# Examining propolis use, social immunity, and food systems transformation to support colony health in honey bees and stingless bees

A Dissertation
SUBMITTED TO THE FACULTY OF
THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Dr. Marla Spivak (advisor)

May 2023

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# Acknowledgements

So many people and so many places came together to make this project possible. I'll start with the bees and beekeepers who took me into their world and changed my life completely. I would also like to thank:

My family, in particular: Héctor, my partner in both life and work. Laura, my sister and co-conspirator. My parents, Anne and Tim, for everything, my brother Jack, and the Minnesota Letschers.

My Minnesota friends, in particular Julia Brokaw, Vivian Wauters, Christy Dolph, Jennifer Nicklay, Kiley Friedrich, Nate Looker, Juliana Vélez, Jordan Pruzenski, and Rafael Contreras, for teaching me the most valuable lessons I learned during graduate school: the importance of friendship and the power of community care.

Science for the People – Twin Cities, and the agroecology communities at the University of Minnesota, for the political education and friendship, and for helping me connect bee research to broader movements for just and sustainable futures.

My advisor, Marla Spivak, and my committee members: Michael Goblirsch, Nick Jordan, Emilie Snell-Rood, and Tim Kurtti.

The Bee Teams that have held this work: the Spivak lab at the University of Minnesota and el Equipo Abejas at El Colegio de la Frontera Sur, with particular appreciation for the individuals who put meticulous care and attention to the colony management, data collection, and laboratory work that went into this project: Gary Reuter, Yuuki Metreaud, Héctor Morales Urbina, Jenny Warner, and Ben Ziegler. Rémy Vandame, Miguel Guzmán Díaz, Erik de Jesus Solorzano Gordillo, Estefhanía López Roblero.

Our collaborators at the United States Department of Agriculture: Michael Simone-Finstrom, Philip Tokarz, Frank Rinkevich, and Quentin D. Read.

The funding organizations that supported me through my PhD: The National Science Foundation, the UMN Entomology Department, the Interdisciplinary Center for the Study of Global Change, the UMN Consortium on Law and Values in Health, Environment & the Life Sciences, and the International Union for the Study of Social Insects.

The childcare providers who made this whole thing possible: Sarah, Geydi, Fran, Karla, Diana, Pola, and the many family members who have created time and space to allow Héctor and I to work and rest.

And, Chai, my two-year-old. Chai didn't help much with the actual work, but he has brought it, and me, much meaning.

I dedicate this dissertation to the bees and their keepers,
with gratitude for the goodness they have given me and
commitment to continued collective efforts,
to transform the food systems we all rely on.

With particular appreciation for Isidro Morales Vazquez
who we love dearly, and lost too soon

# Abstract

As the industrialization of agriculture and other environmental stressors threaten honey bees, stingless bees, and beekeeper livelihoods throughout the world, beekeepers and researchers seek solutions to support bee health. Although many beekeeping practices are designed to support colony health, some inadvertently constrain the natural defenses (or mechanisms of social immunity) that help bees thrive in an unmanaged context. In addition, although most honey bee research seeks to counteract the multiple interacting stressors that cause colony loss, researchers often fail to mention industrial agriculture – the root cause of those stressors – and thus further normalize a major source of bee decline. This dissertation seeks to understand and bolster the natural defenses bees use to support colony health, and to identify ways in which honey bee researchers can reframe their research to contribute to food systems transformation. In Chapter 1, I unpack the relationship between honey bee health and industrial agriculture. I propose steps researchers can take to account for the impacts of this destructive system in our research narratives, and I discuss the uncomfortable questions that surface when we engage in this process. In Chapter 2, I review the use of antimicrobial resin by honey bees and stingless bees for nest construction and defense, and I discuss the ways in which this material contributes, or may contribute, to social immunity in different species. In Chapter 3, I test strategies to stimulate the construction of a robust propolis envelope – a resin-rich structure that wild honey bee colonies build when they nest in hollow tree cavities – in multiple beekeeping contexts. I collaborated with researchers from the United States Department of Agriculture- Agricultural Research Service to assess different surface texture treatments (rough wood boxes, boxes outfitted with propolis traps, and standard, smooth wood boxes) in terms of their ability to stimulate propolis collection, and examined the effect of propolis on colony health, pathogen loads, immune gene expression, bacterial gene expression, survivorship, and honey production. We found that rough wood boxes are the most effective box type for stimulating propolis deposition. The use of rough boxes led to decreased pathogen loads, modulated immune function, and increased colony size. In Chapter 4, I review resin use by stingless bees, specifically. Like honey bees, stingless bees – social, honey-producing bees native to tropical regions – integrate antimicrobial resins in the form of propolis into their colonies. However, the impact of smooth wood box hives on resin collection and the role of propolis in stingless bee colony social immunity have not been examined. In Chapter 5, in collaboration with researchers from the Bee Team at El Colegio de la Frontera Sur, I monitored resin collection and colony development over the course of one year in smooth wood boxes, rough wood boxes modified to mimic hollow tree cavity textures, and thin boxes designed to test the hypothesis that bees use propolis to insulate against temperature change. I also added or removed propolis stores from a second set of colonies and monitored the effect of propolis manipulation on resin foraging and colony development over the course of one year. I found that the use of rough wood boxes leads to increased resin collection, but I did not detect an effect of increased resin collection on colony development. Propolis manipulation in general – and propolis removal specifically – led to increased resin collection, a finding that could have important implications for beekeepers looking to sustainably harvest propolis for medicinal or commercial use.

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# Introduction

Honey bee colony losses occur at staggering rates in the U.S. and in many other parts of the world (Bruckner et al. 2023, Gray et al. 2023). These losses are commonly attributed to a suite of multiple, interacting stressors which include parasites, pathogens, poor nutrition, poor management, and exposure to agrochemicals (Steinhauer et al. 2018, González-Varo et al. 2013, Bretagnolle and Gaba 2015, Goulson 2013, Wu-Smart and Spivak 2016, Alger et al. 2018, Brosi et al. 2017). Efforts to support honey bee health often focus on mitigating these stressors, or on bolstering bees' ability to manage their effects (Shanahan 2022). My dissertation started with the latter. I came into this work as a beekeeper. Prior to entering graduate school, I had spent six years doing bee work in different parts of the world. I was familiar with the realities of colony loss and eager to use scientific methods to develop practical solutions for beekeepers operating at a variety of scales. I began by studying propolis use and social immunity in honey bees and exploring beekeeping practices that allow for the restoration of the propolis envelope.

Propolis is a substance made up of plant resins and small amounts of beeswax (Simone-Finstrom and Spivak 2010, Salatino and Salatino 2021). Resin originates as a plant defense; plants produce resin to protect themselves from herbivores and pathogens (Langenheim 2003). When honey bees nest in hollow tree cavities, they use propolis to fill the cracks and crevices in the cavity walls, creating a continuous layer of propolis called the propolis envelope (Seeley and Morse 1976). The smooth wood boxes where most beekeepers keep their bees contain few cracks and crevices, and do not stimulate much resin collection. Managed honey bees do bring small amounts of propolis into their

nest spaces. They use propolis to fuse bee boxes together, and to fill the gaps between the wooden frames that hold their brood combs and honey combs. The propolis that managed bees deposit in these crannies does not come close to the robust propolis envelopes their wild counterparts construct, but it does sometimes "gum up" beekeeping equipment. Because of this, propolis has long been considered an inconvenience to beekeepers; for years, beekeepers removed propolis from their hives to keep their boxes "clean." These practices pose a problem because, in recent decades, a growing number of studies have shown that propolis supports honey bee colony health (reviewed by Simone-Finstrom and Spivak 2017).

The propolis envelope helps combat a variety of pests and pathogens and thereby contributes to social immunity in honey bees. Social immunity is a term that refers to the many, varied physiological, behavioral, and organizational strategies that social insects use to protect their colonies from parasites and pathogens (Cremer et al., 2018). In light of the benefits of propolis to honey bee health, beekeepers and researchers are now looking for ways to facilitate the construction of the propolis envelope in managed contexts, and thus restore this important aspect of honey bee social immunity.

For my dissertation, I worked with collaborators from the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) to conduct a multi-year study which tested different honey bee hive designs in terms of their ability to stimulate propolis deposition and support colony health in stationary and migratory beekeeping contexts (Chapter 3). We compared propolis deposition and colony health metrics in rough wood boxes, boxes outfitted with propolis traps, and standard, smooth

wood boxes. We found that rough wood boxes performed best in terms of stimulating propolis collection. These boxes resulted in reduced pathogen loads and increased colony size. Importantly, we conducted this experiment in collaboration with the largest commercial beekeeping operation in the United States. This allowed us to trial rough boxes in one of many real-world situations in which they might be useful. Our results support the use of rough boxes as a practical tool to stimulate the construction of a robust propolis envelope. They demonstrate that minor modifications to beekeeping practices can help restore honey bee social immunity in a managed context, which can lead to measurable improvements to honey bee health.

Honey bees are not the only bees who integrate propolis into their nest environments (Chui et al. 2021). Stingless bees collect resin in copious amounts and use this material to build and defend their nests (Roubik 2006). Stingless bees are social, honey producing bees native to tropical regions. They are a diverse group of bees, containing over 600 species which demonstrate many different life history strategies (Roubik 2023). Indigenous and land-based peoples have managed stingless bee colonies for millennia (Chan Mutul et al. 2019). Despite the destructive forces of colonization, which drove decades of decline, the practice of stingless beekeeping persists today. In fact, in recent years, stingless beekeeping has experienced something of a renaissance. As stingless beekeeping expands, beekeepers are increasingly keeping their bees in smooth wood boxes rather than traditional hive types, such as hollow log *jobones* or clay pots.

Before entering graduate school, I spent four years working as an extension educator with the Bee Team at El Colegio de la Frontera Sur (ECOSUR) in Chiapas,

Mexico. During this time, I had the opportunity to witness the rapid expansion of stingless beekeeping in southern Mexico, and I was able to experience one small fraction of the vast and dazzling diversity that exists in and around the world of stingless bees. So when, as a graduate student, I learned that the use of standard, smooth wood honey bee hives inadvertently suppressed honey bees' ability to build a robust propolis envelope, I began to wonder whether transitioning stingless bees from hollow tree cavities to smooth wood boxes might have a similar effect. It seemed to me that honey bee-keepers were working to return their bees to a rough box environment at the very moment that stingless beekeepers were increasingly adopting a smooth one. Could this transition impact the amount of propolis these bees integrated into their nest environment? Would the erosion of resin-rich structures have implications for stingless bee health, as had occurred in honey bees? I reviewed resin use by stingless bees in Chapter 4, and then I explored these questions in Chapter 5, in collaboration with my former colleagues at ECOSUR, with particular support from Miguel Guzmán-Díaz, a stingless beekeeping expert based in Tapachula, Chiapas.

We conducted studies on resin use by the stingless bee species *Scaptotrigona mexicana* in the Soconusco region of Chiapas, from 2019 to 2021. Our goal was to determine whether resin use – particularly the resin (or propolis) that tree-nesting stingless bees use to form the thick walls that surround their nests – supports social immunity in *S. mexicana*, and whether stingless bee management practices (i.e., transitioning colonies from hollow tree cavities to smooth wood boxes) impact the bees' ability to accumulate propolis inside their hives. The first part of this task proved more

complicated than we anticipated. Honey bees are afflicted by myriad stressors, and honey bee health has been a research priority for over a decade, so researchers have developed a keen understanding of the interplay between honey bee pathogens and immune responses (Evans and Pettis 2007, Larsen et al. 2019, Lourenço et al. 2013). To measure the importance of propolis to colony health, for example, honey bee researchers can compare immune gene expression in the presence and absence of propolis (Borba et al. 2015, Simone-Finstrom and Spivak 2010), or challenge colonies with common honey bee pathogens and observe differences resin collection (Simone-Finstrom and Spivak 2012). As discussed in Chapter 2, the same is not true for stingless bees. First of all, propolis is so ubiquitous in stingless bee nest environments that it is not possible to compare colony health in the presence and absence of propolis – for stingless bees, there is no absence. Second, stingless bee colonies are not afflicted by common pathogens the way that honey bees are (Roubik 2023), and studies on stingless bee immune function are only beginning to emerge (Al Naggar et al. 2022, Ravaiano et al. 2018). This made testing the relationship between propolis and stingless bee colony health something of a challenge. Our experimental options were further limited when the COVID-19 pandemic hit three months into our first field season, impacting our ability to run the in-person experiments we had planned. In spite of all this, we were still able to learn a number of things about resin use by *S. mexicana*.[1] We found that housing bees in boxes designed to mimic the inner textures of a hollow tree cavity does result in increased resin collection, and that

[1] This work could not have continued without support from three stellar local researchers who elevated their role in our collaboration when COVID-19 limit my ability to travel: Miguel Angel Guzmán Díaz, Erik de Jesus Solórzano Gordillo, and Estafhanía López Roblero.

when colonies invest extra energy in resin collection to fill cracks and crevices in their hive, this does not negatively impact colony growth or development. It may, in fact, aid in it. Unfortunately, colony growth and development are not very precise proxies for colony health, and we are still a ways from defining whether propolis contributes to stingless bee social immunity in any specific or particular way. I hope that efforts to understand resin collection in stingless bees continue, and that future management practices account for the potential benefits of the natural behaviors that beekeepers and researchers have yet to understand. More than that, though, I hope that future agricultural and land management practices, and social, political, and economic conditions support the health of wild and managed stingless bee colonies so robustly, so completely, that the question of whether propolis is a medicine that stingless bees might need to combat disease never gains practical relevance.

