Social Immunity, Worker Investment, and Fungal Resistance in Ants

by

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Abstract

This thesis examines ant pathogen defense at two levels. Chapter 1 reviews behavioral, chemical, symbiotic, and internal immune defenses, emphasizing that social immunity and individual immunity function as interacting layers rather than replacements for one another. Chapter 2 tests whether colony-size-related worker disinvestment extends from morphology to fungal resistance. After screening nine *Beauveria* isolates against *Solenopsis invicta*, *Beauveria bassiana* ARSEF 2597 was selected for comparative assays across 16 ant species and six conidial concentrations. Susceptibility varied among species and doses, and resistance showed phylogenetic signal at high concentrations. Larger-colony species had reduced body-size-corrected cuticle investment, supporting structural worker disinvestment, but fungal resistance was not consistently predicted by colony size, worker size, or cuticle investment. These results suggest that colony size predicts worker structural investment but not individual fungal resistance.

Artificial Intelligence (AI) Use Disclosure Statement

In preparation of this thesis, artificial intelligence tools were used to support organization of written sections, editing for grammar and clarity, and assistance with code. The author acknowledges full responsibility for the intellectual content of this work and has reviewed and revised all AI-assisted material for accuracy.

Digital Accessibility Disclosure Statement

In the preparation of this thesis, the following digital accessibility tools were used to ensure this document complies with federal requirements: alternative text was created for figures and tables in Microsoft Word. The author acknowledges full responsibility for the intellectual content of this work and has made a good faith effort to comply with digital accessibility requirements in publishing, wherein the nature of the content does not significantly change in order to do so. Furthermore, all content has been reviewed and revised to meet these requirements prior to final publication.

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List of Abbreviations

ARSEF	Agricultural Research Service Collection of Entomopathogenic Fungi
ETD	Electronic thesis or dissertation
PDA	Potato dextrose agar
PGLS	Phylogenetic generalized least squares
RMST	Restricted mean survival time

Chapter 1: Social insect pathogen defense

Introduction

Group living creates important advantages for social organisms, but it can also increase exposure to infectious disease (Schmid-Hempel, 1998; Pie et al., 2004; Kappeler et al., 2015). Individuals that live together often share nest sites, food resources, waste areas, and physical contact networks, all of which can increase opportunities for pathogens and parasites to spread (Schmid-Hempel, 1998; Pie et al., 2004; Kappeler et al., 2015). These risks are especially relevant for social insects. Ant colonies, for example, may contain hundreds to millions of closely interacting individuals that live in stable nests, exchange food through trophallaxis, groom one another, care for brood, and repeatedly contact the same nest surfaces and refuse areas (Schmid-Hempel, 1998; Cremer et al., 2007; Meunier, 2015). In this context, social organization can create conditions that favor pathogen transmission, particularly in humid nest environments where microbes may persist for long periods (Pie et al., 2004; Cremer et al., 2007).

In response to high disease pressures, ants and other social insects have evolved a broad set of colony-level “public health” strategies that reduce pathogen exposure and limit transmission (Traniello et al., 2002; Cremer et al., 2007; Cremer, 2019). Collectively, these terms are referred to as social immunity (Cremer et al., 2007, 2018). Ant disease defense can be grouped into behavioral defenses, external chemical defenses, symbiotic defenses, and internal immune responses, which together act at different stages of pathogen exposure and infection. Many of these strategies mirror those found in human responses to disease, including social distancing (Heinze & Walter, 2010; Pusceddu et al., 2021; Stroeymeyt et al., 2018), the production of antibiotics (Beattie et al., 1986; Currie et al., 1999; Poulsen et al., 2002; Yek & Mueller,

2011), and even vaccination (Konrad et al., 2012). This review frames ant disease defense as a set of interacting layers and uses that perspective to introduce the question tested in Chapter 2, whether worker disinvestment in larger colonies also involves reduced individual fungal resistance.

Behavioral Defenses

The first line of pathogen defense is behavior. Hygienic behaviors function to avoid or remove pathogens before they become systemic infections to protect both individual ants and their colonies as a whole (Cremer et al., 2007, 2018; Stroeymeyt et al., 2014). Ants rely on both individual and collective behaviors to defend themselves against disease. Individual behaviors include grooming and pathogen avoidance, while collective behaviors include interactions among nestmates. Many of these behaviors are supplemented by chemical interventions, such as antimicrobial secretions, that ants secrete externally and apply on themselves, their nestmates, or to the nest in general. These behavioral and chemical defenses form an integrated social immune system that reduces pathogen transmission and infection risk within densely populated ant colonies.

Grooming is one of the best-studied forms of social immunity. Workers remove fungal conidia and other infectious particles from their own bodies or from nestmates before pathogens can establish infection (Currie & Stuart, 2001; Hughes et al., 2002; Reber et al., 2011). Self-grooming is especially important against entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana*, which first attach to the insect cuticle before penetrating into the body. Allogrooming can further reduce

pathogen load because nestmates can clean body regions that exposed workers cannot easily reach themselves (Reber et al., 2011).

Although grooming is often protective, its effects are not always straightforward. In *Formica selysi*, workers exposed to *Beauveria bassiana* increase self-grooming, and nestmates remove conidia through allogrooming before fungal infection becomes established (Reber et al., 2011). In leaf-cutting ants such as *Acromyrmex echinatior*, workers exposed to *Metarhizium anisopliae* also receive increased grooming from nestmates, which can reduce fungal establishment and mortality (Hughes et al., 2002; Walker & Hughes, 2009).

However, grooming may also create risk if infectious material is transferred among nestmates. In *Lasius neglectus* exposed to *Metarhizium brunneum*, fungus-exposed workers increased self-grooming and reduced grooming directed toward healthy nestmates, while allogrooming from nestmates toward exposed workers did not significantly increase. Modeling from this system showed that allogrooming can either reduce or increase disease spread depending on pathogen infectiousness and the effectiveness of social immune defenses (Theis et al., 2015). Thus, the epidemiological effect of grooming depends not only on how often grooming occurs, but also on who grooms whom and on the biology of the host–pathogen interaction.

Brood care provides another important route of behavioral disease defense. Ant brood are often highly vulnerable to infection because they are immobile, aggregated, and dependent on workers for grooming and sanitation. In *Lasius neglectus*, workers disinfect fungus-exposed brood by taking up acidic poison from the acidopore and applying it during brood grooming, which reduces fungal conidial viability (Tragust et al.,

2013). When infection has already progressed, colonies may use a more destructive form of disinfection. Workers can detect infected pupae, remove them from their cocoons, perforate the cuticle, and apply antimicrobial poison before the pathogen completes development and sporulates (Pull et al., 2018). This behavior prevents infected brood from becoming sources of infectious spores inside the nest and has been compared functionally to the removal of infected cells in multicellular organisms (Pull et al., 2018).

Necrophoresis, or the removal of dead colony members, is another widespread hygienic behavior in ants. Wilson et al. (1958) classically showed that corpse-removal behavior is triggered by chemical cues associated with dead nestmates. In the red imported fire ant *Solenopsis invicta*, corpse removal is mediated by chemical cues associated with death and decomposition, and workers remove cadavers from the nest to reduce contact between living nestmates and potentially infectious material (Choe et al., 2009; Howard & Tschinkel, 1976). Broader comparative and experimental work has shown that corpse removal can improve colony survival by reducing pathogen exposure (Diez et al., 2014). In some systems, corpses may also be buried, moved into refuse areas, or otherwise isolated from the main colony, reducing the probability that pathogens spread from dead individuals to workers or brood (Diez et al., 2013, 2014).

Waste management also contributes to colony hygiene. In leaf-cutting ants, waste can include spent fungal substrate, contaminated plant material, dead workers, and microbial pathogens. Species such as *Atta colombica* and *Acromyrmex echinatior* compartmentalize waste into specialized midden chambers or external refuse dumps, physically separating contaminated material from brood chambers and fungal gardens

(Bot et al., 2001; Hart & Ratnieks, 2002). In some leaf-cutting ants, waste-management workers also have restricted task repertoires and limited contact with the rest of the colony (so called “untouchable” castes), which reduces the risk that pathogens associated with refuse will be carried back into cleaner nest areas (Hart & Ratnieks, 2002). This spatial and behavioral separation functions as a form of colony-level quarantine.

Disease defense can also occur through changes in social interactions. In *Lasius niger*, pathogen exposure can alter colony interaction networks by increasing spatial and social separation between foragers and the queen-and-brood core of the colony (Stroeymeyt et al., 2018). This pathogen-induced network reorganization reduces transmission routes to the most valuable colony members and shows that disease defense can involve changes in colony organization, not only direct cleaning behaviors (Stroeymeyt et al., 2018). In *Temnothorax unifasciatus*, moribund workers often leave the nest before death and die in social isolation, reducing contact with nestmates during a period when they may pose a high infection risk (Heinze & Walter, 2010). Ants can therefore reduce disease transmission through colony-level reorganization and through self-removal of infected individuals.

Spatial organization may also contribute to disease defense through nest architecture. Theoretical and empirical work has long suggested that interactions between nest architecture, worker density, and activity patterns influence pathogen transmission in social insects (Naug & Camazine, 2002; Pie et al., 2004; Stroeymeyt et al., 2014). Work on *Lasius niger* provides experimental evidence that ants can actively change nest architecture following pathogen exposure. When colonies were exposed to

Metarhizium brunneum, ants excavated nests with greater spacing between entrances, reduced network density and efficiency, increased network diameter and modularity, and lower chamber centrality. Simulations indicated that these architectural changes reduced pathogen transmission and acted synergistically with self-isolation by exposed workers (Leckie et al., 2025). This form of “architectural immunity” expands the concept of social immunity by showing that ants may modify the physical structure of the nest to reduce epidemic risk.

Fungus-growing ants also use behavioral defenses to protect their cultivated fungal gardens. Attine ants groom and weed their gardens, removing contaminated substrate before specialized fungal parasites such as *Escovopsis* can spread through the mutualistic crop (Currie et al., 2003; Currie & Stuart, 2001). Workers may also use structures such as the infrabuccal pocket to collect, concentrate, and process fungal spores and debris, reducing contamination of the garden (Little et al., 2005). These behaviors are especially important because disease in the fungal garden threatens not only individual workers but also the colony’s food source.

External Immunity

In addition to behavioral defenses, ants use external chemical defenses to suppress microbial growth on the body surface, on brood, within fungal gardens, and in the nest environment. These defenses are especially important because many entomopathogenic fungi, including *Metarhizium anisopliae* and *Beauveria bassiana*, infect insects by attaching to and penetrating the cuticle before proliferating in the hemocoel (Boucias & Pendland, 1998). It is therefore unsurprising that social insects

are often predicted to invest more heavily in antimicrobials than solitary species (Hoggard et al., 2011; Meunier, 2015; Stow et al., 2007). Studies in bees and wasps have found that social species and those that live in larger colonies produce more potent antimicrobials, and research on ants has found that over half invest in broad spectrum antimicrobials. Studies on social insect antimicrobials have driven interest in these insects as new sources of antibiotics in human systems as well as driven interest into the evolution of these disease defense strategies.

One of the most important external defenses in ants is the metapleural gland, a paired exocrine gland unique to ants and widely regarded as a major innovation in ant disease defense (Hölldobler & Engel-Siegel, 1984; Yek & Mueller, 2011). Metapleural gland secretions of some species contain antimicrobial compounds that inhibit bacterial and fungal growth, although their chemical composition varies among species (Yek & Mueller, 2011). In fungus-growing ants, these secretions can inhibit pathogens associated with the fungal garden, including *Escovopsis* and other fungi that threaten the cultivated fungus (Bot et al., 2002; Poulsen et al., 2002). Experimental studies have shown that blocking or impairing metapleural gland function can increase susceptibility to fungal infection, supporting the gland's role as an external antimicrobial defense (Fernández-Marín et al., 2006; Poulsen et al., 2002).

The metapleural gland is not simply a passive source of antimicrobial chemicals. Ants can actively deploy its secretions during pathogen exposure. In fungus-growing ants, workers groom the gland and distribute secretions over the body surface when exposed to fungal pathogens, suggesting that antimicrobial defenses can be behaviorally regulated and directed toward infection risk (Fernández-Marín et al., 2006).

Comparative work further suggests that metapleural gland investment varies across ant lineages and ecological contexts. For example, fungus-growing ants show substantial variation in the size, quantity, and antimicrobial activity of metapleural gland secretions, with derived leaf-cutting ants showing especially strong investment in these defenses (Hughes et al., 2008; Tranter et al., 2015; Yek & Mueller, 2011).

However, the metapleural gland is only one component of ant external immunity. Ants possess several antimicrobial-producing glands, including the metapleural, poison, mandibular, and Dufour's glands (Brough, 1983; Fernández-Marín et al., 2006; Jouvenaz et al., 1972; Maschwitz et al., 1970; Poulsen et al., 2002; Quinet et al., 2012; Tragust et al., 2013). The diversity of these glands suggests that antimicrobial defense has been repeatedly integrated into ant chemical ecology (R. Vander Meer, 2012). In formicine ants such as *Lasius neglectus*, poison-gland secretions rich in formic acid can be used as externally applied antiseptics. Workers disinfect fungus-exposed brood by taking up poison from the acidopore and applying it during grooming, which reduces fungal conidial viability (Tragust et al., 2013). In fire ants such as *Solenopsis invicta*, venom alkaloids also have antimicrobial properties, although their contribution to realized disease resistance should be treated as system-specific rather than assumed to be universal (Blum, 1992; Jouvenaz et al., 1972; R. Vander Meer, 2012). Fire ants also use poison-gland secretions in hygienic contexts, and queens apply venom to newly laid eggs, reducing microbial contamination (Obin & Vander Meer, 1985; Storey, 1990; R. K. Vander Meer & Morel, 1995). Wound care also demonstrates how behavior and chemistry can interact in ant disease defense. In *Megaponera analis*, injured workers receive targeted antimicrobial wound treatment from nestmates, while

Camponotus floridanus, which lacks metapleural glands, can reduce infection risk through wound cleaning and wound-dependent leg amputation (Frank et al., 2023, 2024).

