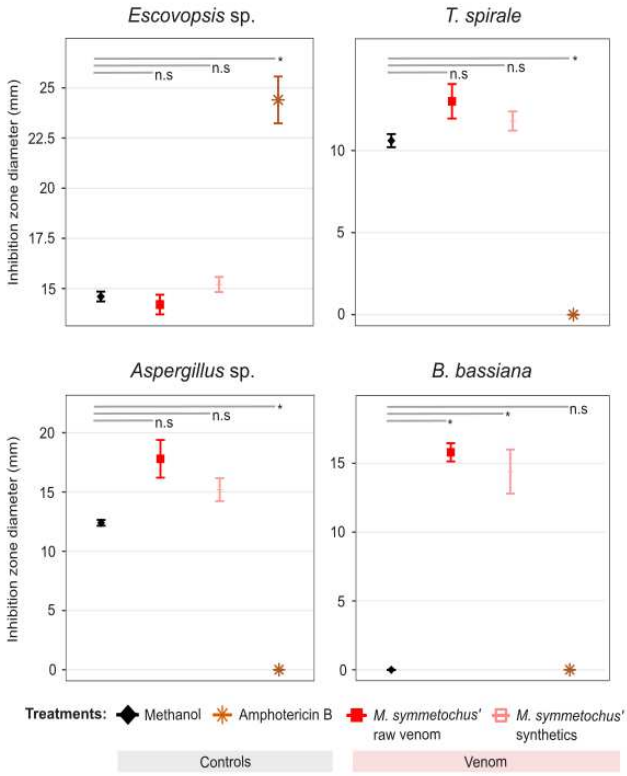


Figure 3: Antifungal activity of the raw venom from M. symmetochus, their synthetic alkaloid ((5Z,8E)-3-butyl-5-hexylpyrrolizine), methanol solvent (negative control), and the antifungal Amphotericin B (positive control) towards four fungi pathogens isolated from S. amabilis nests. The inhibition zone was measured as the halo diameter in mm. Comparisons were made only between the negative control (Methanol) and a single treatment per time (Mann-Whitney U test, α= 0.05). Asterix means significant difference between the respective treatment and the negative control; 'n.s' stands as non-significant.

We found that fungal species varied significantly in their sensitivity to these treatments (Fig. 3). Overall, *M. symmetochus* venom and its alkaloid molecule inhibit common garden contaminants (*Aspergillus* sp.) and an entomopathogen (*B. bassiana*), both relevant threats to host and parasite. However, the mycoparasite *E. weberi* was inhibited only by Amphotericin B. Because *M. symmetochus* venom shows antifungal potential, cleaning behavior involving alkaloidal venom application (Box 3G) likely adds to the protective benefits the host incurs as a result of this symbiotic association.

Distribution and dispersal

Successful dispersal of an obligatory parasite depends on host distribution across space and time (Combes, 2005; Medina & Langmore, 2019). For social parasites, the timing of their reproductive cycle starting with mating and dispersal (reproductive timing) is a key constraint. They must balance the risks associated with emerging too early—when recently founding host colonies are unavailable, with emerging too late—when host colonies are established or are already parasitized. Spatial variation in parasitism rates can also reflect selective pressures (i.e., predation, pathogens, and host density) that determine whether parasites can successfully invade and persist in a given environment (Fischer & Foitzik, 2004; Foitzik et al., 2009; Thompson, 2005).

Megalomyrmex symmetochus have evolved traits that help them overcome challenges related to locating and infiltrating young *S. amabilis* colonies. It is established that (1) young *M. symmetochus* queens follow host trail pheromone (this study), (2) alkaloid venom dampens host aggression (Boot et al., in review), and (3) host workers tolerate the parasite, illustrated by their submissive behaviors (Neupert et al., 2018) (Supplemental Video SV4). These mechanisms of host finding and host management suggest that the stability of this system may be due to a long co-evolutionary history (Sozanski, 2025). Thus, it would not be surprising if parasite reproductive timing is offset from their hosts, emerging at an optimal moment. Kaspari et al. (2001) found that *M. symmetochus* mating flights in Panama peak in May and continue into July in a single annual pulsed reproductive event. Thus, host flights should occur several months prior, providing *M. symmetochus* with a window of opportunity to invade young colonies.