These conditions – the agricultural, social, economic and political conditions that impact bee health – were the focus of the first chapter of my thesis. This chapter, though first in order and importance in this document, was not a part of my initial dissertation plan. I entered graduate school determined to help develop evidence-based management practices that would mitigate honey bee colony losses. However, as I learned more about U.S. beekeeping systems, which are vast, interconnected, and, in large part, industrial, I came to understand that beekeepers and researchers are looking for solutions to improve honey bee health in contexts that do not support their survival. I saw that in order to halt staggering rates of colony loss, those conditions would have to change. I was surprised, and disappointed, to find that although the honey bee research community was keen to

address the multiple interacting stressors that threaten honey bee health, researchers very rarely acknowledged the system that creates and exacerbates these stressors – we rarely acknowledge the impacts of industrial agriculture. I wrote chapter one to call up a conversation that I desperately needed to engage in, and to ask the Dangerous Questions that first shook – and now shape – the way that I understand my work as a beekeeper, researcher, and agroecologist.

Although they operate at different scales, my industrial agriculture analysis is not separate from my efforts to understand propolis use and colony health. In examining the importance of the propolis envelope to honey bee social immunity, we see that distancing bees from their natural behaviors can negatively impact colony health. A number of researchers argue that, in order to support colony health, beekeepers must adopt management practices that restore or at least account for these behaviors (Seeley 2017, Neumann and Blacquière 2017, Brosi et al. 2017). My propolis research supports this view, demonstrating that restoring bees' ability to build a propolis envelope can support honey bee health in measurable ways. My analysis of the impact of industrial agriculture on honey bee health extends this premise – the importance of restoring bees' natural behaviors to support colony health – beyond the apiary, into the broader landscape, into the industrial food systems that drive bee decline. Together, these parallel, overlapping efforts demonstrate that supporting bee health requires not just a better understanding of bee biology, but action, on the part of honey bee researchers, to envision, enact, and defend resilient, diversified agricultural systems.

# Chapter 1: Honey bee health and industrial agriculture: What researchers are missing and why it's a problem[2]

**Abstract:** Industrial agriculture is the root cause of many health problems that honey bees (*Apis mellifera* Linneaus, 1758) face, but honey bee researchers seldom call attention to this fact. We often discuss the stressors that contribute to colony loss (e.g., pathogens, pesticides, poor nutrition), but we rarely talk about where those stressors come from. This is a problem because we cannot resolve honey bee health issues unless we confront the systems that cause them harm. In this forum article, I unpack the relationship between honey bee health and industrial agriculture. I propose steps we can take to reframe our research to account for the impacts of this destructive system, and I discuss the uncomfortable questions that surface when we engage in this process. The goal of this article is to encourage conversation within the honey bee research community around the impacts of industrial agriculture, so that we can fully engage in the transformative change needed to support honey bee health.

## Introduction

In the United States, when honey bee researchers talk about honey bee health, we often start by describing the following problem: honey bee health is precarious, and colony losses occur at unsustainable rates.[3] We then refer to a set of multiple interacting stressors to explain the causes of colony loss (Steinhauer et al. 2018). We point to the four P's: parasites, pathogens, poor nutrition, and pesticides ('Honey Bee Health' 2021).

[2] This chapter was published in the Journal of Insect Science on February 7, 2022.

[3] I use the word 'we' because I am a honey bee researcher and I am part of this learning process, too. After several years as an extension educator and beekeeper, I chose to pursue a Ph.D. because I saw and experienced unsustainable colony loss, and I hoped that research could provide better solutions for beekeepers at all scales. The analysis I share here is centered in the United States, where much of my beekeeping and bee research experience has taken place, though I believe it to be relevant wherever honey bees interact with industrial agriculture.

We note that these stressors are complex and mutually reinforcing (Spivak et al. 2011). We explain, for example that a malnourished colony is more susceptible to parasites and pathogens (Dolezal et al. 2019), and that a diseased colony is less likely to be able to collect the resources it needs for adequate nutrition (Wells et al. 2016, Dolezal and Toth 2018). Next, we reference some of the social, economic, and ecological implications of poor honey bee health and colony loss. We talk about the ways in which this problem negatively affects honey bee wellbeing and beekeeper livelihoods (Goodrich 2019). Sometimes we also mention that the spread of honey bee pathogens could spill over to native bees and other insects, which might negatively impact their health (Mallinger et al. 2017). Taking this one step further, we connect the importance of honey bee wellbeing and beekeeper livelihoods to our agricultural system, the food supply, and global food security (vanEngelsdorp and Meixner 2010).

This narrative frames many of the grants we apply for, the articles we write, and the actions we take to support honey bee health. It is clear cut and widely agreed upon. It is also missing something big. The framing we use to discuss honey bee health highlights the stressors that drive colony loss, but it does not talk about where those stressors come from (see Box 1). In this forum article, I argue that in order to improve the health of honey bees, we, as honey bee researchers, must confront the systems that cause them harm. Here, I discuss the connection between honey bee health and industrial agriculture, a complex eco-social system whose biophysical components are characterized by large-scale monocultures, mechanization, and extensive off-farm inputs (e.g., seeds, chemicals, managed pollinators) (Kovács-Hostyánszki et al. 2017, Petersen-Rockney et al. 2021). I examine the ways that honey bee researchers discuss the causes of colony loss, and reflect on the consequences this messaging has. Finally, I propose options for reframing our research and explore the uncomfortable questions that emerge when we engage in this process. Ultimately, the goal of this paper is to encourage conversation within the honey bee research community around the impacts of industrial agriculture, so that we can fully engage in the transformative change needed to support honey bee health.

## Industrial agriculture negatively impacts honey bee health

The problem of industrial agriculture – also known as intensive, conventional, or modern agriculture – is vast and unwieldy. For the purpose of this article, I will highlight the ways in which the biophysical expression of this system impacts honey bee health.[4]

In non-industrial, low-input, diversified farming systems, complex communities of plants, animals, bacteria, and fungi contribute to ecosystem functions that support sustainable food production (Kremen and Miles 2012, Bommarco et al. 2013). These include vital processes such as pollination, pest control, soil formation, and water regulation (Bacon et al. 2012). To support their function, farmers must manage biodiversity at field, farm, and landscape scales (Petersen-Rockney et al. 2021).

Industrial agriculture is designed around two main goals: 1) increased labor productivity (where the idea is to maximize output per worker) and 2) increased yield (where the idea is to maximize output per plant or animal) (Ellis et al. 2020). Proponents of industrial agriculture argue that farmers must simplify and standardize crop production in order to achieve these goals (Weis 2010, Ellis et al. 2020). This means establishing monocultures and replacing ecosystem services with synthetic fertilizers, pesticides, and other technological fixes (Altieri 1998, Socolar et al. 2021).

The simplification and standardization of agricultural landscapes can support increased yield, but these processes pose some major problems (Tscharntke et al. 2005). First, they undermine biodiversity and erode the ecosystem functions that diverse plants and animals provide, increasing farmer dependence on off-farm inputs (Tilman et al. 2002, Cardinale et al. 2012, Bretagnolle and Gaba 2015). Second, the industrialization of agriculture leads to consequences, or externalities, that extend far beyond crop fields. Some of these externalities include greenhouse gas emissions, viral spillover events,

[4] There are, of course, other problems with industrial agriculture. Many of these problems are rooted in the ways in which this system perpetuates destructive capitalist and colonial projects. The biophysical focus of this paper is not meant to elide these related issues, but to highlight the dynamics that impact honey bee health most directly. For broader analyses on the social, political, and economic components of this sprawling problem, see work by honey bee researchers from the social sciences and humanities (e.g., see Nimmo 2015a; Cilia 2019, 2020).

contaminated water supply, exploitation of workers, and, ironically, food insecurity (Tscharntke et al. 2005, Weis 2010, Kremen and Miles 2012, Montenegro de Wit 2020).

How does the industrialization of agriculture impact honey bee health? In diversified farming systems, farmers rely primarily on wild insects and other animals to pollinate their crops. These pollinators nest in and around agricultural landscapes, and their pollination services support abundant food production (Garibaldi et al. 2013). In industrial agriculture, monocrop landscapes provide limited nesting habitat and forage resources (Dolezal et al. 2016), and pollinators are exposed to an abundance of agrochemicals (Garibaldi et al. 2011, González-Varo et al. 2013). As a result, as agriculture intensifies, the overall abundance and richness of wild pollinators in agricultural landscapes decreases (Kremen et al. 2002, Klein et al. 2007, Garibaldi et al. 2014), and commercial beekeepers bring in honey bees to meet crop pollination needs (Spivak et al. 2011, Bond et al. 2021).

Because they pollinate a wide variety of plants, and because their colonies contain tens of thousands of individuals, honey bees are a relatively effective pollinator to mobilize and massify (vanEngelsdorp and Meixner 2010). When industrial agriculture manufactures a demand for pollination services, industrial beekeeping meets that demand (Cilia 2020). Every year, commercial beekeepers transport more than two million colonies around the United States to pollinate crops like almonds, apples, blueberries, and melons (Goodrich 2019, Bond et al. 2021). Pollination contracts – in which beekeepers rent colonies to growers on a temporary basis to support crop yields – provide a vital source of income for many commercial beekeepers (USDA National Agricultural Statistics Service 2021). These contracts lend some measure of economic stability to an increasingly precarious industry (Goodrich 2019). But, while renting out colonies can be a lifeline for beekeepers, engaging with industrial agriculture is not good for bees (Decourtye et al. 2010, Maderson and Wynne-Jones 2016).

Industrial agriculture – and industrial beekeeping – expose honey bees to the multiple interacting stressors that lead to colony loss (Fig. 1.1) (Colwell et al. 2017, Alger et al. 2018). Monocrop landscapes can provide honey bees with a lot of forage all at once, but the resources they offer are often short-lived and lacking in diversity and nutritional

quality (Di Pasquale et al. 2016). As a result, the proliferation of monocrop landscapes contributes to poor nutrition in honey bees (Decourtye et al. 2010, Durant and Otto 2019). Agrochemicals do further damage. Herbicides kill the so-called weeds that would otherwise provide important forage resources, and can have both lethal and sublethal effects on the bees themselves (Bretagnolle and Gaba 2015, Requier et al. 2015, Abraham et al. 2018, Motta et al. 2018). Fungicides disrupt in-hive microbial communities and affect honey bee metabolism, immune response, and other physiological processes critical to colony function (Cizelj et al. 2016, Kakumanu et al. 2016, Mao et al. 2017, Steffan et al. 2017). Insecticides negatively impact the bees' ability to learn, communicate, and locate their homes, and adversely affect egg-laying and colony development (Goulson 2013, Wu-Smart and Spivak 2016, Mengoni Goñalons and Farina 2018).

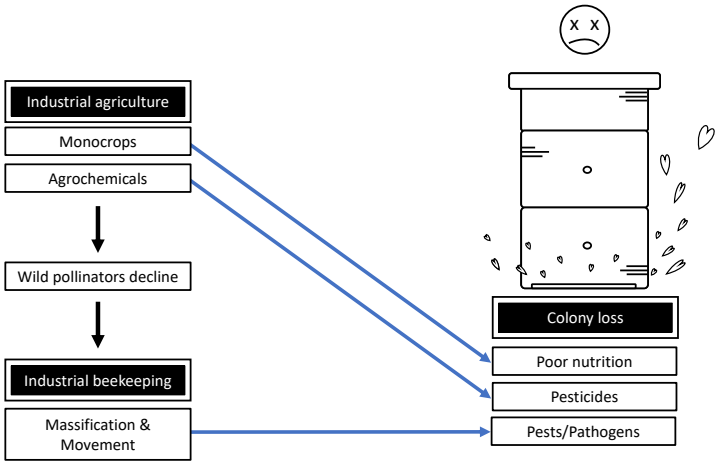


**Figure 1.1** The multiple interacting stressors that negatively impact honey bee health are rooted in and exacerbated by industrial agriculture.