Comparative assays show that external antimicrobial activity varies among ant taxa, but this variation must be interpreted considering the methods used to detect it. In a survey of 20 ant species, Penick et al. (2018) found broad variation in ethanol-extractable surface activity against the bacterial model *Staphylococcus epidermidis*. Nearly 40% of species showed little or no detectable inhibition under those assay conditions, and antimicrobial activity was not clearly predicted by colony size. These results suggest that broad-spectrum, extractable antimicrobial activity is not uniform across ants. However, they do not necessarily mean that species without detectable activity lack antimicrobial defenses altogether. Some defenses may be volatile, such as that found in the Argentine ant (*Linepithema humile)*, which may be used for targeted pathogen control rather than a stable pathogen defensive barrier (Choe et al., 2012; Kesäniemi et al., 2019). More recent work further suggests that measured antimicrobial activity can depend on extraction chemistry and microbial target, although the active compounds and their deployment against natural pathogens remain to be identified (Chon et al., 2025).

External defense can also involve antimicrobial nest materials. Wood ants, such as *Formica paralugubris,* incorporate conifer resin into their nests, and this resin can reduce microbial abundance and inhibit fungal growth within nest material (Castella et al., 2008; Chapuisat et al., 2007; Christe et al., 2003). Wood ants can also enhance the antimicrobial activity of resin by combining it with formic acid from their poison gland,

producing a more potent antimicrobial mixture than either compound alone (Brütsch et al., 2017). This strategy differs from glandular immunity because antimicrobial substances are collected from the environment rather than produced entirely by the ants, but it similarly reduces pathogen pressure before internal immune responses are required. Nest architecture and substrate choice may further influence external disease defense. Waste chambers, refuse piles, and spatial separation between brood areas and contaminated material reduce exposure to pathogens in the nest environment (Bot et al., 2001; Hart et al., 2002).

External antimicrobial defenses often interact with behavioral immunity. Grooming can distribute glandular secretions across the cuticle or onto brood, while waste compartmentalization and corpse removal reduce the pathogen burden that antimicrobial compounds must suppress (Tragust, 2016; Tragust et al., 2013). Because these defenses act before pathogens penetrate the cuticle, they form an important barrier between environmental exposure and internal infection. By suppressing pathogens on the body surface and in the colony environment, external immunity may reduce the frequency and energetic cost of activating internal physiological immune responses.

Symbiotic defenses

In addition to chemical defenses, some ants have been found to develop mutualisms with symbiotic bacteria to aid in pathogen defense. Fungus-growing ants possess some of the best-documented defensive symbioses in insects. Many attine ants maintain associations with antibiotic-producing actinobacteria, especially *Pseudonocardia* and *Streptomyces*, on specialized regions of the cuticle and within the

nest environment (Barke et al., 2010; Cafaro et al., 2010; Currie et al., 1999, 2003). These microbial symbionts produce antimicrobial compounds that suppress *Escovopsis*, a specialized parasite of attine fungal gardens, while helping preserve the ants' cultivated fungus, *Leucoagaricus gongylophorus* (Currie et al., 2003; Haeder et al., 2009; Seipke et al., 2011). This association is not limited to a single microbial partner. *Pseudonocardia* appears to be a more specialized and evolutionarily persistent defensive symbiont in many attine lineages, whereas *Streptomyces* may be repeatedly acquired from the environment and incorporated into colony defenses (Barke et al., 2010, 2011; Caldera & Currie, 2012; H. Li et al., 2018). Some attine ants also have cuticular crypts and other specialized structures that house bacterial biofilms, suggesting morphological adaptation for maintaining defensive microbial communities (Cafaro et al., 2010; Currie et al., 2006; H. Li et al., 2018).

These defensive symbioses can involve chemically diverse and interacting antimicrobial compounds rather than a single antibiotic. For example, *Pseudonocardia* symbionts associated with attine ants produce antifungal compounds such as dentigerumycin and related gerumycins that inhibit *Escovopsis* (Oh et al., 2009; Sit et al., 2015). *Streptomyces* symbionts associated with *Acromyrmex* can produce compounds including candicidins, antimycins, and valinomycins. Combinations of these metabolites may suppress *Escovopsis* more effectively than individual compounds alone while reducing harm to the mutualistic fungal cultivar (Haeder et al., 2009; Schoenian et al., 2011; Seipke et al., 2011). This suggests that microbial defensive symbioses in fungus-growing ants may operate through multi-compound chemical defense, complementing glandular antimicrobials and behavioral garden hygiene.

Internal Immune Responses and Disease Defense Trade-offs

Despite the importance of collective and external defenses, ants also retain internal immune responses that function after pathogens breach external barriers. Like other insects, ants rely on innate immunity, including cellular and humoral responses, to detect and eliminate invading pathogens (Lavine & Strand, 2002; Schmid-Hempel, 2005). For entomopathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana*, internal defenses activate after these pathogens penetrate the cuticle and start to proliferate within the hemocoel (Boucias & Pendland, 1998). These physiological defenses operate at the level of the individual, but they can influence colony-level disease dynamics by limiting pathogen amplification and reducing the likelihood that infected workers become sources of transmission.

Cellular immune defenses in ants are mediated primarily by hemocytes circulating in the hemolymph. These cells participate in phagocytosis, nodulation, and encapsulation of invading pathogens or foreign particles (Lavine & Strand, 2002; Strand, 2008). Small pathogens such as bacteria can be engulfed through phagocytosis, whereas larger targets, including fungal hyphae or parasitoid eggs, may be surrounded by layers of hemocytes through encapsulation (Lavine & Strand, 2002; Strand, 2008). Encapsulation is often followed by melanization, in which phenoloxidase enzymes catalyze melanin deposition around invading organisms. This response can physically isolate pathogens while also generating cytotoxic intermediates that inhibit microbial growth (Cerenius & Söderhäll, 2004).

Melanization, mediated through the prophenoloxidase cascade, is an important component of insect antifungal defense because it targets pathogens that have already entered the body cavity (Cerenius & Söderhäll, 2004; Gillespie and et al., 1997). In ants, phenoloxidase activity and encapsulation responses vary among species, castes, and ecological contexts, suggesting that internal immunity is plastic and shaped by both life history and exposure risk (Baer et al., 2005; Wilson-Rich et al., 2009). In leaf-cutting ants, such as *Acromyrmex echinatior*, immune assays have shown that workers can mount encapsulation and phenoloxidase-related responses, demonstrating that ants retain functional cellular immune defenses despite their extensive reliance on social immunity (Baer et al., 2005).

Humoral immunity also contributes to ant pathogen resistance. Following infection, insects produce antimicrobial peptides and other immune effector molecules that circulate in the hemolymph and inhibit microbial growth (Evans et al., 2006; Schmid-Hempel, 2005). Antimicrobial peptides (defensins, hymenoptaecin-like peptides, abaecin-like peptides, and lysozymes) can target bacteria and fungi by disrupting membranes or interfering with microbial physiology (Evans et al., 2006). Genomic studies have shown that ants retain many conserved immune pathways, including components of the Toll and Imd pathways that regulate antifungal and antibacterial responses in insects (Bonasio et al., 2010; Evans et al., 2006; Gupta et al., 2015). These genomic inventories provide evidence that ants possess the molecular machinery for individual immunity, although they should not be treated as direct evidence of pathogen-induced immune expression unless the ants were experimentally challenged with pathogens.

Reactive oxygen species and oxidative immune mechanisms can also contribute to pathogen defense. During infection, immune activation may generate reactive intermediates that damage microbial cells, but these compounds can also damage host tissues if they are not tightly regulated (Schmid-Hempel, 2005). As a result, oxidative immunity can create trade-offs between pathogen defense and self-damage, metabolism, longevity, or reproduction (Moret & Schmid-Hempel, 2000; Schmid-Hempel, 2005). These costs are particularly relevant in ants because colony members differ in their fitness priorities. Queens must maintain long-term reproduction, workers perform risky tasks, and brood require protection during vulnerable developmental stages.

Internal immune investment can therefore vary across castes and tissues. Queens may experience strong selection for immune protection because of their central role in colony reproduction, but immune activation can also trade off with reproduction and sperm storage (Baer et al., 2006). Studies of ant sperm-storage organs show that some reproductive tissues limit phenoloxidase activity, likely because melanization and oxidative immune reactions could damage stored sperm (Dávila et al., 2015). In leaf-cutting ant queens, sperm-storage organs appear to rely more heavily on targeted antimicrobial defenses than on generalized melanization-associated responses, illustrating that internal immunity can be tissue-specific and constrained by reproductive function (Dávila et al., 2018).

Interaction between social and individual immunity

Experimental studies increasingly show that individual immunity and social immunity can interact. In the leaf-cutting ant *Atta cephalotes*, exposure to *Metarhizium anisopliae* has been associated with hygienic behavior and altered expression of antimicrobial peptides, such as abaecin and defensin, suggesting that individual humoral immunity and collective behavior can respond together during pathogen challenge (Gómez-Díaz et al., 2022). More generally, immune responses may vary among colony members depending on caste, task, pathogen exposure, nutritional condition, and reproductive role. This variation matters because workers, queens, males, and brood experience different risks and contribute differently to colony fitness.

Social living may alter the balance between individual and collective immunity, but ants should not be described as having lost or replaced individual immunity. Earlier hypotheses proposed that social immunity might reduce selection for complex individual immune systems in eusocial insects (Cremer et al., 2007; Evans et al., 2006). However, later genomic and experimental work suggests a more nuanced interpretation: ants retain functional immune pathways, and social, external, and internal defenses likely complement one another rather than substituting for one another (Gómez-Díaz et al., 2022; Gupta et al., 2015). Internal immunity therefore represents the final physiological layer of a broader disease-defense system. When pathogens overcome behavioral sanitation and external antimicrobial barriers, cellular and humoral immune responses remain essential for suppressing infection and limiting pathogen spread within colonies. Understanding how these layers of defense interact across individuals, castes, and

colony organization will be essential for developing a more complete view of disease ecology and immune evolution in ant societies.

These studies suggest that social insect disease defense operates as an integrated, multilayered system in which social immunity complements, rather than replaces, individual immune defenses. Behavioral sanitation, antimicrobial chemistry, defensive symbionts, and internal immune responses can all reduce infection risk, but they operate at different points in the infection process. This creates an unresolved question for comparative work: when colony organization changes, do ants shift investment among these defense layers, or do individual workers maintain similar resistance regardless of colony size? Chapter 2 addresses this gap by testing whether colony size predicts isolated worker resistance to a fungal pathogen after social immune behaviors are removed.

Chapter 2: Comparative pathogen susceptibility in ants

Introduction

Ant colonies vary dramatically in size, ranging from societies composed of only a few workers to superorganisms containing millions of individuals (Burchill & Moreau, 2016; Hölldobler & Wilson, 1990) Understanding the factors that drive the evolution of colony size variation is a central question in sociobiology (Bourke, 1999; Burchill & Moreau, 2016). Recent comparative work has shown that the evolution of larger colony sizes is associated with reduced investment in individual workers, particularly through decreased cuticle investment (Matte et al., 2025; Peeters et al., 2017). Here, cuticle investment refers to the amount of cuticle material produced relative to worker body size, including body-size-corrected cuticle thickness or volume. Colony size may therefore reflect a broader trade-off between producing many low-investment workers and producing fewer, more durable workers. However, worker investment extends beyond morphology. Social insects face strong pathogen pressure and have evolved diverse defenses against infection (L'opez-Uribe et al., 2016; Penick et al., 2018; Schmid-Hempel, 1998; Stow et al., 2007; Stow & Beattie, 2008). Whether the evolutionary reduction in worker investment associated with increasing colony size extends beyond morphology to include individual immunity remains unknown.

Selection for individual-level defenses may be especially strong in small colonies, where the death of a single worker represents a relatively large loss of colony labor (Heinze & Giehr, 2021). Workers in small-colony species may therefore represent greater colony-level investments, favoring costly traits that improve individual survival, such as thicker cuticles, stronger immune responses, or other physiological defenses that reduce infection after pathogen (Matte et al., 2025; Peeters & Ito, 2015). Immune

defenses are energetically expensive to produce and maintain, including cellular and humoral responses, such as melanization, encapsulation, and antimicrobial peptide production (Gonz'alez-Santoyo & C'ordoba-Aguilar, 2012; Schmid-Hempel, 2005; Zuk & Stoehr, 2002). If small colonies favor greater per-worker investment, then they may also favor stronger individual immune defenses. In addition, thicker cuticle may contribute directly to pathogen resistance because it serves as the first barrier against infection. Entomopathogenic fungi, such as *Beauveria* and *Metarhizium*, must penetrate the cuticle to infect the host, so thicker cuticle may provide increased resistance to fungal infection in addition to structural protection (Moret & Moreau, 2012; Ortiz-Urquiza & Keyhani, 2013; Pedrini et al., 2013).

