SOCIAL BEHAVIOR AND THE ECOLOGY OF INFECTIOUS DISEASE

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Infectious disease is a threat for both human and wildlife health. Social behavior can shape the outcomes of disease, and infectious disease can reciprocally shape the evolution of social behavior. Bumble bees, dominant pollinators in temperate ecosystems, are eusocial and have experienced declines associated with pathogens, but we have a limited understanding of how bumble bee social life affects the ecology of disease in pollinator communities. In this thesis, I investigate the relationship between social behavior and infectious disease in the common eastern bumble bee. In Chapter 1, I experimentally test whether disease is a cost of living in larger groups, finding that when infected with a gut parasite larger colonies experienced reduced survival, growth, and reproduction compared to smaller colonies. However, while social behavior can increase the costs of disease, it can also select for cooperative defenses that may counteract these costs. In Chapters 2 and 3 I explore the social defenses used by bumble bees against this gut parasite. First, in Chapter 2, I described a novel element of wild bumble bee nest architecture, a feces-filled secondary cavity, or "outhouse," through field observations of wild colonies in western Alaska. I also observed a fecally-transmitted gut parasite in this region, suggesting that this architecture could serve a hygienic function. Then in Chapter 3 I test this hypothesis with manipulative laboratory experiments, finding that bumble bee colonies modify their nests to create the "outhouse" architecture and that this enables hygienic waste removal behaviors, thereby slowing pathogen transmission, accelerating growth, and increasing reproduction. These results extend our understanding of the consequences

of social behavior on the ecology of infectious disease in plant-pollinator-pathogen communities, and suggest an effective and cost-effective solution for reducing pathogen transmission in managed pollinators. By better understanding the social lives of bees, we will be able to improve predictions of the outcomes of epidemics and develop more effective strategies for their management, in support of pollinator conservation.

BIOGRAPHICAL SKETCH

Leah grew up in rural Maryland, where she became infatuated with all creatures great and small. Her first word was “meow,” in an attempt to communicate with the family cat, and her early efforts at conservation work included “rescuing” backyard crickets from “predators.” As such, nobody has been surprised that her career in science has taken her deeper into animal behavior and conservation work – although she hopes that her methods have become more sophisticated.

As an undergraduate at Dartmouth College, Leah spent most of her time frolicking in the woods and climbing on rocks. She lacked direction for the first two years, and dabbled in cell biology, aquatic toxicology, primate behavior. During her junior year, she harassed Professor Mark Laidre into mentoring her undergraduate honors thesis. This research brought her to the Gulf of Maine and the Osa Peninsula of Costa Rica, where she studied the chemical cues used by marine and terrestrial hermit crabs to locate shells and how these behaviors respond to the “housing” market. After these experiences, Leah could no longer imagine a life for herself outside of science.

After a confusing detour working in a “New York” themed building at a large software company, Leah moved to the real New York state to begin her PhD advised by Professor Rob Raguso in the Department of Neurobiology and Behavior. However, the COVID-19 pandemic had other plans for her, and with the growing importance of infectious disease in human society she soon realized that she wanted to focus her PhD research on infectious disease and social behavior. This led her to the work described in this thesis, with Professor Scott McArt in the Department of Entomology. Through her research, she aims to protect the small creatures that keep our world turning, but remains most motivated by the human capacity for wonder.

I dedicate this thesis to the bees.

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INTRODUCTION

Infectious disease is a global challenge for both wildlife conservation and agriculture. Due to changes in climate, biodiversity, and species introductions, emerging infectious diseases are on the rise (Mahon et al., 2024) and can have significant costs for wildlife populations. For example, the introduction of white-nose syndrome to North America has caused widespread declines across numerous bat species (Hoyt et al., 2021), and the expansion of chytrid fungi has produced historic and devastating rates of amphibian extinction worldwide (Fisher & Garner, 2020). Managed species are also at risk, as high density living conditions (Pitzer et al., 2016) and low levels of genetic diversity (Gibson, 2022) can increase disease in agricultural systems, leading to significant economic costs (Rasmussen et al., 2024). This represents a significant threat both to farmers' livelihoods and to global food security.

Insect pollinators are essential for both wild ecosystems and agriculture (Klein et al., 2007). Bumble bees are particularly important wild pollinators in temperate and arctic ecosystems, where their large bodies and dense pile make them robust to harsh weather and effective transporters of pollen (Heinrich, 1972). Bumble bees also contribute significantly to agricultural pollination; wild bumble bee abundance is correlated with increased pollination in agricultural squash and blueberry fields (Button & Elle, 2014; McGrady et al., 2021), and bumble bee colonies are managed for commercial greenhouse pollination, particularly of buzz-pollinated crops (Morandin et al., 2001). However, bumble bees have experienced declines in recent years due to a combination of stressors including pesticide exposure, habitat loss, climate change, and infectious disease (Goulson et al., 2015; Potts et al., 2010). Thus, understanding the mechanisms driving pathogen transmission in bee communities and how these effects interact with other stressors is a significant goal for pollinator conservation.

Bumble bees host diverse pathogen and parasite species. One such pathogen is *Crithidia bombi*, a fecally transmitted trypanosomatid pathogen that has become a model system for understanding the ecology and evolution of infectious disease (Schmid-Hempel et al., 2019). *C. bombi* negatively impacts bumble bee foraging efficiency (Gegear et al., 2006; Otterstatter et al., 2005), survival (Brown et al., 2003; Conroy et al., 2016), and reproduction (Yourth et al., 2008), although these effects are context-dependent and most negative in bees experiencing additional stressors such as starvation (Brown et al., 2003; Conroy et al., 2016). Like many bee pathogens, *C. bombi* can be transmitted when susceptible individuals forage on contaminated flowers or interact with infected nestmates or materials within the nest. The ways in which floral traits, pollinator traits, and the plant-pollinator network drive pathogen transmission have received increasing attention in recent years (Adler et al., 2018; Figueroa et al., 2019; Graystock et al., 2020; Maurer et al., 2024; Van Wyk et al., 2021), but less is known about within-colony pathogen transmission in social bees (but see: Pinilla-Gallego et al., 2020). Bumble bees are eusocial, living in dense colonies of up to 350 sister workers, and have an annual lifecycle in which colonies die each fall, and newly emerged gynes overwinter alone before starting new colonies in the spring (Goulson, 2010). This lifestyle could have important consequences for the ecology of pathogens in pollinator communities. Moreover, bumble bee pathogens, such as *C. bombi,* survive into the next generation by overwintering in gynes, who are thought to become infected through transmission within their natal nests (Schmid-Hempel, 1998). Thus, in addition to ecological consequences, within-colony transmission dynamics may have important evolutionary consequences for both bumble bees and their pathogens. Greater knowledge of the relationship between social behavior and infectious disease in pollinator communities is needed before we can develop a clearer mechanistic understanding of the ecology of infectious disease

in plant-pollinator communities.

Infectious disease is broadly considered to be a major cost of social life. Group size and density are correlated with pathogen prevalence and intensity across numerous vertebrate species (Cote & Poulin, 1995; Rifkin et al., 2012), and although this relationship is less clear in social insects (Leclerc & Detrain, 2018; Rosengaus & Coates, 1998; Schmid-Hempel, 1998), prior research has shown that within-colony transmission can occur rapidly in laboratory-reared colonies (Pinilla-Gallego et al., 2020).

Social behavior may alter infection in bumble bee colonies in several ways. First, social behavior may increase within-colony transmission by increasing the frequency and duration of interactions between susceptible workers and infectious nestmates or materials (Altizer, Nunn, et al., 2003). Contact networks shape within-colony pathogen (*C. bombi*) transmission in bumble bee (*Bombus impatiens*) colonies (Otterstatter & Thomson, 2007), suggesting that social and interactions within wild bumble bee colonies may accelerate transmission. Additionally, greater numbers of workers in an enclosed nest environment may accelerate interactions with potentially infectious waste materials, such as feces, and this may pose a significant risk for fecal-orally transmitted pathogens like *C. bombi.*

Moreover, the low levels of genetic diversity within bumble bee colonies may increase their exposure or susceptibility to pathogens. Bumble bee colonies are monogynous, and queens typically mate only once, although multiple matings are possible (Estoup et al., 1995; Owen & Whidden, 2013; Payne et al., 2003; Takahashi et al., 2008). Genetic diversity is often inversely correlated with pathogen susceptibility (Altizer, Harvell, et al., 2003; Gibson, 2022; King & Lively, 2012), and experimental studies consistently find this relationship in bees and ants (Baer & Schmid-Hempel, 1999; Hughes & Boomsma, 2004; Reber et al., 2008; Seeley & Tarpy, 2007;

Shykoff & Schmid-Hempel, 1991). Highly related nestmates may also be susceptible to similar pathogens, due to similarities in their immune profiles, behavior, or spatial distribution (Chesnais et al., 2016; Johnson & Hoverman, 2014; Poulin, 2013; Stear et al., 2007)

However, social behavior may sometimes reduce the costs of infectious disease, particularly for highly eusocial, cooperative species (Leclerc & Detrain, 2018; Rosengaus & Coates, 1998; Traniello et al., 2002; Ugelvig & Cremer, 2007). This may be because many eusocial species have evolved cooperative defenses, or "social immunity," to effectively avoid, resist, or eliminate infections (Cremer et al., 2007, 2018; Stockmaier et al., 2021). Social immunity behaviors may be prophylactic, as when ant or honeybee workers use antimicrobial materials in nest construction (Baracchi et al., 2012; Christe et al., 2003; Simone et al., 2009); or they may be induced, such as when workers isolate themselves during infection (Pusceddu et al., 2021). Social organization may also limit pathogen transmission in eusocial insect societies (Naug & Camazine, 2002; Stroeymeyt et al., 2014, 2018).

Most bumble bee species are cavity-nesters, living in subterranean cavities such as abandoned rodent nests (Liczner & Colla, 2019). These spaces set the stage for all within-colony behaviors, interactions, and epidemics. Just as the architecture of human buildings can shape social interactions and environmental conditions, for example by providing adequate space for social distancing or by improving air flow and ventilation (Aliabadi et al., 2011; Emmanuel et al., 2020; Izadyar & Miller, 2022; Megahed & Ghoneim, 2020), nest architecture could have important consequences for interaction patterns, abiotic conditions, and disease in bumble bee colonies. This hypothesis has been supported by theoretical models, although it has not been empirically tested (Pie et al., 2004). If bumble bee nest architecture can alter within-colony pathogen transmission, it also has the potential to have fitness effects, particularly when faced

with virulent pathogens. Although bumble bee nests are not alive, they can be altered through natural selection acting on bumble bee nesting preferences or nest modification behaviors, if these behaviors supply sufficient fitness benefits (Dawkins, 1982; Hansell, 2005). In this way, it is possible that nest structures that adaptively reduce within-colony disease transmission, or "architectural immunity," could be selected for.

In the following chapters, I explore the relationship between social behavior, nest architecture, and infectious disease in the common eastern bumble bee (*Bombus impatiens*). In Chapter 1, I investigate whether social life increases the costs of infectious disease (*C. bombi*) in bumble bees by testing whether survival, growth, and reproduction differ in larger versus smaller bumble bee microcolonies faced with a pathogen. Then, in Chapters 2 and 3, I investigate the cooperative behavioral and architectural defenses that bumble bees use to combat the costs of disease. In Chapter 2, I explore the nest architecture of wild frigid bumble bee (*B. frigidus*) colonies through field observations, finding that wild nests contain a secondary cavity filled with concentrated feces. I hypothesize that this nest architecture was selected for because it facilitates waste removal and hygienic defecation, reducing disease transmission and improving fitness. In Chapter 3, I use manipulative laboratory experiments to test this hypothesis in the common eastern bumble bee (*B. impatiens*). My results extend our understanding of the relationship between social behavior and infectious disease in wild pollinator communities, suggesting that bumble bees may have effective strategies for limiting within-colony transmission. More broadly, these results provide the first empirical evidence that, in addition to shaping the evolution of physiology and behavior, infectious disease can drive the evolution of animal homes.

CHAPTER 1

LARGER GROUP SIZE INCREASES THE COSTS OF DISEASE FOR BUMBLE BEE MICROCOLONIES

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Abstract

Infectious disease is considered a cost of sociality, as increased contact between groupmates increases the risk of disease transmission. However, in cooperative social insect colonies, larger group sizes can provide fitness benefits, and social immunity traits in many social insect species buffer against disease by helping to avoid, control, or eliminate infection. Here, we investigated the relationship between group size and the costs of infectious disease in the common eastern bumble bee (Bombus impatiens) and its gut parasite, Crithidia bombi. We experimentally tested how group size affects survival, prevalence, infection intensity, nest construction, and reproduction for small (15 workers) or large (45 workers) microcolonies inoculated with the same initial prevalence of C. bombi compared to healthy control microcolonies. We found that C. bombi infection increased worker mortality in large but not small microcolonies. Additionally, C. bombi infection reduced nest construction and reproductive output for large but not small microcolonies. Despite higher mortality rates and lower colony performance, there was no difference in C. bombi prevalence in small versus large microcolonies, and C. bombi infection intensity was lower in surviving bees in large microcolonies than in small microcolonies. Our results suggest that infectious disease is a cost of larger group size for this primitively eusocial species.

Significance Statement

Exposure to infectious disease is one major cost of living in larger groups, but for many social insects group size also provides defenses against disease in the form of cooperative "social immunity" behaviors. Prior research on the relationship between group size and disease for insect societies has focused on highly eusocial species, but a better understanding of how these costs and benefits vary across more diverse social systems is needed. Using laboratory experiments, we show that disease is a cost of larger group size for bumble bee microcolonies. When infected with a trypanosomatid parasite, mortality increased only for large microcolonies. Additionally, we show that infection may have greater sublethal consequences in large versus small groups. These results extend our understanding of the costs of group size on disease to primitively eusocial insects.

Introduction

Sociality imposes opposing costs and benefits on the health of animal societies. Exposure to infectious disease is considered one of the major costs of living in larger groups, as group size may increase the local density and contact rates between closely related conspecifics (Cremer et al. 2007; Rifkin et al. 2012; Schmid-Hempel 1998). Meta-analyses have shown that group size has an overall positive effect on parasitism rates in vertebrates, particularly for contagious and environmentally transmitted pathogens, although these effects vary across vertebrate taxa (Cote and Poulin 1995; Rifkin et al. 2012). However, the relationship between group size and disease is less clear in social insects, and research suggests that living in larger groups may reduce the costs of contagious disease for some social insect species (Schmid-Hempel 1998). For example, larger ant (Myrmica rubra) colonies experience lower mortality rates when exposed to the fungus Metarhizium brunneum than their smaller counterparts (Leclerc and Detrain 2018) and termite (Zootermopsis angusticollis) survival is higher when termites infected with the fungus Metarhizium anisopliae are socially housed with uninfected nestmates (Rosengaus and Coates 1998).

Living in larger groups may sometimes reduce the costs of contagious disease because many social species have evolved cooperative behaviors to prevent, slow, or eliminate infection, known collectively as "social immunity" (Cremer et al. 2007; Stockmaier et al. 2021; Stroeymeyt et al. 2014). These behaviors may be prophylactic, as when workers in ant and honeybee colonies incorporate antimicrobial resins or venom into their nests (Baracchi et al. 2012; Christe et al. 2003; Orivel et al. 2001; Simone et al. 2009); or they may be induced by exposure to a pathogen, as when workers identify and remove infected materials or transfer immunity to nestmates (Cremer et al. 2007; Pull et al. 2018; Rosengaus and Coates 1998). Social organization may also alter transmission dynamics in larger groups. When larger groups are more socially compartmentalized than smaller groups, disease may be less likely to spread across the entire group (Nunn et al. 2015; Stockmaier et al. 2021; Stroeymeyt et al. 2014). Indeed, theoretical models suggest that infections will spread more slowly across larger, more modular social networks (Naug and Camazine 2002; Nunn et al. 2015; Pie et al. 2004), although empirical support for this hypothesis is limited (but see Stroeymeyt et al. 2018).

Prior research on group size, social immunity, and disease in insect societies focuses on highly eusocial species with large average group sizes, such as honeybees, ants, and termites (Leclerc and Detrain 2018; Naug 2008; Rosengaus and Coates 1998; Traniello et al. 2002; Ugelvig and Cremer 2007). These species have evolved to live in perennial, highly eusocial, and compartmentalized societies, and thus may face different pressures associated disease and immune investment than primitively eusocial species with annual lifecycles and smaller societies, such as bumble bees. For example, bumble bee colonies display less strict division of labor than more highly eusocial species such as honeybees (Jandt et al. 2009; Johnson 2005), which may preclude the social compartmentalization needed to reduce transmission throughout the group at larger group sizes (Naug and Camazine 2002; Nunn et al. 2015; Pie et al. 2004). A better understanding of the relationship between group size and disease across more diverse

insect societies will provide a broader understanding of the impacts of sociality on infectious disease.

Understanding how the costs of disease experienced by bees are altered by their social biology is also of conservation importance. Bumble bees are important pollinators in temperate and arctic ecosystems but many species are experiencing unprecedented global declines, and contagious disease is among the proposed drivers of these declines (Cameron et al. 2011; Cameron and Sadd 2020; Goulson et al. 2015). Although much research has addressed the ecology of disease transmission between bumble bee foragers and the mechanisms of infection in individual bees (Adler et al. 2018; Cisarovsky and Schmid-Hempel 2014; Figueroa et al. 2019; Gillespie 2010), research on pathogen transmission and the costs of disease within bumble bee colonies is limited (but see Otterstatter and Thomson 2007; Pinilla-Gallego et al. 2020). A better understanding of what social factors contribute to the costs of disease in bumble bee colonies is crucial for understanding the ecology of pathogens in pollinator communities.

Here, we used experimental microcolonies to study the common eastern bumble bee (Bombus impatiens), a generalist bee species that is native to eastern North America and commercially available, and Crithidia bombi (Trypanosomatida; hereafter C. bombi), a trypanosomatid bumble bee intestinal parasite. C. bombi is horizontally transmitted through contact with contaminated feces within the nest or on flowers (Cisarovsky and Schmid-Hempel 2014; Figueroa et al. 2019; Otterstatter and Thomson, 2007) and vertically transmitted from queens to workers (Yourth et al. 2008). It reduces queen survival, overwintering success, colony founding, and reproduction, as well as worker learning and foraging efficiency, and its negative consequences are greater when bees are nutritionally stressed (Brown et al., 2003; Conroy et al., 2016; Fauser et al., 2017; Gegear et al., 2006; Yourth et al., 2008). C. bombi is a widespread pathogen, but there is substantial variation in the prevalence of C. bombi in B. impatiens across different geographic regions and between years, with estimates in North America ranging from 11-80% infected (Adler et al. 2018; Figueroa et al. 2019; Gillespie 2010; Graystock et al. 2020;

Malfi and Roulston 2014). Microcolonies are queenless groups of bumble bee workers that are created in the lab by housing workers together (Klinger et al. 2019). While they cannot produce gynes or new workers, one or more workers will develop ovaries and lay unfertilized male-destined eggs (Klinger et al. 2019). Microcolonies are a useful tool for studying colony-level behavior when working with queenright colonies is not feasible and have been widely used to study the consequences of nutrition and pesticides on colony success (Klinger et al. 2019; Tasei and Aupinel 2008), although they are not perfect proxies for queenright colonies (Van Oystaeyen et al. 2021). We address the implications of using queenless microcolonies in more detail in the discussion.

We investigated how group size affects the costs of disease experienced by microcolonies of B. impatiens infected with C. bombi. We manipulated (1) group size (15 workers versus 45 workers) and (2) infection status (1/3 workers inoculated with C. bombi versus sham control) in queenless laboratory microcolonies and compared the effects of these treatments on survival, prevalence, infection intensity, nest construction, and reproduction. We hypothesized that living in larger groups increases the costs of disease experienced by bumble bee microcolonies. Thus, we predicted that larger microcolonies would experience higher mortality rates, infection rates, and infection loads than their smaller counterparts, and that larger microcolonies would experience a greater per capita effect of infection on nest construction and reproduction than would small microcolonies. One possible mechanism through which living in larger groups may increase the costs of disease is if transmission is greater in larger groups (density-dependent transmission). In contrast, the costs of disease may be more similar across group sizes if disease transmission does not depend on group size (frequency-dependent transmission).

Materials and Methods

(a) Microcolony set-up

Microcolonies were created from workers sourced from 9 commercial bumble bee colonies (Biobest, Leamington, Ontario, Canada). After the microcolonies that experienced contamination were excluded, 8 commercial colonies were represented in the final analyses. To ensure each source colony was not infected with C. bombi, we screened each source colony for infection with C. bombi by randomly selecting, dissecting, and screening the gut contents of five workers from each colony (see "Parasite Screening" below for detailed fecal screening method). All source colonies were free of C. bombi infection at the start of the experiment.

We created queenless microcolonies by randomly selecting 15 (small colony) or 45 (large colony) sister workers from each Biobest colony and rehoming them in new plastic nest boxes with a 15 g pollen patty (Queen boxes provided by Biobest, 15.25 x 15.25 cm). Because C. bombi is spread through contact with feces, each microcolony was connected by a plastic tube to an additional chamber to be used as an "outhouse." The "outhouse" contained cat litter to absorb the bees' feces, and the cat litter was replaced weekly. Providing the bumble bees with the option of defecating in an "outhouse" rather than in their nest box simulated more natural conditions and prevented unrealistic levels of C. bombi transmission due to the buildup of feces inside the colony, as wild bumble bees often defecate outside of the main nest cavity (Pinilla-Gallego et al. 2020; Valdes and Scofield 2023). The groups were given 1 week to establish in the lab, after which all microcolonies had constructed honeypots and developed brood, before inoculation.

All microcolonies were housed in a dark growth chamber at approximately 25 C and 50% RH and were provided sugar-water (30% sucrose) and pollen (sourced from CC Pollen Company, High Desert Bee Pollen Granules) directly to the main nest box. One pollen ball (7 g) was added to each colony 3 days per week, and excess pollen was always visible.

(b) Experimental Design

To assess the effect of group size on the consequences of C. bombi infection, we performed a 2 x 2 factorial experiment crossing microcolony group size (15 workers (small colony) versus 45 workers (large colony) and microcolony infection status (inoculated with C. bombi versus sham inoculation control). For each replicate, microcolonies from the same source Biobest colony were assigned to each level to control for genotype and colony condition. We created 17 replicates of microcolonies matched across the four treatment groups, although only 12 replicates were included in analysis as C. bombi contamination was detected in the control microcolonies of 5 replicates. Each group was given a replicate identifier (replicate ID) that was unique for each source colony and time block combination.

(c) Inoculation

One week after microcolony creation, we prepared a fresh inoculum and administered it to one third of the workers in each C. bombi treatment microcolony. We chose to keep constant the initial prevalence rather than the number of infected bees because we wanted to test against the null hypothesis that colony size did not affect disease transmission. If the spread and persistence of a disease within a colony did not depend on colony size, then disease transmission would have to be frequency- rather than density-dependent. Having the same initial prevalence would then ensure if this null hypothesis was correct, disease prevalence would grow at the same rate in both the small and large colonies. In contrast, the alternative of inoculating the same initial number of infected bees would ensure neither the same growth rate in the prevalence, nor the same number of infected bees (once the number of susceptible bees became limiting) in both small and large colonies under the null hypothesis, making our hypothesis difficult to test. Moreover, because the total number of foragers, and thus the total amount of potential pathogen exposure, increases with colony size, inoculating a similar proportion of infected workers would also provide a biologically relevant approximation of the parasite pressure likely to be experienced in the field by colonies of different sizes. To prepare the inoculum, we randomly selected and dissected 10 workers from an infected source colony, homogenized each intestine in

150 uL of dH2O, and allowed them to sit at room temperature for 4-5 hours, during which the guts settled to the bottom and the C. bombi cells swam up to the supernatant. We transferred 10 uL of the supernatant to a haemocytometer and examined it under a compound microscope at 400x magnification to estimate the number of C. bombi cells per microliter. The supernatant was then diluted with 30% sucrose solution to create an inoculum with 800 C. bombi cells per microliter (Richardson et al. 2015).