Even parasites and pathogens – stressors that seem separate from industrial agriculture – are exacerbated by this system (Welch et al. 2009, Alger et al. 2018). High stocking density leads to heightened pathogen transmission, increased virulence, and

depressed immune response in a variety of industrialized livestock systems (Mennerat et al. 2010, Houshmand et al. 2012, Yarahmadi et al. 2016). Indeed, when honey bees are housed in crowded bee yards, high stocking density contributes to increased pathogen transmission potential, and creates conditions that favor increased virulence (Brosi et al. 2017, Dynes Id et al. 2019). Moreover, migratory practices, the cross-country sale of honey bee 'packages' and nucleus colonies, and the growing popularity of hobby beekeeping bring honey bees – and the pathogens they carry – to all corners of the country. Since pathogen transmission across long distances also contributes to increased virulence, these practices further compound pathogen problems (Brosi et al. 2017). Commercial beekeepers take great care to keep pathogen loads in check, but the conditions of industrial agriculture constantly up the ante. As a result, the spread of parasites and pathogens, on top of poor nutrition, on top of pesticides, makes keeping colonies alive a complicated endeavor.

To review, when honey bee researchers frame honey bee health issues, we often focus on the fact that deteriorating colony health has negative consequences for our agricultural system. But, when we consider the problem of industrial agriculture, we see that colony loss is actually the logical result of the way that we farm, and the way we push honey bees to produce in conditions that are not designed to support their survival (Spivak 2013). When we broaden our framing, we find that industrial agriculture is not the victim of unsustainable colony loss; it is the cause.

This is not actually new information. Sociologists, ecologists, geographers, agroecologists, journalists, and many beekeepers and farmers have provided critical analyses that describe this 'manifestly unsustainable system' (Nimmo 2015a, 2015b, Goulson and Nicholls 2016, Maderson and Wynne-Jones 2016, Suryanarayanan et al. 2018; Cilia 2019, 2020, Durant 2019a, Ellis et al. 2020, McGivney 2020). Many of these analyses explicitly connect honey bee health issues to industrial agriculture (e.g., the 'apis-industrial complex') and to the political, social, and economic structures that underlie this system. These resources are relevant to honey bee research because they help to describe the context in which honey bee health issues are situated. However, we honey bee researchers rarely cite our colleagues across

disciplines. We focus on specific aspects of honey bee health, and we skip the broader context.

Why does this matter? The way we frame a problem shapes the solutions that we implement (see Box 1). When we frame this problem as an issue with honey bee health, rather than an issue with the industrial agriculture system, we undercut our research efforts and lend further support to an unsustainable status quo.

## Failing to Name Industrial Agriculture Undercuts Our Research Efforts

Through years of focused research, honey bee scientists have developed a detailed understanding of many aspects of honey bee biology and colony health. This work often describes or addresses the negative impacts of industrial agriculture, but it seldom names this system explicitly[5] (Tables S1.1 and S1.2). This is a problem because when we attempt to address honey bee health issues without acknowledging industrial agriculture as the underlying driver of colony loss, we run the risk of focusing our energy on partial

[5] In an analysis of the top ten most cited honey bee health articles from the past decade (Web of Science: search terms 'honey bee' and 'health'; see Tables S1.1 and S1.2) for selection criteria and analysis), seven articles discussed the problem of colony loss and the implications this has for agricultural production in the introduction section without acknowledging the ways in which intensive or industrial agriculture contribute to colony loss. One article did not discuss colony loss or agricultural production at all, and instead focused on pesticide toxicity. The two articles that did acknowledge the negative impacts of industrial agriculture in the introduction section were written by authors based at institutions outside of the United States at time of publication.

Articles that were narrowly framed (i.e., articles that did not connect the causes of honey bee colony loss to the expansion of intensive or industrial agriculture) most often concluded by highlighting the need for further research (6/7 articles). Two of these articles also mentioned the importance of taking action to support honey bee health, but the actions they proposed focused on responding to stressors (i.e., improving honey bee management strategies) rather than addressing their root cause (i.e., transforming agroecosystems).

fixes that make it only marginally more possible for honey bees to survive an inhospitable system (Maderson and Wynne-Jones 2016).

Here is another way to put that. The ‘canary in the coalmine’ metaphor is commonly employed to warn of the catastrophic consequences of pollinator demise (Goulson and Nicholls 2016, Hall and Martins 2020, Paffhausen et al. 2021), where honey bees are often (mis-)used as a stand-in for all pollinators (Geldmann and González-Varo 2018). Essentially, the story goes that if honey bees collapse, our food systems will follow. We can extend this metaphor to illustrate the consequences of a framing that focuses on the stressors that cause honey bee disease, without questioning the system that creates those stressors. In this case, if the honey bee is the canary, a narrow framing leads us to focus on the health of the bird instead of its surroundings. We see the canary, we know it is unwell, but instead of evacuating the coalmine and bringing the bird up to the surface for the fresh air that it needs, we scientists are setting up a more permanent camp inside the mine, hooking the canary up to oxygen, running diagnostic tests, supplementing the canary’s diet to elevate its hemoglobin levels, and initiating a program to develop a canary that can survive on $CO_2$. Our efforts may allow the canary to live a little longer, but focusing solely on individual aspects of canary health actually keeps us from asking more fundamental questions: Why are we keeping canaries in coalmines in the first place? Why are we still building coal mines at all?

Attempting to support honey bee health without addressing the root causes of colony loss will not create the change we need. In order to address the larger issue, we must reframe our research. We must name industrial agriculture.

## Reframing our research

As scientists, we reframe our research all of the time. We do this to reach different audiences, tap into different funding sources, and contextualize our work to fit different publications. So, broadening our framing of honey bee health issues to name industrial agriculture as a root cause of colony loss should not be much of a stretch.

Here is one example of what that might look like (Fig. 1.2). When we introduce our research, we start by providing context, we then state the problem, and we talk about how our research will address that problem. Currently, when honey bee researchers talk about honey bee health, we start by stating that honey bees are essential pollinators in agricultural systems; their contribution to crop production is valued at so many billions of dollars. We then describe this problem: colony loss is occurring at unsustainable rates. These losses result from multiple interacting stressors, such as pathogens, pesticides, and poor nutrition. Finally, we talk about how our research will help honey bees or beekeepers manage or overcome one or several of the multiple interacting stressors.

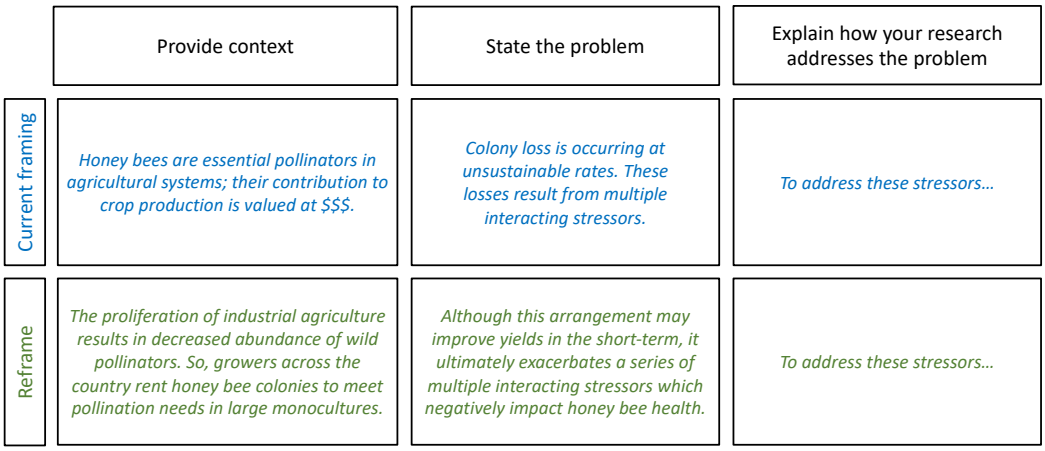


**Figure 1.2.** Reframing honey bee health issues to name industrial agriculture as a root cause of colony loss creates an opportunity for researchers to consider how the actions we take fit into a broader strategy of food systems transformation, and how we can use our research to forward that strategy in a meaningful way.

A hypothetical reframe could look like this: we start by stating that the proliferation of industrial agriculture results in decreased abundance of wild pollinators, so growers across the country rent honey bee hives to meet pollination needs in large monocultures. We then describe this problem: although this arrangement may improve yields in the short-term, it ultimately exacerbates a series of multiple interacting stressors

which negatively impact honey bee health. This is where I stop and notice that shifting my framing *does* change the way I think about the research I am doing. Now that I have named industrial agriculture as a primary driver of colony loss, I must also acknowledge that my specific research focus (resin use and immune function) is unlikely to make much of a difference in honey bee health outcomes, absent structural change. That does not mean my research is useless, but I will have to think more deeply about how my actions fit into a broader strategy to promote honey bee health, and how I can use my research to forward that strategy in a meaningful way.

Changing our framing is simple – I only added a few sentences there – but it is not easy. Why? Engaging with the root causes of colony loss exposes the need for bigger change (Ellis et al. 2020), and big change can be hard to face. This brings us to The Dangerous Questions.

## The Dangerous Questions

The Dangerous Questions invite us to reassess the role of beekeeping and honey bee research in agricultural systems. For example, if we acknowledge that industrial agriculture and industrial beekeeping are bad for honey bee health, and we know that our goal is to move towards a food system that supports bee health, then: what *is* the role of beekeeping in agriculture? If we transform agricultural landscapes in the United States so that they support wild pollinators, and those wild pollinators support crop production, then will beekeeping have a significant role? What if the answer is no, not really? Or, not in a way that could support the livelihoods of the approximately 25,000 apiary workers currently employed in the United States (USDA 2020)?

The dangerous questions do not just impact beekeepers; they affect honey bee researchers as well. In the long-term, if 'saving the honey bee' is less about drilling down on honey bee biology and behavior, and more about food system transformation, then what *is* the role of honey bee research? Does it have a significant role? What if the answer is no, not really? Or, not in its current form? And, in the short term, if honey bee researchers present a critique of the predominant agricultural system in the United States

– the system that currently supports so much of our research – then what happens to our funding?

These questions are dangerous because they represent an existential threat to all those that work within the existing system to support honey bee health. For many honey bee researchers, speaking openly about industrial agriculture may further seem off-limits because engaging with the dangerous questions poses a problem not just for beekeepers, not just for researchers, but for researcher–beekeeper relationships. Researchers may worry that reframing this problem – implicating industrial agriculture and industrial beekeeping in colony loss – will hurt commercial beekeepers. These are people who we work with and care about. Our research is often oriented towards supporting them, and in many ways their work gives our work meaning. If we speak openly about the negative impacts of industrial agriculture, will we alienate the people that work within that system?

To answer this question, I think we have to remember that industrial agriculture is a complex system, one in which all of us – researchers, beekeepers, and farmers alike – are embedded. Beekeepers are acutely aware of the myriad problems that this system poses, and work in their own ways to address them (Maderson and Wynne-Jones 2016, Durant 2019b, Cilia 2020). Describing the impacts of industrial agriculture is not about blame; it is about getting clear about how this system works, so that we can transform it, together. It makes sense to be thoughtful about the way we discuss these issues. It makes sense to acknowledge that, for many, beekeeping is a labor of love, and current conditions make it difficult for bees, beekeepers, and beekeeping businesses to thrive. I think we can do this, while also speaking openly about the root of the problems we collectively face. I believe that beekeepers, researchers, and beekeeper–researcher relationships are capable of holding that complexity. And, that researchers' concern for commercial beekeepers' experience, while valid, should not distract us from also doing the work of understanding the ways in which our own actions – the actions of the honey bee research community – uphold industrial agriculture.