We evaluated three factors to illuminate traits that help explain parasite success. We provide parasitism rate data for each of our sites to illustrate variability of parasite abundance across the landscape. At a single site, we recorded when reproductives were produced in host and parasite colonies to evaluate the timing of reproduction. Finally, we provide measurement data to consider the potential for flight ability and energetic investment in young queens.

Spatial – parasitism rates across sites

To access parasite success across the landscape, we collected parasitism rate data ranging from 2010-2025 from collections across Panama and Costa Rica (Fig. 4). Notably, Plantation Road, our most heavily sampled site, showed 28% parasitism (n = 133), while collections on Barro Colorado Island (BCI) revealed 55% parasitism (n = 40). Rates varied substantially between years (Sarkar et al., in review), however we believe this is likely due to variations in sampling effort across years, not fluctuations in parasitism rate. Like Wheeler (1925), we have observed exceptional years on BCI (2012: 4/7 colonies; 2025: 15/19 colonies). We also identified sites apparently lacking parasites (Bocas: n = 16 colonies; Cerro Azul: n = 13 colonies), though limited sampling precludes strong conclusions about parasite absence.

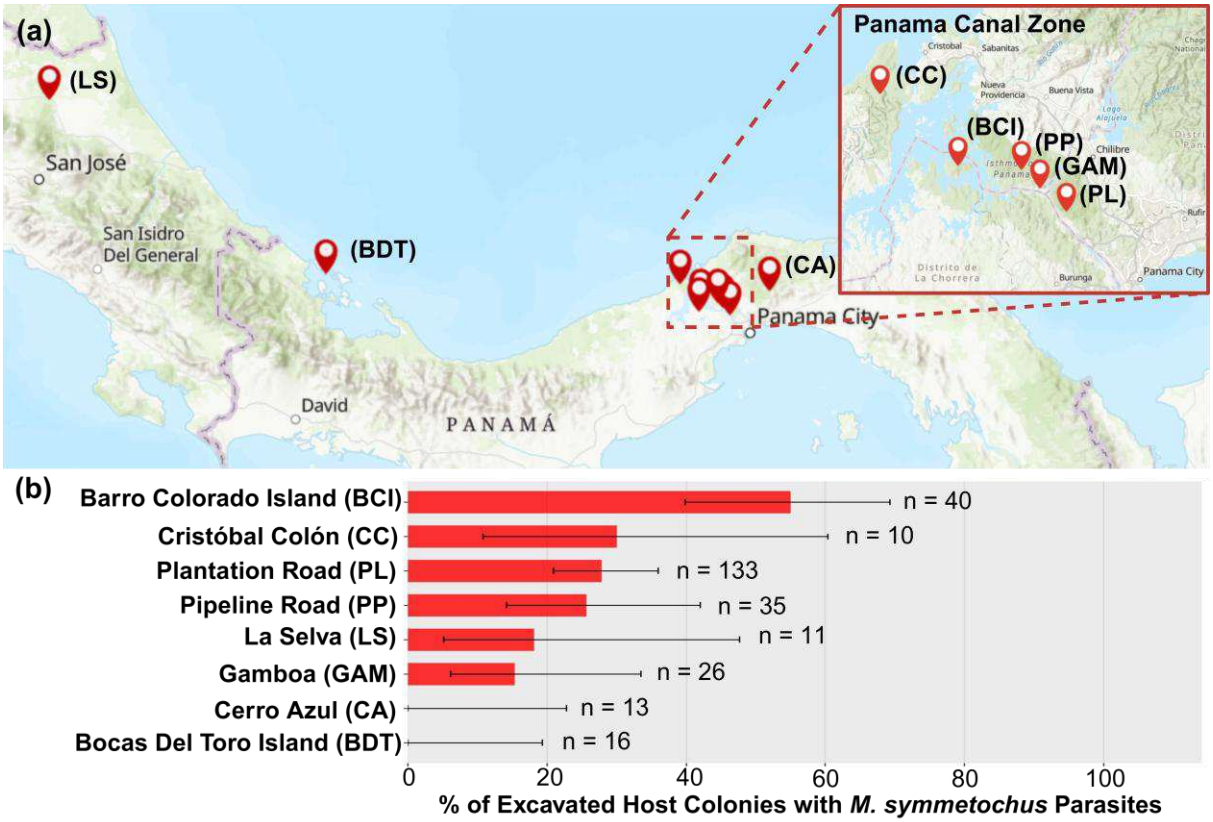


Figure 4. Parasitism rate of *M. symmetochus* on *S. amabilis* colonies across collection sites. (a) Site locations across Costa Rica and Panama. (b) Parasitism rates for each site.

Alate production