One third of the workers from each C. bombi treatment microcolony were randomly selected to be inoculated. Each selected bee was anesthetized with CO2 and 10 uL of the prepared inoculum was pipetted onto its extended proboscis (approximately 8000 cells per bee) (Figueroa et al. 2019, 2021; Richardson et al. 2015). After the bee had consumed the entire inoculum, it was returned to its microcolony. All bees consumed the entire inoculum. The same method was used to "inoculate" control colonies with a sham inoculum of 30% sucrose. Inoculated bees were returned to the colony without being marked and may have been included in subsequent pathogen screening.

The C. bombi used in these experiments was collected in Ithaca, NY from wild B. impatiens workers and sustained in laboratory bumble bee colonies.

(d) Parasite Screening

Mortality and C. bombi infection prevalence were monitored weekly for three weeks. During the third week, all surviving bees were dissected and screened for C. bombi infection intensity.

For the first two weeks after inoculation, we used fecal screening to measure C. bombi prevalence. Each worker was removed from the nest and contained in a small plastic salsa cup until it defecated. Bees that did not readily defecate in the salsa cup were agitated to induce defecation, and bees that never defecated were omitted from analysis. Bees from uninfected

control microcolonies were similarly removed and agitated to control for the stress of the fecal screening procedure.

Fecal samples were transferred to a slide with a 10 uL pipet and five randomly selected transects of each slide were examined under a compound microscope at 400x to assess whether C. bombi was or was not present in the sample.

To ensure that the control microcolonies remained free from C. bombi infection, at the end of the second week of the experiment we screened fecal samples from 5 randomly selected workers from each control microcolony. Five of the early control microcolonies were found to be contaminated with C. bombi, and these microcolonies were excluded from analysis. Because we could not rule out that the inoculated microcolonies in the same replicate groups as the contaminated control microcolonies had also been affected by cross-contamination, we also excluded these microcolonies from analysis. To prevent cross-contamination in subsequent replicates, the microcolonies were housed individually in mesh cages, after which no contamination was detected.

On the third week after inoculation, we dissected all workers in inoculated microcolonies. Each intestine was homogenized, placed in 200 uL dH2O and allowed to settle for 4-5 hours, after which the supernatant was examined with a hemocytometer to quantify C. bombi infection intensity (Richardson et al. 2015). We removed one forewing from each bee and measured the marginal cell length as a proxy for body size (Harder 1982).

(e) Nest Measurements

At the end of the experiment, we weighed each microcolony and counted the number of honeypots as proxies for nest construction. We also counted the number of males and pupae in each microcolony as a measure of reproductive output.

(f) Statistical Analysis

Analyses were conducted using R version 4.2.0 (R Core Team 2022). Plots were produced using the package 'ggplot2' (Wickham 2016). Five replicate groups were excluded from all analyses due to C. bombi contamination. Two individual large, control microcolonies were missing data due to researcher error; one starved when its sucrose feeder became detached from the nest box and was missing all data, and one was missing final performance data due to researcher error. Thus, each analysis had a sample size of n=10-12 replicates per treatment group.

(i) Survival

To investigate the effects of treatment (inoculated versus control) and group size (small versus large), we used a Cox proportional hazard model via the 'survival' package (Therneau and Grambsch 2000; Therneau 2022). The analysis evaluated worker survival by week. The model included treatment, group size, their interaction, and replicate ID as explanatory variables. We used the 'emmeans' package (Length 2022) to perform contrast tests comparing the effects of inoculation within each group size with Bonferroni correction.

Additionally, to assess mortality at the end of the experiment, we used a generalized linear mixed model (GLMM) with a binomial distribution via the 'lme4' package. The model evaluated a binary response variable representing whether each bee was alive or dead at the end of the experiment, and included treatment, group size, and their interaction as fixed effects, and replicate ID as a random effect (Bates et al. 2015). We performed post-hoc contrast tests comparing the effects of inoculation within each group size with the 'emmeans' package, with Bonferroni correction (Length 2022).

Sample sizes for both the survival and final mortality analyses were n=12 for small inoculated microcolonies, n=12 for large inoculated microcolonies, n=12 for small control

microcolonies, and n=11 for large control microcolonies, as one microcolony was lost to starvation

(ii) Microcolony performance

We investigated how group size and infection status affected performance with a linear mixed model (LMM) for each measure of microcolony performance, using the 'lme4' package (Bates et al. 2015). The measures of performance that we analyzed were nest weight and total reproductive output (number of pupae plus number of males at the end of three weeks) on both the total and per capita levels. To calculate per capita measures, we divided the value by the total number of bees at the start of the experiment. For each LMM, group size, treatment, and their interaction were included as fixed effects, and replicate ID as a random effect. We performed Type II Wald chi-square tests to test for significance using the 'Anova' function. Post hoc contrast tests comparing the effects of inoculation within each group size were performed with a Bonferroni correction using the 'emmeans' package (Length 2022). To test whether the assumption of normality of residuals was met, we plotted the residuals in a quantile-quantile plot using the 'ggpubr' package (Kassambara 2020); to test whether the assumption of homogenous residuals was met, we plotted the residuals against the model predictions. To better meet the assumption of homogenous residuals, each measure of performance was log-transformed. Sample sizes for all microcolony performance analyses were n=12 for small inoculated microcolonies, n=12 for large inoculated microcolonies, n=12 for small control microcolonies, and n=10 for large control microcolonies, as one microcolony was lost to starvation and one was lost due to researcher error.

(iii) Infection prevalence

We were unable to screen dead bees for C. bombi, so our measures of infection prevalence were only able to account for the prevalence among surviving bees. First, we tested the effects of group size on the rate of increase in C. bombi prevalence over the course of the experiment. We used a GLMM with a binomial distribution via the 'lme4' package (Bates et al. 2015) and evaluated a binary response variable representing the proportion of infected versus healthy surviving bees, with group size, week, and their interaction as fixed effects and replicate ID as a random effect. A significant interaction between group size and week would indicate a difference in the rate of change in C. bombi prevalence.

Then, we tested the effects of group size on C. bombi prevalence at the end of the experiment. Again, we used a GLMM via the 'lme4' package to evaluate the proportion of infected versus healthy surviving bees as the response variable, group size as a fixed effect, and replicate ID as a random effect.

In both infection prevalence analyses, sample sizes were n=12 for both small and large microcolonies.

(iv) Infection intensity

To assess the effects of group size and worker body size on worker infection intensities, we used a GLMM via the 'glmmTMB' package (Brooks et al. 2017). The analysis evaluated worker infection intensity, including group size and marginal cell length (a proxy for body size) as fixed effects, replicate ID as a random effect, and a truncated negative binomial distribution to account for zero-inflation. We used the 'simulateResiduals' function in the dHARMA package to assess the fit of the distribution (Hartig 2022).

To test whether the body size (represented by marginal cell length) of surviving workers differed in large versus small microcolonies, we used a LMM via the 'lme4' package (Bates et al. 2015). Group size was included as a fixed effect, replicate ID as a random effect, and marginal

cell length (body size) as the response variable. We performed Type II Wald chi-square tests to test for significance using the 'Anova' function.

For each infection intensity or body size analysis, n=12 microcolonies, each with variable numbers of surviving bees, were included in each treatment group.

Results

(a) Survival

Compared to control microcolonies, inoculation with C. bombi increased mortality for large (n=45) microcolonies but not for small microcolonies (Fig. 1; Fig. S1).

Over the course of the experiment, there was no effect of colony size on worker survival in control microcolonies (Cox model, HR = 0.871, 95% CI: 0.601-1.264, z = -0.726, p = 0.468), and there was no effect of C. bombi treatment on worker survival in small microcolonies (Cox model, HR: 1.342, 95% CI: 0.886-2.034, z = 1.389, p = 0.165; Fig. 1a). However, there was a significant interaction between group size and inoculation status (Cox model, Interaction of colony size and inoculation, HR: 1.748, 95% CI: 1.082-2.823, z = 2.282, p = 0.023; Fig. 1a). Post-hoc contrast tests revealed that, while there was no effect of C. bombi infection on survival in small microcolonies (Contrast test, z = -1.389, p = 0.330), C. bombi infection significantly reduced survival in large microcolonies (Contrast test, z = -6.987, p < 0.001; Fig. 1a).

The final mortality results were consistent with the results of the survivorship analysis. There was a significant interaction between group size and inoculation status on the proportion of workers that were alive at the end of the experiment (GLMM, z = 2.309, p = 0.021). C. bombi infection significantly increased mortality rate in large microcolonies (Contrast test, z = -7.272, p < 0.001; Fig. 1b), but not in small microcolonies (Contrast test, z = -1.621, p = 0.210).

(b) Microcolony Performance

For each measure of performance (total nest weight, total reproductive output, per capita nest weight, and per capita reproductive output), there was no interaction between group size and C. bombi treatment (total nest weight: Wald, $\chi2 = 0.976$, $p = 0.323$; total reproductive output: Wald, $\chi2 = 1.712$, $p = 0.191$; per capita nest weight: Wald, $\chi2 = 0.976$, $p = 0.323$; per capita reproductive output: Wald, $\chi2 = 1.712$, $p = 0.191$). Thus, we refit each model without the interaction term to report the main effects of group size and C. bombi treatment.

Group size and C. bombi treatment had significant effects on both total nest weight and total reproductive output (Fig. 2a,b). Total nest weight was significantly greater for large microcolonies than for small microcolonies (Wald, $\chi2 = 138.303$, $p < 0.001$) and significantly lower for inoculated microcolonies than for control microcolonies; (Wald, $\chi2 = 11.028$, $p < 0.001$; Fig. 2a). Although the interaction was not significant, we proceeded with contrast tests, which revealed that total nest weight was not significantly different for small, control microcolonies than for small, infected microcolonies (Contrast test, $t = 1.695$, $p = 0.197$), but was significantly different for large, control microcolonies compared to large, infected microcolonies after Bonferroni correction (Contrast test, $t = 2.889$, $p = 0.013$; Fig. 2a).

Similarly, total reproductive output (number of pupae + number of males) was significantly greater for large microcolonies than for small microcolonies (Wald $\chi2 = 14.573$, $p < 0.001$) and significantly lower for C. bombi treated microcolonies than for control microcolonies (Wald $\chi2 = 6.936$, $p = 0.008$; Fig. 2b). Contrast tests also showed that total reproductive output was not significantly different for small, infected microcolonies than for small, control microcolonies (Contrast test, $t = 1.008$, $p = 0.640$), but was significantly different for large, infected microcolonies compared to large, control microcolonies after Bonferroni correction (Contrast test, $t = 2.665$, $p = 0.023$; Fig. 2b).

Group size and C. bombi treatment also had significant effects on both per capita nest weight and per capita reproductive output (Fig. 2c,d). Per capita nest weight was significantly

lower for C. bombi treated microcolonies than for control microcolonies (Wald $\chi2 = 11.028$, $p < 0.001$; Fig. 2c). However, per capita nest weight was significantly lower for large microcolonies compared to small microcolonies (Wald $\chi2 = 16.076$, $p < 0.001$). Contrast tests revealed no significant difference in per capita nest weight between small, control microcolonies and small, infected microcolonies (Contrast test, $t = 1.695$, $p = 0.197$), and a significant difference for large control microcolonies compared to large, infected microcolonies after Bonferroni correction (Contrast test, $t = 2.889$, $p = 0.013$; Fig. 2c).

Similarly, per capita reproductive output was significantly lower for C. bombi treated microcolonies (Wald $\chi2 = 6.936$, $p = 0.008$) and significantly lower for large microcolonies compared to small microcolonies (Wald $\chi2 = 17.586$, $p < 0.001$; Fig. 2d). Contrast tests also showed no significant difference between small, control, microcolonies and small infected microcolonies (Contrast test, $t = 1.008$, $p = 0.640$), and a significant difference between large, control microcolonies and large, infected microcolonies (Contrast test, $t = 2.665$, $p = 0.023$; Fig 2d). Due to log transformation, the contrast test results are the same for the total colony performance measurements as for their per capita counterparts.

We focused on full reproductive output and nest weight in our analysis, but similar trends were observed across component measurements of number of pupae, number of males, and number of honeypots separately (Fig. S2).

(c) Infection Prevalence

Infection prevalence increased over the course of the experiment, and there was a significant main effect of week on prevalence (GLMM, $z = 8.541$, $p < 0.001$; Fig 3; Fig. S3). Group size had no effect on C. bombi prevalence: there was no significant interaction between group size and week, suggesting the rate of change in C. bombi prevalence was not different in small versus large microcolonies (GLMM, $z = -0.701$, $p = 0.483$; Fig. 3a). Similarly, group size

did not have a significant effect on C. bombi prevalence at the end of the experiment (GLMM, z = 0.475, p = 0.634; Fig. 3b).

(d) Infection Intensity

Infection intensity was significantly lower for surviving workers in large microcolonies compared to workers in small microcolonies (GLMM, z = -2.981, p = 0.003; Fig. 4a; Fig. S4). Additionally, there was a significant main effect of worker body size (represented by marginal cell length) on infection intensity (GLMM, z = 3.323, p<0.001; Fig. 4b). Finally, surviving workers in large microcolonies were marginally smaller than surviving workers in small microcolonies (Wald, $\chi 2 = 3.805$, p = 0.051; Fig. 4c).

Discussion

In this study, we found that bumble bees experienced greater rates of mortality when living in larger groups, but not when living in smaller groups, when faced with the same initial C. bombi exposure rate. We observed a similar trend for each measure of microcolony performance, with greater reduction in nest weight and total reproduction for larger microcolonies compared to small microcolonies. These results suggest that C. bombi imposes greater costs on larger bumble bee microcolonies than on smaller microcolonies. Despite this, we found lower C. bombi infection loads in larger compared to smaller bumble bee microcolonies and we did not observe a difference in C. bombi prevalence in surviving bees in small versus large microcolonies. However, because our screening methods cannot detect C. bombi infection in dead bees, our estimates of C. bombi prevalence may be underestimates of true infection rates, particularly in colonies with high rates of mortality. Thus, we cannot rule out the possibility that true infection rates may differ in large versus small microcolonies and we cannot confidently draw conclusions on C. bombi transmission mechanisms. We propose that the greater costs of C.

bombi infection in larger microcolonies could be explained (1) if living in larger groups imposes additional stresses on bumble bees that could make them both more vulnerable to and better hosts for C. bombi, or (2) there are greater rates of transmission and subsequent mortality in larger microcolonies.

Theoretical models and empirical research in highly eusocial ants (Myrmica rubra) and termites (Zootermopsis angusticollis) have found that reduced disease susceptibility can be an advantage of sociality (Leclerc and Detrain 2018; Naug and Camazine 2002; Pie et al. 2004; Rosengaus and Coates 1998; Schmid-Hempel 1998). Our differing results may be due to differences in the underlying social structure or life cycle of bumble bees. Bumble bee colonies are primitively eusocial and relatively small (Goulson 2010), while Myrmica rubra and Zootermopsis angusticollis live in larger highly eusocial societies. The larger size of ant and termite colonies could increase the pressure on these species to evolve social immunity behaviors or could make them more efficient at employing social immunity behaviors. Bumble bees also differ in that their colonies are shorter-lived, with annual rather than perennial lifecycles (Goulson 2010). Shorter lived species are generally hypothesized to invest less in immunity than longer lived species, as they may experience greater benefits to rapidly investing in growth and reproduction and lower costs of parasitism (Boots et al. 2013; Johnson et al. 2012; Martin II et al. 2006; Previtali et al. 2012).

Despite having lower rates of C. bombi-induced mortality, workers in small microcolonies had significantly higher C. bombi loads than workers in large microcolonies. One potential explanation for this is that the higher mortality rate in the large microcolonies reduced the number of bees with high levels of infection. In particular, if the bees that were inoculated with C. bombi had higher mortality rates in the large compared to small microcolonies, the greater C. bombi loads observed in small microcolonies could have been driven by greater numbers of surviving initially infected bees in those small microcolonies, compared to in the large microcolonies. We are unable to rule out this possibility because the initially inoculated

bees were not marked and thus were included at unknown proportions in all screening and analysis.

Alternatively, prior research has found that access to dietary pollen and nectar significantly increases C. bombi loads in individual bees, despite reducing mortality (Brown et al. 2003; Conroy et al. 2016). Thus, nutritional stress could account for both the greater mortality and the lower C. bombi loads observed in bees in larger colonies. Although the microcolonies in our experiment had ad libitum access to food, social factors such as resource guarding or scramble competition could reduce access to pollen or sucrose in larger microcolonies. Competition could also increase the metabolic or physiological demands on workers in larger microcolonies, indirectly increasing nutritional stress (Gobin et al. 2003). There are several mechanisms by which host nutritional stress could affect gut parasites: (1) by reducing the direct availability of nutrients for the parasite, (2) by negatively affecting host quality, or (3) by reducing host metabolism or activity (Conroy et al. 2016; Schmid-Hempel 1998).

Importantly, we focused on queenless microcolonies in this study. Microcolonies are a useful model when it is not feasible to work with full colonies, and they have been widely used to study the effects of pesticides and nutrition on colony success (Klinger et al. 2019). However, their behavior can differ from that of queenright colonies in important ways (Klinger et al. 2019; Tasei and Aupinel 2008; Van Oystaeyen et al. 2021). Queenless bumble bee colonies experience greater reproductive conflict and aggression than queenright colonies, because workers compete to develop ovaries and lay their own male-destined eggs (Kocher and Grozinger 2011; Lopez-Vaamonde et al. 2007; Princen et al. 2020). As workers in queenless colonies divert more energy and resources toward competition and reproduction, they may reduce their investment in both individual and social immune defenses (Gobin et al. 2003; Schwenke et al. 2016). Indeed, queenless acorn ant (Temnothorax curvispinosus) colonies perform fewer collective behaviors and are more susceptible to an entomopathogenic fungus than are queenright colonies (Keiser et al. 2018). These differences may be especially pronounced at larger group sizes, as ovarian

development, worker aggression, and the number of workers sharing reproduction all increase with group size in bumble bee colonies (Amsalem and Hefetz 2011; Cnaani et al. 2007).

While queenless colonies are not perfect proxies for queenright colonies, queenless bumble bee colonies may be more common than is typically acknowledged and therefore deserving of more attention. Estimates on the rate of queenlessness in wild bumble bee colonies are limited, largely because most bumble bee species nest in subterranean cavities that are difficult to locate and study (Liczner and Colla, 2019). However, in four studies that have carefully excavated and recorded the demographics of bumble bee colonies, a large proportion were found to be queenless: 8 of 14 Bombus terrestris colonies were queenless (O'Connor et al. 2013); 6 of 11 Bombus deuteronymus colonies were queenless (Takahashi et al. 2010); 2 of 7 Bombus ignites colonies were queenless (Takahashi et al. 2008); and 4 of 10 Bombus frigidus colonies were queenless (Valdes and Scofield 2023). This suggests that queenless colonies may be more common and ecologically important than is widely thought. If so, our use of queenless microcolonies to study the impacts of disease may have similar ecological importance as a model for queenless colonies as it does for queenright colonies.

Finally, our study compares only two colony sizes, 15 workers versus 45 workers, and is not able to assess the relationship between group size and disease beyond these two discrete colony sizes. Additionally, mature queenright bumble bee colonies often reach over 300 bees, a size far outside the maximum group size tested in this study (Cnaani et al. 2002). It is possible that while microcolonies with 45 workers are more susceptible to disease than microcolonies with 15 workers, this relationship may be non-linear, and the trend may change or reverse at very high or very low group sizes. While our study represents a starting point for understanding the relationship between group size and disease in bumble bees, research across a broader continuum of group sizes is necessary to explain a more complicated, non-linear relationship.

Overall, our results demonstrate that living in larger groups can increase the costs of disease experienced by bumble bee microcolonies. These results extend prior research on the

contexts in which bumble bees are more susceptible to C. bombi infection, showing that the negative consequences of C. bombi infection depend on social as well as individual factors (Conroy et al. 2016). These results also build on prior research on the relationship between group size and disease that had previously been concentrated in highly eusocial insects, suggesting that group size may come with greater costs in this primitively eusocial species. Future research across taxa is needed to resolve when group size may serve as a risk or protection against infectious disease (Nunn et al. 2015; Rifkin et al. 2012). Finally, while our results show greater costs of disease in larger bumble bee microcolonies, they show similar infection rates and higher infection loads in smaller colonies. These patterns suggest several mechanisms by which group size could shape disease dynamics in bumble bees, and future research investigating which mechanisms may drive these observed patterns will provide broader insight into how group size and social behavior shape the consequences of infectious disease for social insects.

Figure 1: Infection with *C. bombi* increases mortality for workers in large, but not small bumble bee microcolonies. N=12 for small inoculated colonies, n=12 for large inoculated microcolonies, n=12 for small control microcolonies, and n=11 for large control microcolonies, as one microcolony was lost to starvation. (a) Survival curve showing worker mortality in *C. bombi*-inoculated vs. uninoculated small and large colonies during the full three-week experiment. Blue, control; Orange, inoculated; Solid line, small (15 bees); Dotted line, large (45 bees). (b) Proportion of workers dead at the end of the experiment in each treatment group. Each point represents one microcolony.

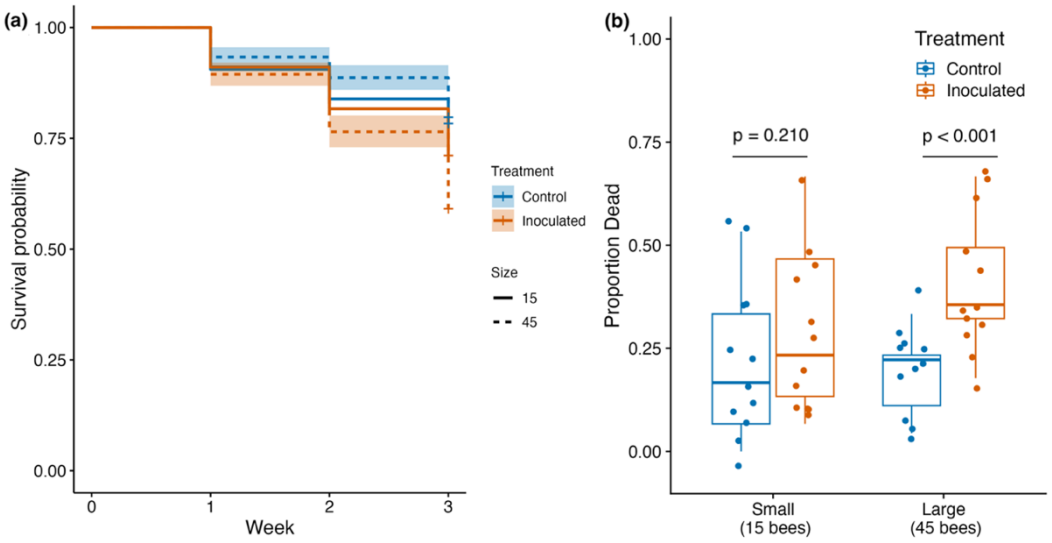


Figure 2: Infection with *C. bombi* significantly reduces measures of performance for large, but not small microcolonies, on both the total and per capita levels. N=12 for small inoculated colonies, n=12 for large inoculated microcolonies, n=12 for small control microcolonies, and n=10 for large control micocolonies, as two microcolonies were lost to starvation and researcher error. For each figure, each point represents one microcolony.