If small colonies favor increased investment in individual immunity, then large colonies may instead rely more heavily on collective defenses. Social insects possess a diverse suite of colony-level defenses, collectively termed "social immunity," that reduce pathogen transmission and infection risk through allogrooming, sanitation, and other cooperative behaviors (Cremer et al., 2007; Liu et al., 2019; Meunier, 2015). Because these defenses emerge from interactions among nestmates, their effectiveness may increase with colony size and social complexity (Leclerc & Detrain, 2018; Scharf et al., 2012). Under such conditions, selection on costly individual immune defenses may be relaxed because disease protection is increasingly provided by the colony rather than the worker. Large colonies may also better tolerate worker loss through replacement, reducing the fitness consequences of individual mortality. In extreme cases, infected workers may even leave the colony or become socially isolated, limiting pathogen spread to nestmates at the expense of their own survival (Heinze & Walter, 2010;

Stroeymeyt et al., 2018). Consequently, species with large colonies may exhibit lower individual resistance when workers are isolated and challenged with a pathogen.

Here, we tested whether evolutionary shifts in colony size are associated with changes in individual resistance to fungal infection across ant species. To isolate individual immunity from colony-level disease defenses, we challenged workers individually with a fungal pathogen. After screening multiple strains of *Beauveria* spp. to identify a highly virulent isolate, we exposed workers from 16 ant species to a range of pathogen concentrations. We then examined whether colony size, worker size, and cuticle thickness predicted survival following pathogen exposure. If the evolution of larger colonies is associated with reduced investment in individual workers, then species with larger colonies should exhibit lower individual resistance to fungal infection, indicating that the evolutionary reduction in worker investment associated with large colonies extends beyond morphology to encompass individual immunity.

Methods

Study species and colony collection

To test how resistance to entomopathogenic fungi varies among ants, we collected workers from 16 species representing 15 genera and four ant subfamilies: Ponerinae (*Brachyponera chinensis, Odontomachus haematodus*), Dolichoderinae (*Dorymyrmex bureni, Linepithema humile*), Formicinae (*Nylanderia fulva, Prenolepis imparis*), and Myrmicinae (*Aphaenogaster fulva, Crematogaster ashmeadi, Cyphomyrmex rimosus, Monomorium minimum, Pheidole obscurithorax, Pogonomyrmex badius, Solenopsis invicta, Solenopsis molesta, Temnothorax curvispinosus, and Trachymyrmex*

septentrionalis). This sampling represents approximately 19% of Nearctic ant genera and 44% of Nearctic ant subfamilies, providing broad regional taxonomic coverage Colonies were collected from sites in Alabama, Florida, and Georgia, USA (Table 1). Workers were separated from nest debris and maintained in plastic nest boxes containing artificial nest tubes constructed from 15-mL centrifuge tubes partially filled with water and plugged with cotton to provide moisture. Prior to experimental trials, workers were acclimated for 24 h at 25 ± 2°C under a 12:12 h light cycle. During this period, colonies were provided with water, 20% sucrose solution, frozen beetle larvae *(Zophobas morio*), and an artificial diet prepared following modified methods of Bhatkar and Whitcomb (1970).

Beauveria strain selection

To identify a focal *Beauveria* isolate for subsequent cross-species comparisons, we screened nine isolates using the red imported fire ant, *Solenopsis invicta*. All isolates originated from the USDA Agricultural Research Collection of Entomopathogenic Fungi (ARSEF; Ithaca, New York, USA; **Table 2**). The collection included eight isolates of *Beauveria bassiana* and one isolate of *Beauveria brongniartii*. Each isolate was cultured on potato dextrose agar (PDA) plates for 14 days at 28°C. Following methods commonly used for *B. bassiana* insect bioassays (Johnson et al., 2020), conidia were harvested from actively sporulating cultures by adding 10 mL of sterile deionized water containing 0.02% Tween 80 and gently scraping the culture surface with a sterile scalpel. The resulting suspension was transferred to a sterile 50-mL centrifuge tube and vortexed to disperse conidia. The suspension was then allowed to settle briefly to

separate large hyphal fragments from the conidial suspension, after which the upper portion was carefully removed and standardized using hemocytometer counts of viable conidia.

To quantify conidial concentration, a 1:10 dilution was prepared by combining 50 µL of conidial suspension with 450 µL of sterile 0.02% Tween 80 solution. A second dilution was then prepared by combining 100 µL of the diluted suspension with 400 µL of 0.4% trypan blue solution (Corning, Corning, NY, USA), resulting in a total dilution factor of 50. Ten microliters of the diluted sample were loaded into a hemocytometer, and viable conidia (unstained) were counted in the four large corner squares of the central grid. Concentrations were calculated from the mean count across the four squares using standard hemocytometer procedures and adjusted for dilution. Suspensions were then diluted with sterile deionized water containing 0.02% Tween 80 to a final concentration of 1×10^8 conidia mL^{-1}.

Minor workers of *S. invicta* (head width 0.5–0.8 mm) from five colonies were exposed to each fungal isolate at a concentration of 1×10^8 viable conidia mL^{-1} using a whole-body immersion assay similar to previous entomopathogenic fungal bioassays ((J. Li et al., 2016); **Fig. 1**). For each colony, eight workers were assigned to each of the nine *Beauveria* isolates and a control treatment, resulting in a maximum total of 80 workers per colony (**Table 3**). Prior to exposure, conidial suspensions were vortexed to resuspend settled conidia. Workers were handled with sterile featherweight forceps and immersed individually in 500 µL of conidial suspension for 3–5 s (fungal dose refers to the concentration of the suspension used for exposure rather than the exact number of conidia retained on each worker). Following exposure, workers were briefly placed on

dry filter paper to remove excess liquid and transferred to individual 35-mm Petri dish arenas lined with damp filter paper. Each arena contained a cotton ball saturated with 20% sucrose solution and a 1 × 1 cm fragment of water-soaked cosmetic sponge to maintain moisture. Arenas were ventilated through a 7-mm opening in the lid covered with 80-mesh metal screen, sealed with parafilm, and maintained at 25°C in complete darkness. High humidity was maintained by placing an open container of water within each incubator, resulting in a mean relative humidity of 92 ± 2% as measured using a Hygrochron iButton® datalogger (Maxim Integrated; San Jose, CA, USA).

Survival was assessed every 24 h for seven days. Following each mortality check, arenas containing dead ants were removed from the incubator to prevent fungal sporulation and cross-contamination among treatments. Because workers were housed individually, removal of dead ants occurred after survival was scored and did not affect the survival environment of other workers within an arena. To verify that mortality was associated with fungal infection, 10 workers from each colony × strain combination were retained in their original arenas and monitored for an additional three days at 25°C. Cadavers were then examined for the characteristic white conidial growth of *Beauveria*. Colony × strain combinations were retained for analysis only if fungal outgrowth was observed in at least 70% of examined cadavers. Ants that died within 24 h of exposure were excluded from analyses to minimize the influence of handling-related mortality. Ants that died within 24 h of exposure were excluded from analyses to minimize the influence of handling-related mortality. This cutoff provides a conservative approach for excluding handling-related deaths, although it could remove rare cases of extremely rapid pathogen-associated mortality.

Cross-species comparison of fungal susceptibility

Based on the strain-selection assay, *B. bassiana* isolate ARSEF 2597 was selected for all subsequent cross-species comparisons because it produced consistently high mortality. Between two and five colonies were tested for each ant species. Workers from each colony were exposed to one of six conidial concentrations (1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, or 1×10^3 viable conidia mL^{-1}) or a control treatment consisting of sterile deionized water containing 0.02% Tween 80. For each colony, eight workers were assigned to each treatment, resulting in a maximum of 56 workers per colony when all concentrations were represented (**Table 4**). Workers were exposed, housed, incubated, and monitored using the procedures described for the strain-selection assay. To minimize cross-contamination, treatments were applied from the lowest to highest conidial concentration, and forceps were sterilized between colonies and concentration treatments.

Trait correlates of fungal susceptibility

To examine factors associated with variation in fungal susceptibility among species, we quantified traits related to colony size, worker size, and worker investment. We then tested for relationships between these traits and species-level susceptibility to *B. bassiana*.

Colony size: Mature colony size estimates for each species were obtained from published literature sources (**Table 5**). Estimates were intended to reflect mature colony size and were based on the best available published estimate for each species. Because these values were compiled from heterogeneous literature sources, they may

differ in whether they were based on direct counts, nest excavations, or broader population estimates. Colony size was therefore treated as an order-of-magnitude comparative trait and $\log_{10}$-transformed prior to analysis because values spanned several orders of magnitude among species.

Body size: Worker size was quantified using three complementary metrics: dry mass, Weber's length, and body volume. Dry mass was measured for five workers from each colony using a Mettler-Toledo XA105DU analytical balance (0.01 mg precision; Mettler-Toledo, Columbus, OH, USA). For polymorphic species, only minor workers were measured to improve comparability among taxa. Weber's length was measured from specimen images available through AntWeb (Fisher, 2026) as the diagonal length of the mesosoma from the anterior margin of the pronotum to the posterior margin of the propodeum. Body volume estimates were obtained from previously published micro-CT analyses of ant morphology (Matte et al., 2025). When body volume measurements were unavailable for focal species, values from the closest available species in the Matte et al. (2025) dataset were used.

Cuticle investment: Cuticle thickness was measured using three-dimensional micro-CT datasets from the Antscan repository (Katzke et al., 2026). Scans were downloaded as TIFF image stacks and imported into 3D Slicer version 5.10.0 (Pieper et al., 2004). Voxel dimensions were calibrated using scan metadata provided by Antscan. To isolate the cuticle layer, scans were segmented using a threshold-based workflow following the Antscan protocol. Briefly, voxels corresponding to cuticle were identified using a grayscale threshold range of 120–255, and non-cuticular structures were removed using island-based filtering. Segmented image stacks were then oriented to

obtain a longitudinal section through the body midline. For each species, cuticle thickness was measured from the segmented cuticle layer at standardized locations on the head (midline between the eyes), pronotum (thickest region visible in longitudinal section), and first gastral tergite (thickest region visible in longitudinal section). When scans were unavailable for a focal species, measurements were obtained from the closest available congener. These substitutions assume that cuticle traits are more similar within genera than among more distantly related taxa, but they also reflect a practical limitation of available micro-CT data. All substitutions used for cuticle-thickness measurements are listed in **Table 6** to make these assumptions transparent.

Cuticle volume estimates were obtained from the comparative micro-CT dataset of Matte et al. (2025). When direct measurements were unavailable for focal species, values from the closest available species were used as described above using the same species substitutions as used for body volume described above. To account for the strong allometric relationship between cuticle volume and body volume, cuticle investment was quantified as the residuals from the regression of log10(cuticle volume) against log10(body volume). Positive residual values indicate greater cuticle investment than expected for a worker of a given size, whereas negative residual values indicate reduced cuticle investment.

Within-species effects of worker size on fungal susceptibility

To determine whether larger workers exhibit greater resistance to fungal infection within a species, we conducted an additional experiment using the red imported fire ant, *S. invicta*. Workers from five colonies were separated into minor (head width 0.5–0.8 mm)

and major (head width >1.0 mm) worker castes and exposed to the same six concentrations of *B. bassiana* isolate ARSEF 2597 and control treatment used in the cross-species susceptibility assay. Exposure, housing, incubation conditions, mortality monitoring, and confirmation of fungal infection followed the procedures described above.

Statistical analyses

All analyses were conducted in R version 4.6.0 (R Core Team, 2026). Survival analyses were performed using the 'survival' package (Therneau, 2026; Therneau & Grambsch, 2000), Kaplan–Meier curves were visualized using the 'survminer' package (Kassambara et al., 2026), and restricted mean survival time (RMST) was calculated using the 'survRM2' package (Uno et al., 2014, 2022). Phylogenetic comparative analyses were implemented using the 'ape' (Paradis & Schliep, 2019) and 'nlme' packages (Pinheiro et al., 2026; Pinheiro & Bates, 2000).

Beauveria strain-selection assay: For the *Beauveria* strain-selection assay, survival of workers exposed to each fungal isolate was compared with control workers using Cox proportional hazards models. Hazard ratios and 95% confidence intervals were estimated for each isolate relative to the control treatment. Because multiple isolates were compared simultaneously, *p*-values were adjusted using the false discovery rate (FDR) procedure.

Cross-species susceptibility assay: For the cross-species susceptibility assay, survival following fungal exposure was analyzed using Kaplan–Meier survival curves, Cox proportional hazards models, and restricted mean survival time (RMST). Cox

proportional hazards models were used to compare survival among fungal concentrations within species. Fungal concentration refers to the concentration of the suspension used for exposure rather than the exact number of conidia retained on each worker. To facilitate comparisons among species, survival was summarized as RMST over a seven-day observation period ($\tau = 7$ days). RMST represents the average survival time during the observation period and was selected because it provides an intuitive measure of resistance that does not require the proportional hazards assumption.

Species-level RMST values were estimated separately for each fungal concentration. Differences in RMST among species and fungal concentrations were evaluated using linear models with species and concentration as fixed effects. Pairwise comparisons among fungal concentrations were conducted using Tukey-adjusted estimated marginal means. To assess whether susceptibility exhibited phylogenetic structure, phylogenetic signal in species-level RMST was quantified separately for each fungal concentration using Blomberg's K. All phylogenetic analyses were conducted using a species-level phylogeny derived from the ant Tree of Life (Economo et al., 2018), which was pruned to include the focal taxa or their closest available substitutes. Branch lengths were retained from the source tree, and the resulting tree was used to estimate Blomberg's K and to fit Brownian-motion PGLS models. Species rankings among concentrations were compared using Spearman rank correlations.