## Holding Complexity

It is difficult for me to confront the broader systems that lead to such massive colony loss, in part because of the implications that a reframe might have for my life and work. The scope of my research is limited. Like so many scientists, I have specialized. I have focused on one tractable problem, hoping to make a small amount of positive change. I am not an expert in agricultural systems. What can a scientist studying honey bee immune health contribute in the face of such a massive and tangled problem? Three important things: First, I can do my best to direct my research to support honey bee health within our current system. Second, I can engage with interdisciplinary scholarship and diverse knowledge systems to better understand the context in which my work is situated. Third, I can directly describe the origin of the problems that my research attempts to address. The benefits of the first action will not have much impact unless we connect with the second, and actualize the third (Mortensen and Smith 2020). So, here is the call to action. Honey bee researchers: name industrial agriculture in the grants you apply for, in the articles you write, and in the actions you take to support honey bee health. When you talk about colony loss, when you list the multiple interacting stressors, explain where those stressors come from. Take a closer look at industrial agriculture, and name the problems it presents, so that, collectively, we can move towards transforming this system.

This may not seem like much, or it may seem like too much. But, when we consider the massive harms that industrial agriculture imposes on individuals, communities, and living systems, we find that telling the truth in honey bee research is both necessary and the barest of minimums. And, if turning towards The Dangerous Questions is uncomfortable, turning away from them represents its own existential threat. When we normalize industrial agriculture, we are not just pushing honey bees to survive a system that does not support their survival. It is much more than that. When honey bee researchers describe the conditions of industrial agriculture without calling into question the system that creates them, we lend legitimacy to the erroneous idea that industrial agriculture is an immutable system, when it is actually only one of many forms of food

production (Kloppenburg 1991, Rosset and Altieri 2018, Carlisle et al. 2019). When we fail to acknowledge the broader context contributing to colony loss, we protect that toxic system from actual transformation (Montenegro de Wit and Iles 2016). We are stuck making things work when we should be making them change, and the consequences of these actions extend far beyond honey bee health, to native bees, greenhouse gas emissions, viral spillover events, exploitation of workers, food insecurity, and beyond.

Fortunately, there are ways forward. Beekeepers, farmers, individuals, communities, and organizations in the United States and all over the world are working to envision, enact, and defend alternatives to industrial agriculture (Maderson and Wynne-Jones 2016, Mier y Terán Giménez Cacho et al. 2018), and to realize the social, political, and economic changes that must accompany their widespread implementation (e.g., 'Agrarian Trust' 2021, Calo et al. 2021). These efforts are supported by ample research which demonstrates that so-called 'alternative' farming systems (e.g., diversified farming systems, regenerative agriculture, agroecological systems, and Indigenous and traditional farming systems) support abundant food production (Tscharntke et al. 2012, Kremen and Merenlender 2018) and can help to repair many of the harms imposed by industrial agriculture (Petersen-Rockney et al. 2021). Efforts to enact these alternatives are inherently interdisciplinary. They connect food systems transformation to broader social and political movements for justice (e.g., see Indigenous land and seed sovereignty initiatives ('Indigenous Seed Keepers Network' 2020, 'Reparations' 2021) and efforts to eradicate racism from the food system (e.g., 'Soul Fire Farm' 2021)). When honey bee researchers recognize industrial agriculture as the root cause of honey bee health issues, we open ourselves to the opportunity to collaborate meaningfully in these movements, and contribute to the future that must be built. We add our voices to the growing chorus that knows, and insists, that industrial agriculture is not the only way. It is one way. It is a way that we made. It is a thing we can change. The question is whether we open up and allow that change to happen *through* us, or dig in our heels until that change happens *to* us.

# Chapter 2: Resin use and social immunity in honey bees and stingless bees[6]

**Abstract:** Honey bees (Apini) and stingless bees (Meliponini) use plant resins for a variety of purposes within their nest spaces. Resin use is particularly well-studied in *Apis mellifera*, which surrounds its nest with a continuous layer of resin-rich propolis. The resulting propolis envelope serves to waterproof the hive interior and supports honey bee social immunity by stabilizing immune system function and mitigating pathogen threats. *Apis florea* and *A. dorsata* are also known to collect resin in small amounts, but resin use by these and other *Apis* species is less well studied. Resin use by stingless bees is comparatively extensive, but is rarely the specific subject of investigation. Stingless bees use resin to build brood comb, honey pots, pollen pots, and a number of other nest structures, and to defend their nests from predators. Although resin use is integral to stingless bee colony function, it is unclear whether resin also serves to support social immunity in stingless bees. In this chapter, we review the ways in which resin contributes to nest structure, defense, and social immunity in Apini and Meliponini, and we discuss connections between bee health, agricultural systems, and social immunity research.

## Introduction

Social organization presents a variety of advantages for eusocial insects like honey bees and stingless bees. In social insect colonies, individuals work together to construct highly complex nest structures and mount effective defense strategies. However, the crowded nest environments where many social insects live also facilitate pathogen spread. Thus, social insects must manage immune responses at both the individual and social level. The term 'social immunity' refers to the physiological, behavioral, and organizational

[6] This chapter was co-authored by myself (Maggie Shanahan), Michael Simone-Finstrom, and Marla Spivak for publication in a forthcoming book on stingless bee propolis and cerumen. Dr. Simone-Finstrom took the lead on the *Apis* section, I took the lead on the Meliponini section and editing, and Dr. Spivak contributed to social immunity framing and editing.

strategies that social insects use to protect their colonies from parasites and pathogens (Cremer et al. 2018). The collection of antimicrobial resins is one example of a behavior that social insects use to support colony health (Simone-Finstrom and Spivak 2010). Plants produce resins to protect themselves from herbivores and disease (Langenheim 2003). Honey bees and stingless bees collect these resins and mix them with varying amounts of beeswax and other substances to form materials like propolis and cerumen (Shanahan and Spivak 2021). In this chapter, we review the ways in which these resin-rich materials contribute to nest structure, defense, and social immunity in Apini and Meliponini.

## Use of plant resins by Apini

Honey bees (bees in the genus *Apis*) collect resins to varying degrees, largely based on nesting strategies, genetic predisposition and environmental conditions. *Apis florea* and *A. dorsata* collect resin in small amounts, while *A. mellifera* can forage for and deposit profuse amounts of resin in the hive (Crane 1990; Seeley and Morse 1976). *A. cerana* is noted not to collect resin at all, even in areas where *A. mellifera* collects resin. Less is documented about resin use by other *Apis* species. Within *A. mellifera*, some races collect more resin than others. For example, it is often noted that *A. m. caucasica* colonies naturally collect substantial amounts of resin (Crane 1990; Kekeçoğlu et al. 2020). In some regions, beekeepers select for resin collection since propolis can represent a value-added product for the apicultural industry. For instance, breeding has further enhanced already demonstrable levels of resin collection and propolis use by *A. m. scutellata* colonies in Brazil (Manrique and Soares 2002; Nicodemo et al. 2013; 2014). The availability of resinous resources plays an additional role in the amount and diversity of resins that bees are able to forage (Drescher et al. 2014; Drescher et al. 2019; Abou-Shaara and Eid 2019; Orth et al. 2022). When resin-producing plants are scarce, bees occasionally turn to other sources of terpene-rich substances (e.g., asphalt or caulking) or rob resin from other colonies (Ribbands 1953; Simone-Finstrom and Spivak 2010).

A description of resin foraging and handling by *A. mellifera* was provided by Meyer (1956) with a more detailed analysis of in-hive behaviors provided by Nakamura and

Seeley (2006) (previously reviewed in Simone-Finstrom and Spivak 2010; Simone-Finstrom et al. 2010). In general, a very small proportion of the foraging force collects resin (Nakamura and Seeley 2006; Mountford-McAuley et al. 2021). Honey bees appear to demonstrate fidelity to resin foraging for at least one full day, and often several days, but can switch to pollen or nectar (Nakamura and Seeley 2006). Resin foragers pack resin on their corbiculae after removing it from its source with their mandibles. Upon returning to the hive, foragers rely on other bees to remove resin loads from their corbiculae (Meyer 1956), which is different from pollen foragers that remove their pollen loads themselves. It has been suggested that Western honey bees have enzymes that reduce adhesion of propolis to the mouthparts (Saccardi et al. 2022), and perhaps these fluids are mixed with resins to varying degrees in the hive (Dvykaliuk et al. 2022). In *Apis* spp., once resins are brought to the hive, manipulated by the bees, and often mixed with beeswax, they are termed propolis. Over the years, it has become increasingly clear that resin collection and its use as propolis by honey bees is a complex behavior whose multimodal use is affected by numerous intra-colony and environmental factors. Since it is potentially quite influential in a colony's health and productivity, we need to investigate the various drivers underlying resin collection and propolis deposition to reveal how and why honey bees incorporate plant-produced resins in the nest.

## *Nest construction*

The primary function of resin use by *Apis* workers has long been thought to be in nest construction, particularly for *A. mellifera* (Fig. 2.1). This is less so for open-nesting bees (e.g., *A. florea*, *A. dorsata*), whose nests consist of a single comb attached to a shrub, tree limb, or cliff wall (Crane 1990). For Western honey bees, propolis likely helps improve the nest site and promote a homeostatic hive environment (review by Simone-Finstrom and Spivak 2010). Feral colonies that nest in tree cavities use resin to fill in cracks and crevices (Seeley and Morse 1976), and both managed and feral colonies use propolis to limit the size of the nest entrance (Haydak 1953; Ghisalberti 1979). When nesting in hollow trees, Western honey bees line the entire nest interior with a thin layer of propolis, which has

been termed the “propolis envelope” since it encloses the occupied areas of the hive (Seeley and Morse 1976). The propolis envelope functions to fill in gaps and crevices (e.g., Seeley and Morse 1976; Hodges et al. 2019), waterproof the hive walls, and prevent the growth of microbes that could decay the wood surface or infect the bees, their food, and living space (Seeley and Morse 1976; Visscher 1980). Because of its waterproofing and insulating properties, it has been suggested that lining the entire nest cavity with propolis facilitates more efficient evaporative cooling, which helps regulate temperature and humidity (Clark 1918). Additionally, when bees use propolis to fill cracks and smooth walls, this creates a site for secure comb attachment. Thus, when colonies varnish parts of nest interior with propolis, this may also indicate that the area is ready for comb building. (Seeley and Morse 1976). One sign of the importance of propolis as an indicator of a higher quality nesting space is that propolis has been shown to be an attractant for honey bees searching for a new hive location.

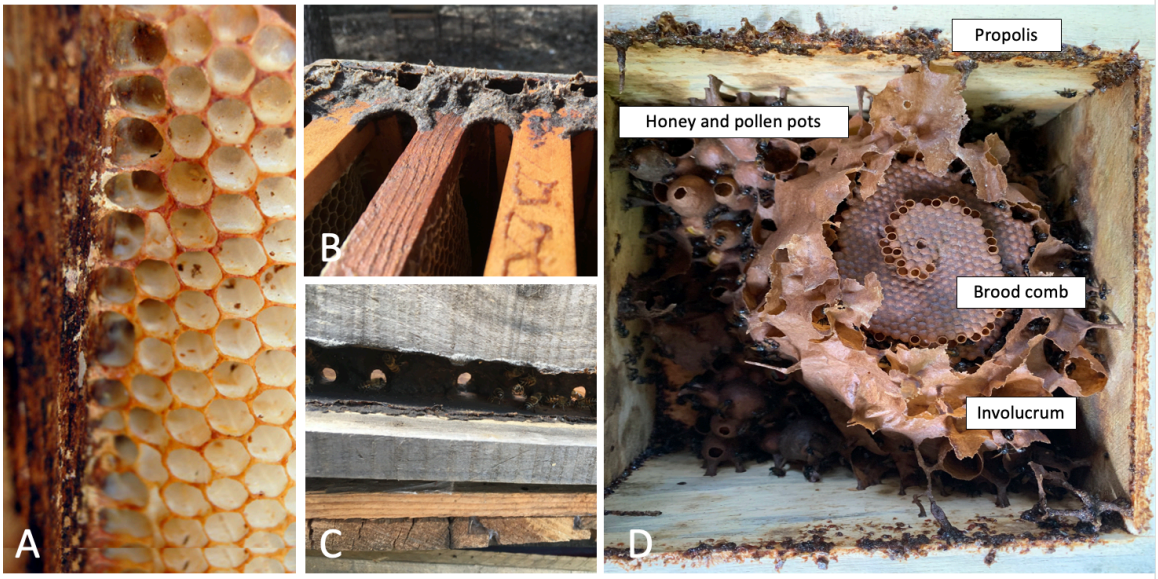


**Figure 2.1.** *Apis mellifera* incorporate resin into their nests in a variety of ways, occasionally lining wax combs with resin (A), and using propolis to seal cracks and crevices (B) or reduce nest entrances (C). (D) Stingless bees mix resin with beeswax to form cerumen and use this material to build nest structures such as brood comb, honey and pollen pots, and the involucrum. They also often seal their hives with propolis.