To assess temporal patterns in alate production, we excavated chambers from 66 colonies in the Panama Canal Zone between May 2022 and November 2023. Non-parasitized *S. amabilis* colonies (n=55) produced male or female alates (or both) between the months of May and October (note: no collections occurred November – April due to the difficulty of excavating colonies in dry soil). In contrast, among 11 parasitized *S. amabilis* colonies, only one produced males in September (Fig. 5c), whereas non-parasitized host colonies started producing males as early as June. Because we surveyed only 1-3 chambers per colony (non-lethal colony collection), our results may be an underestimation. However, in our many years of observing parasitized colonies in the lab and field, it is rare to see host colonies produce reproductives while parasitized. When female alates do appear, they may be castrated by the parasite workers (Box 3B; Adams et al., 2012; Boudinot et al., 2013), thereby forcing them to becoming part of the worker caste. Interestingly, we found that *M. symmetochus* alate production peaked between May and July as expected (Kaspari et al., 2001). These temporal patterns suggest that the host species does not have a single pulsed reproductive bout but instead produces reproductives for several months, similar to another fungus growing ant species, *Cyphomyrmex costatus* (Kaspari, Pickering, Longino, et al., 2001).

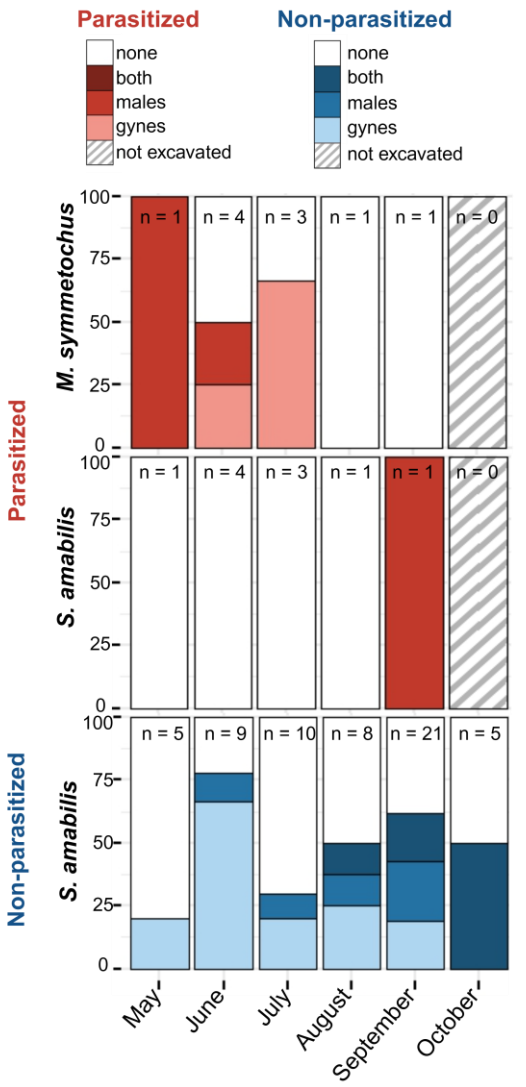


Figure 5. Excavation survey of *M. symmetochus* and *S. amabilis* alates in Panama during the wet season. Y-axis represents the percentage of excavated *S. amabilis* colonies with alates present. Red indicates alate data collected from parasitized *S. amabilis* colonies.