To evaluate how fungal susceptibility varied across fungal concentrations, species-level RMST values were calculated separately for each dose. Analyses focused primarily on the stock concentration (1×10^8 conidia mL^{-1}), which produced the greatest

among-species variation in survival. To assess the robustness of observed patterns, colony-size analyses were repeated across all additional fungal concentrations.

Colony size and worker investment: To determine whether region-specific cuticle thickness measurements captured biologically meaningful variation in worker investment, Pearson correlations were calculated among head, thorax, and gaster cuticle thickness measurements and independent estimates of cuticle volume and relative cuticle investment from Matte et al. (2025). Because head and thorax thickness measurements were strongly correlated with one another and with independent estimates of cuticle investment, subsequent analyses focused on their mean value.

To test whether colony size was associated with worker investment, ordinary least-squares (OLS) regression and phylogenetic generalized least-squares (PGLS) regression were used with worker morphological traits as response variables and colony size as the predictor. Body-size-corrected cuticle investment was quantified as residual cuticle volume obtained from a linear regression of log10(cuticle volume) on log10(body volume). Body-size-corrected cuticle thickness metrics were calculated as residuals from regressions relating mean head–thorax thickness to worker body-size metrics. Alternative size corrections were compared, and dry weight provided the strongest fit and was therefore used for subsequent analyses. PGLS models incorporated a Brownian-motion correlation structure to account for shared evolutionary history among species.

Colony size, worker investment, and fungal susceptibility: To examine whether fungal resistance was associated with colony size and worker investment, species-level RMST values at the stock concentration were related to colony size and morphological

traits using both OLS and PGLS regression. Colony size was log10-transformed prior to analysis. Predictor variables included colony size, dry weight, Weber's length, body volume, head thickness, thorax thickness, mean head–thorax thickness, cuticle volume, residual cuticle volume, and body-size-corrected cuticle thickness metrics. PGLS models incorporated a Brownian-motion correlation structure to account for shared evolutionary history among species. Because multiple trait predictors were evaluated, isolated significant associations were interpreted cautiously and were considered biologically meaningful only if supported by consistent patterns across related traits and analytical approaches.

Within-species worker-size assay: To determine whether worker size influences fungal susceptibility within species, survival of major and minor *S. invicta* workers exposed to the fungal concentration series was compared using Cox proportional hazards models and Kaplan–Meier survival analyses. Hazard ratios and 95% confidence intervals were estimated for each worker caste and fungal concentration.

Results

Beauveria *isolates vary in virulence against fire ants*

All nine *Beauveria* isolates significantly increased mortality in *Solenopsis invicta* workers relative to the Tween-water control (all FDR-corrected $p < 0.01$; **Table 7**; **Fig. 2**). However, virulence varied substantially among isolates, with hazard ratios ranging from 4.36 to 14.78 relative to controls. The most virulent isolate was *Beauveria bassiana* ARSEF 2597, which increased mortality risk 14.8-fold relative to control workers (hazard ratio [HR] = 14.78, 95% CI = 7.41–29.49). Other highly virulent isolates included ARSEF

2860 (HR = 13.29, 95% CI = 6.34–27.84) and ARSEF 3097 (HR = 10.49, 95% CI = 4.15–26.51). Because ARSEF 2597 consistently produced the highest mortality among the isolates tested, it was selected for all subsequent cross-species susceptibility assays.

Cross-species susceptibility varies among species and fungal concentrations

Susceptibility to *Beauveria bassiana* differed significantly among ant species and fungal concentrations (**Fig. 3**). A global Cox proportional hazards model revealed strong effects of both species (χ^2_{15} = 456.37, $p < 0.001$) and fungal concentration (χ^2_6 = 453.44, $p < 0.001$). Likewise, species-level restricted mean survival times (RMST) varied significantly among species ($F_{15,75}$ = 8.04, $p < 0.001$) and concentrations ($F_{5,75}$ = 55.11, $p < 0.001$).

Across species, survival declined monotonically with increasing fungal concentration: mean RMST decreased from 6.22 ± 0.11 days at 10^3 conidia mL^{-1} to 4.30 ± 0.20 days at the stock concentration (10^8 conidia mL^{-1}), with all pairwise comparisons involving the stock concentration remaining significant after Tukey adjustment (all $p < 0.001$). The stock concentration also produced the greatest among-species variation in susceptibility, with RMST values ranging from 3.18 to 6.52 days among species (range = 3.34 days). Moreover, susceptibility showed significant phylogenetic signal at both the stock concentration (Blomberg's K = 0.94, p = 0.036) and at 10^7 conidia mL^{-1} (K = 0.98, p = 0.030), whereas lower concentrations exhibited no detectable phylogenetic structure (**Table 8**). Closely related species therefore tended

to exhibit similar levels of susceptibility when challenged with the highest fungal concentrations.

Despite substantial changes in overall mortality, species rankings were broadly consistent across fungal concentrations (**Fig. 4**). Species rankings at the stock concentration were most strongly correlated with rankings at 10^7 conidia mL^{-1} (Spearman $\rho = 0.71$) and 10^6 conidia mL^{-1} ($\rho = 0.69$), indicating that species exhibiting high resistance at one concentration generally remained among the most resistant species at other concentrations (**Fig. 5**). Because the stock concentration generated strong mortality, resulted in the greatest among-species variation, and retained detectable phylogenetic signal, all subsequent comparative analyses focused on susceptibility measured at 10^8 conidia mL^{-1}.

Colony size is associated with worker cuticle investment

Before testing colony-size relationships, we evaluated whether region-specific cuticle thickness measurements captured variation in worker investment. Head and thorax cuticle thickness were strongly correlated with one another ($r = 0.95$, $p < 0.001$) and with independent estimates of cuticle volume ($r = 0.67$, $p = 0.004$) and cuticle investment ($r = 0.70$, $p = 0.002$) derived from Matte et al. (2025). In contrast, gaster thickness showed no relationship with either cuticle volume or cuticle investment (all $p > 0.35$; **Table 9**). Because head and thorax thickness provided the strongest correspondence with independent measures of cuticle investment, subsequent analyses focused on their mean value.

Worker body size did not vary systematically with colony size **(Fig. 6**): dry weight, Weber's length, and body volume all showed weak and nonsignificant relationships with colony size in both OLS and phylogenetically corrected analyses (all $p > 0.3$; **Table 10**). Likewise, raw measures of cuticle investment, including mean head + thorax thickness and cuticle volume, exhibited only weak negative relationships with colony size (all $p > 0.1$; **Table 10**).

However, both cuticle volume and cuticle thickness scaled strongly with worker body size: cuticle volume was strongly associated with body volume (OLS $\beta = 1.046$, $R^2 = 0.923$, $p < 0.001$), and mean head + thorax thickness increased significantly with worker dry weight (OLS $\beta = 0.011$, $R^2 = 0.498$, $p = 0.0023$). We therefore used residual cuticle volume and residual cuticle thickness as body-size-corrected measures of worker investment. Residual cuticle volume (cuticle volume corrected for body volume) declined significantly with colony size in both OLS ($\beta = -0.101$, $p = 0.015$) and PGLS models ($\beta = -0.074$, $p = 0.038$; **Fig. 6**). Residual head + thorax thickness (corrected for dry weight) showed a similar pattern but did not reach statistical significance (OLS $\beta = -0.0033$, $p = 0.068$; PGLS $\beta = -0.0027$, $p = 0.083$). These results indicate that species with larger colonies invest less cuticle material in individual workers despite exhibiting no reduction in worker body size.

Colony size and worker investment do not predict susceptibility

Despite the association between colony size and worker cuticle investment, neither colony size nor worker morphological investment predicted resistance to fungal infection (**Fig. 7**). At the stock concentration (10^8 conidia mL^{-1}), species-level restricted mean

survival time (RMST) was not associated with colony size, body volume, cuticle volume, or body-size-corrected cuticle volume in either OLS or phylogenetically corrected analyses (all $p > 0.25$; **Table 11**). Likewise, no significant relationships were detected for cuticle thickness or body-size-corrected cuticle thickness metrics (all $p > 0.09$; **Table 11**).

Among worker body-size measures, Weber's length exhibited a positive relationship with RMST in the non-phylogenetic analysis (OLS: $p = 0.030$), but this relationship was no longer statistically significant after accounting for phylogenetic relatedness (PGLS: $p = 0.091$). Moreover, dry weight and body volume showed no evidence of association with RMST (all $p > 0.09$; **Table 11**), suggesting that the Weber's length result was not a consistent predictor of fungal resistance and likely reflects either shared evolutionary history or multiple-testing effects rather than a biologically meaningful relationship.

Analyses across all fungal concentrations produced a similar pattern. Colony size was not associated with RMST at concentrations of 10^3, 10^4, 10^5, 10^7, or 10^8 conidia mL^{-1} (all $p > 0.07$). A negative relationship was detected at 10^6 conidia mL^{-1} in the phylogenetically corrected analysis (PGLS: $\beta = -0.260$, $p = 0.024$), with a similar but nonsignificant trend in the corresponding OLS model ($\beta = -0.211$, $p = 0.074$). However, this relationship was not observed at adjacent concentrations and did not remain significant after correction for multiple comparisons.

Sensitivity power analysis indicated that the primary species-level OLS regressions had 80% power to detect large relationships between fungal resistance and predictor traits. With 16 species, the minimum detectable effect was approximately $R^2 =$

0.36, equivalent to $|r| = 0.60$, indicating that the study was well powered to detect large associations but had limited power to detect moderate or small effects. Because Matte et al. (2025) suggested that fungus-growing ants may deviate from the general worker trade-off pattern, we conducted a biological sensitivity analysis excluding the two fungus-growing species in our dataset, *Cyphomyrmex rimosus* and *Trachymyrmex septentrionalis*. Excluding these species did not qualitatively alter the results: the negative relationship between colony size and cuticle investment remained, whereas fungal resistance remained unrelated to colony size or cuticle investment.

Within-species differences in worker size do not predict pathogen resistance

Worker size did not significantly affect survival of *S. invicta* workers at any fungal concentration (**Fig. 8**; **Table 12**). Hazard ratios comparing large and small workers ranged from 0.40 to 1.19 across concentrations, but none differed significantly from unity (all $p > 0.05$). The strongest trend occurred at 10^4 conidia mL^{-1}, where larger workers exhibited lower mortality risk than smaller workers (HR = 0.40, 95% CI = 0.15–1.06, $p = 0.066$). However, this pattern was not observed at adjacent concentrations and did not remain significant after correction for multiple comparisons.

Discussion

Although larger ant societies invest less in the structural construction of individual workers (Matte et al., 2025; Peeters et al., 2017), this reduction in worker investment does not appear to extend to individual immune resistance. Across species, colony size was negatively associated with body-size-corrected cuticle investment, supporting previous suggestions that workers in large colonies are built with fewer structural resources. In contrast, susceptibility to the fungal pathogen *Beauveria bassiana* varied significantly among species but was not consistently predicted by colony size, worker body size, or cuticle investment. Likewise, within the red imported fire ant (*Solenopsis invicta*), major and minor workers exhibited similar survival rates following fungal exposure despite pronounced differences in body size, again supporting no change in individual immune investment associated with greater worker investment. Overall, evolutionary reductions in worker investment appear to be concentrated in structural traits rather than in mechanisms that determine survival following pathogen exposure.

Species that live in small colonies are expected to invest more heavily in individual workers because each worker represents a larger fraction of colony labor. We found support for this hypothesis in morphology, where workers from small-colony species invested more heavily in cuticle after controlling for body size, but we found no evidence that this greater investment extended to individual immunity. Species with larger workers or thicker cuticles were not more resistant to *Beauveria*, and major workers in *S. invicta* were not more resistant than minor workers despite their substantially larger size. Thus, small-colony species appear to invest in physical durability, but not in enhanced pathogen resistance. This goes against the hypothesis

that ants that live in smaller colonies should invest more in the protection of individual workers from pathogens. Our work suggests that these workers are more protected from physical harm but not from disease.

Despite differences in cuticle investment among ants, cuticle thickness itself did not determine resistance to *B. bassiana*. This result is notable because entomopathogenic fungi such as *Beauveria* and *Metarhizium* infect insects externally, attaching to the cuticle before penetrating into the body using a combination of mechanical pressure and cuticle-degrading enzymes (Ortiz-Urquiza & Keyhani, 2013; Pedrini et al., 2013; Qu & Wang, 2018). Because the cuticle represents the first barrier encountered by the pathogen, thicker cuticles could theoretically impede infection by increasing the amount of material that must be breached. For example, knockdown of cuticular protein genes in the northern armyworm, *Mythimna separata*, reduced cuticle thickness and increased mortality following *B. bassiana* infection, suggesting that cuticle structure can contribute to fungal resistance in some insects (W. Li et al., 2026). However, our results suggest that cuticle thickness alone is insufficient to explain variation in resistance among ant species. Instead, susceptibility could depend on other aspects of immunity, such as physiological immune responses that act after fungal penetration.

In contrast to small-colony species, ant species that live in large colonies are expected to rely more heavily on collective defenses, and may therefore display lower individual immunity. We found no support for this hypothesis. Colony size did not predict susceptibility to *Beauveria* infection, despite substantial variation in resistance among species. This result suggests that the evolutionary reduction in worker investment

associated with larger colonies does not extend to individual resistance against fungal pathogens. More broadly, our findings indicate that the relationship between sociality and disease defense may not be linear. Comparative studies have shown that transitions from solitary to social living are often associated with increased investment in disease defenses. Antimicrobial activity increases with sociality in bees and with social complexity in wasps (Hoggard et al., 2011; Stow et al., 2007), supporting the hypothesis that heightened disease transmission in group-living species favors stronger front-line defenses. However, patterns within already eusocial lineages are less consistent: López-Uribe et al. (2016) found that encapsulation responses declined with increasing sociality across insect lineages, whereas Penick et al. (2018) found no relationship between colony size and antimicrobial investment across ants, despite variation in colony size spanning several orders of magnitude. These results suggest that once eusociality has evolved, further increases in colony size do not necessarily require greater or lesser investment in any single component of immunity.