In addition to the role of propolis in hive structure, it has become clear that propolis, or at least some propolis compounds, are incorporated in wax combs (Pusceddu et al. 2021). However, this seems to be highly variable and more needs to be known about its function and impact. Propolis appears to mainly be used on the edges or rims of cells (Chauvin 1992; Strehle et al. 2003; Tautz 2008) and at the site of comb attachment (Marletto and Olivero 1981; Simone-Finstrom and Spivak 2010). One hypothesis is that propolis deposits are placed on the rims of cells as a potential inhibitory signal to stop building, since removing the propolis rim can cause the comb to be reconstructed (Chauvin 1992). Given the multiple functions of propolis in the nest interior, there are likely many roles that propolis plays with respect to its presence in comb that remain to be investigated fully (but see Pusceddu et al. 2021).

### *Defense*

One of the most consistent uses of resin in nest construction is as a mechanism for overall colony defense. Nest entrance restriction using propolis helps regulate microclimate within the nest, but it also prevents some intruders from invading the nest cavity. One study of Cyprian honey bees (*A. mellifera cypria*) noted that this population utilizes two different strategies to contend with attacks from the predatory hornet *Vespa orientalis* (Papachristoforou et al. 2011). Some *A. mellifera cypria* colonies maintain wide, open entrances lined with guards that rapidly attack invaders. Other "retreater" colonies create a propolis wall to restrict nest entrances, thereby preventing hornet access to the hive and facilitating colony defense (Papachristoforou et al. 2011). The differences in these defensive traits appear to have a genetic basis, and "retreater" colonies consistently rebuild their propolis walls if damaged (Papachristoforou et al. 2011).

Using resins as a barrier against predators is also a common feature in *A. florea* nests. *A. florea* colonies are fully exposed; their nests consist of a single comb attached to and hanging from a branch in the open. Ants are a common natural enemy for these bees, and *A. florea* colonies place a sticky resin ring around the branch leading to the nest to

prevent ant attack (Seeley et al. 1982, Crane 1990; Duangphakdee et al. 2005). This resinous barrier repels or traps ants, thus preventing them from invading the comb. A similar nest entrance defense has been noted in *A. cerana japonica*, which uses plant materials to mask pheromones deposited by *Vespa* predatory hornets (Fujiwara et al. 2016). When *A. cerana japonica* are exposed to *Vespa,* foragers collect and chew leaves and other plant parts, return to their colony, and spread the "odorous plant material" on the outside of the nest entrance (Fujiwara et al. 2016, 2018). While this plant material may not be pure resin, it likely contains some similar plant-derived compounds. Because *A. cerana* does not construct a propolis envelope within its nest, such an application outside the nest is the clearest case of resin or non-nutritive plant material use known for the species.

Western honey bees also utilize propolis to reduce the impact of other pests. *Apis mellifera* entombs—or mummifies—mice and other invaders that die or are killed within the nest with propolis (Hoyt 1965; Simone-Finstrom and Spivak 2010). Although the documentation of resin use by *A. dorsata* remains sparse, it has been noted that they, too, coat foreign objects with resin (cited in Seeley and Morse 1976). *Apis mellifera* also covers stored pollen with a layer of propolis when the pollen has been contaminated with certain pesticides, namely the fungicide chlorothalonil (vanEngelsdorp et al. 2009). Most notably, *A. mellifera* uses propolis as a means to restrict small hive beetles to the fringes of the nest, thereby prohibiting their reproduction. This behavior was first termed "social encapsulation" and it was thought that beetles were encased in propolis by the bees (Neumann et al. 2001; Ellis et al. 2003). However, Ellis and colleagues (2004) later suggested that the beetles are merely confined in propolis-laden crevices and then patrolled by guard bees, thus not actively corralled and imprisoned in propolis by the workers. Nonetheless, propolis can help to control small hive beetles and can greatly reduce their impact on colonies.

## *Social immunity*

Understanding the role of propolis in colony construction and nest defense has long been the primary focus of research on resin use by *Apis*. Propolis does itself mean "in front

of" (pro-) "the city" (-polis). However, over the last decade, research has elucidated a combination of direct and more subtle effects that propolis exerts on colony health. Since propolis is more fully incorporated into the hive structure of Western honey bees, much of this research has focused on *A. mellifera*. As a whole, these works have clarified that propolis use in honey bees is a mechanism of social immunity (Cremer et al. 2007) and is a behavioral form of disease and parasite resistance.

Propolis use likely evolved primarily to support nest construction, then further experienced positive selection as a type of "preventive care" strategy of social immunity that focuses on sanitizing the nest interior and promoting generalized resistance to parasites and pathogens (Cremer et al. 2018). The mechanistic role of propolis use with respect to colony health is multifaceted. *A. mellifera* forage for resins constitutively or prophylactically, and resin collection can also be induced by the presence of particular pathogens and parasites (Simone-Finstrom and Spivak 2012; Drescher et al. 2017; Pusceddu et al. 2019, review by Simone-Finstrom 2017; Spivak et al. 2019). In each case, however, the effect of propolis appears to primarily result in prevention or reduction of infection or infestation. This is best understood as a preventive care strategy. Various studies, previously reviewed by Simone-Finstrom and Spivak (2010) and Simone-Finstrom and Spivak (2017), document the role of propolis against brood pathogens infecting honey bee larvae, such as the bacterial agents causing American foulbrood (Lindenfelser 1968; Antunez et al. 2008; Bastos et al. 2008; Wilson et al. 2015; Borba et al. 2017) and European foulbrood (Murray et al. 2022; Simone-Finstrom et al. unpublished data), and the fungus *Ascosphaera apis* that causes chalkbrood (Simone-Finstrom et al. 2012; Wilson et al. 2015). Additional work has indicated potential effects of propolis against the parasitic mite *Varroa destructor* (Garedew et al. 2002; Popova et al. 2014; Drescher et al. 2017; Pusceddu et al. 2021) and common viruses that are transmitted from *Varroa* to bees and among bees themselves (Drescher et al. 2017). The effect of propolis on *Varroa* and viruses in field colonies appears to be context-dependent and inconsistent among studies (e.g., Borba et al. 2015; Drescher et al. 2017). Inhibitory effects of propolis on the microsporidian gut parasite *Nosema* (*Varimorpha*) spp.) have also been documented in experimental studies after feeding bees propolis, though propolis consumption is not typically thought to be a

natural route of exposure. Work on *Nosema* has been conducted with *A. mellifera* (Arismendi et al. 2018; Burnham et al. 2020; Mura et al. 2020; Naree et al. 2021), *A. florea* (Suwannapong et al. 2011), and *A. cerana* (Yemor et al. 2015). Given the clear impact of propolis against various pathogens, and because collection of resin is both prophylactic and inducible, foraging for these materials has been described as a type of social medication (Spivak et al. 2019).

Although the direct effects of propolis against pathogens and parasites are notable, the presence of propolis in the nest environment also impacts bees in less direct ways. The first study to investigate the potential function of propolis as a type of social (colony level) immunity determined that bees in propolis-enriched hives reduced individual bee investment in immune function; this study also provided evidence of altered microbiota (Simone et al. 2009). These findings were corroborated in subsequent studies (e.g., Borba et al. 2015). A reduction in immune function is hypothesized to be beneficial for two reasons. First, a constantly upregulated immune system is costly to maintain and can reduce colony fitness (Evans and Pettis 2005). Second, the modulation of immune gene expression can lead to enhanced immune function when bees are challenged (Simone-Finstrom and Spivak 2017; Borba 2017; Turcatto et al. 2018). In addition, when propolis-enriched colonies are exposed to the pathogenic bacteria that cause the brood disease American foulbrood, the presence of propolis leads to increased antimicrobial activity in the royal jelly fed to larvae (Borba et al. 2017). Further studies are needed to determine if propolis components leach into the brood food from volatiles or from the wax comb, or if a heightened or reallocated investment in immune molecules is produced by nurse bees in these scenarios.

Propolis-enriched nest environments appear to have a somewhat stabilizing effect on honey bee immune function at the colony level. In this way, propolis functions not only to establish a homeostatic nest environment but also to maintain social homeostasis with respect to bee physiology. Borba et al. (2015) found that the immune gene expression among colonies enriched with propolis is less variable over the season, relative to colonies maintained without a propolis envelope. This demonstrates that propolis in the nest space creates stability or consistency in the bees' base immunity investment. Recent data from a

study on a commercial beekeeping operation confirmed this finding (Simone-Finstrom, Shanahan, Spivak, unpublished data), and it is a phenomenon worthy of further exploration. The concept of propolis as a mechanism to maintain social homeostasis also extends to the influence of propolis exposure on microbial communities associated with honey bees. An investigation into the gut microbiota from bees in propolis-enriched environments determined that bees from propolis-rich colonies had more similar microbiota community structure, compared to those from propolis-poor colonies (Saelao et al. 2020). Interestingly, a similar result was found with respect to the microbiota community structure found in honey bee mouthparts (Dalenberg et al. 2020). Mechanistic questions regarding how the presence of propolis in the nest environment influences bee microbiota and how this may in turn also influence immune function warrant continued study.

Considering that *A. cerana* does not apply resin within the nest cavity and other *Apis* species may apply resin only to the comb attachment surface (i.e., it is not known if resin or resin compounds are incorporated into the wax combs), it is unclear if resin use is a form of social immunity in these bees. It is worth exploring the immune defenses and/or life history strategies of these species, especially in cavity nesting *A. cerana*, that are used to maintain colony health and homeostasis in lieu of resin collection.

## Use of plant resins by Meliponini

Like honey bees, different species of stingless bees (tribe Meliponini) use resin in different ways. But, while *Apis* spp. can survive without resin, its use is both obligate and extensive for many stingless bees. Notably, although resin is ubiquitous in stingless bee nest spaces and is central to both nest construction and defense, little is known about whether and how resin figures into stingless bee social immunity.

Resin foragers make up an estimated 1–3% of *A. mellifera*'s foraging force (Mountford-McAuley et al. 2021), but for some stingless bee colonies, resin foragers exceed even pollen foragers in number (Nascimento and Nascimento 2012; Leonhardt et al. 2009; Leonhardt et al. 2014). A variety of intrinsic and extrinsic factors impact resin

collection, including colony size, developmental stage, temperature, humidity, and resource availability (Biesmeijer and Slaa 2004). Stingless bees are known to increase resin collection in response to external threats, but the impact of pathogen pressure on resin collection has yet to be explored. Out on the landscape, resin foragers use visual and olfactory cues to identify resin sources (Leonhardt et al. 2010). They respond to and discriminate between highly specific combinations of volatile mono- and sesquiterpenes, leading researchers to speculate that stingless bees are even more selective toward resin sources than they are toward nectar sources (Leonhardt et al. 2014). Like *A. mellifera*, stingless bees use their mandibles to gather resin from plants and pack this material onto their corbiculae to carry it back to the nest (Gastauer et al. 2011). Some stingless bee species induce injury in plants to stimulate resin secretion; in some cases, this behavior is so common that certain species have been deemed agricultural pests (López-Guillón et al. 2019). Back at the nest, resin foragers remove resin from their corbiculae, sometimes with assistance. Like *A. mellifera*, they are thought to use a lubricating substance to facilitate resin removal (dos Santos et al. 2009). After removal, resin is utilized in a variety of nest structures.

### *Nest construction*

Like *A. mellifera*, many stingless bee species incorporate resin into brood comb and use it to build the propolis-rich envelopes that often surround their nests (Roubik 2006). However, while *A. mellifera* combs are made primarily of self-produced wax, most stingless bee nest structures are built using cerumen (Fig. 2.1) (Roubik 2023). Cerumen is a mixture of beeswax and plant resins. It is malleable and durable, and old cerumen is often recycled to form new structures within the nest. Although the relative proportions of beeswax and resin found in cerumen vary across species (Roubik 2006; Schwarz 1948), the fact that cerumen almost always contains resin means that this material permeates most parts of stingless bee nest spaces (except possibly *Schwarzula* spp. (Carmago and Pedro 2002) and *Austroplebeia australis* (Milborrow et al. 1987)). Honey and pollen are stored

in resin-rich pots, brood are immersed in resin-rich combs, and stingless bee adults are in constant contact with structures that contain a considerable amount of resin.