<u>*Morphology – comparing host and parasite queens*</u>

We hypothesize that selection—driven by the spatial aggregation of host colonies—may act against aerial dispersal in *M. symmetochus* parasite queens because dispersing by wing may risk landing outside of the host patch (Cronin et al., 2013; Helms & Godfrey, 2016). In contrast, the host should have strong flight muscles corresponding with a large thorax volume (Stille, 1996) so they can mate in mixed leks and disperse from their natal, and sometimes parasitized, populations (Møller et al., 2004; Peeters & Ito, 2001; Suhonen et al., 2010) as predicted by host-parasite dispersal theory (Møller et al., 2004). We measured MicroCT images to compare the host and parasite species. The mesosoma of

Sericomyrmex gynes is 2.28 ± 0.18 (mean, SD; n = 3) times the size of the head compared with *Megalomyrmex* where this proportion is very similar (2.23 ± 0.13, mean/SD; n = 3). In other words, in both species the thorax is 69% of the head and mesosoma mass.

Our results regarding reproductive production timing and gyne morphology were surprising. Because *M. symmetochus* and most other sympatric tropical ant species have a strong pulsed flight phenology that occurs during the rainy season (Donoso et al., 2022; Kaspari, Pickering, & Windsor, 2001) we expected to see a similar pattern in *S. amabilis*. Instead, alates are produced for several months, suggesting continuous flight activity. This leads one to wonder if the reproductive timing of *S. amabilis* is driven by the high level of parasitism in the population.

Young guest ant queens mate on the ground, have limited dispersal, and invade another ant species nest with a constant food supply (host brood and garden). Thus, we would not expect a large thorax that stores fat and has extensive musculature in the parasite. On the other hand, our observations of this and other guest ant species provide a different perspective. We have found that young guest ant queens withhold their own reproduction while the host colony grows, much like the guest ant *M. adamsae* (Adams et al., 2012). Therefore, she may indeed need fat reserves to sustain her. Measuring muscles and fat reserve composition could help explain if muscles are supporting flight in *S. amabilis* and if fat reserves are supporting heath/sustenance in *M. symmetochus*.

It would be interesting to see if the phenology and morphology patterns hold in *S. amabilis* populations where pressures from parasites are lower (e.g., Bocas del Torro Island, Cerro Azul) or environmental factors differ. Local ecological conditions could influence parasite success, consistent with geographic mosaic theory (Thompson, 1994, 1999, 2005). Reductions in host fecundity at the colony level could reduce the number of potential host colonies in the future, creating the conditions for boom-bust dynamics similar to those in other host-parasite systems (Hudson et al., 1998; MacDonald & Brisson, 2022).

Conclusion

Despite contemporary trends seen in research institutions that de-emphasize natural history in light of theoretical, laboratory, or simulation-based research (Nanglu et al., 2023), we choose to step-back and observe our system as a whole. The geographic mosaic theory of coevolution emphasizes the coevolutionary dynamics between interacting species (Thompson, 1994, 1999, 2005). With this in mind, we demonstrate how host communication systems are exploited, beginning on the forest floor surface, and how interspecies communication continues within the depths of a *S. amabilis* host colony. Our work illuminates the interconnectedness of relationships, how they begin, are maintained, and change over space and time. The endlessness of describing the complexities of symbiotic species networks can be rewarding and always leads to more questions than answers.

As with any species interaction, it is important to consider the fitness impacts of the coevolving organisms and the costs and benefits of their communication systems. While