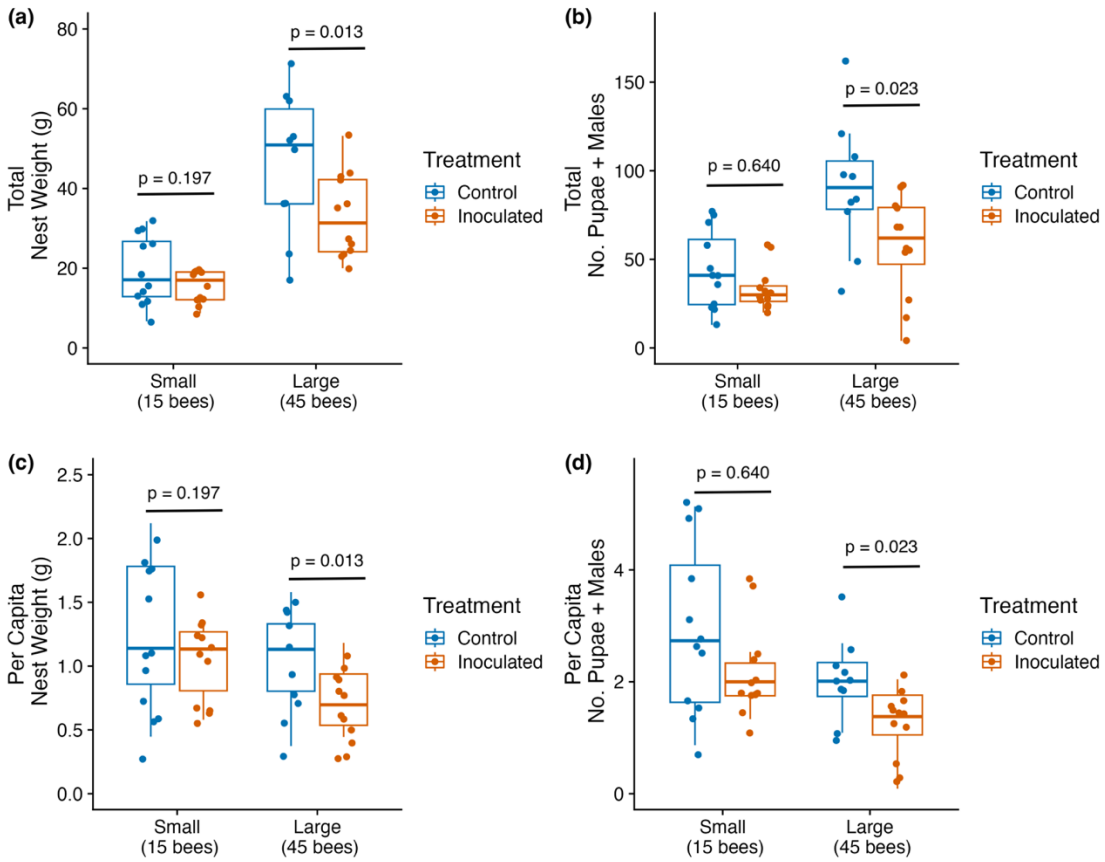


Figure 3: There was no difference in the proportion of surviving workers infected with *C. bombi* in small versus large microcolonies. N=12 for all treatment groups. (a) Shows the proportion of surviving workers infected with *C. bombi* over three weeks for small (blue) and large (orange) microcolonies. Each point one microcolony at the timepoint, and trendlines represent a generalized linear model. (b) Shows the proportion of surviving workers infected with *C. bombi* in each microcolony at the end of the experiment.

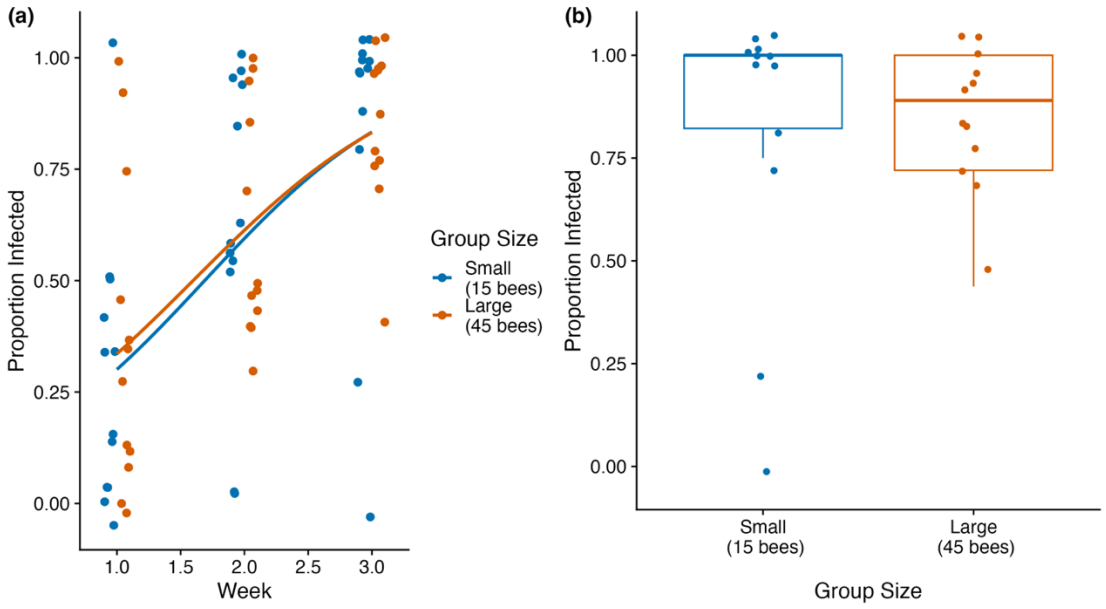


Figure 4: *C. bombi* infection intensity is greater for bumble bee workers in small versus large microcolonies, and infection intensity is correlated with differences in body size. N=12 microcolonies, each with variable numbers of surviving bees, were included in each treatment group. Each point represents one bee and only bees with detectable *C. bombi* infection were included. (a) *C. bombi* infection intensity in small versus large microcolonies. Each point represents the number of *C. bombi* counted in one bee at the end of the experiment plotted on a log scale. (b) The relationship between bumble bee worker size and *C. bombi* infection intensity. The trend line represents a generalized linear model fit with a negative binomial distribution. (c) Marginal cell length in small versus large microcolonies. Marginal cell length is a proxy for body size.

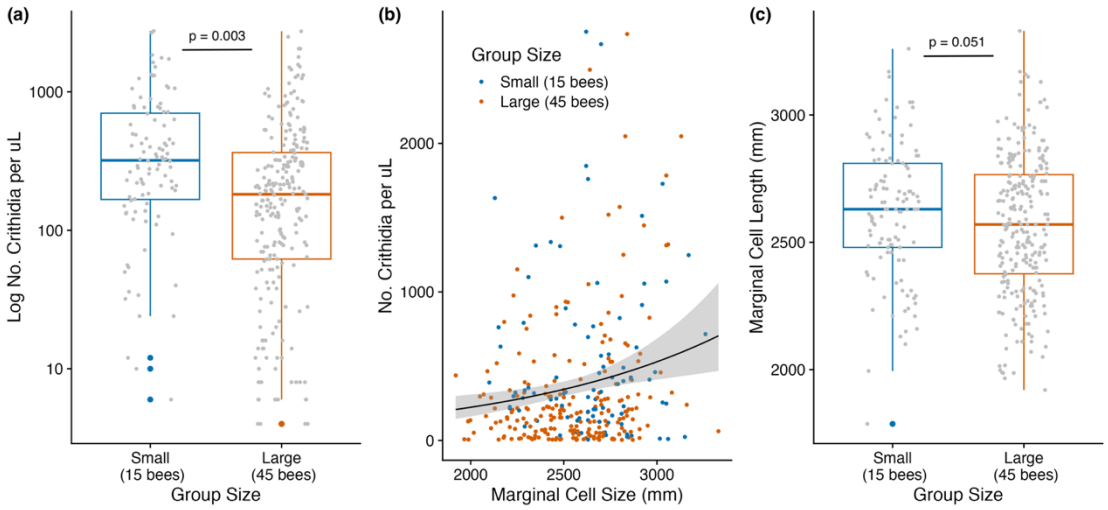


Figure S1: Survival for workers over the three weeks of the experiment. Each panel represents one matched set of microcolonies and each line represents one microcolony (Blue, small microcolonies (15 bees); Orange, large microcolonies (45 bees) Control; Dotted line, *C. bombi* treatment).

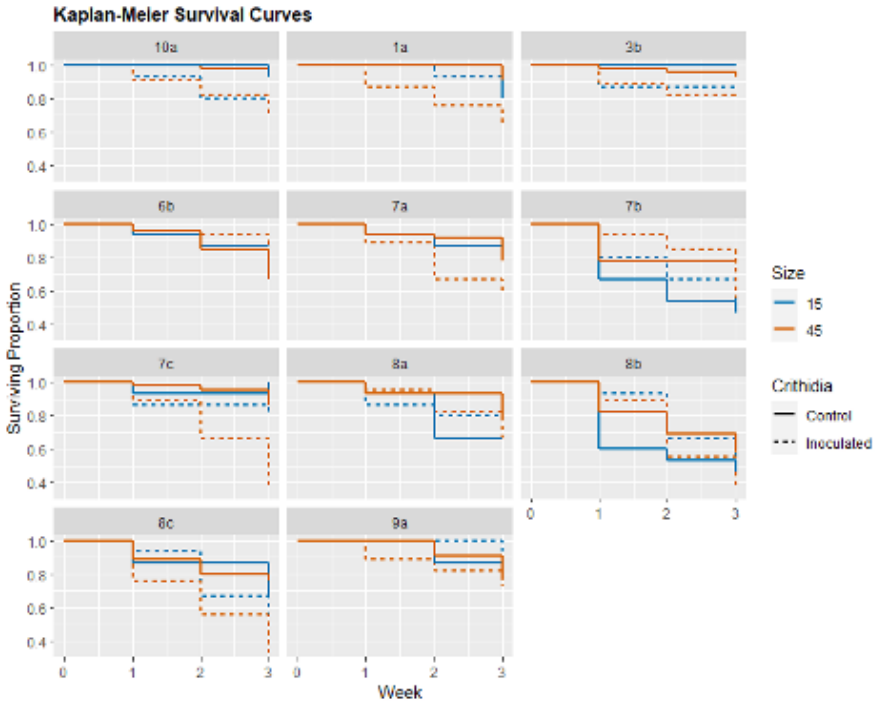


Figure S2: Effect of colony size (small (15 bees) versus large (45 bees)) and treatment (control versus *C. bombi* inoculated) on each proxy for colony performance measured. N=12 for small inoculated colonies, n=12 for large inoculated colonies, n=12 for small control colonies, and n=10 for large control colonies, as two microcolonies were lost to starvation and researcher error. Each point represents one colony at the end of the three week experiment.

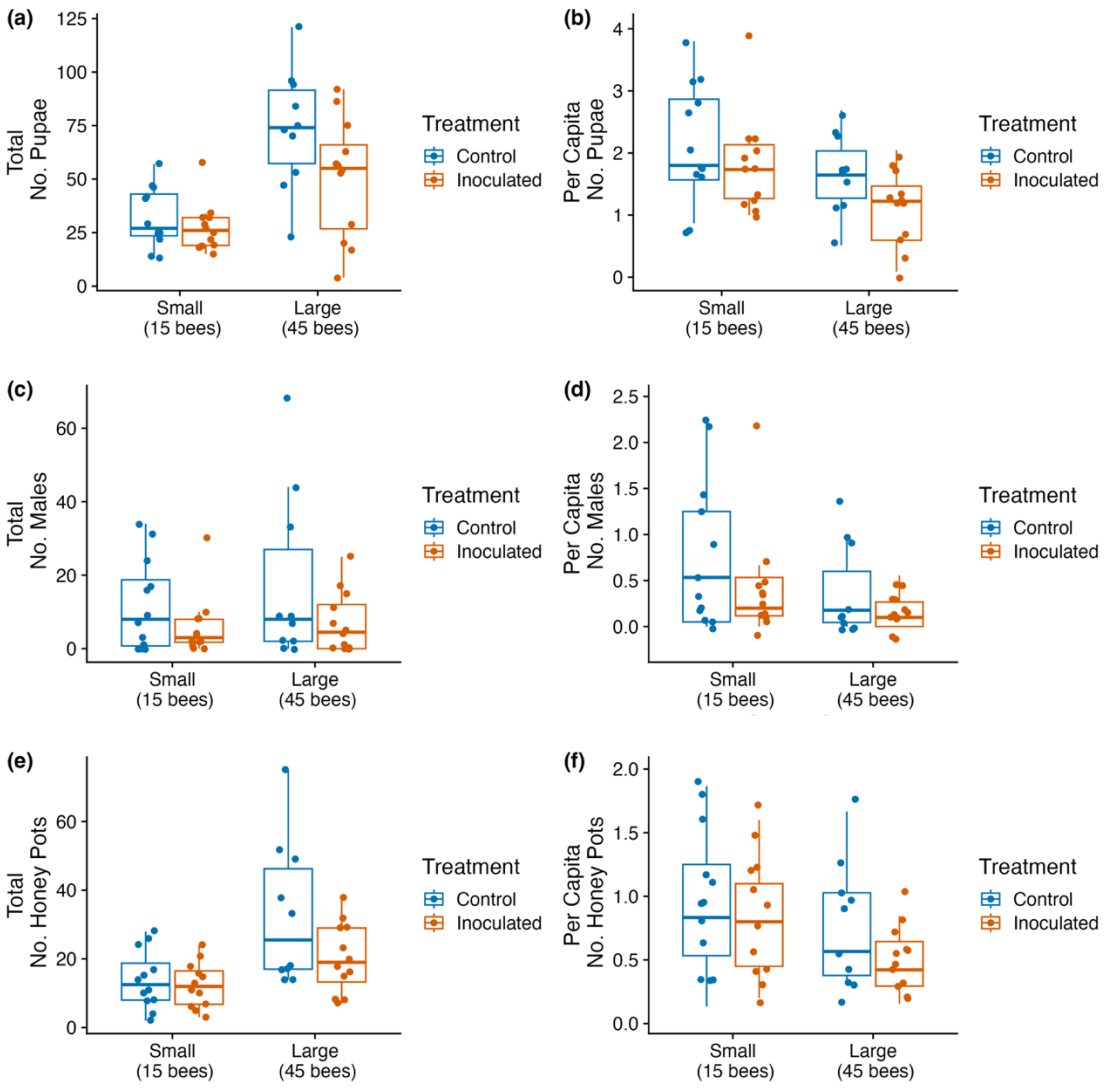


Figure S3: Shows the proportion of workers that tested positive for *C. bombi* infection in each matched pair of microcolonies over the three weeks of the experiment. Each panel represents one matched pair of microcolonies and each line represents one microcolony (Blue, small (15 bees); Orange, large (45 bees)).

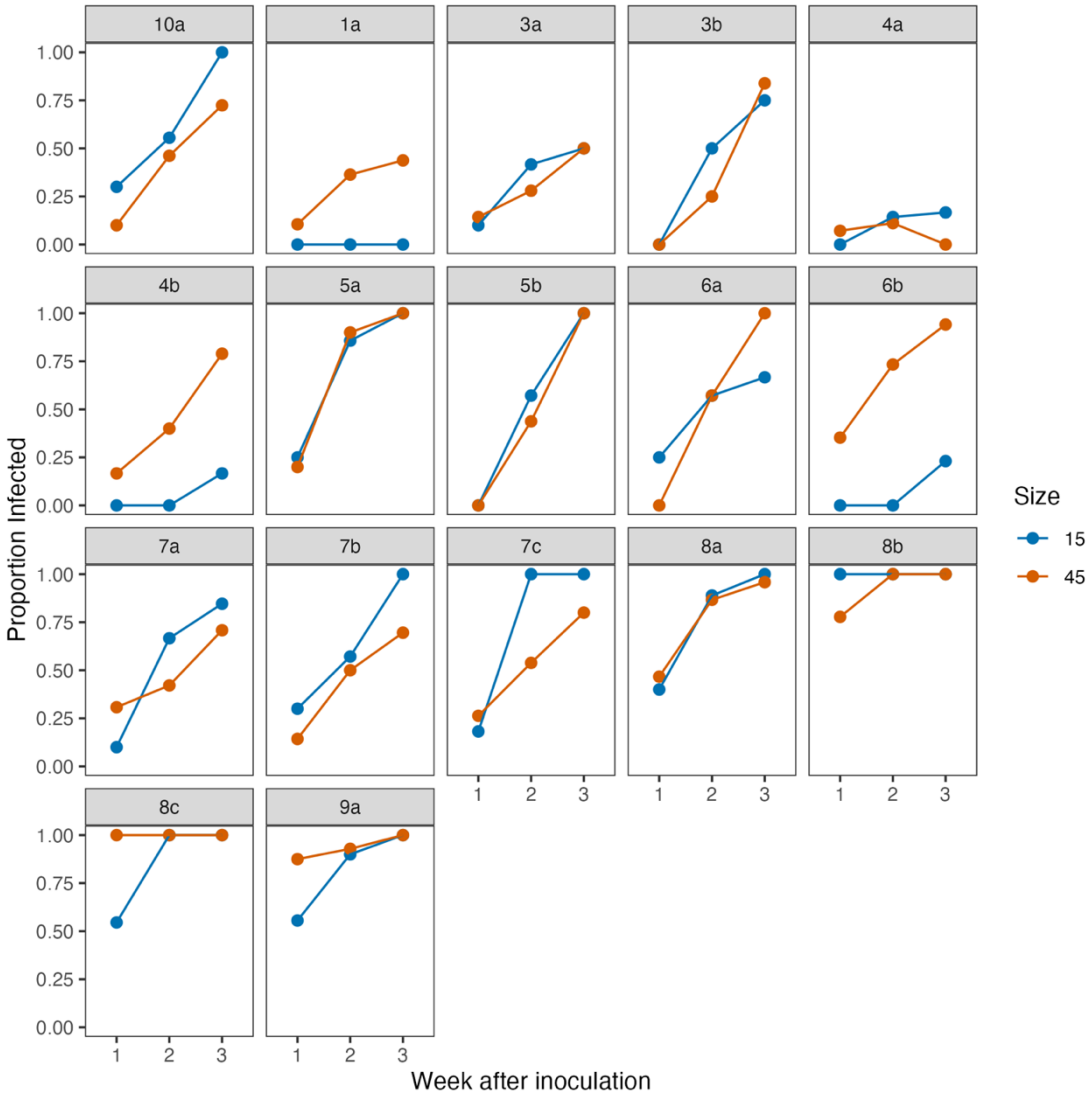
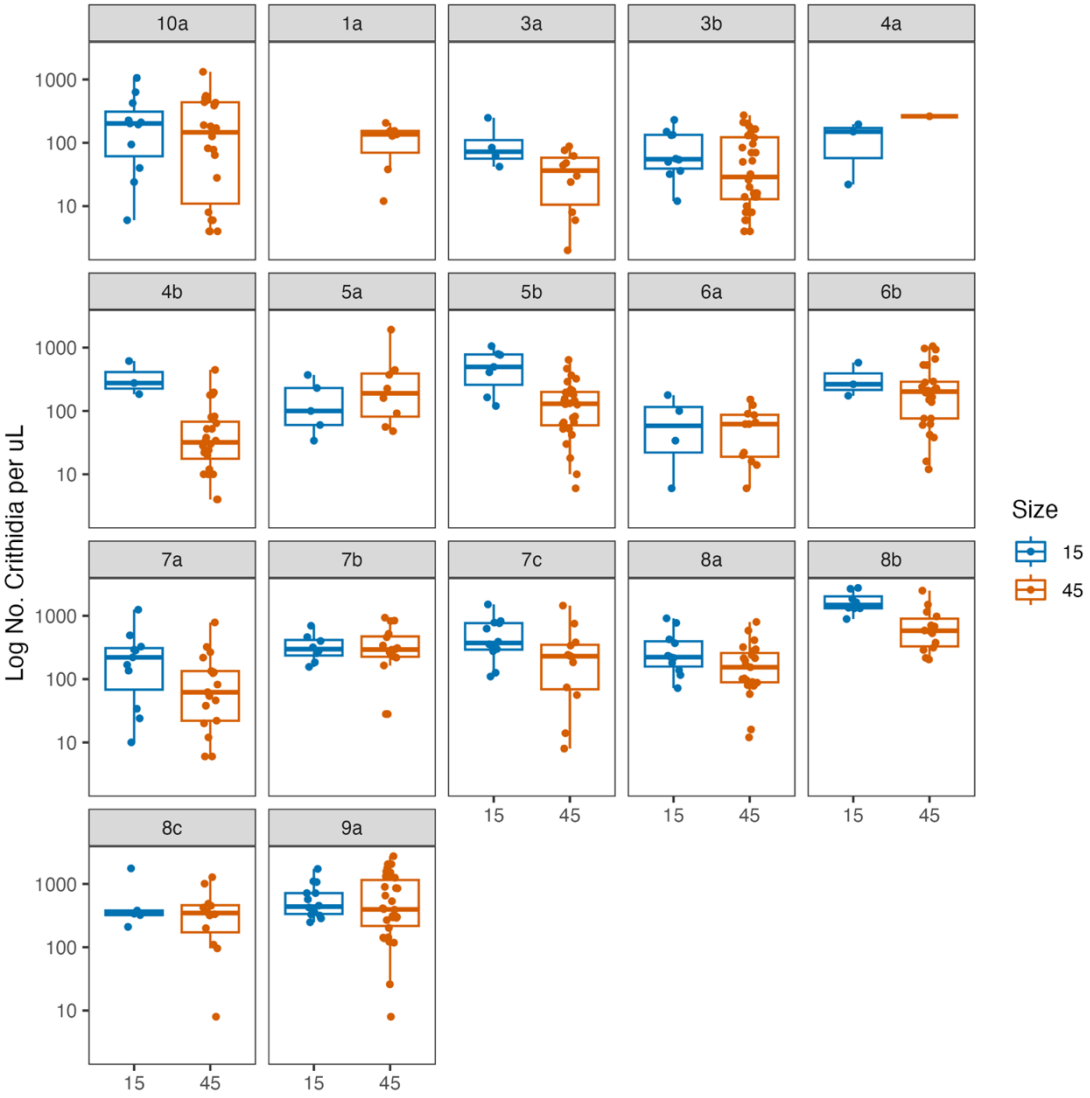


Figure S4: Infection intensities of workers infected with *C. bombi* in small (15 bees) versus large (45 bees) microcolonies, omitting workers that were not infected. Each panel represents one matched pair of microcolonies, each boxplot represents one microcolony, and each point represents one bee. Points are plotted on a log scale.



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Data availability

The datasets generated and analyzed in this study and the scripts used for data analysis in the present study are available on Github: https://github.com/LeahValdes/Bumblebee-group-size-and-disease

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CHAPTER 2

NEST OBSERVATIONS REVEAL HYGIENIC ARCHITECTURE IN AN ARCTIC BUMBLE BEE SPECIES

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Abstract

Many bumble bee species are declining worldwide. Disease and habitat loss are two contributors to these declines, yet little is known about disease transmission within bumble bee colonies or bumble bee nesting habitat requirements. These gaps are especially wide for Arctic species, as prior bumble bee research focuses disproportionately on the contiguous United States, southern Canada, and Europe. Here, we describe the nesting habitat, spatial distribution, and nest architecture of ten frigid bumble bee (*Bombus frigidus*) nests found in the low Arctic in western Alaska. In these nests, we observed a novel nest architecture, the "outhouse," that consisted of an additional subterranean cavity containing concentrated fecal materials and observed that the bees continued to defecate in one or a few locations outside of the main nest cavity when transplanted to the laboratory. Finally, we measured fecal-borne trypanosome parasites in frigid bumble bee workers in this region. Together, our findings provide natural history information on this Arctic

bee species and suggest that the outhouse architecture could function as a social immunity trait to reduce the transmission of fecal-borne pathogens within the nest.