One explanation for the absence of a relationship between colony size and fungal resistance is that increasing colony size does not simply relax selection on individual defenses. Larger colonies may experience greater pathogen exposure because they contain more workers, maintain larger nests, and generate more frequent interactions among nestmates (Cremer et al., 2007; Naug & Camazine, 2002; Pie et al., 2004; Schmid-Hempel, 1998). Under these conditions, individual resistance may remain important even as colonies evolve additional forms of social immunity. Rather than replacing individual defenses, collective defenses may augment them. In fire ants, workers exposed to entomopathogenic fungi survive substantially longer when

maintained in groups than in isolation, demonstrating that social interactions can enhance resistance beyond what individuals achieve alone (Qiu et al., 2014). Similar effects have been reported in other social insects, where grooming, sanitation behaviors, antimicrobial secretions, and the removal or isolation of infected individuals reduce pathogen transmission and improve colony survival (Cremer et al., 2007; Meunier, 2015; Stroeymeyt et al., 2018). These findings suggest that social immunity frequently operates as an additional layer of defense rather than a substitute for individual immunity.

A limitation of this study is the relatively small number of species included in the comparative analyses. Experimental measurements of fungal susceptibility are time-intensive, limiting the number of species that can be practically examined. Consequently, our analyses were powered to detect only relatively large relationships between colony size and fungal resistance, and more moderate evolutionary trends cannot be excluded. Despite our smaller sample, several observations were qualitatively consistent with the predicted pattern. For example, the trap-jaw ant *Odontomachus brunneus*, which has relatively small colonies, large workers, and among the greatest cuticle investment in our dataset, also exhibited the greatest resistance to *B. bassiana*. Another potential source of uncertainty concerns the fungus-growing ants. Because these species have unusually thick cuticles for their colony sizes (Matte et al. 2025), they could have weakened the expected relationship between colony size and worker investment. Nevertheless, removing them from the analyses produced the same overall conclusions.

If colony size is not the primary determinant of individual resistance, what explains the large differences among species? One possibility is that ant species rely on different combinations of disease-defense strategies rather than a single defense that scales predictably with colony size. This interpretation is consistent with Bos et al. (2019), who found pronounced differences in susceptibility to *B. bassiana* and *Metarhizium brunneum* among 12 ant species even when workers were maintained in groups that allowed social interactions. Interestingly, species exhibiting higher rates of autogrooming also experienced higher mortality following fungal exposure. Bos et al. suggested that grooming may be most effective under lower pathogen exposure, but species rankings in our study remained broadly consistent across fungal concentrations, suggesting that behavioral defenses alone are unlikely to explain differences in resistance across species. Likewise, Bos et al. (2019) found that experimentally blocking the antimicrobial-producing metapleural gland did not significantly reduce survival *in Formica sanguinea* workers exposed to *B. bassiana*, suggesting that metapleural-gland activity alone may not explain interspecific differences in fungal resistance. Future work integrating different disease-defense traits across species will be critical for understanding why susceptibility varies so markedly among ant lineages.

Our results suggest that evolutionary increases in colony size are associated with reduced structural investment in workers but not reduced resistance to fungal pathogens. Small-colony species invested more heavily in cuticle, yet neither cuticle investment nor colony size predicted susceptibility to *Beauveria* infection. Thus, the evolutionary reduction in worker investment associated with large colonies appears to be concentrated in morphology rather than individual disease resistance. Instead,

pathogen resistance likely emerges from multiple interacting defenses that vary among lineages. Understanding how these alternative disease-defense portfolios evolve may provide a more productive framework for explaining variation in disease resistance across ant societies than models based on any single defense mechanism alone.

Table 1. Collection information for ant colonies used in this study. For each colony, the table lists the colony identifier, species, collection date, and geographic coordinates of the collection site.

Colony	Species	Collection Date	Coordinates
FulColony1	*Aphaenogaster fulva*	2025-06-21	32.57955, -85.42272
FulColony2	*Aphaenogaster fulva*	2025-07-10	32.57955, -85.42272
FulColony3	*Aphaenogaster fulva*	2025-07-10	32.57955, -85.42272
FulColony4	*Aphaenogaster fulva*	2025-07-28	32.57955, -85.42272
ChiColony1	*Brachyponera chinensis*	2025-07-19	32.59469, -85.48982
ChiColony2	*Brachyponera chinensis*	2025-07-19	32.59469, -85.48982
ChiColony3	*Brachyponera chinensis*	2025-08-21	32.59469, -85.48982
ChiColony4	*Brachyponera chinensis*	2025-08-21	32.59469, -85.48982
AshColony1	*Crematogaster ashmeadi*	2025-07-28	32.59546, -85.49184
AshColony2	*Crematogaster ashmeadi*	2025-09-21	32.60440, -85.48298
AshColony3	*Crematogaster ashmeadi*	2026-03-19	32.60053, -85.48906
AshColony4	*Crematogaster ashmeadi*	2026-03-27	32.59598, -85.48275
AshColony5	*Crematogaster ashmeadi*	2026-03-27	32.59598, -85.48275
RimColony1	*Cyphomyrmex rimosus*	2025-10-25	32.47972, -85.61077
RimColony2	*Cyphomyrmex rimosus*	2025-10-25	32.47972, -85.61077
RimColony3	*Cyphomyrmex rimosus*	2025-10-25	32.47972, -85.61077
RimColony4	*Cyphomyrmex rimosus*	2025-10-25	32.47972, -85.61077
BurColony1	*Dorymyrmex bureni*	2025-09-03	32.58795, -85.48219
BurColony2	*Dorymyrmex bureni*	2025-09-03	32.58795, -85.48219
BurColony3	*Dorymyrmex bureni*	2025-09-20	32.47972, -85.61077
BurColony4	*Dorymyrmex bureni*	2025-09-24	32.58734, -85.48292
HumColony1	*Linepithema humile*	2025-08-03	32.59469, -85.48982
HumColony2	*Linepithema humile*	2025-08-03	32.59469, -85.48982
HumColony3	*Linepithema humile*	2025-08-03	32.59469, -85.48982
HumColony4	*Linepithema humile*	2025-08-03	32.59469, -85.48982
HumColony5	*Linepithema humile*	2025-08-03	32.59469, -85.48982
MinColony1	*Monomorium minimum*	2025-08-30	32.59469, -85.48982
MinColony2	*Monomorium minimum*	2025-08-30	32.59469, -85.48982
FulColony1	*Nylanderia fulva*	2026-05-05	30.28706, -87.62372
FulColony2	*Nylanderia fulva*	2026-05-05	30.28706, -87.62372
FulColony3	*Nylanderia fulva*	2026-05-05	30.28706, -87.62372
HaeColony1	*Odontomachus haematodus*	2026-05-03	30.93188, -86.87217
HaeColony2	*Odontomachus haematodus*	2026-05-03	30.93188, -86.87217
HaeColony3	*Odontomachus haematodus*	2026-05-03	30.93188, -86.87217
HaeColony4	*Odontomachus haematodus*	2026-05-04	30.93188, -86.87217
HaeColony5	*Odontomachus haematodus*	2026-05-03	30.93188, -86.87217
ObsColony1	*Pheidole obscurithorax*	2025-09-10	32.59981, -85.48333
ObsColony2	*Pheidole obscurithorax*	2025-09-14	32.47972, -85.61077
ObsColony3	*Pheidole obscurithorax*	2025-09-14	32.47972, -85.61077
ObsColony4	*Pheidole obscurithorax*	2025-09-28	32.47972, -85.61077
BadColony1	*Pogonomyrmex badius*	2025-11-11	31.98476, -85.43654
BadColony2	*Pogonomyrmex badius*	2026-05-03	30.93188, -86.87217
BadColony3	*Pogonomyrmex badius*	2026-05-04	30.93188, -86.87217
BadColony4	*Pogonomyrmex badius*	2026-05-03	30.93188, -86.87217
BadColony5	*Pogonomyrmex badius*	2026-05-03	30.93188, -86.87217
ImpColony1	*Prenolepis imparis*	2026-02-12	32.59469, -85.48982
ImpColony2	*Prenolepis imparis*	2026-02-12	32.59469, -85.48982
ImpColony3	*Prenolepis imparis*	2026-02-12	32.59469, -85.48982
ImpColony4	*Prenolepis imparis*	2026-02-12	32.66004, -85.48493
ImpColony5	*Prenolepis imparis*	2026-02-12	32.66004, -85.48493
InvColony1	*Solenopsis invicta*	2025-06-15	32.59747, -85.48102
InvColony2	*Solenopsis invicta*	2025-06-21	32.59747, -85.48102
InvColony3	*Solenopsis invicta*	2025-06-28	32.59747, -85.48102
InvColony4	*Solenopsis invicta*	2025-06-29	32.59747, -85.48102
InvColony5	*Solenopsis invicta*	2025-06-29	32.59747, -85.48102
InvColony6	*Solenopsis invicta*	2025-06-29	32.59747, -85.48102
MolColony1	*Solenopsis molesta*	2026-04-01	32.47972, -85.61077
MolColony2	*Solenopsis molesta*	2026-04-01	32.47972, -85.61077
CurColony1	*Temnothorax curvispinosus*	2025-10-21	32.66004, -85.48493
CurColony2	*Temnothorax curvispinosus*	2025-10-21	32.66004, -85.48493
CurColony3	*Temnothorax curvispinosus*	2025-10-25	32.66004, -85.48493
SepColony1	*Trachymyrmex septentrionalis*	2025-11-11	31.98476, -85.43654

Colony	Species	Collection Date	Coordinates
SepColony2	*Trachymyrmex septentrionalis*	2026-04-21	32.59578, -85.48336
SepColony3	*Trachymyrmex septentrionalis*	2026-05-03	30.93188, -86.87217

Table 2. *Beauveria* isolate information.

Species	Strain	Isolation source	Order	Family	Isolate Origin	Collection Year
Beauveria bassiana	ARSEF 1520	Nymph, Lygus sp.	Hemiptera	Miridae	France: Prades, Pyrénées-Orientales	1984-06-27
	ARSEF 1564	Pupa, Hyphantria cunea	Lepidoptera	Arctiidae	Italy: Villa Cadè, Reggio Emilia, Emilia-Romagna	1984-03
	ARSEF 2597	Hyblaea puer on teak, Tectona grandis	Lepidoptera	Hyblaeidae	India	1988-09-12
	ARSEF 2860	Schizaphis graminum on spring wheat	Hemiptera	Aphididae	USA: Parma, Idaho	1987-07-17
	ARSEF 3097	Anthonomus grandis	Coleoptera	Curculionidae	USA: Rio Grande Valley, Texas	1990-10-18
	ARSEF 4305	Soil	N/A	N/A	Australia: Fairfield, Epping Forest, Tasmania	1994-06-06
	ARSEF 5078	Larva, Galleria mellonella	Lepidoptera	Pyralidae	USA: Cranberry bog, Grayland, Washington	1994-01-17
	ARSEF 8028	Anthocoris nemorum from Urtica dioica / stinging nettle habitat	Hemiptera	Anthocoridae	Denmark: Bakkegården, Copenhagen, Tåstrup, Zealand	2002-06-25
Beauveria brongniartii	ARSEF 617	Larva, Melolontha melolntha	Coleoptera	Scarabaeidae	France	1981-05-27

Table 3. Replication for the *Beauveria* strain-selection assay. Rows represent individual *Solenopsis invicta* colonies, and values indicate the number of workers exposed to each *Beauveria* isolate and control treatment.

	B. bassiana								*B. brongniartii*	
Colony	ARSEF 1520	ARSEF 1564	ARSEF 2597	ARSEF 2860	ARSEF 3097	ARSEF 4305	ARSEF 5078	ARSEF 8028	ARSEF 617	**Control**
Colony 1	8	0	7	8	0	8	0	0	0	8
Colony 2	8	0	8	8	0	8	0	0	0	8
Colony 3	5	0	6	7	0	8	0	0	0	8
Colony 4	7	0	6	6	0	6	0	0	0	8
Colony 5	0	6	6	0	7	0	6	6	7	8
Colony 6	0	6	7	0	5	0	8	7	8	8
Colony 7	0	6	5	0	7	0	8	8	8	7
Colony 8	0	7	7	0	8	0	8	7	7	8
Colony 9	6	8	14	8	6	7	6	6	6	15

Table 4. Replication for the cross-species susceptibility assay using *Beauveria bassiana* isolate ARSEF 2597. Rows represent individual colonies grouped by species, and values indicate the number of workers exposed to each conidial concentration and the control treatment. Individual workers served as experimental units and were nested within colonies. Colony-level nonindependence was accounted for in the survival analyses, while species and fungal concentration were the primary grouping variables used to evaluate variation in susceptibility.