we did not study coevolution directly, we see positive signs that fungus-growing ants and *Megalomyrmex* social parasites have likely been coevolving for approximately 57 million years (Sozanski, 2025), and the behavioral evidence presented here suggests that this long history has resulted in a remarkable degree of chemical fluency between the host and parasite. We have shown that young, mated queens follow the host trail pheromone while seeking a nest to invade, and that parasite workers, males, and gynes all respond to host alarm pheromone indicating that *M. symmetochus* guest ants have tapped into the host's communication systems, eavesdropping on host-derived signals to navigate both host location and danger avoidance. The host ants, in turn, show signs of recognition of the parasite communication system (Boot et al. in review). Like the invading *Gnamptogenys* raider ants (Adams et al., 2013), the fungus-growing host ants alter their behavior when exposed to or within the vapor space of a *M. symmetochus* venom (Neupert et al., 2018). This suggests the host is sensitive to the guest ant's venom as a signal, not just after direct contact but from a distance (Boot et al., in review). Behavioral traits are shaped both by genetically-determined species-specific recognition signals and the experiential refinement of recognition through repeated encounters (Bey et al., 2025; Errard et al., 2008). This reciprocal chemical recognition capacity is consistent with the broader framework of social parasite coevolution, in which host recognition systems and parasite chemical strategies evolve in response to one another over time (Brandt, Foitzik, et al., 2005; Lorenzi, 2006; Nash et al., 2008). Regardless of whether this recognition is innate or learned, these two species have established a behavioral repertoire for interactions. Identifying specific compounds, concentration-dependent responses, and fitness consequences for both parties, is an important direction for future work.

Host-parasite Conflict and Cooperation

We have collected several parasitized colonies with over 10 nest chambers, suggesting these two species cohabit peacefully for many years. However, we have found injured guest ants in the field and observed direct conflict in the lab. Peaceful cohabitation can be disrupted with perturbations, that seems more frequent when the parasites are producing reproductives (Adams Pers. Obs.). We have, however, observed young queens grow their colonies side-by-side for nearly 3 years. Despite its muted CHC profile, the guest ant is detected but tolerated by the host (Neupert et al., 2018). Overtime, as the two colonies grow, the host has the possibility of a rebellion due to sheer numbers. One significant reproductive consequence for the host is the direct consumption of gyne larvae, and later, castration of *S. amabilis* gynes by the guest ants (Box 3H; Boudinot et al., 2013). With their wings removed, *S. amabilis* gynes are forced to stay within the nest. Interestingly, males are left untouched and disperse as usual. Direct consumption and manipulation of *S. amabilis'* reproductive caste by *M. symmetochus* may explain why we see few *S. amabilis* host alates in parasitized colonies.

It is not surprising to see that the guest ants protect their host colony due to their obligate dependence (Adams et al., 2013; Combes, 2005). Through direct and indirect interactions, protective symbionts prevent the success of competing parasites (Brownlie & Johnson, 2009; Haine, 2008; Hopkins et al., 2017). For example, when a host harbors guest ants,

raiding *G. hartmani* parasite scouts are aggressively attacked. They may also avoid a raid presumably due to guest ant-derived odors (Adams et al., 2013). *Gnamptogenys* raiders are generalist parasites (Dijkstra & Boomsma, 2003) and an altercation with *M. symmetochus* may shift the raider's preference to a different host species, negatively impacting alternative host species at the community level (Hopkins et al., 2017). *Gnamptogenys hartmani* are frequently found occupying the nest of the sympatric host *Mycetomoellerius mikromelanos* (Dijkstra & Boomsma, 2003; Adams Pers. Obs.). These community-level ecological processes caused by protective symbionts can have profound impacts on the population dynamics of a host species or lead to extinction cascades in the community (e.g., ants protecting scales on Christmas Island; O'Dowd et al., 2003). This punctuates the importance of studying a system using an integrative framework to understand how host symbiotic species networks are formed, maintained and influenced through ecological interactions.