Introduction

Many bumble bee species are in decline worldwide, and disease and habitat loss are two major contributors to these declines (Goulson et al. 2015, Cameron et al. 2011). Social insects may be especially vulnerable to disease because individuals live at high densities and are often closely related (Schmid-Hempel 1998, Cremer et al. 2007). Consequently, many species of social insects have evolved collective "social immunity" behaviors to reduce transmission, infection load, and fitness costs associated with infection (Schmid-Hempel 1998, Cremer et al. 2007). Although social immunity behaviors are well-studied in highly eusocial insects (honey bees, ants, and termites), less is known in primitively eusocial species such as bumble bees. However, social immunity behaviors that have been observed in bumble bees include removing corpses to the outside of the nest, guarding the nest entrance against intruders, and transmitting beneficial gut microbiota to nestmates (Jandt et al. 2009, Koch and Schmid-Hempel 2011, Munday and Brown 2018, Walton et al. 2019). A better understanding of within-colony disease transmission and defenses is necessary for understanding and predicting the consequences of disease for pollinator communities.

One group of parasites implicated in bumble bee declines are trypanosome intestinal parasites, such as *Crithidia spp*. *Crithidia* parasites are widespread across the United States and Europe and infect many bumble bee species, although there is significant regional variation in prevalence (Gillespie 2010, Cordes et al. 2012, Malfi and Roulson 2014, Adler et al. 2018, Figueroa et al. 2023). Infection harms foraging ability, colony founding success, and survival,

particularly for stressed bees (Brown et al. 2000, Otterstatter et al. 2005, Figueroa et al. 2023). Although *Crithidia* parasites are transmitted through contact with contaminated feces both within the colony and on flowers, prior research has focused disproportionately on floral transmission. Less is known about the consequences of within-colony hygienic behavior and nest architecture for how this fecal-borne parasite is transmitted within colonies (Schmid-Hempel 1998, Figueroa et al. 2023, but see Otterstatter and Thomson 2007, Pinilla-Gallego et al. 2020).

Healthy honey bees defecate almost exclusively outside of their nests, and this is thought to serve social immunity by reducing the accumulation of potentially harmful materials inside the nest (Seeley 1995, Cole et al. 2021). In contrast, bumble bees and stingless bees often defecate inside their nests, sometimes in a designated area (Michener 1974). Published descriptions of bumble bee nest box designs used for numerous species include an additional vestibule near the nest entrance where the bees defecate, and including the vestibule in the nest box design helps to prevent the accumulation of feces inside the nest (Sladen 1912, Alford 1975, Pomeroy and Plowright 1980). Moreover, laboratory observations have suggested that providing *B. impatiens* with an additional cavity prevents the accumulation of feces and slows *C. bombi* transmission within the nest, suggesting that hygienic defecation behavior could play a role in reducing within-colony disease transmission (Pinilla-Gallego et al. 2020). However, observations of defecation behavior in wild bumble bee colonies are needed to better understand the ecological importance of this behavior.

Habitat loss is another a major contributor to bee declines, and extensive research has investigated bumble bee forage habitat requirements. However, nesting habitat is also an essential and potentially limiting resource for bumble bees (Goulson 2015, Richards 1978, Osborne et al. 2008). Despite this, knowledge of bumble bee nesting behavior is challenged by

the difficulty of locating nests in the field. Many bumble bee species nest in tall grasses above ground or in abandoned rodent burrows below ground, making them difficult to detect visually (Kells et al. 2003, Liczner and Colla 2019). Methods for systematic nest detection have been developed but are time-intensive and typically biased toward common species and agricultural landscapes (e.g., Pugesek and Crone 2021, Osborne et al. 2008, O'Connor et al. 2012, Rao and Skyrm 2013, Liczner and Colla 2019). Consequently, although published descriptions of bumble bee nests exist for a number of species, these reports are usually opportunistic and typically describe only a few (n=1-3) excavated nests (e.g., Hoffmann et al. 2004, Koch and Cane 2022, Boone et al. 2022, Martinet et al. 2022).

Research on the nesting biology of Arctic bumble bee species is especially limited (Liczner and Colla 2019). Nests of many European species with ranges in the subarctic and arctic have been described (e.g., *B. soroeensis, B. lucorum, B. magnus, B. hypnorum, B. pratorum, B. alpinus*), and like temperate bumble bee nests, many of these nests were found in tufts of grass, abandoned rodent or bird nests, or cavities in man-made structures (Loken 1973). However, these descriptions are brief and anecdotal in nature (Loken 1973). More recently, Martinet et al. (2022) describe one nest of *B. lapponicus*, a common circum-Arctic bumble bee species, that was found in an abandoned rodent burrow in Sweden.

In the North American Arctic, descriptions of natural bumble bee nests are lacking for many species (Liczner and Colla 2019, but see Milliron and Oliver 1966). Nesting preferences and behavior have been studied for some species, although much of this research uses artificial domiciles in lieu of natural nests (Hobbs 1964, 1965, 1967, Richards 1978). This research shows species-specific variation in nesting behavior and preferences. *Bombus (Pyrobombus) frigidus* Smith, 1954 (Hymenoptera: Apidae), an early-emerging North American bumble bee species that

is found in tundra and boreal forest regions from western Alaska to eastern Canada, as well as in the Rocky Mountains, showed more flexibility in its nesting preferences compared to other *Pyrobombus* species (Hobbs 1967, Richards 1978, Vogt et al. 1994, Williams et al. 2014). *B. frigidus* established nests in artificial domiciles that were underground, on the surface, and above-ground (Hobbs 1967, Richards 1978). This research informs our understanding of these Arctic species' nesting preferences, but observations of natural nests are needed to supplement these findings.

Here, we report the nesting habitat and spatial distribution of ten frigid bumble bee, *B. frigidus*, nests found in the low Arctic on the Seward Peninsula of Alaska. We describe the nest architecture of these *B. frigidus* nests, including a novel "outhouse" structure and hygienic defecation behavior. Additionally, we screened bumble bee workers across the Seward Peninsula for trypanosome parasites. These findings provide natural history information on the nesting behavior of this subarctic bumble bee species, and we hypothesize that the outhouse architecture may function to reduce the spread of disease within the nest.

Locating and describing nests

Two methods were used to locate bumble bee nests in different environments on the Seward Peninsula. To locate nests in a Nome neighborhood, Icyview (Site 1), we obtained

permission from residents to survey for nests on their land. We then walked random transects while observing the ground and built structures for bumble bee activity.

To identify potential nest sites in the tundra, we walked transects along the Kougarok Road (Nome-Taylor Highway) between June 20th and 27th 2021 (Figure 1). Every 5-10 miles we walked two transects of approximately 100 paces, perpendicular to each side of the road, while scanning for bumble bee activity. Transects were only walked on land on which we had permission to sample: land owned by the Sitnasuak Native Corporation, State of Alaska, or the Bureau of Land Management.

Nests were found by auditorily observing underground buzzing or visually observing bees entering or emerging from the ground. Nests were then found by feeling either substrate-borne vibrations or the change from cold, wet moss to a warm, dry nest area. After the first nest was located at a site, we searched for additional nests nearby by walking random transects within 200m of the nest for 1 hour.

The *B. frigidus* colonies showed defensive behaviors during nest collection but were less aggressive than wild bumble bee colonies observed in the northeastern United States (*B. impatiens*, *B. griseocollis, B. fervidus*). Disturbing the *B. frigidus* nests provoked buzzing from the bees, and a few workers emerged onto the nest surface and oriented their abdomens upward. No bees flew up from the nest and we did not receive any stings during nest collection.

We located a total of 10 nests, all of which were *B. frigidus* colonies. Nests were located in the Icyview neighborhood of Nome and at 2 transect sites along the Nome-Taylor Highway. In the Icyview neighborhood, we found four nests at two different locations 0.4 km apart (n=1 and n=3 nests respectively). Along the Nome-Taylor Highway, we found nests at two transect sites,

Site 2 (n=1 nest) and Site 7 (n=5 nests). At both Site 1 and Site 7, nests were found close together: three of the four nests at Site 1 were found within 0.5 m of each other, while all five nests at Site 7 were found within 40 m of each other.

Nest site locations varied in their environment, plant cover, and physical substrate (Table 1, Figure 2b). Site 1 was located within a neighborhood surrounded by tundra, but nests were found in clumps of grass planted atop gravel fill (n=3) and one nest was found in the fiberglass insulation in an exterior shed wall. Site 2 was located under a mix of native grasses, sedges, and wild Iris along the highway roadside, and the nest was enclosed in a cavity approximately 8 cm under the soil. Site 7 was located in open tundra among stands of Alaska willows (*Salix spp.*). These nests were covered in small dome-shaped canopies built from sphagnum moss and various lichen species and were located 3-6 cm below the surface of the matted sphagnum moss, above the soil (Figure 2c). Although we did not observe nest construction behavior, the canopies neatly covered each nest, suggesting that the bees may have actively constructed and maintained the nest canopies. We did not observe a wax canopy covering the nests or supporting the canopies.

Nests were located in the field between June 21st and June 27th, and were collected and transported to a laboratory in Riverside, CA for further study on July 4th 2021. Only bees that were inside the nest at the time of excavation were transported to the lab, but nests were collected on a cold and rainy day to maximize the number of bees inside each nest at the time of collection. After collection, the bees in each nest were censused by caste (queen/gyne, worker, or male), pinned, and donated to the University of Alaska Entomology Collection (UAA). Photos of vouchers from each colony were identified by Leif Richardson and all pinned specimens were independently identified by Jessica Rykken.

Outhouse architecture and behavior

During nest collection, the vegetation surrounding the nest was removed to expose the nest. Within each nest, we observed an additional smaller cavity adjacent to the primary nest cavity, which contained concentrated fecal materials (Figure 3a). This "outhouse" cavity was within the same substrate (dried moss, grass, or fiberglass insulation) as the primary nest cavity, but clearly separated by a barrier of the substrate. While each nest was approximately 6-9 cm in diameter, each outhouse was considerably smaller, approximately 3-4 cm in diameter. We did not observe any fecal materials in the nest area outside of the "outhouse" cavity. Although the main nest cavity was noticeably warmer and drier to the touch than the surrounding substrate, the "outhouse" cavity was colder and appeared wetter than the surrounding area.

Three colonies survived transport to the lab and were allowed to acclimate for 3 days, after which every nest was photographed to document where in the nest boxes the bees had defecated. We also visually inspected the bottom of each box for feces. The bees showed strong defecation site fidelity; defecation was concentrated in 1-2 sites on the edge of their cages, outside of the main nest cavities (Figure 3b).

Parasite Prevalence

In addition to nest collection, we opportunistically collected n=50 *B. frigidus* bees across the Nome Road System on the Seward Peninsula and screened each bee for trypanosome gut

parasites via light microscopy. Each bee's intestine was dissected, homogenized in 200 µL dH2O, and allowed to settle for 2 hours. The supernatant was then examined with a hemocytometer to observe whether trypanosome gut parasites were present within each bee's intestine (Richardson et al. 2015). Trypanosome parasites were identified in 29 of the 50 bees screened for parasites (prevalence of 58%).

Conclusions

Our discovery of a larger sample of *B. frigidus* nests (n=10) provides natural history information on the distribution and diversity of wild *B. frigidus* nests that is rarely available for bumble bee species, particularly in the Arctic. The nests were found across a variety of habitats (residential, roadside, tundra), substrates (grass, fiberglass insulation, sphagnum moss), and positions (underground, under the substrate surface, on the surface) indicating plasticity in this species' nesting requirements and a potential tolerance for anthropogenic habitat. This finding is consistent with previous manipulative work using artificial domiciles to study *B. frigidus* nesting behavior, in which *B. frigidus* established nests in artificial domiciles in different positions (underground, on the surface, above ground) and habitat types (open, transition, woods) (Hobbs 1967, Richards 1978).

In addition, the nests were heterogeneously distributed across survey sites. Nests were aggregated at a minority of sites, with four nests at Site 1, one nest at Site 2, five nests at Site 7, and no nests at the remaining seven transect sites. The aggregated distribution of nests across sites could reflect the distribution of preferred nesting habitat, abiotic conditions, floral resources, or preexisting nesting cavities. Nests were also heterogeneously distributed within our

sites; three nests were found within just 0.5 m of each other at Site 1. Bumble bees often nest opportunistically in abandoned rodent nests and may require specific nesting materials such as dried grasses or rodent fur, and as a result may be constrained by the distribution of potential nest sites or materials (McFrederick and LeBuhn 2006, Richards 1978). Better understanding what traits or resources allow some sites to support larger numbers of bumble bee nests is important for habitat conservation and for understanding how nesting resources will be affected by ecological change.

The aggregated distribution of nests could also have important consequences for social and reproductive behavior. Workers in many bumble bee species are known to drift to other colonies, where they may produce their own offspring (Blacher et al. 2013, Zanette et al. 2014). The close proximity of *B. frigidus* nests may elevate levels of drift and social parasitism in these colonies.

Most interestingly, we describe a novel element of wild bumble bee nest architecture, a damp feces-filled secondary nest cavity, hereafter "the outhouse," and demonstrate that workers continue to behaviorally isolate their feces outside of the main nest cavity when deprived of a physical outhouse structure. Published descriptions of designs for artificial bumble bee nest boxes include additional cavities that reduce the accumulation of feces within the nest, and laboratory bumble bee colonies of many species defecate on the perimeter of their enclosures or in additional cavities (Sladen 1912, Michener 1974, Alford 1975, Pomeroy and Plowright 1980, Pinilla-Gallego et al. 2020). Thus, although our observations are limited to *B. frigidus,* it is possible that this is a more general trait of wild bumble bee colonies. Moreover, to our knowledge, an outhouse structure has not before been described in a wild bumble bee nest. Our observation of an outhouse structure in wild bumble bee nests supports that prior laboratory

observations are ecologically relevant to wild bumble bees, and suggests that this behavior may also be present in species not routinely reared in the lab.

It is not clear whether the bees constructed the outhouse cavities or used preexisting cavities on their nests' perimeters. Bumble bee queens across many species dig hibernacula to use as overwintering sites, suggesting that bumble bees could be capable of digging to alter their nest (Liczner and Colla 2019). Additionally, many bumble bee species construct canopies made of dried grasses and leaves over their nests, demonstrating that they actively alter their nest architecture (Taylor and Cameron 2003, Hines et al. 2007). However, nest construction is energetically costly and time consuming for many animal species, and it is not clear to what extent bumble bee species are able to excavate their nests (Sladen 1912, Plath 1924, Laidre 2018). Future behavioral research could clarify whether *B. frigidus* actively constructs outhouse cavities in their nests.

Although honey bees primarily defecate outside, an underground outhouse chamber may be more important for arctic bees, as they face relatively cold temperatures that may limit them from leaving the nest for longer periods of time (Cole et al. 2021, Burns et al. 2022). Summer temperatures characteristically range from 7-9° C in the low arctic and 9-12° C in the subarctic (Burns et al. 2022). These temperatures approach the critical thermal minimums of other bumble bee species (*B. huntii*, *B. bifarius*, and *B. sylvicola*), suggesting that cold temperatures could be a stressor for bumble bees in these regions (Oyen et al. 2016).

Finally, we propose that the outhouse could serve a social immunity function for *B. frigidus*. Bumble bees are hosts to multiple species of trypanosome gut parasite that are transmitted via contact with infected fecal materials, and our study confirmed that these parasites are present in *B. frigidus* workers on the Seward Peninsula.

In the continental United States, many studies have reported *Crithidia* rates in bumble bee species. There is substantial variation both within and across species, as well as across space and time, with estimates ranging from 0-82% infected (Gillespie 2010, Cordes et al. 2012, Malfi and Roulson 2014, Figueroa et al. 2021). A nationwide survey comparing *C. bombi* rates across 36 species in the United States found reported that *C. bombi* prevalence was usually lower than 10% for the majority of bumble bee species, but highest in *B. mixtus* and *B. impatiens* populations (32%, N=68; 26.7%, N=45, respectively). Interestingly, this study also reported a *C. bombi* prevalence of 25% for *B. frigidus* (N=12) (Cordes et al. 2012). In comparison, we found that 58% of the *B. frigidus* workers screened on the Seward Peninsula were infected with trypanosome parasites. However, an important caveat is that our visual screening method was not able to identify *Crithidia* parasites to the species level, potentially inflating the detection rate compared to studies that specifically measure *C. bombi* prevalence. Nonetheless, the infection rates that we measured suggest that *B. frigidus* is often parasitized by trypanosome parasites. Defecating outside of the main nest cavity could reduce the transmission of these and other fecal-transmitted parasites among nestmates.

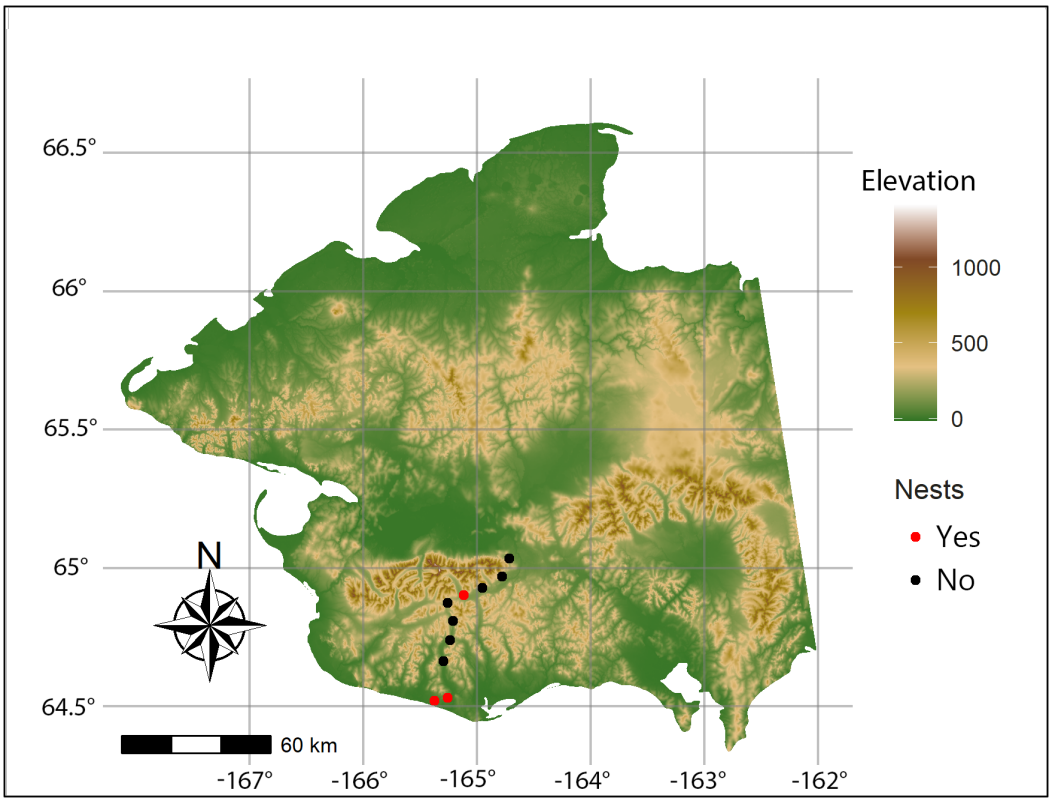
Social immunity behaviors have been well studied in eusocial insect species, and hygienic defecation behaviors and nest architecture are likely involved in social immunity in honey bees, ants, and termites (Weiss 2006, Cremer et al. 2007, Rosengaus et al. 2011, Cole et al. 2021). For termites, fecal materials may aid disease resistance: the dampwood termite (*Zootermopsis angusticollis*) and the Formosan termite (*Coptotermes formosanus*) line their nests with fecal materials that have antifungal properties (Rosengaus et al. 1998, Chouvenc et al. 2018). For many other eusocial insect species, hygienic behaviors isolate fecal materials away from the main nest cavity. For example, black garden ants (*Lasius niger*) defecate in distinct

fecal patches in the corners of their nests; *Platythyrea punctata* foragers primarily defecate outside the nest while nest workers defecate in waste middens within the nest; and honey bees almost exclusively defecate outside the hive (Seeley 1995, Czaczkes et al. 2015, Bernadou et al. 2018). Similarly, the common red ant (*Myrmica rubra*) removes more pathogenic fecal waste more quickly than less pathogenic waste (Pereira et al. 2020). Although the consequences of these behaviors for disease transmission have not been directly studied in these species, it is plausible that they function to reduce the spread of harmful pathogens within the nest (Weiss 2006, Cremer et al. 2007, Cole et al. 2021). Our research suggests that hygienic defecation behaviors may also be a component of social immunity for primitively eusocial bumble bees. Future experimental research should clarify whether the outhouse effectively reduces disease transmission for individual colonies and how these effects affect disease transmission in the broader pollinator community.

Table 1. Habitat and location of every nest, as well as the number of queens/gynes, workers, and males found in each.

Colony	Site	Landscape	Position	Substrate	GPS	Queens /Gynes	Workers	Males
1	1	Neighborhood	Surface	Tufts of grass	64.5194, -165.3728	0	4	0
2	1	Neighborhood	Surface	Tufts of grass	64.5194, -165.3729	2	0	0
3	1	Neighborhood	Surface	Tufts of grass	64.5194, -165.3728	0	2	3
4	1	Neighborhood	Under surface	Fiberglass insulation	64.5211, -165.3715	5	11	0
5	2	Roadside	Under ground	Soil, under grass	64.5316, -165.2607	1	10	0
6	7	Tundra	Under surface	Sphagnum moss	64.9016, -165.1155	0	9	0
7	7	Tundra	Under surface	Sphagnum moss	64.9016, -165.1161	2	10	0
8	7	Tundra	Under surface	Sphagnum moss	64.9018, -165.1160	0	13	2
9	7	Tundra	Under surface	Sphagnum moss	64.9016, -165.1158	3	13	4
10	7	Tundra	Under surface	Sphagnum moss	64.9016, -165.1162	1	5	2

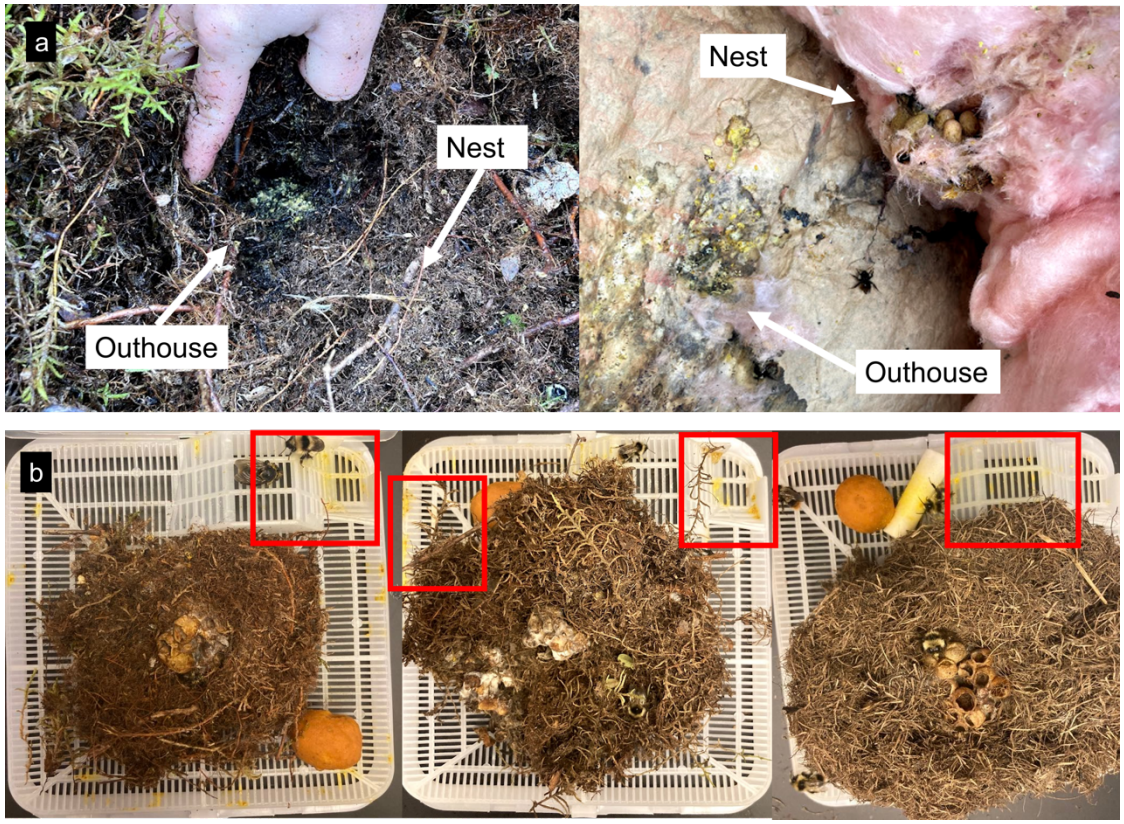
Figure 1 Nests were located on the Seward Peninsula of Alaska in the Icyview neighborhood of Nome and on a transect along the Kougarok Road (Nome-Taylor Highway). Each point represents a transect site, and red points represent sites where nests were located (Sites 1, 2, and 7). This figure was created by and used with permission from Carl St. John.