Colony	1×10^8	1×10^7	1×10^6	1×10^5	1×10^4	1×10^3	Control
Aphaenogaster fulva							
Colony 1	8	8	8	8	8	5	8
Colony 2	8	7	8	8	8	8	8
Colony 3	7	6	7	8	6	8	7
Colony 4	8	8	8	8	8	8	8
Brachyponera chinensis							
Colony 1	8	8	8	7	8	7	8
Colony 2	8	7	7	7	7	8	8
Colony 3	8	8	8	8	8	8	8
Colony 4	8	8	8	8	7	8	8
Crematogaster ashmeadi							
Colony 1	8	8	8	8	8	8	8
Colony 2	8	8	8	8	8	8	8
Colony 3	8	8	8	8	8	8	8
Colony 4	7	6	7	8	7	7	7
Colony 5	8	8	8	7	7	8	8
Cyphomyrmex rimosus							
Colony 1	8	8	6	8	8	8	6
Colony 2	8	8	8	8	8	8	7
Colony 3	8	8	8	8	8	8	8
Colony 4	8	8	8	8	8	8	8
Dorymyrmex bureni							
Colony 1	8	8	8	8	8	8	8
Colony 2	8	8	8	8	8	8	6
Colony 3	8	8	8	8	8	8	8
Colony 4	7	8	8	8	8	7	7
Linepithema humile							
Colony 1	6	7	6	6	7	7	7
Colony 2	9	9	8	9	8	9	10
Colony 3	4	8	10	9	9	10	9
Colony 4	6	9	7	9	8	3	7
Colony 5	8	10	10	10	10	9	10
Monomorium minimum							
Colony 1	8	8	8	7	6	8	8
Colony 2	8	8	8	8	8	8	8
Nylanderia fulva							
Colony 1	7	6	6	8	5	6	5
Colony 2	8	8	8	8	8	8	8
Colony 3	7	7	8	8	8	8	8
Odontomachus haematodus							
Colony 1	8	8	8	8	8	8	8
Colony 2	8	8	8	8	8	8	8
Colony 3	8	7	7	8	8	8	8
Colony 4	8	8	7	8	8	8	8
Colony 5	8	8	8	8	8	8	8
Pheidole obscurithorax							
Colony 1	8	8	8	8	8	8	7
Colony 2	7	7	5	5	5	7	7
Colony 3	7	7	8	7	7	7	7
Colony 4	8	8	8	8	8	8	6

Colony	1×10^8	1×10^7	1×10^6	1×10^5	1×10^4	1×10^3	Control
Pogonomyrmex badius							
Colony 1	7	8	7	6	7	7	5
Colony 2	7	6	8	6	6	8	8
Colony 3	6	8	7	8	4	7	8
Colony 4	7	8	8	8	8	8	8
Colony 5	7	7	5	6	8	8	6
Prenolepis imparis							
Colony 1	7	8	8	8	8	8	8
Colony 2	7	8	7	8	8	8	8
Colony 3	8	8	8	8	8	7	8
Colony 4	7	8	7	7	7	8	8
Colony 5	7	8	8	4	8	8	8
Solenopsis invicta							
Colony 1	10	9	8	9	10	0	10
Colony 2	8	7	8	7	7	6	8
Colony 3	6	8	8	7	8	8	6
Colony 4	7	7	7	7	7	6	8
Colony 5	8	7	8	6	7	6	8
Colony 6	6	8	7	8	7	7	7
Solenopsis molesta							
Colony 1	2	6	8	7	8	7	7
Colony 2	5	7	8	8	6	8	8
Temnothorax curvispinosus							
Colony 1	8	8	8	8	8	8	8
Colony 2	8	8	8	8	7	7	8
Colony 3	8	8	8	8	8	8	7
Trachymyrmex septentrionalis							
Colony 1	8	8	8	8	8	8	8
Colony 2	7	7	8	8	7	8	8
Colony 3	7	8	8	8	8	6	8

Table 5. Colony-size estimates and literature sources used in cross-species trait analyses.

Species	Colony size	Reference
Aphaenogaster fulva	280	Headley 1949
Brachyponera chinensis	1,000	MacGown 2009
Cyphomyrmex rimosus	67	Murakami & Higashi 1997
Dorymyrmex bureni	1,000	King & Porter 2007
Linepithema humile	150,000	Beckers et al. 1989
Monomorium minimum	3,000	Van Pelt 1958
Pheidole obscurithorax	10,000	King & Tschinkel 2007
Solenopsis invicta	220,000	Kaspari & Vargo 1995
Crematogaster ashmeadi	4,693	King & Porter 2007
Prenolepis imparis	3,370	Kaspari & Vargo 1995
Trachymyrmex septentrionalis	223	Seal & Tschinkel 2007
Pogonomyrmex badius	4,300	Tschinkel 2004
Odontomachus haematodus	500	Beckers et al. 1989
Nylanderia fulva	16,000	Sharma et al. 2025
Temnothorax curvispinosus	84	Geraghty et al. 2007
Solenopsis molesta	3,000	Thompson 1990

Table 6. Species substitutions used for AntScan cuticle-thickness measurements and Matte et al. body-volume and cuticle-volume estimates.

Focal species	AntScan species used	AntScan specimen code	Matte et al. species used	Matte et al. specimen code
Aphaenogaster fulva	*Aphaenogaster japonica*	OKENT0105435	*Aphaenogaster japonica*	OKENT0105436
Brachyponera chinensis	*Brachyponera luteipes*	OKENT0105013	*Brachyponera sennaarensis*	CASENT0744933
Cyphomyrmex rimosus	*Cyphomyrmex rimosus*	CASENT0744354	*Cyphomyrmex rimosus*	CASENT0744354
Dorymyrmex bureni	*Dorymyrmex* sp. insanus group	CASENT0741426	*Dorymyrmex pyramicus*	CASENT0744260
Linepithema humile	*Linepithema humile*	OKENT0105450	*Linepithema humile*	OKENT0105451
Monomorium minimum	*Monomorium intrudens*	CASENT0744527	*Monomorium floricola*	CASENT0741441
Pheidole obscurithorax	*Pheidole obscurithorax*	CASENT0744317	*Pheidole pieli*	OKENT0105142
Solenopsis invicta	*Solenopsis invicta*	CASENT0744384	*Solenopsis invicta*	OKENT0105176
Crematogaster ashmeadi	*Crematogaster pinicola*	CASENT0709291	*Crematogaster teranishii*	OKENT0105413
Prenolepis imparis	*Prenolepis naoroji*	CASENT0745648	*Paratrechina zanjensis*	CASENT0744773
Trachymyrmex septentrionalis	*Trachymyrmex carinatus*	CASENT0709287	*Mycetomoellerius holmgreni*	CASENT0744498
Pogonomyrmex badius	*Pogonomyrmex badius*	CASENT0709280	*Pogonomyrmex naegelii*	CASENT0744359
Odontomachus haematodus	*Odontomachus haematodus*	CASENT0744388	*Odontomachus haematodus*	CASENT0744388
Nylanderia fulva	*Nylanderia jaegerskioeldi*	CASENT0744760	*Nylanderia jaegerskioeldi*	CASENT0744760
Temnothorax curvispinosus	*Temnothorax curvispinosus*	CASENT0709276	*Temnothorax congruus*	OKENT0105444
Solenopsis molesta	*Solenopsis jacoti*	CASENT0744087	*Solenopsis desecheoensis*	CASENT0744889

Table 7. Cox proportional hazards analysis comparing mortality of *Solenopsis invicta* workers exposed to nine *Beauveria* isolates relative to the Tween-water control. Hazard ratios greater than 1 indicate increased mortality risk relative to controls. P-values were corrected for multiple comparisons using the Benjamini-Hochberg false discovery rate procedure. Significance codes: *** p < 0.001, ** p < 0.01, * p < 0.05.

Isolate (ARSEF)	Hazard Ratio	95% CI	FDR-corrected *p*
B. bassiana 2597	14.78	7.41–29.49	<0.001 ***
B. bassiana 2860	13.29	6.34–27.84	<0.001 ***
B. bassiana 3097	10.49	4.15–26.51	<0.001 ***
B. bassiana 4305	9.42	4.01–22.11	<0.001 ***
B. bassiana 1520	7.73	4.20–14.24	<0.001 ***
B. bassiana 1564	7.31	2.89–18.48	<0.001 ***
B. bassiana 8028	5.33	1.87–15.17	0.0017 **
B. bassiana 5078	4.62	2.09–10.23	<0.001 ***
B. brongniartii 617	4.36	1.93–9.85	<0.001 ***

Table 8. Restricted mean survival time (RMST), among-species variation, and phylogenetic signal across *Beauveria bassiana* concentrations. RMST values represent mean survival time across species over a seven-day observation period ($\tau = 7$ days). Tukey groups are based on pairwise comparisons among dose means from the global RMST model; concentrations sharing a letter do not differ significantly. Phylogenetic signal was assessed using Blomberg's K. Significance codes: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Beauveria **concentration (conidia mL^{-1})**	**Mean RMST (days)**	**Among-species SD**	**RMST range (days)**	**Tukey group**	**Blomberg's K**	***P*-value**
10^3	6.22	0.44	1.73	a	0.510	0.820
10^4	6.07	0.53	2.04	a	0.465	0.914
10^5	6.06	0.48	1.71	a	0.573	0.660
10^6	5.91	0.47	1.90	a	0.689	0.292
10^7	5.21	0.74	2.84	b	0.982	0.030 *
10^8 (stock)	4.30	0.79	3.34	c	0.943	0.036 *

Table 9. Pearson correlations among cuticle thickness measurements and independent estimates of cuticle investment from Matte et al. (2025). Significance codes: *** p < 0.001, ** p < 0.01, * p < 0.05.

Comparison	***r***	***P***
Head vs. Thorax thickness	0.95	<0.001 ***
Head + Thorax thickness vs. Cuticle volume	0.67	0.004 **
Head + Thorax thickness vs. Cuticle investment	0.70	0.002 **
Gaster thickness vs. Cuticle volume	-0.12	0.66
Gaster thickness vs. Cuticle investment	-0.25	0.36

Table 10. Relationships between colony size and worker body size and cuticle investment. Ordinary least squares (OLS) and phylogenetic generalized least squares (PGLS) models tested whether worker morphological traits decline with increasing colony size. Worker body size was quantified using dry weight, Weber's length, and body volume. Cuticle investment was quantified using mean head + thorax thickness, cuticle volume, residual head + thorax thickness corrected for worker dry weight, and residual cuticle volume corrected for body volume. Significance codes: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, • $p < 0.1$.

Trait category	Trait	OLS β	OLS *p*	PGLS β	PGLS *p*
Body size	Dry weight	-0.070	0.683	0.048	0.762
Body size	Weber's length	-0.035	0.555	0.011	0.845
Body size	Body volume	-0.148	0.324	-0.058	0.658
Cuticle investment	Mean head + thorax thickness	-0.0040	0.115	-0.0022	0.323
Cuticle investment	Cuticle volume	-0.256	0.107	-0.135	0.332
Cuticle investment	Residual head + thorax thickness (dry-weight corrected)	-0.0033	0.068 •	-0.0027	0.083 •
Cuticle investment	Residual cuticle volume (body-volume corrected)	-0.101	0.015 *	-0.074	0.038 *

Table 11. Relationships between fungal resistance and colony size and worker investment at the stock *Beauveria* concentration (10^8 conidia mL^{-1}). Ordinary least squares (OLS) and phylogenetic generalized least squares (PGLS) models tested whether species-level RMST was associated with colony size, worker body size, or worker cuticle investment. RMST was calculated over a seven-day observation period (τ = 7 days). Significance codes: *** p < 0.001, ** p < 0.01, * p < 0.05, • p < 0.1.

Trait category	Predictor	OLS β	OLS *P*	PGLS β	PGLS *P*
Colony size	Colony size	-0.143	0.495	-0.091	0.621
Body size	Dry weight	0.531	0.094 •	0.391	0.206
Body size	Weber's length	1.900	0.030 *	1.460	0.091 •
Body size	Body volume	0.412	0.260	0.084	0.824
Cuticle investment	Head thickness	16.8	0.458	15.9	0.500
Cuticle investment	Thorax thickness	13.2	0.495	8.43	0.670
Cuticle investment	Head + thorax thickness	15.2	0.472	12.2	0.582
Cuticle investment	Cuticle volume	0.371	0.269	0.030	0.932
Cuticle investment	Residual cuticle volume	0.104	0.933	-0.637	0.623
Cuticle investment	Residual thickness (dry-weight corrected)	-17.4	0.560	-16.5	0.584

Table 12. Effects of worker size on survival following *Beauveria bassiana* exposure in *Solenopsis invicta*. Hazard ratios (HR) are from dose-specific Cox proportional hazards models comparing large workers to small workers. HR < 1 indicates lower mortality risk for large workers. Significance codes: *** p < 0.001, ** p < 0.01, * p < 0.05, • p < 0.1.