Microbial Manipulations

The microbiota of fungus-growing ants and their cultivars form a dynamic community that is also shaped by ecological processes. The attines manipulate the garden microfungal community through weeding and cleaning behaviors used against entomopathogens and mycopathogens (Currie & Stuart, 2001; Goes et al., 2022). The garden bacterial community is likewise actively manipulated by the ants, such as the maintenance of Entomoplasmatales and Enterobacteriales symbionts (Bringhurst et al., 2024), but also passively manipulated through supplying substrate that are selectively metabolized by particular bacteria, facilitating growth (Barcoto et al., 2025). The parasites can also manipulate the microfungal community through dispersing their venom alkaloids. We show that raw venom and synthetic (5Z,8E)-3-butyl-5-hexylpyrrolizidine are effective at suppressing the growth of entomopathogens, suggesting the parasites may contribute to colony-level social immunity beyond defense against raider ants. Collectively, the microbes produce a rich landscape of volatile and non-volatile secondary metabolites with distinct chemical profiles (Aguilar-Colorado & Rivera-Chávez, 2023; Silva-Junior et al., 2017). Ants can discriminate between entomopathogenic fungi (Goes et al., 2022) as well as core bacterial symbionts (Zhang et al., 2007), mediating the structure of the microbial community. However, it is currently unclear in most cases what specific compounds the ants detect and react to. Characterizing volatile metabolites produced by fungi and bacteria integrated within this symbiotic network represents a logical next step, making the chemical ecology of this system a critical frontier for future investigation.

Both bacterial and fungal microbiota also interact. Functionally redundant bacteria can compete against each other, such as antimicrobial-producing strains of *Amycolatopsis* that produces nocamycin V, which suppresses the growth of *Pseudonocardia*, (Hansen et al., 2022). Such interactions ultimately influence the fitness of the overall symbiosis and may impact long-term host-associations (i.e., vertical transmission. Other microbial interactions enhance overall symbiosis health. For example, *Burkholderia* from higher attines (e.g., *Atta*, *Sericomyrmex*, *Paratrachymyrmex*) produces pyrrolnitrin and burkholdine, which suppresses both transient (i.e., *Aspergillus*) and mycopathogenic

microfungi (Francoeur et al., 2021). Garden bacteria can also promote the growth of other microbes, likely through the production of autoinducer compounds (Mukherjee & Bassler, 2019), but these actions are strain-specific, resulting in an intricate network of antagonistic and cooperative microbial relationships (Martiarena et al., 2023). Complicating this is the environmental variation in the microbial community composition (Bringhurst et al., 2023), resulting in population-specific networks of microbial interactions for each species.

Geographic mosaic

If guest ant social parasites coevolve with their host (e.g., *M. symmetochus* with *S. amabilis*; *M. adamsae* with *M. mikromelanos* and *M. zeteki*), we would expect a geographic mosaic of co-adapted populations that can serve as natural experiments for understanding reciprocal coevolution within a multipartite symbiosis. Each population would face differing selective pressures dependent on the composition of the local symbiotic species network. Reciprocal pressures among those that are in conflict or have mutualistic gains are expected to be context and frequency dependent (Thompson, 1999). The composition of the local symbiotic species network will shape the direction and intensity of these pressures. Including host and parasite dispersal dynamics in this framework will be essential for interpreting how symbiotic networks are assembled and maintained across space and time. Future population genomics research on host and guest ant species is underway. We aim to understand the interconnectedness and gene-flow of parasitized populations, to unveil hidden genetic mosaics.

The *M. symmetochus* – *S. amabilis* – microbe system is an especially compelling case for applying the geographic mosaic framework, as the outcome for these interactions (host tolerance, reproductive cycles, and host or parasite defense against pathogens/invaders) likely vary across *M. symmetochus'* geographic range (Gomulkiewicz et al., 2000). In conservation, preservation of symbiotic networks of organisms requires the protection of geographically distributed populations to maintain the genetic health of all members. Carthey et al. (2020) argue that the relevant biological unit for conservation is not the individual organism but the holobiont (the host together with its full complement of microbial and symbiotic partners), and that conservation efforts which focus solely on the host species risk preserving an incomplete version of the necessary community members (Carthey et al., 2020). Losing *M. symmetochus* from a population does not only remove a parasite, it potentially dismantles a co-evolved defensive partnership, leaving the host vulnerable to virulent ant- and microbial- antagonists. Effective conservation of this system therefore requires attending to the full spatial extent of the interaction network, protecting not just *S. amabilis* and *M. symmetochus* colonies across their range, but the geographically variable assemblages of guests, garden microbes, and local enemies within where coevolutionary processes are actively unfolding.

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