Map credit: Used with permission from Carl St. John

Figure 2 (a) *B. frigidus* worker. (b)Two landscapes where nests were located: the residential neighborhood of Site 1 (top) and the tundra landscape of Site 7 (bottom). (c) Two nests housed in different substrates: grasses at Site 1 (top) and sphagnum moss at Site 7 (bottom). Photo credit: L. Valdes.

Figure 3 (a) Outhouse architecture containing concentrated yellow fecal materials found in wild *B. frigidus* nests in two different substrates: tundra (left) and fiberglass insulation (right). (b) The three *B. frigidus* colonies that were housed in the lab. Red rectangles mark where the bees defecated outside of the main nest cavity. Photo credit: L. Valdes.



Open Research Statement

Data and supporting information for this project are available at the following location: https://zenodo.org/doi/10.5281/zenodo.7888360

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CHAPTER 3

ARCHITECTURAL IMMUNITY INCREASES FIRTNESS IN BUMBLE BEE COLONIES UNDER PATHOGEN STRESS

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Summary

The built environment can have profound consequences for organisms' health. This relationship is well known in human society; modern architects often incorporate design elements that protect against disease transmission. However, how the relationship between architecture, behavior, and infectious disease extends to non-human animals is less understood. Understanding this relationship has the potential to reveal general principles driving architectural immunity and the evolution of animal architecture more broadly. Here, we investigate whether nest architecture provides an adaptive defense against a trypanosomatid pathogen (*Crithidia bombi*) and a fungal pathogen (*Aspergillus flavus*) in bumble bees (*Bombus impatiens*). We describe bumble bee nest modification, finding that laboratory colonies excavate their nests to create additional cavities resembling structures found in natural colonies. We hypothesized that this contributes to architectural immunity by creating peripheral "outhouse" spaces in which to isolate waste (feces and corpses), thereby reducing exposure to contagious materials, slowing pathogen transmission, and increasing fitness. We tested this hypothesis with manipulative laboratory experiments, finding that the "outhouse" architecture enables hygienic waste removal behaviors, thereby slowing *C. bombi* transmission and reducing *A. flavus* prevalence. Most importantly, this architecture accelerated colony growth and provided fitness benefits, and these effects were correlated with *A. flavus* prevalence. These results demonstrate that architecture can shape the outcomes of epidemics in social species, and suggest that infectious disease may play an important role in driving the evolution not only of animal behavior but also of animal homes.

Introduction

Architects often incorporate elements of design to protect against infectious disease in the built environment. Ventilation, exposure to sunlight, and spatial compartmentalization are widely used by humans to defend against airborne pathogens (Aliabadi et al., 2011; Emmanuel et al., 2020; Izadyar & Miller, 2022; Megahed & Ghoneim, 2020), waste removal infrastructure defends against water-borne pathogens (Hussein, 2022; Melosi, 2008), and building modifications such as window screening and closed eaves make houses less permeable to insect vectors (Kirby et al., 2009; Lindsay et al., 2003; Tusting et al., 2016). Less is known about how non-human animals use architecture to protect against infectious disease. The architecture of social insects, which is often shaped by the collective behaviors of individuals (Di Pietro et al., 2024; Franks & Deneubourg, 1997; Khuong et al., 2016; Theraulaz et al., 2003) rather than centralized design, offers comparative insight and has received increasing interest from architects and biologists alike (Ireland & Garnier, 2018). Research investigating the relationship between architecture, behavior, and infectious disease in non-human animals has the potential to reveal general principles driving architectural immunity and the evolution of animal architecture more broadly (Pinter-Wollman et al., 2018).

For social cavity-nesting insects, such as bees and ants, nest architecture may create conditions that increase pathogen exposure and transmission. These species nest within confined cavities, which may increase contact rates between nestmates (Schmid-Hempel, 1998). This is particularly salient for bumble bees, which often nest in subterranean cavities such as abandoned rodent nests (Liczner & Colla, 2019). However, organisms' nests represent an "extended phenotype," (Dawkins, 1982; Laidre, 2021), and natural selection can alter nest architecture by acting on species' nest preferences or construction behaviors (Hansell, 2005; Tschinkel, 2021;

Weber et al., 2013). Thus, if disease poses a significant challenge, nest structures that reduce within-colony transmission could confer a fitness benefit. However, to our knowledge, this hypothesis has not been empirically tested.

It has been hypothesized that nest architecture could reduce disease transmission by altering the physical environment, constraining social organization, or facilitating hygienic behaviors. One class of behaviors that could be altered by nest architecture is waste removal. While many species dispose of waste outside the nest (e.g. honeybees: (Michener, 1974); leafcutter ants: (Julian & Cahan, 1999); fire ants: (Howard & Tschinkel, 1976)), wild social insect nests often include chambers containing concentrated waste materials (e.g. ponerine ants: (Peeters et al., 1994), suggesting that architecture may facilitate nest hygiene by providing colonies with a separate space in which to deposit waste without venturing outside. Waste removal behaviors are broadly hypothesized to contribute to social immunity by reducing exposure to hazardous materials, and indeed corpse removal and nest hygiene have been shown to improve worker survival in healthy fire ants and in theoretical models (Diez et al., 2014; Fefferman et al., 2007). However, empirical evidence testing the hypothesis that waste midden architectures reduce pathogen transmission in social insects is lacking. Filling this gap may reveal novel empirical evidence that nest architecture can adaptively shape the outcomes of epidemics in animal societies, as well as a mechanism driving this effect, and would suggest that infectious disease could act as a selective force in the evolution of animal architecture.

Given recent pollinator declines caused in part by pathogens (Cameron et al., 2011; LeBuhn & Vargas Luna, 2021; Potts et al., 2010), understanding how pathogens are transmitted is a major goal for pollinator conservation. Bumble bees are dominant wild and agricultural pollinators that host numerous pathogens that can be transmitted both on flowers (Adler et al.,

2018; Figueroa et al., 2019) or within the nest, but prior research has focused mainly on floral transmission (but see: (Otterstatter & Thomson, 2007; Pinilla-Gallego et al., 2020; Valdes et al., 2025)*Crithidia bombi* (Trypanosomatida; hereafter *C. bombi*) is a widely distributed fecal-transmitted trypanosomatid pathogen that can negatively impact foraging ability, colony founding, survival, and reproduction (Brown et al., 2003; T. J. Conroy et al., 2016; Gegear et al., 2006; Schmid-Hempel et al., 2019). Because *C. bombi* is transmitted via contact with infected fecal materials, defecation behavior and nest architecture may be particularly important for controlling its transmission within bumble bee colonies. Indeed, observations of 10 wild bumble bee (*Bombus frigidus*) nests in a region with high rates of trypanosomatid infection revealed that each nest contained an additional vestibule filled with concentrated fecal materials (Valdes & Scofield, 2023), suggesting this nest architecture and behavior could play a role in controlling pathogen transmission in wild colonies.

Additionally, *Aspergillus spp*. are ubiquitous, opportunistic fungal pathogens that represent a threat to both human and animal health (Becchimanzi & Nicoletti, 2022; Seyedmousavi et al., 2015). They are found in soil and decaying vegetation worldwide and have been associated with diverse bee species (Batra et al., 1973; Becchimanzi & Nicoletti, 2022; Seyedmousavi et al., 2015). Among bees, the genus *Aspergillus* has been best studied in honeybees, in which it can infect all developmental stages and is the causative agent of stonebrood (Batra et al., 1973; Foley et al., 2014). We identified incidental *A. flavus* infections in 14 of our experimental colonies, providing a rare opportunity to study the effects of *A. flavus* within bumble bee colonies, as well as the efficacy of architectural defenses against this pathogen.

In the present study, we investigate whether infectious disease could drive the evolution of nest architecture. We hypothesize that this could occur if the presence of a peripheral "outhouse" cavity architecture in bumble bee nests facilitates the removal of hazardous waste materials such as feces and corpses, thereby reducing pathogen transmission and improving fitness (Figure 1). We used manipulative laboratory experiments to test this hypothesis, focusing on *C. bombi* and *A. flavus.* We address the following questions:

(1) Do bumble bees modify nest architecture to create peripheral cavities for waste?

(2) Do bumble bees use peripheral nest cavities for waste removal when infected?

(3) Do waste removal behaviors reduce within-colony *C. bombi* transmission?

(4) Does nest architecture improve fitness by reducing pathogen transmission?

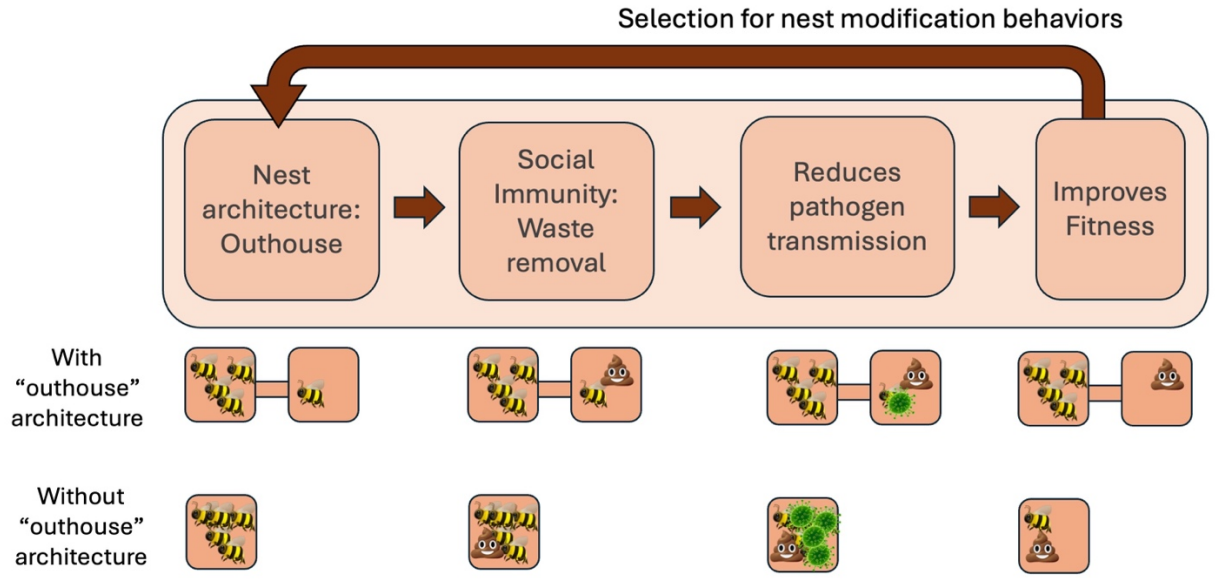

Figure 1. We hypothesized that spatial architecture of bumble bee nests contributes to architectural immunity. This could occur if the presence of a peripheral "outhouse" cavity facilitates the removal of hazardous waste materials such as feces and corpses, thereby reducing pathogen transmission, improving fitness, and driving selection for the nest modification behaviors that produce this structure.

Results

Question 1: Do bumble bees modify nest architecture to create peripheral cavities for waste?

For beneficial nest architectures to be ecologically relevant and selected for in wild bumble bee colonies, bumble bees must be able to modify their nests. To test this hypothesis, we housed bumble bee microcolonies in nest boxes that were half-filled with florist foam and observed whether they altered the nest interior by digging tunnels or cavities in the foam (Figure S1). Seven of the eight microcolonies (87.5%) modified their foam blocks. Nest modifications included tunnels and cavities excavated on the top or bottom of the foam blocks (Figure 2a). We hypothesized that bumble bees used these excavated cavities as waste middens, or "outhouses." Supporting this hypothesis, we found a greater proportion of the area under the foam block contained feces than under the original nest area ($t(6) = 2.997$, $p = 0.012$; Figure 2b, 2c).

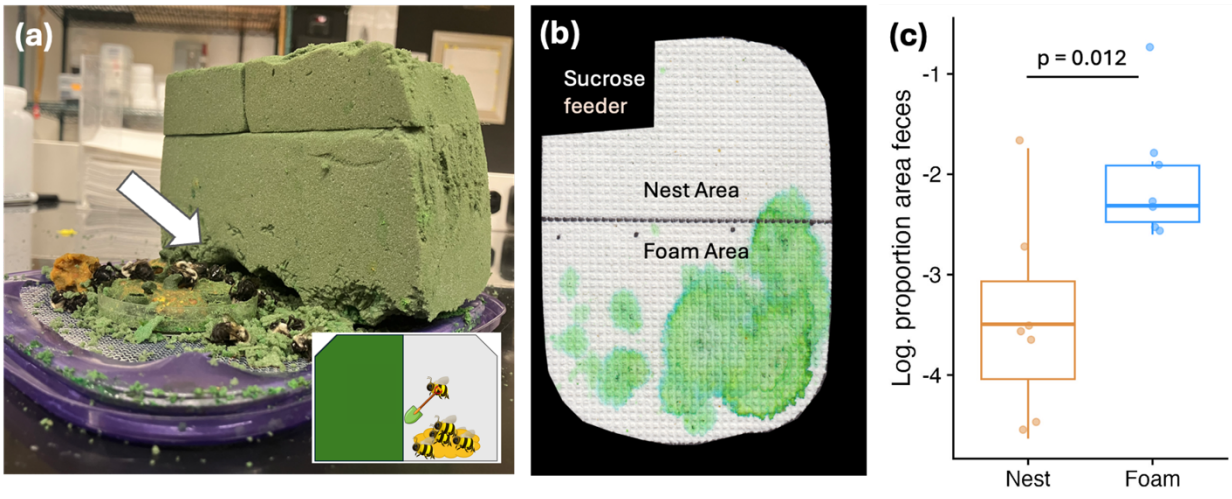

Figure 2. Bumble bees remodel their nests the create peripheral chambers in which they are more likely to defecate. **a**. Shows a foam block that was modified by a bumble bee microcolony to create a peripheral chamber nest. **b**. Defecation patterns (green coloring) for one colony at the end of the experiment. "Nest Area" was located under the original open nest cavity, and "Foam Area" was located under the foam

block. **c**. The concentration of feces in each area after nest modification for each microcolony that modified their foam block (n = 7). A greater proportion of the area under the foam block contained feces than under the original nest area (p = 0.012).

Question 2: Do bumble bees use peripheral nest cavities for waste removal when infected?

To further test the hypothesis that bumble bees used these excavated cavities as waste middens, we tested whether infected and uninfected bumble bee colonies use additional cavities for the removal of feces or corpses with two laboratory experiments. In the first experiment, bumble bee microcolonies were housed in nest boxes containing prefabricated "outhouse" cavities, and we tested whether bees preferred to defecate in the outhouse vs. nest when inoculated with *C. bombi* or a sham control (Figure S2). Both treatment groups defecated significantly more in the outhouse than in the nest (*C. bombi*: t = 3.414, df=6.08, p=0.014; Control: t = 4.967, df=6.54, p=0.002; Figure 3a).

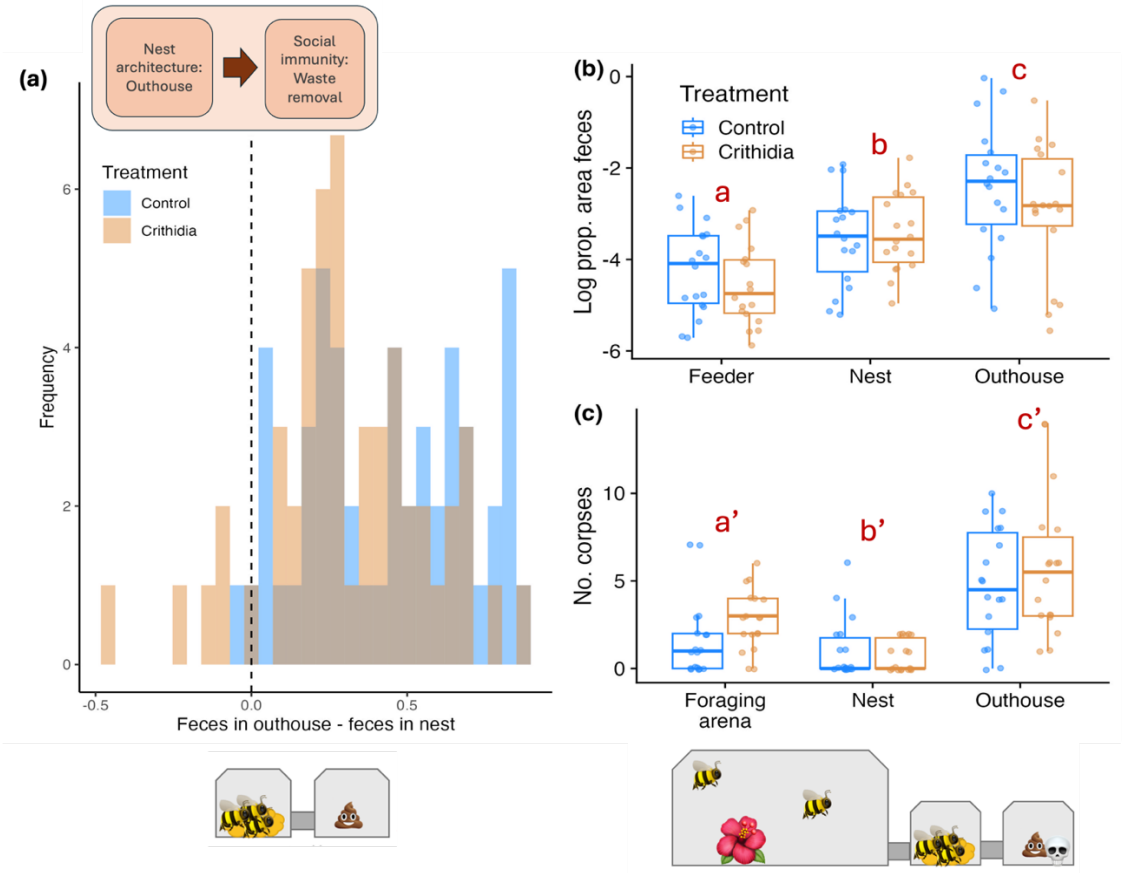

Figure 3. Feces and corpses were more likely to be found in the "outhouse" chamber than in any other area for both *C. bombi* and sham control inoculated microcolonies. Letters indicate statistically significant differences. **a**. The distribution of the differences in concentration of feces in the outhouse versus the nest cavity for sham control and *C. bombi* inoculated microcolonies (n = 18). **b**. The log-transformed concentration of feces in each location (n = 18). **c**. The number of dead bees in each location (n = 18).

We performed a second experiment to extend our description of waste removal to include corpses and feces in colonies with access to an outside foraging arena (in addition to a nest and outhouse). In this experiment, we established microcolonies in nest boxes and inoculated them with *C. bombi* or a sham control, as above, but also provided them with access to a foraging arena (Figure S3). The bees defecated in the outhouse cavity more than in the nest or feeding areas (Wald, $\chi^2 = 47.170$, $p < 0.001$; Contrast test, Feeder-Nest: $t = -3.183$, $p = 0.006$; Feeder-Outhouse: $t = -6.862$, $p < 0.001$; Nest-Outhouse: $t = -3.679$, $p = 0.001$; Figure 3b). Similarly, corpses were more likely to be located in the outhouse cavity (Wald, $\chi^2 = 108.656$, $p < 0.001$; Nest-Outhouse: $z = -9.243$, $p = 0.001$; Foraging arena-Outhouse: $z = -6.538$, $p < 0.001$; Foraging arena-Nest: $z = 4.218$, $p < 0.001$; Figure 3c). Whether or not the bees were inoculated with *C. bombi* did not influence these patterns (location x *C. bombi* treatment interaction: $p > 0.1$ in each case).

Question 3: Do waste removal behaviors reduce within-colony C. bombi *transmission?*

Having established that bumble bee colonies remove feces and corpses into the "outhouse" cavity, we next tested which of these waste removal behaviors contribute to social immunity by reducing or preventing *C. bombi* transmission. To test this, we performed a manipulative experiment in which *C. bombi*-infected feces or corpses were introduced to either the nest box or outhouse box of bumble bee microcolonies (Figure S4). When infected feces were added to the nest box, colonies were significantly more likely to become infected than when feces were added to the "outhouse" box (Fisher's exact test, $p < 0.001$; Figure 4a). There was no difference in the number of infected colonies when infected corpses were added to the nest versus "outhouse" box ($p = 1$). We observed each colony for a 20-minute observation period

to measure the number of times workers contacted infected feces in each location. There was a significant overall effect of location (nest or outhouse) on the number of times workers contacted infected feces (Wald, $\chi^2 = 243.38$, $p < 0.001$; Figure 4b). Pairwise comparisons revealed that contact rates significantly differed between all three locations, and were greatest when feces were located in the center of the nest and least when they were in the "outhouse" (Contrast test, Nest-Nest edge: $z = 10.80$, $p < 0.001$; Nest-Outhouse: $z = 11.85$, $p < 0.001$; Nest edge-Outhouse: $z = 5.404$, $p < 0.001$).

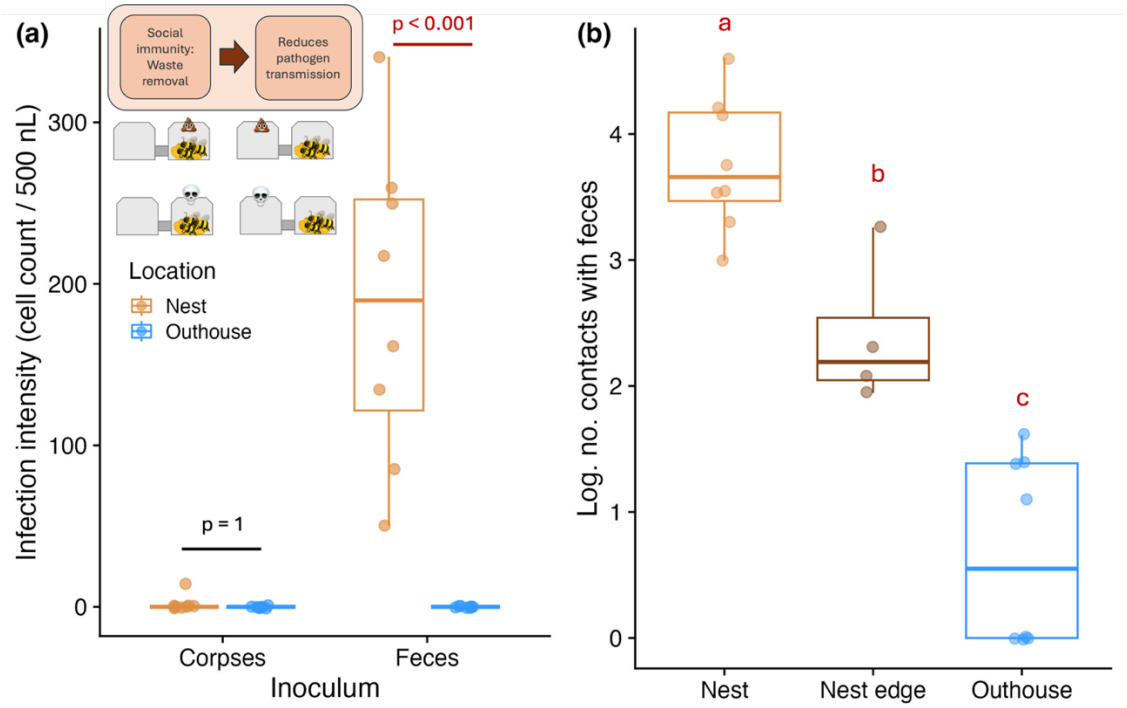

Figure 4. Increasing the concentration of infected feces within the nest increases pathogen exposure and infection. **a.** The average *C. bombi* infection intensity measured in bumble bee microcolonies that were inoculated with *C. bombi*-infected feces or corpses added to the primary nest box or "outhouse" box (n = 8). 10 workers were screened from each microcolony. **b.** The number of times workers contacted a contaminated feces droplet in a 20-minute observation period. Each point represents one microcolony (Nest: n = 8; Nest edge: n = 4; Outhouse: n = 8).