In addition to using resin to make cerumen, stingless bees incorporate resin into their nests as "deposit-resins" (resin caches containing viscous resin), propolis, and geopropolis (a mixture of resin, soil, silt, and/or sand particles) (reviewed by Shanahan and Spivak 2021). Propolis and geopropolis are used – sometimes in conjunction with other materials such as mud, seeds, wood, and feces – to build structures like the nest entrance and batumen. The batumen is somewhat analogous to the propolis envelope that *A. mellifera* constructs, in that it is a waterproof structure thought to help control fungal growth (Wille and Michener 1973). However, the batumen is most often a thick wall at the nest extremes: the upper and lower limits to the nesting area in a cylindrical tree cavity, and the area around the single bee-sized hole of the nest entrance. And, unlike the honey bee propolis envelope, the stingless bee batumen can take many forms. *Lining batumen*, a thin, continuous resin lining generally measuring less than two millimeters in thickness, is quite similar in form to *A. mellifera*'s propolis envelope. By contrast, *exposed batumen* (the outer layer that surrounds exposed or partially exposed nests), *batumen plates* (thick, resinous walls that surround stingless bee nests in hollow cavities, sometimes measuring up to ten centimeters thick) and *laminate batumen* (multiple layered resinous sheets) are larger, more substantial structures that often contain large quantities of resin (Wille and Michener 1973).

Although *A. mellifera* and most stingless bee species use resin in similar parts of the hive (e.g., the propolis envelope, brood comb rims, and nest entrances), there are differences both in the amount of resin these groups use, and in the centrality of resin to colony function. In contrast to *A. mellifera*, most stingless bee species could not even begin to build their nests without resin, let alone defend them.

## *Defense*

Resin is an important component of colony defense for many stingless bee species. Like *A. mellifera*, many stingless bees use resin to immobilize and engulf invaders

(Halcroft et al. 2011, Greco et al. 2010). However, many stingless bees take resin-based defenses several steps further. Some species bombard predators with sticky resin in a behavior known as resin-daubing (resin-daubing, when applied inside the nest, can result in mummification) (Halcroft et al. 2011). Similar to *A. florea*, other species use resin droplets to build sticky resin barriers (Fig. 2.2), which prevent would-be invaders from entering their nests (e.g., genera *Lepidotrigona, Scaura, Tetragona, Tetragonula,* and *Trigonisca*) (Roubik 2006). The resin-derived terpenoid compounds found within these structures serve to repel predators (Leonhardt and Blüthgen 2009). The predators that are not repelled often become trapped in their attempts to breach the sticky barrier.

Hardened resin and cerumen are also used to barricade nest entrances to ward off attack. *Melipona panamica, M. flavolineata*, and other *Melipona* species block their entrances using small resin balls (1-1.5 cm diameter) which they keep close to the nest entrance for colony defense (Roubik 2006; Nunes et al. 2014). When the colony detects a threat, the bees roll the hardened spheres into the narrow entrance tube, and use fresh resin to fasten it in place.

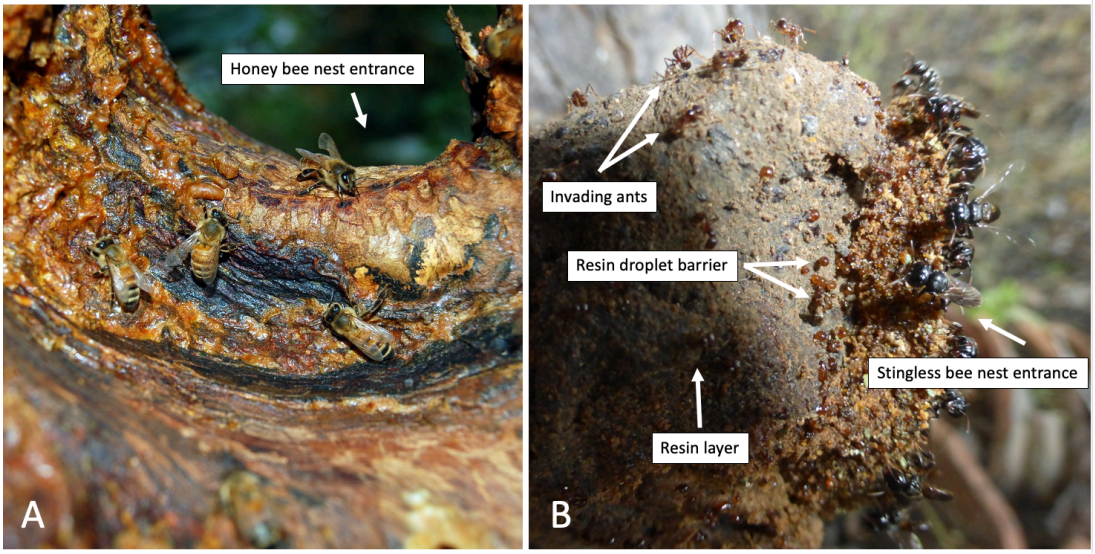


**Figure 2.2.** (A) Particularly when nesting in hollow tree cavities, *A. mellifera* often surrounds its nest entrance with a layer of resin. (B) Some stingless bee species use resin droplets to form a protective barrier that prevents ants and other predators from breaching the nest entrance.

Some stingless bee species carry visible amounts of resin on their bodies, a behavior thought to provide some form of protection. *Tetragonula carbonaria* coats its cuticle with resin, so that the whole body is sticky (Wenzel 2011). *Tetragonsica angustula* has also been observed with a thin layer of resin covering its legs, head, and thorax, and *Tetragonula melanocephala* nectar foragers are known to depart their nests carrying resin in their corbiculae (Jones et al. 2012; Leonhardt and Blüthgen 2007). *Melipona subnitida* workers routinely equip themselves with resin as they leave the hive, carrying both hardened and fresh resin in their corbiculae (Harano et al. 2020). Studies have shown that, when disturbed, the number of *M. subnitida* workers leaving the nest bearing resin increases from 11% to 90%, with the majority carrying hardened resin. Researchers speculate that these resin loads could provide individual protection, repelling predators that would otherwise eat the resin-bearers (Harano et al. 2020). Alternatively (or additionally), resin loads may serve as a collective defense; once a predator consumes a resin-bearing bee, the foul taste of the resin load might discourage further predation.

In a more subtle example of resin-wearing, many stingless bee species incorporate resin-derived compounds in their cuticular chemical profile (Leonhardt et al. 2011a; Leonhardt et al. 2011b). This behavior has been found to deter predators (Leonhardt et al. 2015), and to influence both nestmate and non-nestmate interactions. A series of behavioral assays showed that predator ants are more repelled by *T. carbonaria*, a bee whose cuticular compounds are 50% resin-derived, than by *Austroplebeia australis,* a bee whose cuticular compounds are just 1% resin-derived. Washing the bees to remove these compounds diminished the ants' preference, suggesting that the resin-derived compounds do contribute to stingless bee defense. Stingless bees are the only social insects known to enrich their cuticular chemical profile with resin-derived compounds (Leonhardt et al. 2015). To our knowledge, this has not been studied in *Apis*, though it is possible that *A. mellifera* also acquires resin-derived compounds from its nest environment, and this could contribute in some way to the bees' individual or collective defenses.

## *Social immunity*

While stingless bees use resin in a number of ways to defend their nest from predators, it is unclear how much resin contributes to stingless bee pathogen defense. Given the ubiquity of resin in stingless bee nests, and the role of resin in supporting social immunity in *A. mellifera*, it seems likely that resin fosters stingless bee social immunity either directly – by mitigating pathogen pressure – or indirectly – by shaping microbial communities or modulating stingless bee immune function. This has yet to be experimentally confirmed.

Testing the effect of resin on stingless bee social immunity is difficult for a couple of reasons. First, since resin is fundamental to the construction and defense of stingless bee nests, resin-free nest spaces do not exist. Therefore, it is not possible to compare stingless bee health outcomes in the presence and absence of resin, as has been done for *A. mellifera* (e.g., Simone et al. 2009). Manipulating the amount of resin in colony nest spaces or comparing health outcomes in naturally high-resin and low-resin colonies may provide some insight into the role of resin in supporting colony health. However, even in the best of cases, it is difficult to disentangle the specific effects of resin on colony health from its general importance to colony function. For example, is a high-resin colony thriving because resin supports social immunity, or because resin is an important resource for construction and defense, and therefore high-resin colonies possess a resource advantage?

Second, in contrast to *A. mellifera* – a highly managed species plagued by many pathogens – there are few documented examples of pathogens afflicting stingless bee colonies (except see Heard 2016; as cited by Leonhardt et al. 2017; Roubik 2023). Stingless bee propolis *has* been shown to inhibit the growth of numerous microbes, and its antiviral properties are well-documented in human medicine (reviewed by Bankova and Popova 2007; Zulhendri et al. 2021). Because of this, researchers have long speculated that resin may have some effect on the microbes (pathogenic, beneficial, and otherwise) present within stingless bee nests, as occurs in *A. mellifera* colonies (Roubik 1989; Dalenberg et al. 2020; Saeloa et al. 2020). Since the use of antimicrobial resins is integral to stingless bee nest construction, and since many stingless bee species inhabit tropical environments

where microbes abound, it would follow that resin helps shape the microbial communities found in stingless bee nests, but this has yet to be tested directly. Since few pathogens are known to affect stingless bees, the antimicrobial, antifungal, and antiviral activity of stingless bee resin is most often tested against human or *A. mellifera* pathogens, and the role of resin in protecting stingless bees from the pathogens that could affect them remains poorly understood.

In any analysis of resin use and social immunity, and especially when comparing honey bee and stingless bee colony health, it is important to keep in mind the disparate contexts in which these groups experience disease. In industrialized agricultural systems, and particularly in the United States, honey bee colonies are often managed in large numbers, at high densities, and transported long distances to provide pollination services in monocrop landscapes (Shanahan 2022). These conditions can be detrimental to honey bee health and may facilitate the emergence and spread of myriad parasites and pathogens (Brosi et al. 2017). At present, stingless bees remain comparatively healthy. Many stingless bee colonies are managed in low numbers and at low densities, and the practice of renting and transporting stingless bee colonies for pollination services is relatively uncommon (Cham et al. 2019; except see Khalifa et al. 2021). However, stingless bee management has intensified in recent years as has the illegal transport and trade of stingless bee colonies (Carvalho 2022; dos Santos et al. 2022; Quezada-Euan et al. 2022), and multiple pathogens commonly found in honey bees are now present in stingless bee populations (Guzman-Novoa et al. 2015; Alvarez et al. 2018; Macías-Macías 2020). If stingless bee management continues to intensify, and if stingless bee colonies are integrated as inputs in agricultural systems at a large scale, these bees may be exposed to the kind of pathogen pressure honey bees experience in the United States and in other industrialized agricultural systems.

In this evolving context, there is much to be gained by examining honey bee and stingless bee systems side by side, not just to determine the role of resin in supporting colony health in a diseased state, but also to help shape agricultural and ecological systems that support bee health in general. And, while the role of resin in supporting stingless bee social immunity remains unknown, its importance to stingless bee nest architecture, defense, and honey bee colony health is clear. As we pursue a deeper understanding of

resin use by stingless bees and honey bees, beekeepers and researchers should also work to prioritize the conservation of temperate and tropical landscapes rich in resin secreting plants (Drescher et al. 2014) to ensure honey bees and stingless bees have access to the resins that help their colonies survive and thrive.

# Chapter 3: Thinking inside the box: Restoring the propolis envelope facilitates honey bee social immunity[7]

**Abstract:** When wild honey bee colonies (*Apis mellifera*) nest in hollow tree cavities, they coat the rough cavity walls with a continuous layer of propolis, a substance comprised primarily of plant resins. Studies have shown that the resulting "propolis envelope" leads to both individual- and colony-level health benefits. Unfortunately, the smooth wooden boxes most commonly used in beekeeping do little to stimulate propolis collection. As a result, most managed bees live in hives that are propolis-poor. In this study, we assessed different surface texture treatments (rough wood boxes, boxes outfitted with propolis traps, and standard, smooth wood boxes) in terms of their ability to stimulate propolis collection, and we examined the effect of propolis on colony health, pathogen loads, immune gene expression, bacterial gene expression, survivorship, and honey production in both stationary and migratory beekeeping contexts. We found that rough wood boxes are the most effective box type for stimulating propolis deposition. Although the use of rough wood boxes did not improve colony survivorship overall, *Melissococcus plutonius* detections via gene expression were significantly lower in rough wood boxes, and viral loads for multiple viruses tended to decrease as propolis deposition increased. By the end of year one, honey bee populations in migratory rough box colonies were also significantly larger than those in migratory control colonies. The use of rough wood boxes did correspond with decreased honey production in year one migratory colonies but had no effect during year two. Finally, in both stationary and migratory operations, propolis deposition was correlated with a seasonal decrease and/or stabilization in the expression of multiple immune and bacterial genes, suggesting that propolis-rich environments contribute to hive homeostasis. These findings provide support for the practical implementation of rough box hives as a means to enhance propolis collection and colony health in multiple beekeeping contexts.