Beauveria concentration (conidia mL^{-1})	**N**	**HR (large vs. small)**	**95% CI**	***P***
10^8	61	0.72	0.43–1.21	0.219
10^7	60	0.81	0.46–1.44	0.467
10^6	58	0.97	0.50–1.89	0.929
10^5	61	1.19	0.53–2.69	0.671
10^4	62	0.40	0.15–1.06	0.066 •
10^3	59	0.70	0.29–1.70	0.436

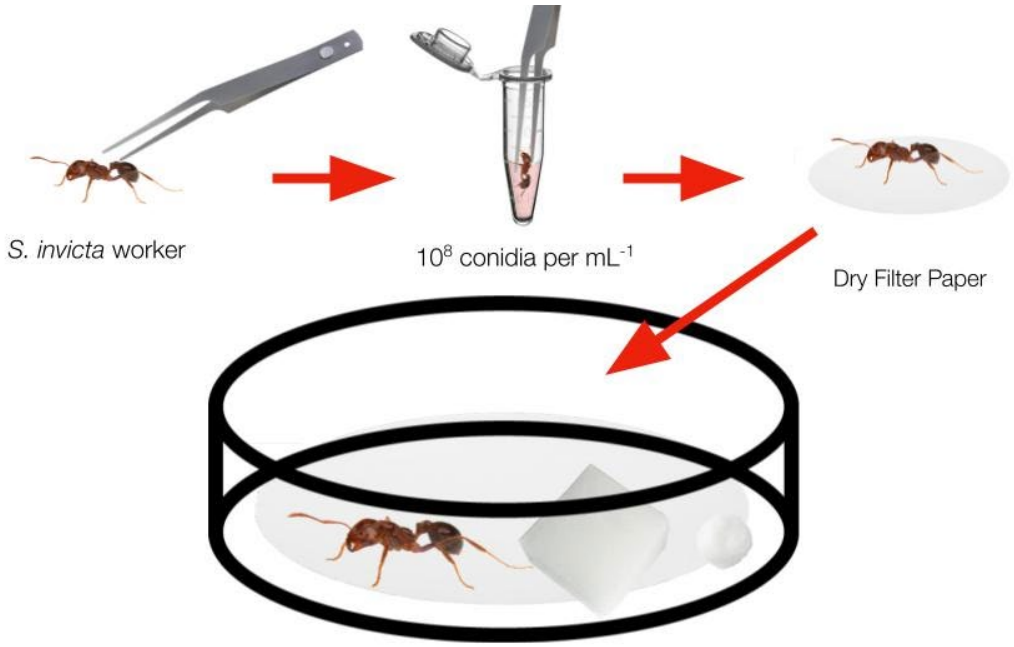

Fig. 1. Experimental chamber used for individual fungal-exposure assays. Workers were exposed to *Beauveria* suspensions by whole-body immersion and then housed individually in ventilated Petri dish arenas containing damp filter paper, a sucrose source, and a water-soaked sponge to maintain humidity. This setup isolated individual workers from nestmates, allowing survival to be measured without the influence of colony-level social immune behaviors.

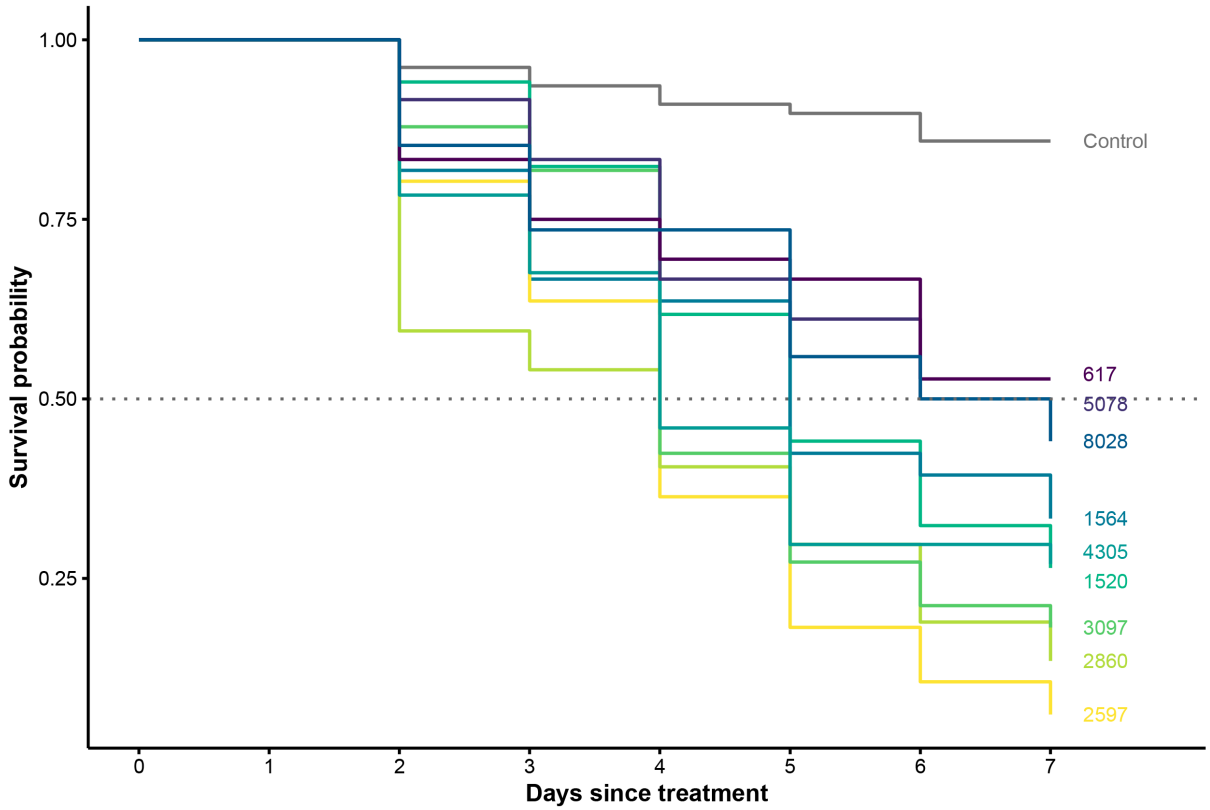

Fig. 2. Virulence of *Beauveria* isolates against *Solenopsis invicta* workers. Survival curves show mortality over seven days for workers exposed to nine *Beauveria* isolates and a Tween-water control. All isolates increased mortality relative to the control, but *B. bassiana* ARSEF 2597 produced the strongest effect and was selected for the cross-species susceptibility assay. The full 0–7 day RMST range is shown to preserve the absolute scale of the seven-day survival assay.

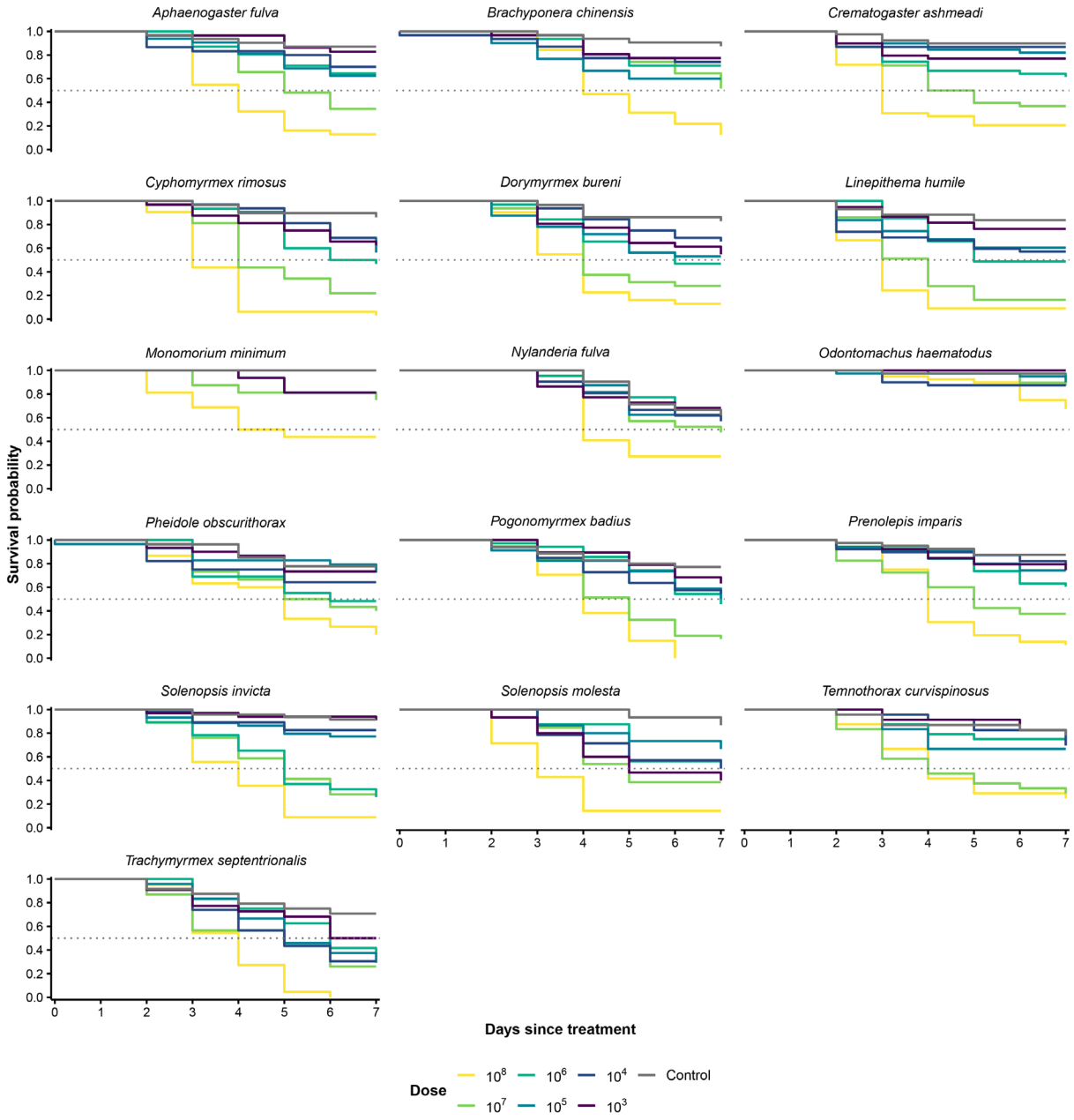

Fig. 3. Survival of ant workers exposed to *Beauveria bassiana* ARSEF 2597 across species and fungal concentrations. Kaplan–Meier survival curves show that mortality increased with conidial concentration and varied among ant species. These patterns indicate that the fungal-exposure assay captured biologically meaningful variation in susceptibility across both pathogen dose and host species.

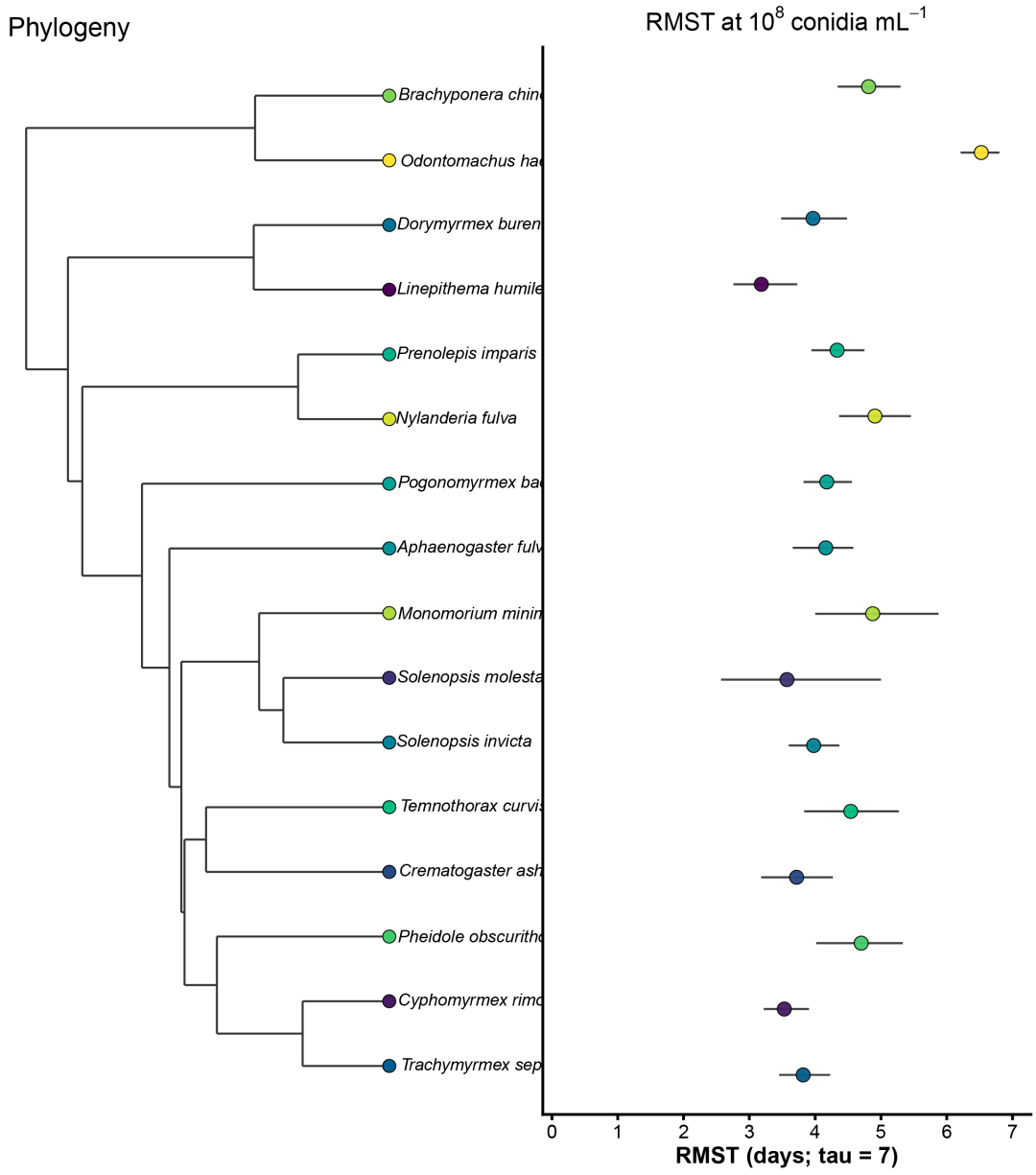

Fig. 4. Species-level resistance to *Beauveria bassiana* across fungal concentrations. Restricted mean survival time (RMST) was calculated over the seven-day assay period for each species and concentration. Higher RMST values indicate greater survival and lower susceptibility. Species rankings were broadly similar across higher fungal concentrations, but the magnitude of survival differences depended on dose, showing

that susceptibility was context dependent rather than fixed across all pathogen pressures.