Question 4: Does nest architecture improve fitness by reducing pathogen transmission?

We hypothesized that this architecture contributes to architectural immunity, increasing colony fitness by reducing pathogen transmission. We tested this hypothesis with a 2 x 2 factorial experiment crossing nest architecture (with or without an additional cavity) and initial inoculation rate (3% versus 30% inoculated with *C. bombi*). Each colony was weighed, and a sample of workers was screened for *C. bombi* weekly for 6 weeks. At the end of the experiment, colonies were dissected and censused to measure reproduction (Figure S5).

We first test whether the presence of an additional nest cavity slows pathogen transmission. Access to an additional cavity significantly slowed *C. bombi* transmission for colonies inoculated with a high (30%) initial inoculation rate (Figure 5b), but not for colonies inoculated with a low (3%) initial inoculation rate (Figure 5a). There were no main effects of initial inoculation rate or cavity treatment (Inoculation rate: Wald, $\chi^2 = 1.609$, $p = 0.205$; Cavity treatment: Wald, $\chi^2 = 2.656$, $p = 0.103$), but contrast tests revealed that in colonies with high (30%) initial inoculation rates, there was a significant difference in *C. bombi* infection rate in colonies with versus without additional cavities ($z = 5.04$, $p = 0.023$), while there was no difference within colonies with low (3%) initial inoculation rates ($z = 1.61$, $p = 0.486$). Neither access to an additional cavity nor initial inoculation rate affected the final *C. bombi* infection intensities measured in infected workers (Figure S6; Cavity treatment: Wald, $\chi^2 = 0.810$, $p = 0.368$; Inoculation rate: Wald, $\chi^2 = 1.709$, $p = 0.191$).

We detected incidental *Aspergillus flavus* infections in 14 colonies (Figure S7). *Aspergillus flavus* is a widespread, opportunistic pathogen that can infect bees at every

developmental stage and causes stonebrood in honeybees (Batra et al., 1973; Becchimanzi & Nicoletti, 2022; Foley et al., 2014). Of colonies with detected *A. flavus* infection, prevalence was also significantly lower in colonies with an outhouse (t = 3.780, df = 4.237, p = 0.009; Figure 5c).

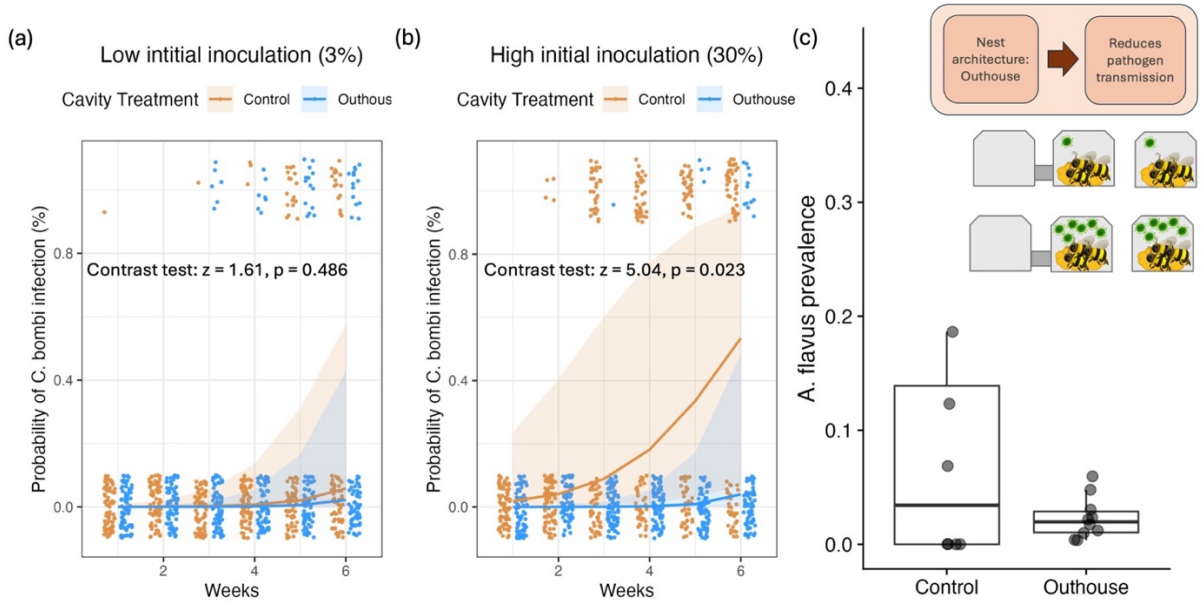

Figure 5. Access to an "outhouse" significantly reduced *C. bombi* and *A. flavus* in full queenright bumble bee colonies. **a.** The change in *C. bombi* prevalence over time in colonies with access to an "outhouse" cavity (blue) versus control (orange) colonies in the low (3%) initial inoculation group and **b.** in the high (30%) initial inoculation group. Each point represents one bee that is either infected (1) or uninfected (0) with *C. bombi*. Trendlines represent GLMM predictions, and the shaded areas represent the 95% confidence interval; n=5 colonies per treatment, 15 bees per colony per timepoint. **c**. *A. flavus* prevalence measured across all individuals (larvae, pupae, workers, males, gynes) at the end of the experiment (Control: n = 8; Outhouse: n = 10).

The presence of an additional nest cavity effectively reduces pathogen transmission, but for this nest architecture to be selected for it must also provide a fitness advantage. Thus, we next tested whether the presence of an additional nest cavity improved colony growth and reproduction. Colonies with access to an "outhouse" cavity grew more quickly than control colonies (cavity treatment:week: Wald, $\chi^2 = 36.432$, $p < 0.001$) and experienced greater overall colony weight (cavity treatment: Wald, $\chi^2 = 25.246$, $p < 0.001$), but there was no main effect of initial inoculation rate (inoculation rate: Wald, $\chi^2 = 0.418$, $p = 0.518$; Figure 6a). Colonies with access to an additional cavity also had greater reproduction than control colonies (adults: Wald, $\chi^2 = 18.643$, $p < 0.001$; reproductives: Wald, $\chi^2 = 5.665$, $p = 0.017$; males: Wald, $\chi^2 = 8.908$, $p = 0.003$; Figure 6b, 6c, 6e). However, there was no effect of cavity treatment on the number of daughter queens, or "gynes" (gynes: Wald, $\chi^2 = 1.672$, $p = 0.196$; Figure 6d), and no effect of initial inoculation rate on any measure of reproduction (adults: Wald, $\chi^2 = 0.021$, $p = 0.884$; reproductives: Wald, $\chi^2 = 0.186$, $p = 0.667$; gynes: Wald, $\chi^2 = 0.016$, $p = 0.898$; males: Wald, $\chi^2 = 0.185$, $p = 0.667$).

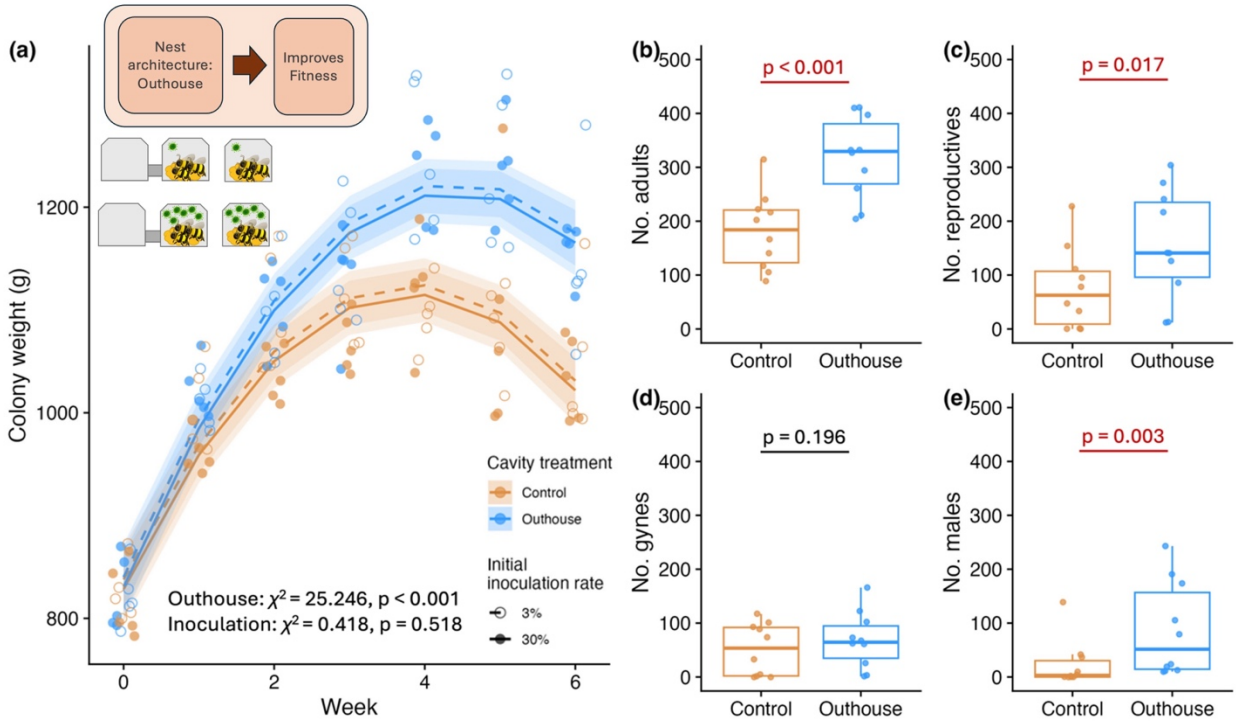

Figure 6. Colony growth and reproduction were significantly greater in colonies with the "outhouse" architecture. **a.** Colonies with the "outhouse" architecture (blue) grew more quickly and reached greater maximum weights compared to control colonies (orange), but there was no difference between colonies (yellow) with low (3%) initial inoculation rates (open circles, dotted line) versus high (30%) initial inoculation rates (closed circles, solid line); n = 5 colonies per treatment group. **b**. The total number of adult bees (gynes + males + workers), **c**. reproductives (gynes + males), **d**. gynes, and **e**. males in each colony at the end of the experiment. P-values represent general linear model results.

Finally, if the fitness benefits of the additional cavity architecture are caused by architectural immunity, we would predict there to be a negative relationship between fitness and infection rate. There was a significant negative relationship between *A. flavus* prevalence and colony growth ($F(1,12) = 10.482$, $p = 0.007$; Figure 7b) and the number of adults (males, gynes, and workers) present at the end of the experiment ($F(1,12) = 10.327$, $p = 0.007$; Figure 7b), while there was no significant relationship between the number of reproductives (males and gynes) and *A. flavus* prevalence ($F(1,12) = 0.729$, $p = 0.410$; Figure 7b). Conversely, there was no relationship between *C. bombi* prevalence and colony growth or reproduction (colony growth: $F(1,18) = 0.947$, $p = 0.343$; number of reproductives: $F(1,18) = 0.008$, $p = 0.932$; number of adults: $F(1,18) = 1.435$, $p = 0.247$; Figure 7a). This suggests high virulence of *A. flavus* compared to *C. bombi*, which we observed via numerous *A. flavus*-infected cadavers (Figure S7).

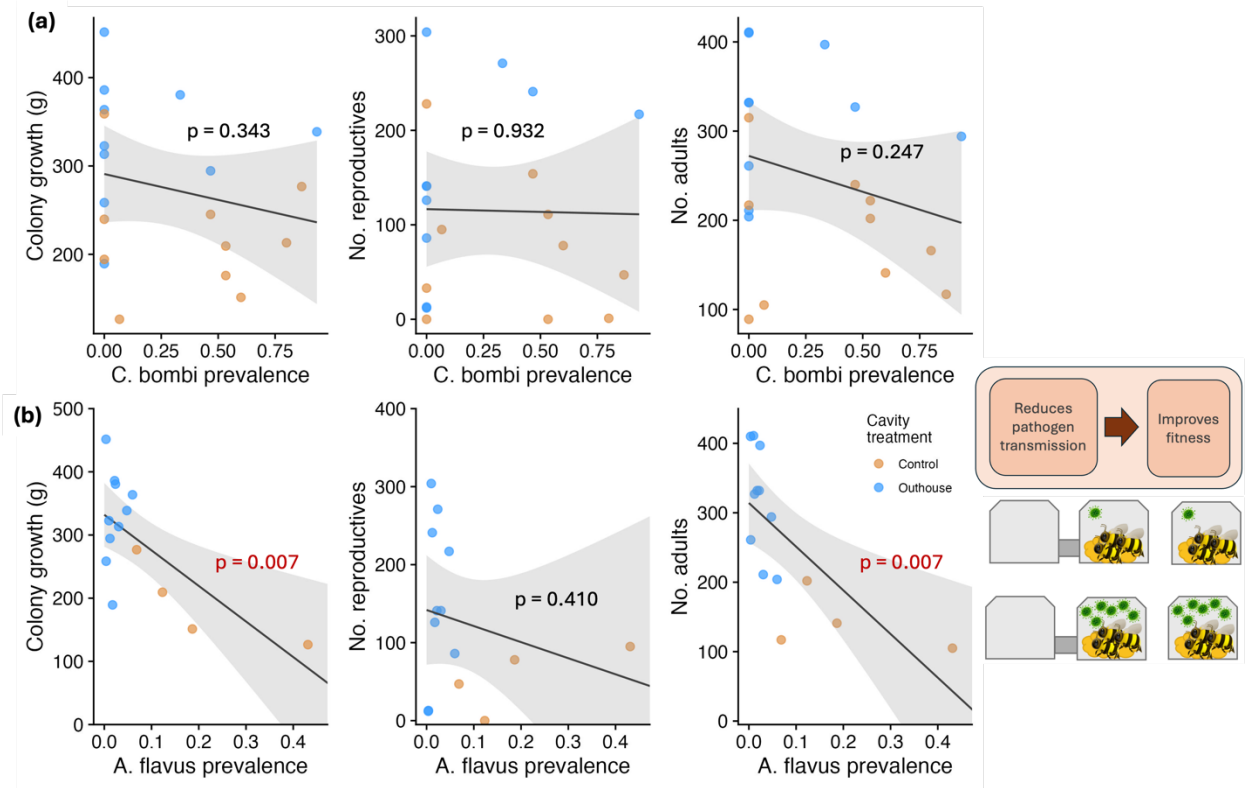

Figure 7. **a**. There was no relationship between *C. bombi* prevalence and colony growth, total number of reproductives (males + gynes), or total number of adults produced by the end of the experiment (Control: n = 10; Outhouse: n = 10). **b**. There was a negative relationship between *A. flavus* prevalence and colony growth and the number of adults produced in colonies with detected *A. flavus* infection, but there was no relationship with total number of reproductives (Control: n = 4; Outhouse: n = 10). Each point represents one queenright colony and trendlines represent linear model predictions.

Discussion

Our results provide empirical evidence for architectural immunity in bumble bees, showing that architecture can shape the outcomes of epidemics by enabling waste-removal behavior and providing physical separation from infectious materials. This design principle is well-understood by human architects; archaeological remains of latrines date back to the origins of agriculture (Mitchell, 2015), and modern homes universally contain bathrooms. Our results illustrate that bumble bees have achieved a convergent solution to combat infectious disease, employing simple architectural separation to segregate the group from its waste. Further, this strategy confers fitness benefits that could select for the nest modification behaviors that create this architecture. These findings suggest that infectious disease may play an important role in shaping the evolution of animal architecture.

Importantly, although the "outhouse" architecture reduced *C. bombi* transmission and increased colony performance in the nest architecture experiment, we did not observe a relationship between final *C. bombi* prevalence and colony growth or reproduction, indicating that *C. bombi* was not solely responsible for this effect. However, incidental infections with *Aspergillus flavus* were detected in 14 experimental colonies. Of the colonies with incidental *A.*

flavus infections, *A. flavus* prevalence was also greater in control colonies, revealing that the "outhouse" architecture may similarly protect against fungal transmission. We observed significant negative relationships between *A. flavus* prevalence and both colony growth and the total number of adult bees, as well as a negative trend between *A. flavus* prevalence and the number of reproductives produced. These results suggest this fungal pathogen may in part be responsible for the reduction in colony performance experienced by colonies without the "outhouse" architecture. Because we terminated the experiment at a single timepoint rather than monitoring all colonies until they naturally senesced, our reproduction measurements represent a snap-shot in time rather than cumulative lifetime reproduction, and may consequently underestimate the true variation in lifetime reproduction across treatment groups. Prior studies have consistently found that colony size is strongly correlated with reproductive success (Imhoof & Schmid-Hempel, 1999; Müller & Schmid-Hempel, 1992; Owen et al., 1980). Thus, the reproduction trends we observed may have been more pronounced had we observed the colonies until their natural senescence.

C. bombi can reduce survival and reproduction in bumble bees, suggesting that mechanisms that reduce infection should also increase fitness, but its virulence often depends on nutritional or social context (Brown et al., 2003; Conroy et al., 2016; Valdes et al., 2025). Thus, it is possible that our experimental colonies did not experience sufficient stressors, such as food limitation, for observable fitness effects associated with *C. bombi* alone. However, while research on *Aspergillus* in bumble bees is limited, this genus is the causative agent of stonebrood in honeybees (Batra et al., 1973; Foley et al., 2014), and *A. flavus* is a particularly virulent species that significantly reduces the survival of both larval and adult honeybees (Foley et al., 2014). We observed high mortality in *A. flavus*-infected colonies, with larval and adult cadavers

harboring stonebrood-like symptoms (Figure S7). *Aspergillus* fungi are also considered opportunistic and are more virulent in stressed hosts (Foley et al., 2012). Thus, it is possible that the greater rates of *C. bombi* infection in control colonies increased their susceptibility to *A. flavus*, or that additive effects of *C. bombi* and *A. flavus* coinfections were responsible for the observed fitness effects of the "outhouse" architecture.

In addition to documenting architectural immunity in bumble bees, our study supports one main mechanism for this effect: waste removal. Bumble bees isolated both feces and corpses in the "outhouse" structure, and we found that the location of feces was responsible for reducing *C. bombi* transmission. Bumble bee workers experienced reduced contact rates with infected feces when those feces were relocated in the "outhouse" versus the nest, and these lower contact rates corresponded with dramatically lower *C. bombi* infection rates. Thus, waste removal is a powerful behavioral defense against this pathogen. Relocating feces to the "outhouse" reduced contact rates more than relocating them to the edge of the nest, and *C. bombi* transmission was significantly greater in colonies with no "outhouse" compared to colonies with access to an additional "outhouse" cavity, further indicating that the spatial separation provided by this architecture is more effective for reducing *C. bombi* transmission than waste removal behaviors alone. These findings reveal that, by shaping spatial behavior and contact patterns, nest architecture can have more profound consequences for the outcomes of infectious disease in animal societies than hygienic behavior alone.

Several non-mutually exclusive mechanisms may also be contributing to the effectiveness of the "outhouse" architecture for reducing *C. bombi* transmission. For example, there were differences in the abiotic conditions of the primary nest cavity versus "outhouse" cavity that could alter the viability or infectivity of *C. bombi* cells or *A. flavus* spores (Figure S8), infected

workers may be more likely to socially isolate themselves outside of the primary nest cavity (T. E. Conroy & Holman, 2022), and theory suggests that increasing compartmentalization could reduce social transmission (Pie et al., 2004). While our study represents a first step in demonstrating architectural immunity in a social insect species, future work could expand our understanding of the potentially diverse mechanisms by which architecture shapes behavior and infectious disease.

Infectious disease is one factor that has been implicated in the decline of both managed and wild pollinators (Cameron & Sadd, 2020; Goulson et al., 2015; LeBuhn & Vargas Luna, 2021). Our results reveal that nest architecture may combat these vulnerabilities, providing a simple and potentially cost-effective method for controlling disease and increasing pollination services in bumble bees managed for agriculture, while also reducing the risk of spillover into wild pollinators (see also: Alford, 1975; Pomeroy & Plowright, 1980; Sladen, 1912). Commercial bumble bee colonies are shipped worldwide for greenhouse tomato pollination (Osterman et al., 2021; Velthuis & Van Doorn, 2006), and the addition of a simple "outhouse" cavity in commercial nest boxes could make these colonies viable for longer, improving pollination services saving money for growers. Additionally, managed bumble bees (*B. terrestris,* and *B. impatiens*) often experience high rates of infection and regularly escape from the greenhouses (Whittington et al., 2004), which can lead to pathogen spillover from managed to wild bumble bee populations (Colla et al., 2006; Graystock et al., 2013; Otterstatter & Thomson, 2008). Consequently, more effective methods for controlling pathogens in managed bees may support the conservation of native wild bees. Finally, in addition to economic and ecological benefits, this finding may have important human health implications. *Aspergillus* spp. cause localized infections, fatal disseminated diseases, and allergic reactions in humans, and are

particularly dangerous for immunocompromised hosts (Seyedmousavi et al., 2015). By reducing *Aspergillus* infections in managed bumble bee colonies, this method could improve the health and safety not only of bees, but also of the growers who work with them.

By performing these experiments in the lab, we were able to isolate within-colony transmission and control for confounding environmental variables such as temperature, floral community, and pesticide exposure, but an important limitation of this laboratory setup was that the colonies were not allowed to forage in the pathogen transmission experiments. Wild bumble bee foragers often defecate while flying or on flowers outside the nest (Figueroa et al., 2023), but in our experiments all workers were forced to defecate within the nest structure. However, many bumble bee workers never forage and remain within the nest for their entire lives, and even foragers are less likely to fly during inclement weather (Goulson et al., 2002; Heinrich, 1972; Wang et al., 2024). The workers that do not leave the nest must defecate somewhere within the nest, and indeed, we found that workers continued to defecate in the "outhouse" cavity even when given access to an external foraging cage. Thus, our results provide valuable insight into the qualitative effects of hygienic behavior and architecture on infection outcomes for the majority of workers that defecate within the nest.

Animal architecture is an extended phenotype that can be shaped by natural selection (Dawkins, 1982; Hansell, 2005; Laidre, 2018; Mainwaring & Hartley, 2013). Animals construct structures to protect from predators and competitors, regulate their physical environment, capture prey, and communicate (Hansell, 2005). As awareness of the role that parasites and pathogens play in shaping the evolution of animal physiology and behavior grows, it has also been hypothesized that defense against disease may drive the evolution of architecture (Pinter-Wollman et al., 2018). Our results reveal novel empirical evidence for this hypothesis, extending

a principle that is well-understood in humans to the nest structures and behaviors of social insects, which are shaped by natural selection and collective behavior rather than by centralized design. We hypothesize that this architecture and its associated hygienic behavior may mitigate the costs of infectious disease that are widely associated with the transition to sociality, and hope future research will deepen our understanding of the evolutionary interactions between infectious disease, social behavior, and architecture.