[7] This chapter was submitted for publication to *Plos One* on April 6, 2023 in collaboration with co-authors Michael Simone-Finstrom, Philip Tokarz, Frank Rinkevich, Quentin D. Read, and Marla Spivak.

**Key words:** honey bees, colony health, beekeeping, propolis, resin, social immunity

## Introduction

Although many beekeeping practices are designed to support colony health, some inadvertently constrain the natural defenses (or mechanisms of social immunity) that help honey bees (*Apis mellifera*) thrive in an unmanaged context (Brosi et al. 2017, Loftus et al. 2016, Seeley 2019). When external conditions are favorable (i.e., when colonies have access to abundant floral resources and are exposed to few external stressors), constraining these defenses may not significantly impact colony health. However, many honey bee colonies face conditions that are far from favorable (Goulson et al. 2015, vanEngelsdorp and Meixner, 2010). In the U.S. and around the world, industrial agriculture increases bees' exposure to agrochemicals (González-Varo et al. 2013) and pathogens (Brosi et al. 2017, Zhu et al. 2014), and limits access to diverse forage resources (Decourtye et al. 2010, Durant and Otto 2019), leading to high levels of colony loss (reviewed by Shanahan 2022). These stressors impact both large-scale, migratory beekeeping operations – where colonies providing pollination services participate directly in industrial agriculture – and stationary, small-scale apiaries, which may interface with industrial agriculture less directly (Bruckner et al. 2023). While restoring honey bees' natural defenses will not address the full spectrum of stressors that currently cause colony loss, recovering these health-supportive behaviors could represent one valuable step towards improved honey bee health (Neumann and Blacquière 2016). Propolis collection is one example of a natural defense that could be integrated by beekeepers working at a variety of scales to improve colony health.

Honey bees collect antimicrobial resins produced by plants (Bankova et al. 2018, Langenheim 2003), and mix this material with beeswax to make propolis, which serves multiple purposes inside the hive (Simone-Finstrom and Spivak, 2010). When wild honey bee colonies nest in hollow tree cavities, the cracks and crevices found inside the tree stimulate bees to lay down a continuous layer of propolis, called the "propolis envelope"

(Nakamura and Seeley 2006, Seeley and Morse 1976). However, the smooth wood boxes that most beekeepers use have few cracks and crevices and do little to stimulate propolis collection (Borba et al. 2015). Moreover, since propolis gums up beekeeping equipment, propolis collection has long been considered a sticky inconvenience, and over time beekeepers have selected against propolis collection traits, particularly in the U.S. (Simone-Finstrom and Spivak 2010). As a result, most managed bees live in hives that are propolis-poor. This is concerning because a growing body of evidence suggests that propolis is an important part of a colony's social immunity and could reduce the impact of some of the stressors that threaten honey bee health both within and beyond industrialized agricultural landscapes (Simone-Finstrom et al. 2017).

Propolis-rich environments have been shown to support honey bee colony health in a variety of ways (reviewed by Simone-Finstrom and Spivak 2010, Simone-Finstrom and Spivak 2017). In addition to modulating immune gene expression and improving colony strength and survivorship, propolis may help mitigate pathogen impacts (Borba et al. 2015, Drescher et al. 2017, Simone-Finstrom et al. 2017). One study demonstrated that honey bee colonies increased resin-foraging when infected with the fungal parasite *Ascosphaera apis*, and chalkbrood infection was reduced in hives painted with a propolis extract solution (Simone-Finstrom and Spivak 2012). In another study, when propolis extract was applied to larval rearing cells in amounts similar to those found in brood comb, the survival and reproduction of *Varroa* mites was decreased compared to propolis-free controls (Pusceddu et al. 2021). The colony-level implications of this effect are unclear. When propolis was added to one set of colonies and removed from another to create propolis-rich and propolis-poor hive environments, no significant differences in mite infestation were observed, though propolis did appear to interfere with the transmission of Deformed wing virus (DWV), which could have important implications for colony health (Drescher et al. 2017). Lastly, propolis may help mitigate *Nosema ceranae* infection (*Vairimorpha ceranae*; Tokarev et al. 2020), as bees fed with a propolis extract had significantly reduced *V. ceranae* spore loads (Arismendi et al. 2018, Mura et al. 2020, Naree et al. 2021). Though honey bees are not known to consume propolis directly, honey does contain numerous propolis-derived compounds, and these

may help protect bees against pathogens, toxins, and other important stressors (Mao et al. 2013; Berenbaum and Calla, 2021).

There is also evidence that propolis has a stabilizing effect on the honey bee microbiome. Multiple studies have shown that bees from propolis-rich environments (i.e., hives whose surface textures are modified to encourage propolis collection) tend to have more consistent (i.e., less diversity, lower abundance) microbial communities, and bees from propolis-poor environments tend to host a greater diversity of microbiota (Dalenberg et al. 2020, Saelao et al. 2020). The biological significance of this effect is unknown, but a study comparing honey bee mouthpart microbiomes in propolis-rich and propolis-poor conditions suggests that propolis promotes the growth of putatively beneficial microbes, and may mitigate the growth of opportunistic microbes that trigger the production of antimicrobial peptides and other honey bee immune defenses (Dalenberg et al. 2020). If dysbiosis negatively impacts honey bee health, as studies of the honey bee gut have suggested (Anderson and Ricigliano 2017), then the stabilization of microbial communities in propolis-rich environments could help explain why the presence of propolis supports bee resistance to external stressors.

Although abundant laboratory and colony-level evidence demonstrates that the propolis envelope supports honey bee health in a variety of ways, this natural tool for honey bee defense has yet to be integrated into commercial beekeeping operations. Borba et al. (2015) made important strides in this direction, demonstrating that placing commercially produced plastic propolis traps on the interior walls of bee boxes stimulates bees to build a natural propolis envelope, which leads to measurable improvements in colony health. Unfortunately, plastic propolis traps are bulky. When attached to the inner walls of a beehive, they take up space and make it difficult to maneuver frames. They can also be expensive to implement on a large scale (US$11.50/propolis trap (Mann Lake) x four traps/colony to cover the inner walls of just one brood chamber = US$46.00/colony), and this may represent a significant barrier for commercial beekeepers. In recent years, surface texture treatments like rough wood, saw kerfs, screen walls, and grooved aluminum plates have been tested by both bee researchers and beekeepers (France et al. 2019, Fares et al. 2008, Hodges et al. 2018). These textures do stimulate propolis

deposition in beehives (Hodges et al. 2018) and could represent a viable alternative to propolis traps. However, their impacts on colony health have not yet been tested, nor has the effect of increased propolis deposition been examined in a real-world commercial beekeeping setting.

Our study addressed two main questions: (1) How do rough wood boxes compare to boxes outfitted with propolis traps in terms of their ability to stimulate propolis collection? And (2) can rough wood boxes support colony health in both stationary and migratory commercial beekeeping contexts? To answer these questions, we compared propolis deposition and colony health in rough wood boxes, boxes outfitted with propolis traps (proven to support colony health by Borba et al. 2015), and smooth wood control boxes. We also collaborated with a large commercial beekeeping operation, which allowed us to evaluate propolis deposition and colony health in rough wood and control boxes in a migratory beekeeping context over multiple years. Lastly, we conducted landscape analyses to shed some light on potential differences in the diversity and abundance of resin resources in the areas surrounding stationary and migratory beekeeping yards.

## Materials and Methods

### *Colony set-up*

We evaluated propolis deposition and colony health across multiple hive types in stationary (2019-2020) and migratory (2019-2020, 2020-2021) beekeeping contexts. In our stationary yard, we compared three texture treatment types: 1) plastic propolis traps (Mann Lake Ltd, MN, USA, part no. HD370) stapled to the four interior walls of each standard Langstroth-size deep hive body, following Borba et al. (2015) (propolis trap boxes), 2) roughened wood boxes (Propolis Hive Company, MN, USA) specially constructed to provide bees with a highly texturized interior surface (rough boxes), and 3) standard, smooth hive boxes scraped clean prior to installation to remove all visible traces of propolis (control boxes) (Fig. 3.1). Rough boxes contained 0.3175 cm wide by 0.3175

cm deep grooves cut vertically and spaced every 0.635 cm on the interior surface of all four walls of the hive body. The inner surface of these grooved hive bodies was not planed or sanded and remained rough.

This proof-of-concept experiment allowed us to determine whether rough boxes were as effective as previously tested propolis trap boxes in stimulating propolis collection and supporting colony health. Additionally, we evaluated the rough box design in a migratory beekeeping operation, monitoring propolis deposition and colony health over the course of two years.

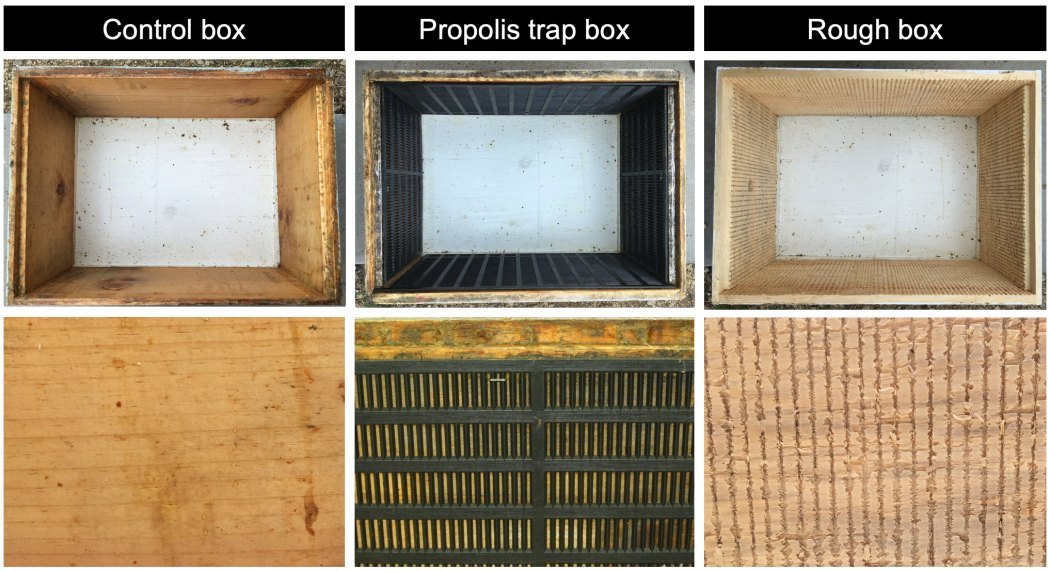


**Figure 3.1. Interior hive surface textures modified to stimulate propolis collection.** Three box types were evaluated: unmodified, smooth wood boxes ("control boxes"), smooth wood boxes outfitted with plastic propolis traps ("propolis trap boxes"), and boxes with rough, grooved interior walls ("rough boxes"). Control boxes consisted of previously used standard bee boxes scraped clean prior to installation to remove all visible traces of propolis. Propolis trap boxes contained propolis traps cut to hive body dimensions, stapled to all four interior hive walls, following Borba et al. (2015). Rough boxes (Propolis Hive Company, MN, USA) contained deep vertical grooves measuring 0.3175 cm wide by 0.3175 cm. Grooves were cut every 0.635 cm into the interior surface of all four walls of the deep hive body. The inner surface of these grooved hive bodies was not planed or sanded so it was rough, even slightly splintered.

**Stationary colonies**

The stationary component of this study was conducted at Carver Park Reserve, MN, USA (44.885776, -93.703419). Packages (Olivarez Honey Bees, Inc, California, USA) (n = 38) containing Saskatraz queens were introduced in April of 2019 in 10-frame Langstroth hive boxes featuring the three surface texture treatments described above (12 rough box hives, 12 propolis trap hives, and 14 control hives).