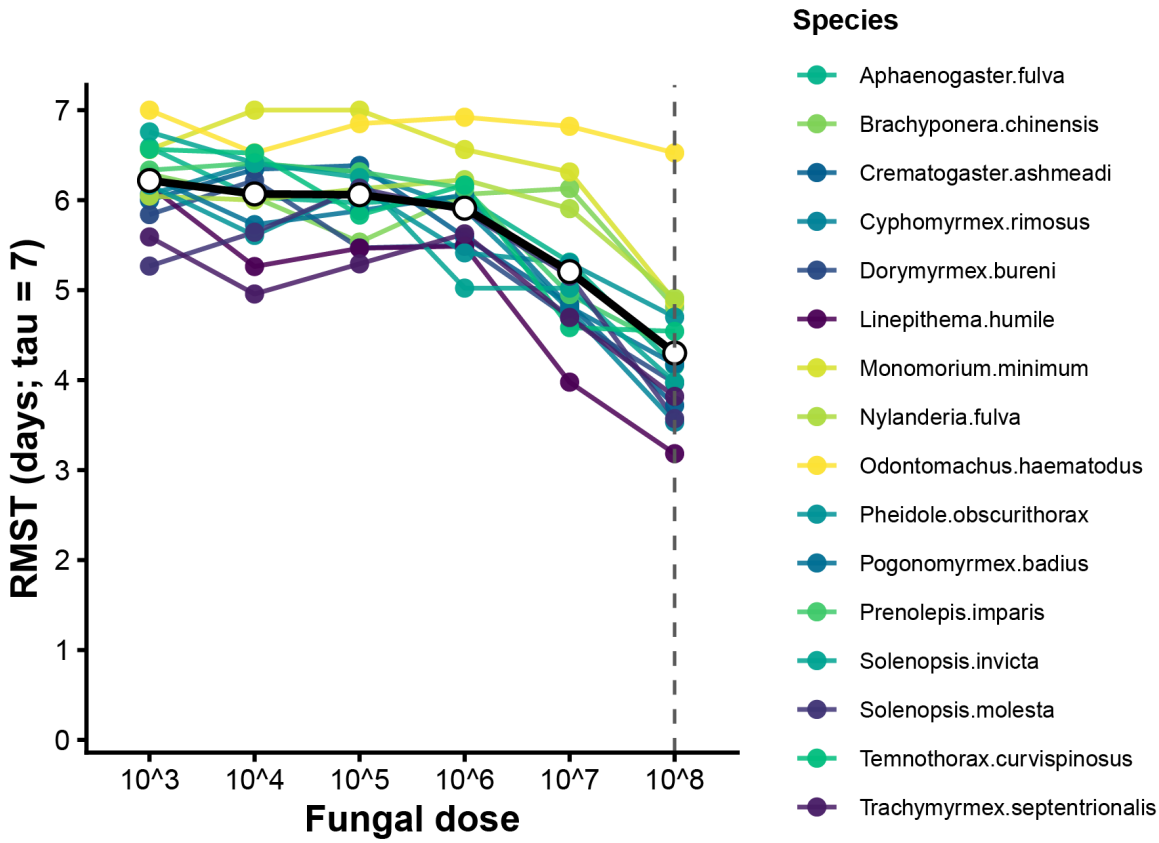

Fig. 5. Species survival responses across fungal doses. Although overall survival declined as fungal concentration increased, species rankings were broadly consistent across doses, indicating that species with relatively high resistance at one concentration generally remained among the more resistant species at other concentrations. The dashed vertical line marks the stock concentration, 10^8 conidia mL^{-1}, which produced strong mortality and was used for subsequent comparative analyses of fungal resistance.

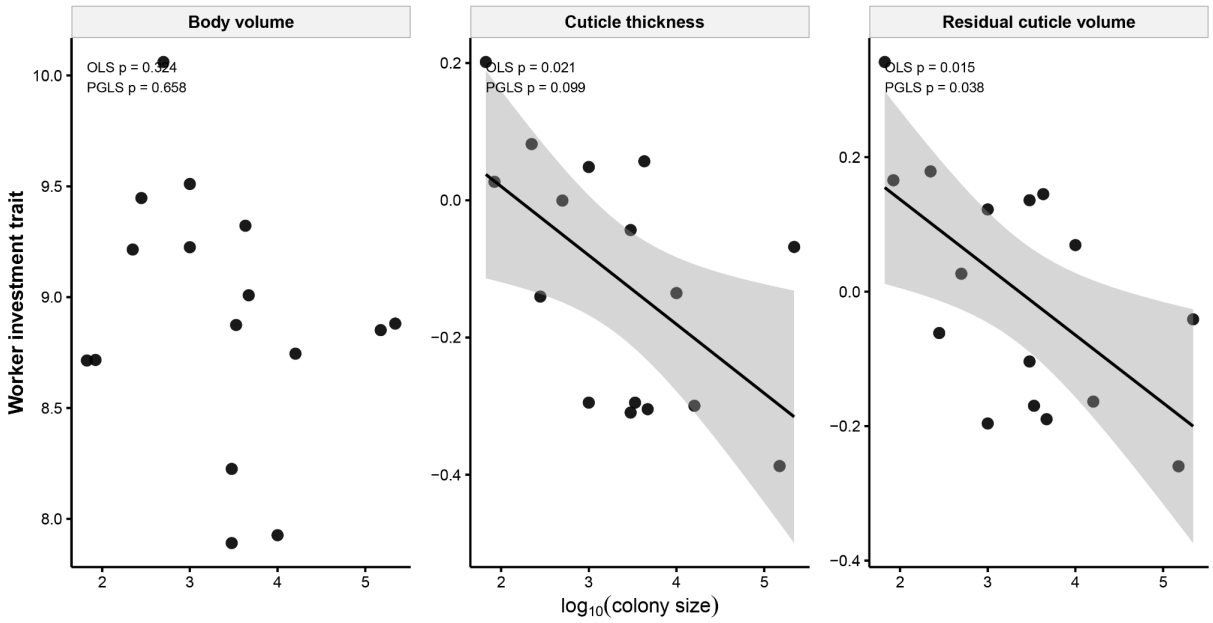

Fig. 6. Relationships between colony size and worker morphological investment. Worker body size did not decline systematically with colony size, but body-size-corrected cuticle investment declined in species with larger colonies. These results support the hypothesis that larger ant societies produce workers with reduced structural investment rather than simply producing smaller workers.

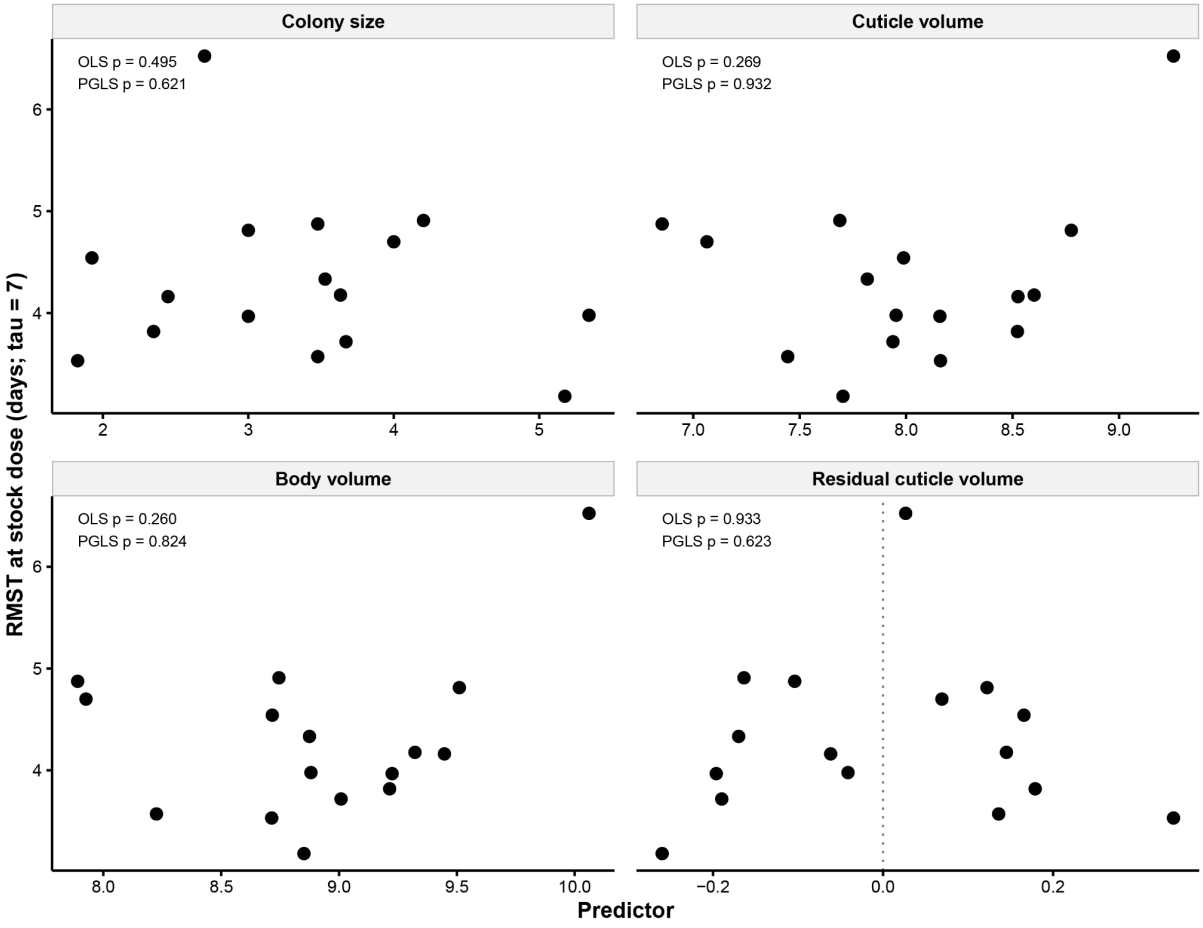

Fig. 7. Relationships between fungal resistance and colony size, worker body size, and cuticle investment. Species-level RMST at the stock *Beauveria* concentration was compared with colony size and worker morphological traits using ordinary least-squares and phylogenetically corrected models. Fungal resistance was not consistently predicted by colony size, body size, cuticle volume, or body-size-corrected cuticle investment, indicating that structural worker investment did not explain interspecific variation in susceptibility.

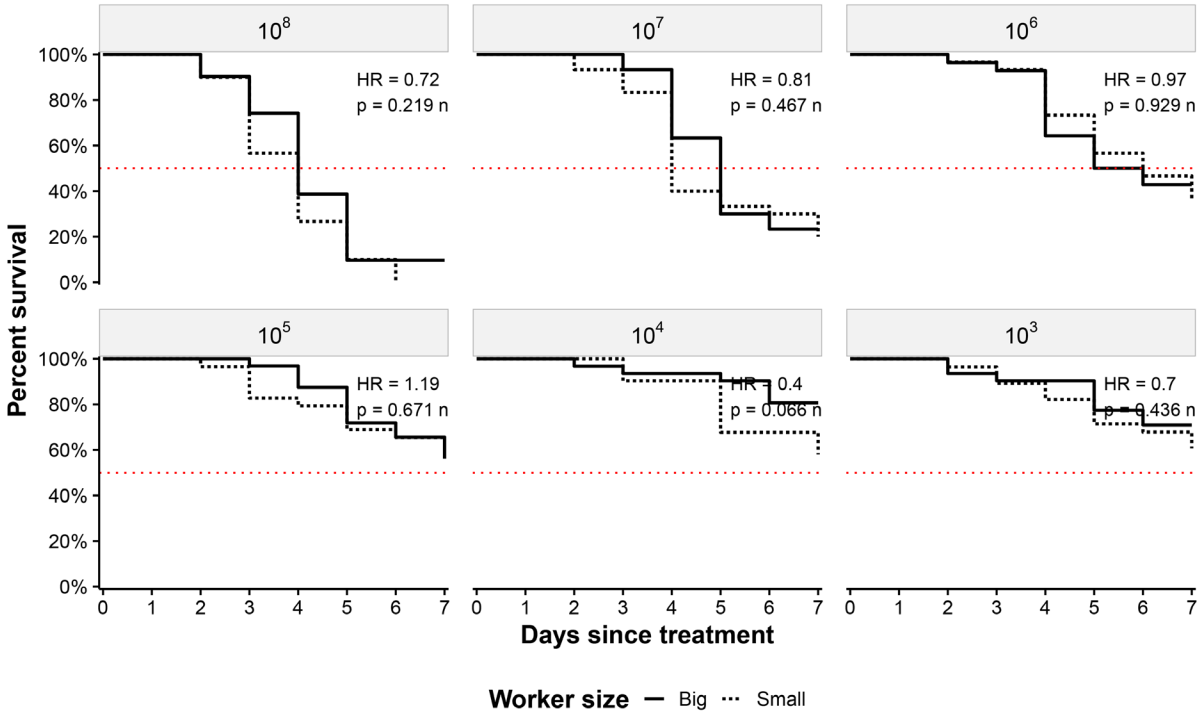

Fig. 8. Effects of worker size on *Beauveria* susceptibility within *Solenopsis invicta*. Survival of large and small workers was compared across fungal concentrations using dose-specific Cox proportional hazards models. Worker size did not significantly affect survival at any concentration, indicating that major workers were not consistently more resistant to fungal infection than minor workers.

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