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Declaration of interests

The authors have no conflicts of interest to declare.

STAR Methods

Experimental model and study participant details

Study system

We studied the common eastern bumble bee (*Bombus impatiens*) and its intestinal pathogen, *Crithidia bombi*. *B. impatiens* is a widespread generalist social bee species that is native to eastern North America (Williams et al., 2014). It lives in annual colonies and nests in

below-ground cavities, such as abandoned rodent nests (Goulson, 2010; Liczner & Colla, 2019). All colonies used in this study were commercial colonies sourced from Koppert Biological Systems (Howell, MI). In the lab, colonies were housed at 25°C and 25-50% RH and had ad libitum access to 30-40% sucrose solution and pollen.

Bumble bees and *C. bombi* are an emerging model system for disease ecology research. *C. bombi* is a trypanosomatid pathogen that is horizontally transmitted through contact with contaminated materials within the nest or on flowers (Cisarovsky & Schmid-Hempel, 2014; L. L. Figueroa et al., 2019) and vertically transmitted from queens to workers (Yourth et al., 2008). It reduces queen survival, colony founding, and reproduction, as well as worker foraging efficiency, particularly when bees are nutritionally stressed (Brown et al., 2003; T. J. Conroy et al., 2016; Gegear et al., 2006; Yourth et al., 2008). *C. bombi* is widespread, but there is dramatic variation in its prevalence in *B. impatiens*, with estimates ranging from 11-80% infected across North America (Adler et al., 2018; Figueroa et al., 2019; Gillespie, 2010; Graystock et al., 2020; Malfi & Roulston, 2014).

We used experimental microcolonies throughout this study. Microcolonies are queenless groups of workers that are created by housing sister workers together (Klinger et al., 2019). In the absence of a queen, one or more workers will develop ovaries and lay unfertilized male-destined eggs (Klinger et al., 2019). While they are not perfect proxies for queenright colonies (Van Oystaeyen et al., 2021), microcolonies are useful tools for studying colony-level processes when working with queenright colonies would not be possible, and have been widely used in nutrition and pesticide research (Klinger et al., 2019; Van Oystaeyen et al., 2021).

Method details

C. bombi inoculation

We used the following inoculation protocol adapted from Richardson et al. (2015) to inoculate bumble bees with *C. bombi* in each experiment. To prepare the inoculum, we dissected the midgut from 10 workers from an infected source colony (*C. bombi* source was acquired from wild bumble bee workers collected in Ithaca, NY), homogenized each midgut in 150 uL of dH_2O, and allowed them to sit at room temperature for 4-5 hours, during which the *C. bombi* cells swam up to the supernatant. We examined 10 uL of the supernatant on a haemocytometer under a compound microscope at 400x magnification to estimate the number of *C. bombi* cells per microliter. The supernatant was then diluted with 30% sucrose to create an inoculum of the intended concentration (Richardson et al. 2015). Each bee to be inoculated was removed from its nest, anesthetized with CO_2 for 15 seconds, and fed 10 uL of the prepared inoculum. The bees consumed the entire inoculum as they awoke from anesthetization. This method was also used to "inoculate" control colonies with a sham inoculum of 30% sucrose.

Microcolony Setup

To create each microcolony, N=25 sister workers were randomly selected from one queenright commercial colony and rehomed in a plastic nest box with 15 g of pollen and ad libitum access to 30% sucrose solution. A 5 g pollen ball was added to each microcolony every Monday, Wednesday, and Friday, and the microcolonies were monitored to ensure they always had access to pollen. Before the start of each experiment, each queenright colony was screened for *C. bombi* infection by randomly selecting five workers, dissecting and homogenizing their midgut, and observing the gut contents under a compound microscope at 400x; *C. bombi* was not

detected in any queenright colonies. All microcolonies were kept in a dark growth chamber at approximately 25°C and 25-50% RH, except when otherwise stated.

Image analysis. We used the following method to quantify the amount of feces in each part of the nest. We used the ImageJ image processing software to measure the total and proportion surface area of each area that had absorbed colored fecal materials. Each liner was digitally scanned with a Canon LiDE 210 scanner. To measure the total number of pixels, the perimeter of the sampling area was selected with the Polygon Selection tool and measured with the Measure command. To measure the number of colored pixels, the Color Thresholding tool was used to select pixels in the HSB color space in the following ranges based on the color of food dye used in the experiment: Green: (Hue: 40-120; Saturation: 40-255; Brightness: 0-255); Red: (Hue: 0-255; Saturation: 35-255; Brightness: 0-255). The Measure command was used to measure the number of pixels that met these conditions. The number of colored pixels was divided by the total number of pixels to estimate the proportion of the surface area of the liner containing colored feces.

Question 1: Do bumble bees modify nest architecture to create peripheral cavities for waste?

We first tested whether bumble bees can modify their nests and describe these architectural modifications, to ensure that the structures in our study are ecologically relevant (Figure S1). To describe bumble bee nest modification behavior, we established n=8 bumble bee microcolonies in small (10.5 x 14.5 cm) nest boxes. The bees from each microcolony were sourced from a different commercial colony. The microcolonies were given three days to establish in the small nest boxes and on the fourth day each microcolony was rehomed into a

larger (20 x 14 cm) nest box that was half-filled with florist foam (Oasis, model # EN111832), such that the open area was the same size as the original smaller nest box. We then observed whether the microcolonies modified the nest by digging tunnels or cavities in the foam.

To measure defecation in the nest and under the foam, and thus whether the bees were defecating in the modified nest cavities, we fed each microcolony green-colored sucrose, which was prepared by mixing 50 drops of green food dye with 1 gallon of 30% sucrose. An absorbent liner was placed under each box to absorb the feces that passed through the mesh floor on the third through fourth days after the colonies were transferred into the larger box. We then used the image analysis protocol (above) to quantify the amount of feces under the open nest area versus under the foam.

Question 2: Do bumble bees use peripheral nest cavities for waste removal when infected?

In the following experiments, we investigate the mechanism driving the fitness and immunity benefits provided by the additional nest cavity architecture. We hypothesize that these structures serve as waste middens, or "outhouses," providing a hygienic space for bees to remove contagious waste materials such as feces and corpses, reducing exposure to these materials within the nest. We first address this hypothesis by testing whether bumble bees use these structures for waste removal.

Within-nest waste removal experiment

This experiment tests whether bumble bee microcolonies defecate outside their primary nest cavity, when inoculated with *C. bombi* or sham control (Figure S2). We set up n=18 pairs of microcolonies (see *Microcolony Setup* above). Each plastic nest box contained two cavities: one nest cavity (dimensions: 13.3 cm x 13.3 cm) in which the bees built their nests and had access to

sucrose and pollen, and one additional empty cavity (dimensions: 13.3 cm x 13.3 cm). The cavities were connected to each other by a plastic tube and both cavities had mesh floors to allow feces to pass through. For each replicate, microcolonies were paired from the same Biobest source colony to control for genotype, age, and colony condition. One week after establishing microcolonies, we inoculated all workers in the *C. bombi* treatment group with *C. bombi* (10 uL inoculum of 800 cells/uL) and all workers in the control group with a sham 30% sucrose inoculum (see *C. bombi Inoculation* above).

Ten days after inoculation, we screened 10 uL of feces collected from five bees in every microcolony for *C. bombi* infection to ensure that the *C. bombi* treatment groups had sufficient levels of *C. bombi* infection and that the control microcolonies were free of *C. bombi*. If *C. bombi* was identified in at least 4 of the 5 bees screened in the treatment group, we started data collection. If fewer than 4 of the 5 of the treatment group bees' feces contained *C. bombi*, we waited 5 more days and then repeated the screening. After 15 days, *C. bombi* was identified in at least 4 of the 5 bees screened in every *C. bombi* treatment group microcolony.

To measure defecation in the nest and outhouse chambers, we fed each microcolony green food-colored sucrose and placed an absorbent liner under each box to capture the feces that passed through the mesh floors. After the start of data collection, each liner was changed every two days for six days, for three repeated measurements of defecation in each chamber. To quantify the amount of defecation in each cavity (nest, outhouse), we used the image analysis method described in the nest modification experiment.

Foraging cage waste removal experiment

To extend our description of bumble bee waste removal behavior to colonies with access to an outside foraging area, we provided two-chambered bumble bee microcolonies with access to a foraging cage and compared the proportions of (a) feces and (b) corpses in the nest cavity, in the additional "outhouse" cavity, and on an artificial flower feeder (feces) or in the foraging cage (corpses) for microcolonies that were inoculated with *C. bombi* versus a sham control (Figure S3). Each plastic microcolony cage contained two cavities (cavity dimensions: 13.3 cm x 13.3 cm) connected by a plastic tube.

We set up 19 pairs of microcolonies (see *Microcolony Setup* above) in blocks of 4-6 microcolonies (2-3 matched pairs) at a time. We performed the experiment in repeated blocks because greenhouse space limitations precluded performing all replicates at the same time. One week after establishment, we inoculated all workers with a 10 uL inoculum of either *C. bombi* (1000 cells/uL) or a sham sucrose control (see *C. bombi Inoculation* above). One *C. bombi* treatment colony died before the start of the experiment, and one control colony abandoned its nest in the foraging cage, so 18 microcolonies per treatment were included in the analysis.

8-10 days after inoculation, we screened 10 uL of feces collected from five bees in every microcolony for *C. bombi* infection using the same method as in Experiment 1.

In the greenhouse, each microcolony was connected to its own foraging cage (dimensions: 74.6 cm x 45.7 cm) containing an artificial feeder with both 200 mL of 40% sucrose treated with 35 drops of red food coloring, and a dish containing 10 g pollen. The artificial feeder, nest cavity, and outhouse cavity were lined with an absorbent liner on which red-colored feces were clearly visible. The microcolonies were then left to forage freely for 5-6

days, after which the experiment was terminated. After the experiment, the image analysis protocol (above) was used to quantify the amount of feces in the nest, outhouse, and feeder areas.

Question 3: Do waste removal behaviors reduce within-colony C. bombi transmission?

Finally, if the "outhouse" architecture reduces pathogen transmission through waste removal, waste removal must reduce or prevent pathogen transmission. To test whether feces or corpse removal behaviors reduce *C. bombi* transmission in bumble bee colonies, we performed a laboratory experiment in which we manipulated the location (nest or "outhouse") of *C. bombi*-infected waste (feces or corpses) in two-chambered bumble bee microcolonies and compared *C. bombi* prevalence across each treatment (Figure S4).

Microcolonies were housed in two-chambered nest boxes and kept in the growth chamber at approximately 25°C and 25-50% RH with ad-libitum access to 40% sucrose and pollen. We established n=8 matched groups of 4 microcolonies, for a total of 32 microcolonies. Each microcolony was established with 20 workers. Workers within each matched group of four microcolonies were sourced from the same commercial source colony to control for genotype effects, and each matched group was sourced from a different commercial colony to ensure that replicates were independent.

The microcolonies were given 24 hours to establish in the lab before the start of the manipulation. Microcolonies within each matched group were randomly assigned to one treatment group and inoculated with (1) *C. bombi*-infected feces (40 uL, 8,000-24,000 cells/40 uL) or (2) one *C. bombi*-infected corpse placed in the center of the nest or "outhouse" cavity. Inoculations occurred once per day for 5 days Infected feces were acquired by isolating workers from the infected laboratory colony in small cups, chilling them to 5°C to stimulate defecation,

and pipetting fecal droplets into a microcentrifuge tube. Infected corpses were acquired by selecting workers from a *C. bombi* infected laboratory colony and screening their feces to confirm infection, before anaesthetizing and euthanizing the bees via decapitation. 10-11 days after the last day of the manipulation, 10 bees from each microcolony were randomly selected, dissected, and screened for *C. bombi* via microscopy.

To determine how the location of infected feces alters contact rates, on the last day before dissection we added 5 uL of *C. bombi* infected feces to the center of the nest or center of the "outhouse," according to the microcolony's treatment group (n = 8). Black food coloring was added to the feces to improve visibility, and each microcolony was recorded under red light for at least 20 minutes (Camera: Canon VIXIA HF R800). During the second block of the experiment, an additional 5 uL of feces were added on the edge of one corner of the nest box for colonies in the nest inoculation group (n = 4). To score each video, we counted the number of times a worker contacted the fecal droplet during a 20-minute period.

Question 4: Does nest architecture improve fitness by reducing pathogen transmission?

We hypothesized that this architecture contributes to architectural immunity, increasing colony fitness by reducing pathogen transmission. We tested this hypothesis with a 2 x 2 factorial experiment crossing nest architecture nest architecture (one-cavity nest versus two-cavity nest) and initial inoculation rate (3% versus 30% of workers inoculated with *C. bombi;* Figure S5). This experiment focuses on two widespread pathogens: *C. bombi* and *A. flavus.*

We used 20 queenright *B. impatiens* colonies sourced from Koppert Biological Systems (dimensions: 25 cm x 22 cm), each of which was pre-screened for *C. bombi* infection by collecting and examining 10 uL of feces from 5 randomly selected bees under a compound

microscope at 400x magnification. For the duration of the experiment, the colonies were kept in a dark growth chamber at approximately 25°C and 25-40% RH and had ad libitum access to pollen and 30% sucrose. An additional "outhouse" box (dimensions: 40 cm x 28 cm) filled with clay cat litter (Special Kitty ™ natural clay non-clumping cat litter) was connected by a plastic tube to 10 randomly selected colonies. The clay cat litter was replaced with paper cat litter (Vibrant Life ™ natural paper pellet cat litter) after week 2 to control high levels of fungal (*Aspergillus flavus*) contamination.

C. bombi inoculation. The colonies were given 11-14 days to acclimate before inoculation, after which each colony was inoculated at either a low initial *C. bombi* prevalence (~3% inoculated) or high initial prevalence (~30% inoculated). Because the colonies had variable numbers of workers that could not be reliably counted, we first estimated the number of bees in each colony by taking two photos of each colony. The number of bees in each photo was counted by two researchers and these four counts were averaged for the final estimate of colony size. For each colony, we inoculated either 3% or 30% of this group size estimate. Using the inoculation method described above (*C. bombi Inoculation*), we randomly selected bees from each colony to be inoculated with a 10 uL inoculum of 1000 cells/uL.

C. bombi screening. Beginning 1 week after inoculation, we randomly selected and screened 15 workers from each colony weekly for 6 weeks, after which colonies started producing reproductives and worker populations declined. Each bee was removed from the primary nest box and anesthetized with CO_2, after which its midgut was dissected, homogenized in 200 uL dH_2O, and allowed to settle for at least 2 hours. The concentration of *C. bombi* cells in the supernatant was measured on a haemocytomter under a compound microscope at 400x magnification. Additionally, because *C. bombi* infection load is influenced by bee size, we

measured the marginal cell of one forewing from each bee as a proxy for body size, using ImageJ (Nooten & Rehan, 2020; Owen, 1988; Van Wyk et al., 2021).

Colony performance. Each colony was weighed once per week. For colonies in the "outhouse" treatment, only the primary nest cavity was weighed. Additionally, after each colony was frozen at -20^{o}C at the end of the experiment and all workers, gynes, and males were counted.

Aspergillus flavus. We detected unintended *A. flavus* infections in 14 colonies (Figure S7). *A. flavus* is a widespread, opportunistic pathogen that can infect bees at every developmental stage and causes stonebrood in honeybees (Batra et al., 1973; Becchimanzi & Nicoletti, 2022; Foley et al., 2014). To quantify these infections, we counted the number of dead individuals (larvae, pupae, workers, males, or gynes) growing visible *A. flavus* fruiting bodies at the end of the experiment.

Quantification and statistical analyses

Analyses were performed with R version 4.2.0 (R Core Team 2022) and plots were produced using the package 'ggplot2' (Wickham 2016). The 'simulateResiduals' function in the 'DHARMa' package was used to test the model assumptions (Hartig 2022).

Question 1: Do bumble bees modify nest architecture to create peripheral cavities for waste?

A paired one-tailed t-test was used to test whether there were more feces in the area under the foam than under the original nest cavity in the colonies that had modified the foam block. Observations were paired by microcolony and the response variable was the difference between the log-transformed proportion of the area under the foam and the area under the original nest cavity that contained green feces.

Question 2: Do bumble bees use peripheral nest cavities for waste removal when infected?

Within-nest waste removal experiment

To investigate how the amount of feces differed in the nest versus outhouse cavity and whether this changed with *C. bombi* infection, we fit a linear mixed model with the 'lme4' package. For this, we paired the nest and outhouse cavities from the same microcolonies and subtracted the amount of feces in the nest from the amount of feces in the outhouse. We used this measure of the difference in the amount of defecation in the outhouse versus the nest cavities as the response variable, treatment (*C. bombi* inoculated versus sham control) as the fixed effect, and block and source colony as random effects. To test whether there were more feces in the outhouse than in the nest cavity for either the *C. bombi* inoculated and control colonies, we compared the difference in the amount of feces in the outhouse versus the nest to zero using the 'emmeans' function (Lenth 2022). To test whether the amount of feces in either cavity significantly differed for *C. bombi* inoculated colonies compared to control colonies, Type II Wald chi-square tests were performed using the 'Anova' function.

Foraging cage waste removal experiment

Feces. To investigate how the amount of feces differed between the (1) nest cavity, (2) outhouse cavity, and (3) artificial feeder, and whether this changed with *C. bombi* infection, we fit a linear mixed model (LMM) with the 'lme4' package. We included the total number of colored pixels as the response variable, log transformed to meet the model assumptions of constant variance and normality of residuals. We included treatment (*C. bombi* inoculated versus sham control), area (main nest cavity, outhouse cavity, or feeder), and their interaction as fixed effects, as well as total sucrose consumption. Source colony and block were included as random effects. Because

the interaction was not significant, we refit the model without the interaction to report main effects. Type II Wald chi-square tests were performed using the 'Anova' function to test for significant differences. Contrast tests were performed using the 'emmeans' function (Lenth 2022) to compare the amount of feces between each pairwise combination of areas (feeder vs. nest; feeder vs. outhouse; nest vs. outhouse), using the Tukey method to adjust for multiple tests.

Corpses. To test whether the locations of corpses varied with *C. bombi* infection, we used the 'glmmTMB' package to fit a generalized linear mixed model (GLMM) with a poisson distribution. Location (nest, outhouse, or foraging arena), *C. bombi* treatment (inoculated versus control), and their interaction were included as fixed effects, and source colony and block were included as random effects. Type II Wald chi-square tests were performed using the 'Anova' function to test for significant differences. Finally, contrast tests performed with the 'emmeans' function to compare the number of corpses in each pairwise combination of areas, using Bonferroni correction to adjust for multiple tests.

Question 3: Do waste removal behaviors reduce within-colony C. bombi transmission?

Infection. To test whether the likelihood of bumble bee microcolonies becoming infected with *C. bombi* differed when infected feces or corpses were added to the nest or "outhouse" cavity, we used four Fisher's exact tests, using the Bonferroni method to adjust for multiple tests. First, we tested for differences between all four treatment groups. Subsequently, we performed three pairwise comparisons: Feces in the nest versus feces in the "outhouse," feces in the nest versus corpses in the nest, and corpses in the nest versus corpses in the "outhouse."

Contact rate. To test whether contact rates between workers and infected feces differed when infected feces were added to the nest, edge of the nest, or "outhouse" cavity, we used a

generalized linear mixed model with a Poisson distribution and number of contacts as the response variable, location (nest, nest edge, or "outhouse") as a fixed effect, and source colony as a random effect. Type II Wald chi-square tests were performed using the 'Anova' function to test for significant differences, and contrast tests were performed with the 'emmeans' function for pairwise comparisons between locations, using the Tukey method to account for multiple tests.

Question 4: Does nest architecture improve fitness by reducing pathogen transmission?

C. bombi prevalence. We fit a generalized linear mixed model with a binomial distribution, using the 'glmmTMB' function, to assess how nest architecture and initial inoculation rate affected *C. bombi* prevalence over time. The response variable was infection status (a binary value representing whether *C. bombi* was detected in an individual bee), and architecture treatment (one or two cavities), initial prevalence (3% or 30% infected), the week of sampling, and their 3-way interaction were included as fixed effects. Marginal cell length (a proxy for body size) was included as an additional fixed effect, and colony was included as a random variable. Type II Wald chi-square tests were performed using the 'Anova' function to test for significant differences. The 'testTemporalAutocorrelation' function in the dHARMA package was used to test for temporal autocorrelation, grouping data within each week. Finally, contrast tests were used to compare probability of infection in colonies with versus without "outhouses" within each initial infection rate treatment (Length 2022).

C. bombi intensity. To assess how nest architecture and initial inoculation rate affected *C. bombi* infection intensity in bees with detectable *C. bombi* infection, we used a generalized linear mixed model, implemented with the 'glmmTMB' function. We focus only on final (week 6) infection intensity in our analysis, because we did not have enough infected bees across all treatment groups to assess trends in *C. bombi* infection intensity over time (Figure S6). Infection intensity

(the number of *C. bombi* cells counted per bee) was included as the response variable; architecture treatment, initial inoculation rate, and their interaction were included as fixed effects; marginal cell length (a proxy for body size) was included as an additional fixed effect, and colony identity was included as a random variable. The model was fit with a truncated negative binomial distribution, as bees with no detected *C. bombi* were excluded from the analysis. Type II Wald chi-square tests were performed using the 'Anova' function to test for significant differences. Contrast tests were used via the 'emmeans' package to compare infection intensity in colonies with versus without outhouses within each initial inoculation rate treatment group. The interaction between cavity treatment and initial inoculation rate did not have a significant effect on *C. bombi* infection intensity (Wald, $\chi^2 = 3.055$, $p = 0.081$). Thus, we removed the interaction term to report the main effects.

Weekly growth. If the "outhouse" architecture improves colony performance, we predict that colonies with access to "outhouses" will grow faster and reach greater maximum weights than control colonies. To test this prediction, we fit a linear mixed model with the 'lme4' package. The response variable was total colony weight, and architecture treatment, week, their interaction, and initial inoculation rate were included as fixed effects. Because the relationship between week and colony weight was curved, we also included the quadratic term I(Week^2). Finally, colony was included as a random effect. Type II Wald chi-square tests were performed using the 'Anova' function to test for significant differences, and the 'testTemporalAutocorrelation' function in the dHARMA package was used to test for temporal autocorrelation, grouping data within each week.

Fitness. If the "outhouse" architecture improves colony performance, we would also predict that colonies with access to "outhouses" would have greater reproductive output than control

colonies. We tested whether architecture affected several measures of reproduction: the numbers of adults (workers + males + gynes), reproductives (males + gynes), gynes, and males produced by the end of the experiment. For each response variable, we fit a general linear model with a negative binomial distribution using the 'glmmTMB' function, with architecture treatment, initial inoculation rate, and their interaction as main effects. Type II Wald chi-square tests were performed to test for significant differences.

Aspergillus flavus. Because our experimental colonies experienced high levels of *A. flavus* infection, we performed several analyses to test whether A. flavus infection was correlated with nest architecture and colony performance. First, if the "outhouse" slows established A. flavus transmission, of the colonies with detected *A. flavus* infection, we would predict prevalence to be greater in control colonies. We used a one-tailed t-test to test this prediction, with *A. flavus* as the response variable and cavity treatment as the explanatory variable.

Infection and performance. If the "outhouse" architecture improves colony performance by reducing *C. bombi,* we would predict colonies with lower *C. bombi* prevalences to experience greater colony growth and greater reproduction. To test this prediction, we used the 'lme4' package to run three linear models with colony growth (final nest weight – initial nest weight), final number of adults (workers + gynes + males), and final number of reproductives (gynes + males) respectively as response variables, and final *C. bombi* prevalence as the explanatory variable. However, if *A. flavus* infection is driving the observed differences in colony growth and reproduction, we would predict colonies with greater *A. flavus* prevalence to experience less colony growth and reproduction. We tested this prediction with three linear models, fit with colony growth, final number of adults (workers + gynes + males), and final number of reproductives (gynes + males) respectively as response variables, and final *A. flavus* prevalence

observed across all individuals (larvae, pupae, workers, males, gynes) as the explanatory variable, in colonies in which *A. flavus* infection was detected. Two colonies were excluded from these analyses due to missing data. The interactions between cavity treatment and initial inoculation rate had no effect on any measure of reproduction, so we removed the interaction to report main effects (adults: Wald, $\chi^2 = 0.408$, $p = 0.523$; reproductives: Wald, $\chi^2 = 0.200$, $p = 0.655$; gynes: Wald, $\chi^2 = 1.649$, $p = 0.199$; males: Wald, $\chi^2 = 0.411$, $p = 0.521$).

Supplemental Materials

Figure S1. Experimental setup addressing Question 1: Do bumble bees modify nest architecture to create peripheral cavities for waste? In this experiment, bumble bee microcolonies were housed in nest boxes that were half-filled with florist foam. Modification of the foam blocks and defecation patterns were observed.

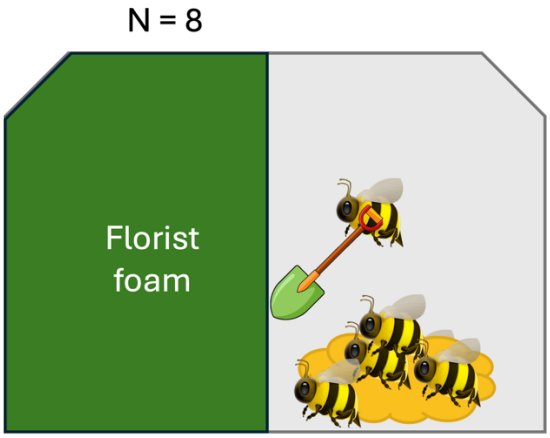


Figure S2. Setup for first experiment addressing Question 2: Do bumble bees use peripheral nest cavities for waste removal when infected? Bumble bee microcolonies were established in two-chambered nest boxes, and defecation patterns were observed for colonies inoculated with either *C. bombi* or a sham control.

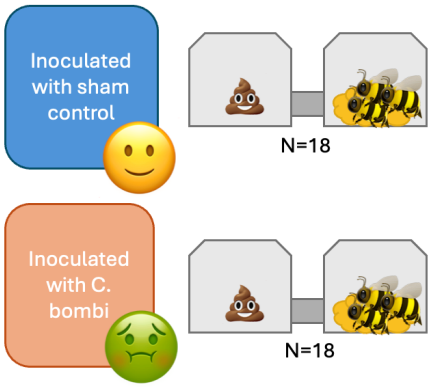


Figure S3. Setup for second experiment addressing Question 2: Do bumble bees use peripheral nest cavities for waste removal when infected? Bumble bee microcolonies were established in two-chambered nest boxes and given access to a foraging cage under natural light conditions. Defecation patterns were observed for colonies inoculated with either *C. bombi* or a sham control.

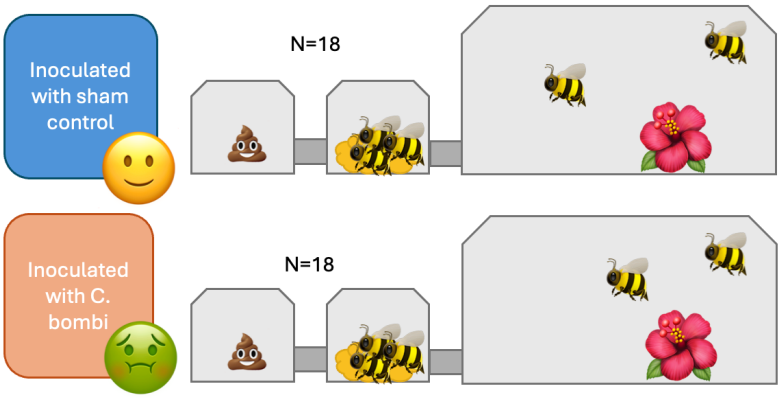


Figure S4. Experimental setup addressing Question 3: Do waste removal behaviors reduce within-colony *C. bombi* transmission? All microcolonies were established in two-chambered cages, and this experiment had a 2 x 2 factorial design, crossing the type of inoculum (infected feces or corpses) and the location of the inoculum (added to the nest or peripheral chamber). The number of times workers contacted the fecal inoculum in each area was observed over a 20-minute observation period. At the end of the experiment, a sample of 10 workers from each colony were screened for *C. bombi*.

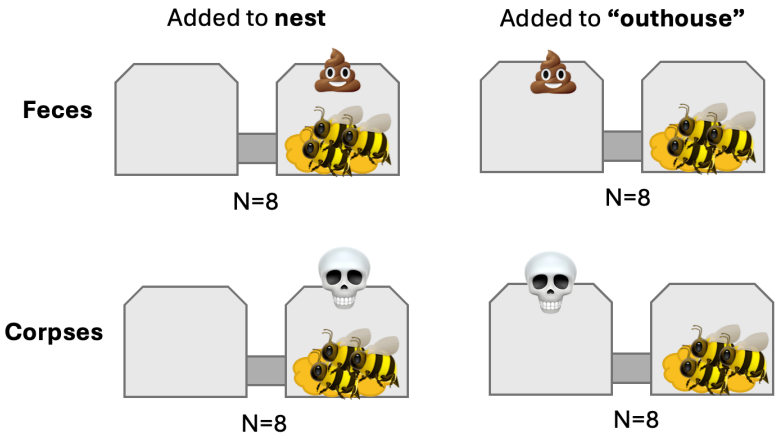


Figure S5. Experimental setup addressing Question 4: Does nest architecture improve fitness by reducing pathogen transmission. This experiment had a 2 x 2 factorial design, crossing nest architecture (with or without an additional cavity) and initial inoculation rate (3% versus 30% inoculated with *C. bombi*). Each colony was screened and weighed for *C. bombi* weekly for the 6 week experiment, and censused after the experiment was terminated.

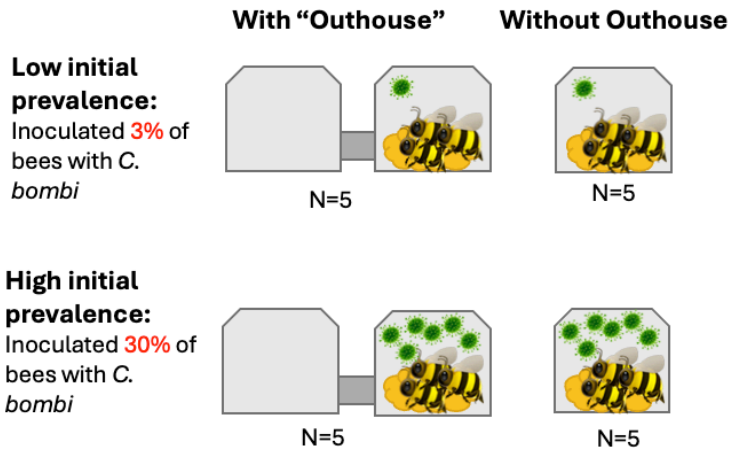


Figure S6. Measures of *C. bombi* infection intensity for workers in colonies with an "outhouse" (yellow) versus control colonies (blue) in the **a.** low (3%) initial inoculation treatment and **b.** high (30%) initial inoculation treatment. Each point represents one worker. 15 workers from n = 5 colonies per treatment group were screened, and only workers with detectable *C. bombi* infection are included. c. Final measures of infection intensity in colonies with detectable *C. bombi* infection. Each point represents one worker, and letters represent colony means.

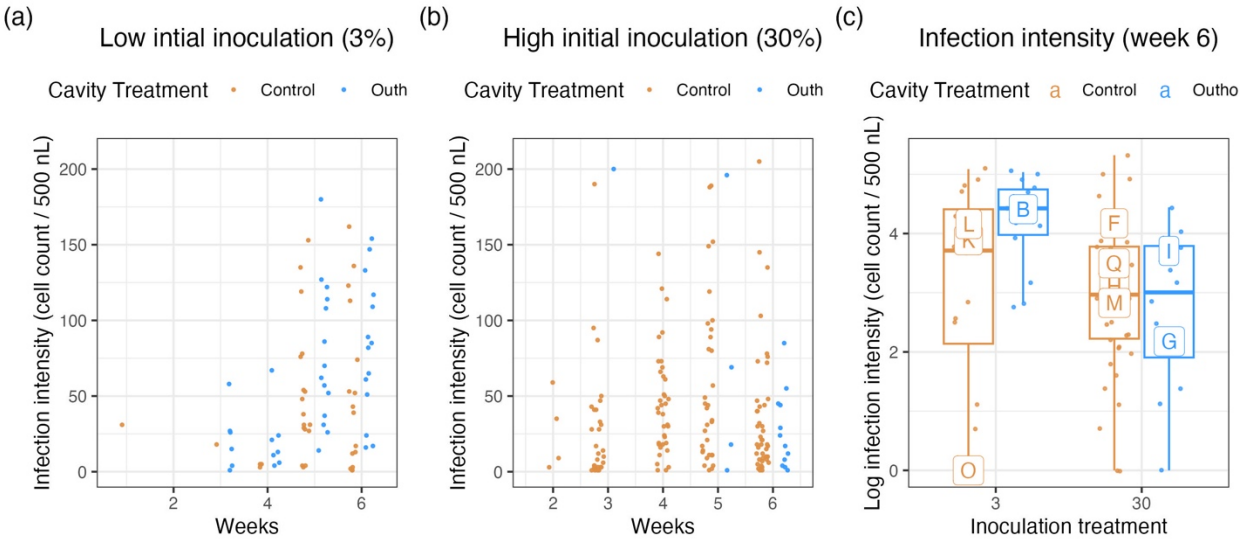


Figure S7. *Aspergillus flavus* infection in a bumble bee **a**. pupae and **b**. worker corpse.

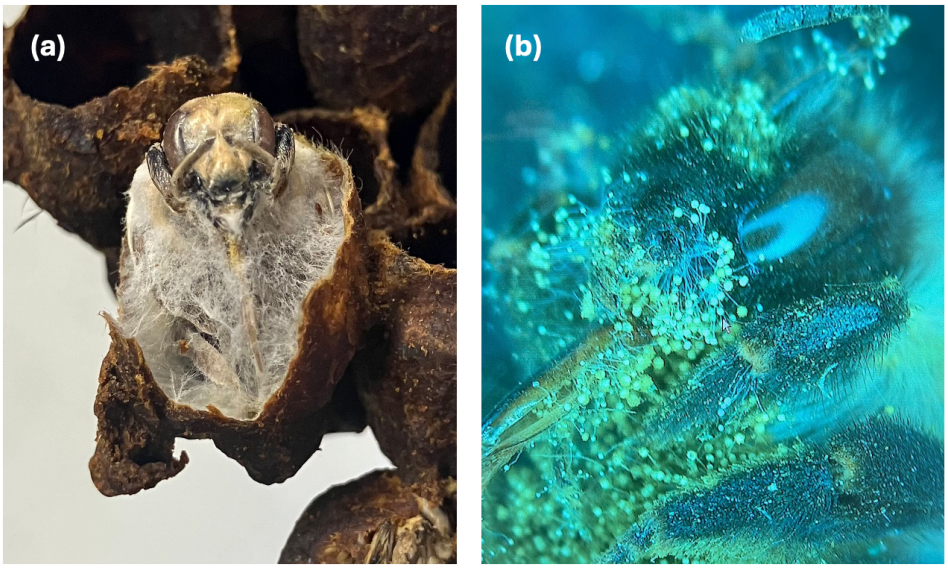
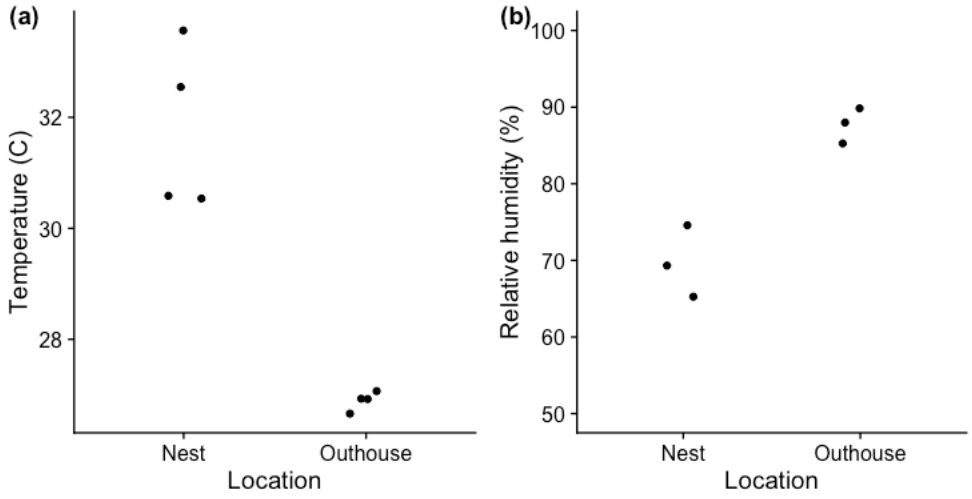


Figure S8. **a**. Temperature (n = 4) and **b**. relative humidity (n = 3) measurements for the nest cavity versus "outhouse" cavity from colonies in the "outhouse" treatment group.



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DISCUSSION

From the individual to population level, social behavior and disease dynamics are shaped by the environments in which animals live. The relationship between the built environment, behavior, and disease is well-understood in human societies (Pinter-Wollman et al., 2018). In the mid-nineteenth century, urban planners aimed to reduce overcrowded and unsanitary conditions in cities through zoning – spatially separating residential, commercial, and industrial areas – and architects combatted infectious diseases such as tuberculosis through housing designs that increased airflow and sunlight (Campbell, 2005; Corburn, 2005; Pinter-Wollman et al., 2018). While these changes responded to the urgent challenges associated with infectious disease, some of them have introduced new challenges that have contributed to modern chronic diseases associated with physical inactivity and poor diet, such as diabetes and heart disease (Schilling & Linton, 2005). The trade-offs associated with architectural defenses against disease in human societies illustrate that architecture does not represent a one-size-fits-all approach to combating disease, and instead produces highly variable context-dependent effects. A better understanding of these effects across diverse ecological, social, and pathological contexts may provide valuable insight into the general principles driving the interactions between architecture, social behavior, and disease.

One aspect of the spatial environment that can shape disease is individual density, or the number of individuals in the nest. Group density (number of individuals per unit space) and group size (total number of individuals) are often highly correlated, making it difficult to untangle the causative effects of either. However, has been hypothesized that group size and density may increase the frequency or duration of social contacts between nestmates, thereby

directly increasing rates of transmission of infectious pathogens (Anderson & May, 1978; Cote & Poulin, 1995; Rifkin et al., 2012). Group size or density may also increase species' susceptibility to disease indirectly, by increasing social stress or conflict (Iguchi et al., 2003). These effects may vary dramatically across species or individuals, based on their unique social requirements, and both overcrowding (humans: Wells & Harris, 2007) and social isolation (rhesus macaques: Hannibal et al., 2017) have been observed to elevate stress and alter disease outcomes. Overall, a correlation between group size, density, and disease has been observed across vertebrate species (Cote & Poulin, 1995; Rifkin et al., 2012), but evidence in social insects is less clear (Schmid-Hempel, 1998); in some highly eusocial insect species, group size is correlated with reduced costs of disease, perhaps due to increases in the efficacy or efficiency of social immunity behaviors (Hughes & Boomsma, 2004; Leclerc & Detrain, 2018; Traniello et al., 2002).

In Chapter 1, we found that bumble bee microcolonies with greater numbers and densities of workers experienced greater mortality and reduced reproduction when inoculated with a fecally-transmitted pathogen compared to their smaller counterparts, indicating that individual density can increase the costs of infectious disease for this species. We did not find evidence for increased pathogen transmission in larger microcolonies, suggesting that worker density may increase the costs of disease not by directly increasing rates of transmission, but rather by indirectly increasing social stress or reproductive conflict. However, due to the different rates of mortality observed in small versus large microcolonies, we were not able to rule out the possibility that there was increased pathogen transmission in larger microcolonies, and future research is necessary to confirm this mechanism. Regardless of the mechanism driving this effect, our discovery that worker density significantly increases the costs of infectious disease for

bumble bee microcolonies suggests that nest architectures that increase worker crowding increase the risks of infectious disease for this social, cavity nesting species.

While Chapter 1 revealed that the spatial environment can constrain social life in ways that can increase susceptibility to disease, organisms may be able to combat these costs and prevent or reduce pathogen transmission by restructuring their spatial environments. Humans are able to modify their spaces to reduce disease: for example, plexiglass barriers were deployed broadly during the COVID-19 pandemic to limit the spread of airborne droplets. However, understanding who designs and constructs the built environment, what building methods or materials they employ, and the cumulative costs involved, is necessary for understanding when and how the built environment can be restructured to reduce disease (Pinter-Wollman et al., 2018). Similarly, nest modification can be energetically or temporally costly for non-human animals (De Gasperin et al., 2016; Mainwaring & Hartley, 2013), and in some cases physical or energetic constraints may preclude architectural modifications that could otherwise reduce pathogen susceptibility or transmission. Despite this, if restructuring the nest provides sufficient fitness benefits – for example, by providing defenses against a highly virulent or prevalent pathogen – the behaviors associated with that restructuring could be selected for. Empirical research into nest modification in non-human animals is limited, and no prior study has documented whether non-human animals modify their nest structures to defend against disease.

In Chapters 2 and 3, we investigate whether bumble bees restructure their nest structures to reduce pathogen transmission. We hypothesized that bumble bees modify their nests to create a peripheral cavity adjacent to the primary nest cavity, which facilitates waste removal behaviors, reduces disease transmission, and improves fitness, ultimately driving selection for nest modification behaviors. In Chapter 2, we observed 10 wild bumble bee (*Bombus frigidus*) nests,

finding that all of the nests contained an additional cavity filled with concentrated fecal materials. This natural history observation provided the first evidence for hygienic architecture in wild bumble bee colonies, and laid the foundation for the ecological relevance of the laboratory experiments described in Chapter 3. In Chapter 3, we extensively tested this hypothesis with manipulative laboratory experiments, finding that bumble bees (*Bombus impatiens*) actively modify the interior structure of their nests, and that this restructuring facilitates waste removal behaviors, reduces pathogen transmission, and increases reproductive success. Thus, while Chapter 1 suggested that nest architecture can constrain behavior and increase the costs of disease, Chapters 2 and 3 reveal that wild bumble bee colonies are ultimately able to manipulate the relationship between architecture, social behavior, and infectious disease within their nests, effectively leveraging this relationship to reduce disease. This study provides the first empirical evidence for architectural immunity – the use of the spatial environment to reduce infectious disease transmission – in a non-human animal species.

While this thesis explores the relationships between architecture, disease, and social behavior in bumble bees, these relationships are highly context-dependent and shaped by species' ecology and evolution. We found that bumble bees modify their nests to create an interior waste chamber within the nest. In contrast, it is known that healthy honeybees defecate exclusively outside the nest (Drummond, 2022). Why some species use interior waste chambers to dispose of waste while others dispose of waste outside the nest is an open question, but several hypotheses related to the species' ecological or evolutionary context could explain these differences.

First, species that nest in moist environments that are more suitable to pathogens may be more likely to dispose of waste in an interior waste chamber because such a chamber can be effectively closed off to confine pathogens. Conversely, species that nest in xeric environments

may be more likely to dispose of waste outside, where hot, dry conditions neutralize the risks posed by pathogens. Comparative work in leaf cutting ants has found that species that live in wet environments are more likely to dig waste middens within the nest, while those from xeric environments are more likely to dispose of waste outside the nest (Farji-Brener et al., 2016), suggesting that ecological context may have important consequences for waste removal behaviors and architectures. However, the causative mechanisms driving these relationships and whether they extend to social bees remain untested. Alternatively, species that live in harsh or dangerous environments may be more likely to dispose of waste in designated interior waste chambers, to reduce the costs or risks associated with venturing outside the nest, while species that nest in more moderate environments may experience fewer costs associated with disposing of waste outside the nest. Bumble bees are important pollinators in arctic, subarctic, and alpine ecosystems, and are particularly adapted to cold and wet environments (Heinrich, 1972), while honeybees are associated with mediterranean climates (Han et al., 2012). As such, disposing of waste within the nest may be more important for bumble bees, protecting them from exposure to cold, wet conditions, than for honeybees. Finally, differences in hygienic behaviors or architectures may not be adaptive, and instead physical or morphological constraints may limit their evolution. For example, species that nest in rigid structures may be less able to modify the architecture of their nests than species that nest in malleable substrates. If this is true, honeybees may defecate outside the nest simply because modifying the wood cavities (Seeley, 2019) they nest within is not possible or is prohibitively costly, while bumble bees are able to excavate cavities in their soil nest cavities (Liczner & Colla, 2019) relatively easily.

Species' waste removal behaviors and architectures may also be shaped by their movement ecology and social behavior. Because nest modification can be time- and energy-

intensive (De Gasperin et al., 2016; Hansell, 2005; Mainwaring & Hartley, 2013), species that exhibit strong site fidelity or nest in one location for long periods of time may experience greater cumulative benefits from investing in nest hygiene or modification, while species that move more frequently may not remain in one nest long enough for the benefits associated with hygienic nest modification to accrue. Additionally, species that nest in one location for longer periods of time may experience greater costs associated with waste accumulation compared to species that move frequently and leave waste behind at past sites (Weiss, 2006). The increased need for behavioral and architectural hygiene in larger, more stationary societies is exemplified by humans: latrine structures date back to the origins of sedentary communities and agriculture, as the transition to larger community sizes and a more sedentary lifestyle necessitated the development of new structures to accommodate and control the corresponding increase in the volume of and proximity to waste (Antoniou et al., 2016; Mitchell, 2015). However, despite the intuitive nature of these hypotheses, whether movement ecology and social behavior are correlated with behavioral or architectural hygiene in non-human animals remains an open question, and future comparative work is needed to fill this gap.

Finally, while this thesis explores the relationships between nest architecture, social behavior, and infectious disease in individual bumble bee colonies, these effects may have important cascading consequences at the community or population level. Consequently, it is possible that nesting ecology could shape host and pathogen evolution. For example, species that nest in substrates that can be modified to create waste midden structures may experience selection for more transmissible and less virulent fecal-orally transmitted pathogens, as transmission events may occur less frequently in hosts with effective architectural defenses; or host species that nest in substrates that cannot be modified to create architectural immunity

defenses may evolve more effective physiological immunity. However, these hypotheses remain purely speculative, and future research is necessary to reveal whether nesting ecology could drive differences in host or pathogen evolution.

This thesis provides the first empirical evidence for architectural immunity in a non-human animal species, revealing that infectious disease can drive the evolution not only of hygienic behaviors, but also of animal homes. We first found that bumble bees' nesting environment can increase the costs of infectious disease. As spatial constraints increase worker density in colonies with greater numbers of workers, mortality increased and reproduction decreased, when colonies were faced with pathogen pressure. In this way, bees' nesting and social environment can increase the costs of disease. However, we also found that bumble bees are not passive occupants of their spaces, and are able to modify their nesting environment in ways that facilitate waste removal, reduce pathogen transmission, and improve fitness. This reveals that the nest environment can also provide adaptive defenses and benefits against disease in this species. While our work in bumble bees provides the first evidence for the importance of architecture in shaping infectious disease dynamics, as well as a case study for these effects, our focus on bumble bees is not yet sufficient to provide broad general insight into the context-dependent drivers of these relationships. For this, future comparative research across more diverse ecological, social, and pathological contexts is needed. However, as the spatial and architectural environment has often faded into the backdrop and has been historically overlooked in studies of behavior and disease, this research provides an important reminder that social behavior and disease dynamics are crucially shaped, constrained, and selected for by the environments in which animals live.

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