**Migratory colonies**

The migratory component of this experiment was conducted in collaboration with Adee Honey Farms. Queenless colony divisions were created in 10-frame deep Langstroth hive boxes with smooth hive walls with two frames of sealed brood, two empty combs, four combs of honey and pollen, and a plastic frame feeder in southern Mississippi in March 2019. Queens were grafted from breeder queens selected from within the operation. Queen cells were installed into queenless colony divisions one day before emergence. Newly emerged queens were allowed to open mate in an exclusive drone saturation area established by the beekeeper. Colonies were inspected in late April 2019 to ensure mating success as identified as colonies that had areas of sealed brood consistent with time from emergence and mating. Queens were paint-marked for later identification and assessment of queen replacement events. Colonies were inspected for amount of sealed brood and adult bee population (frames of bees). A total of 120 colonies were included in year one of this study (2019-2020); these were standardized according to amount of sealed brood and adult bee population. The control colonies (n=60) were housed in the existing hive bodies with smooth interior walls. Experimental colonies (n=60) were transferred to rough box hive bodies (rough boxes) as described above. Control and rough box colonies were housed in 10-frame deep Langstroth boxes in which the bottom two boxes contained eight frames of drawn comb with a 5 cm wide deep frame feeder. Colonies in the second year of this study (2020-2021) were set up and standardized following the

same practices, but housed in rough boxes derived from the first year of the study which already contained some amount of propolis.

## *Colony management*

### Stationary colonies

Colonies were given routine management on a bi-weekly basis during the growing season from spring 2019 to spring 2020. In April, new package bees were fed pollen substitute (Mann Lake Ltd, MN, USA, part no. FD374) and 50% w/v sugar syrup. As colonies grew, a second hive body with the corresponding surface texture treatment was added. In June and July, medium supers (no texture treatment) were added for honey storage as needed. Colonies were inspected regularly for disease and treated for *Varroa* mites in late August/early September (Formic Pro, two treatments). Colonies were fed 50% w/v sugar syrup in the fall and wrapped with Bee Cozy Winter Wraps (Mann Lake Ltd, MN, USA, part no. WT160) in October. Colonies that survived winter were noted in spring of 2020.

### Migratory colonies

All management of colonies followed the cooperating beekeeper's standard practices. Colonies were initiated in southern Mississippi in March 2019 and maintained until they were transported to South Dakota in early May 2019. In South Dakota, colonies were distributed among four different apiaries. Each apiary contained 15 control and 15 experimental colonies as well as 34 colonies unrelated to the study for a total of 64 colonies per apiary. Honey supers placed above the two-box brood chamber in both the control and rough box colonies were 10-frame Langstroth boxes with smooth walls. Boxes in both the control and experimental groups were added and removed at the discretion of the beekeeper throughout the season. Following the beekeeper's management strategy, colonies in the study were provided 50% w/v sugar syrup in the feeders and a 500g proprietary protein supplement patty upon arrival to South Dakota in

May 2019. Supplemental feeding of syrup and protein supplements were provided at the beekeeper's discretion throughout the season. Honey was harvested in mid-August 2019. Immediately after honey harvest, all colonies were condensed to two boxes, provided syrup and a supplemental protein patty, and treated for *Varroa* mites. Colonies were inspected immediately after honey harvest. Colonies were transported from South Dakota to holding yards in California in late October 2019 where they were provided syrup and supplemental protein patties at the beekeeper's discretion. Colonies were moved into almond orchards in early February 2020 and inspected in mid-February 2020. Colonies were returned to Mississippi in mid-March 2020 and inspected for the final time in late March 2020. Colonies were managed in the same manner in year two of this study, from 2020-2021.

### *Landscape composition*

To determine whether differences in propolis deposition between stationary and migratory colonies corresponded to differences in resource availability, we characterized the landscapes surrounding each of the apiary locations used during year one (one stationary yard, four migratory yards). Landscape data was pulled from the USDA-NASS Cropscape database's 2019 Cropland Data Layer (https://nassgeodata.gmu.edu/CropScape/). A circle with a 2.5-mile radius was drawn around each apiary (corresponding to honey bees' typical foraging range), and land use statistics were calculated within these defined areas of interest. Land use types were sorted into the following categories: grass and pasture, forest and shrubs, water, herbaceous and woody wetlands, developed, corn and soy, and other crops. Proportional land use was calculated by dividing each category's acreage by the total acreage within the 2.5-mile radius. Apiary locations are not disclosed in order to protect the privacy of the beekeeper who participated in this study.

## *Colony-level measurements*

### Stationary colonies

Colonies were assessed in August of 2019 and monitored for survival through the spring of 2020. In August, frames of bees were counted for both the top and bottom hive body. Queen status (i.e., whether the colony contained a living queen, and whether this queen was the same queen the colony had at the beginning of the experiment) was ascertained, and brood pattern was evaluated on a scale from 1 to 3 (1 = poor, 2 = fair, 3 = good). Brood frames were inspected for signs of brood disease (e.g., American foulbrood, European foulbrood and chalkbrood) and parasitic mite syndrome. Honey supers (i.e., the boxes located at the top of the hive where the bees store excess honey) were removed and weighed. Propolis deposition was measured using a visual scoring system (see above). Only strong, healthy colonies (30/38 colonies) with greater than twelve frames of brood and no signs of parasitic mite syndrome or brood disease were scored for propolis deposition and used for immune gene expression analysis.

### Migratory colonies

In year one, colonies were inspected in April of 2019 during colony establishment in southern Mississippi, in August of 2019 immediately after honey harvest in South Dakota, in February of 2020 during almond pollination in California, and in March of 2020 in southern Mississippi after almond pollination. Adult bee population (frames of bees) and amount of sealed brood were visually estimated using standard methods (Delaplane et al. 2013). The status of the queen bee was determined by the presence or absence of a paint-marked queen. *Varroa* infestation was measured by collecting approximately 300 bees from frames of sealed brood into a 1L ziptop bag and transporting them back to the USDA-Honey Bee Lab in Baton Rouge where *Varroa* were dislodged by shaking the bees in soapy water on an oscillating table shaker for >30 minutes. The number of *Varroa* and honey bees in the sample were counted and *Varroa*

infestation was calculated as the number of *Varroa* mites per 100 bees. Honey production (lbs/colony) was measured by weighing each honey super containing and subtracting the weight of an empty honey super. Propolis deposition was measured using a visual scoring system (see above). Colony survivorship was measured in February of 2020, when migratory colonies were transported to California for almond pollination. Survivorship was calculated as the number of colonies remaining in the study in February relative to the starting number of colonies (n=120 overall, n=60 in each of the two treatment groups). Measuring survivorship in migratory operations can be complicated, since beekeepers regularly combine or otherwise alter weak colonies. Thus, the discontinuity of a colony could signal either a colony death or a management intervention. Brood was inspected qualitatively and disease and brood issues were noted. Signs of European foulbrood (EFB) infections were noted in March of 2020 and EFB infection scores were calculated per brood frame following established protocols (0 = no cells, 1 = less than 10 cells, 2 = 11-100 cells, 3 = more than 100 cells).

Travel and work restrictions due to the COVID-19 pandemic limited the scope of work performed in year two of the migratory study. Colony establishment was performed in March and April 2020 as described above. The initial inspection in Mississippi in April 2020 only included data on frames of bees and frames of brood. Honey production was measured in South Dakota in September 2020. Queen status, frames of brood, frames of bees, brood pattern (ranked 1-5 with 5 being a solid brood pattern and 1 being poor), were measured during almond pollination in California in February of 2021. Propolis deposition score was measured for surviving colonies that were transported back to Mississippi in March of 2021.

### *Propolis deposition scoring*

In year one, for both stationary and migratory colonies, propolis deposition was assessed within one week of collecting bee samples for gene expression analysis. Frames were removed from hive bodies, and all four walls of the second deep were photographed

(Fig. 3.2). For propolis trap colonies, traps were removed from the hive walls, and photographs were taken both of the wall and of the detached trap, in order to account for all propolis deposited. Propolis deposition was evaluated following Hodges et al. (2018). Four observers scored each photo on a scale from one to ten, where one is 0-10% wall coverage and ten is 90-100% wall coverage (Fig. S3.1), and an average score was obtained for each box (Fig. S3.2).

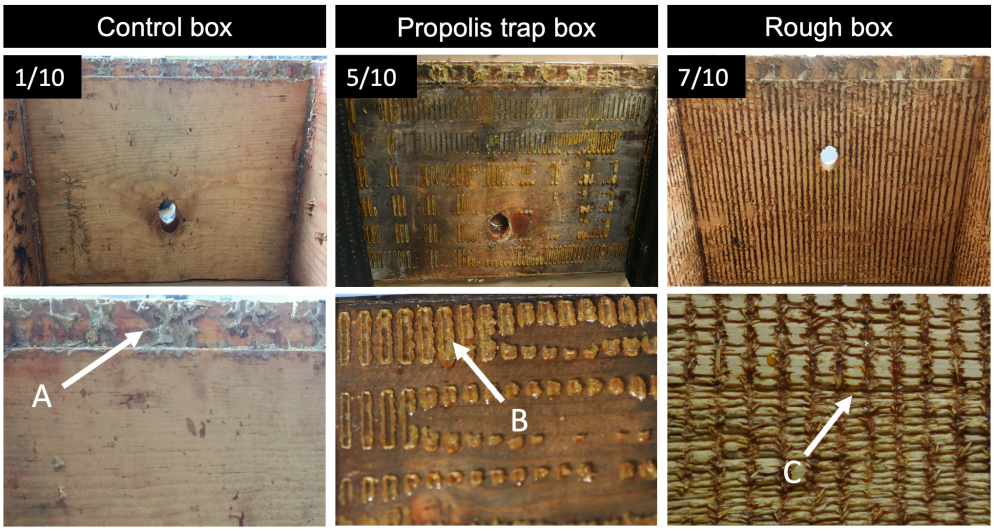


**Figure 3.2. Propolis deposition scoring.** The interior walls of control, propolis trap, and rough box colonies were photographed, and propolis deposition was scored on a scale from 1-10, where one is 0-10% wall coverage and ten is 90-100% coverage. In control colonies, propolis was primarily deposited on frame rests (A), the ledges that support the frames that bees build combs on. In propolis trap colonies, propolis was deposited in the small, rectangular holes in the propolis traps; these rectangular deposits (B) generally remained fixed to the hive walls even after propolis traps were removed. In rough box colonies, propolis was deposited in the cracks and crevices that covered the hive walls (C).

### Stationary colonies

Propolis deposition was scored in August of 2019, after colonies had been established for four months.

**Migratory colonies**

Propolis deposition was scored at three time points. Year one colonies were scored in August of 2019 and February of 2020. A subset of year two colonies (which were established in the same control and rough boxes used for year one bees) were scored in March of 2021 after they returned to Mississippi from almond pollination. COVID-19 restrictions during 2020-2021 made it impossible to monitor propolis deposition on a more regular basis in migratory colonies.

## *Sample collection for gene expression analysis*

**Stationary colonies**

Newly emerged bees (aged approximately one day) were paint-marked on the thorax using enamel paint and recovered from the colony six days later. Twenty 7-day-old bees were collected per colony, stored in Falcon tubes on dry ice, and then transferred to a -80C freezer until processing. Seven-day-old bees were used because immune expression is less variable in young bees; variation in immune gene expression increases when bees leave the hive to forage (Amdam et al. 2005, Simone et al. 2009).

*Migratory colonies.* At each sampling interval described above, a sample of approximately 300 bees from frames of sealed brood were collected into a 1L ziptop bag and placed immediately on dry ice in a cooler while in the field. Samples were transported on dry ice back to the USDA Honey Bee lab in Baton Rouge LA where they were stored at -80C until molecular analyses could be conducted.

## *Real-time PCR methods*

### Stationary colonies

RNA was extracted from individual whole bee samples (20 bees/colony) at the University of Minnesota Bee Research Facility. Bees were homogenized in microcentrifuge tubes using a pestle. RNA was extracted using the reagent TRIzol (Ambion, Austin, TX, USA), following the protocol recommended by the manufacturer (Evans et al. 2013). A NanoDrop2000 (Thermo Scientific Inc., Grand Island, NY, USA) was used to determine the quality and quantity of the RNA extracted. DEPC treated water was added to samples to normalize RNA concentration at 100ng/µl. Samples were stored at -80C and shipped to USDA facility in Baton Rouge, Louisiana for cDNA synthesis and qPCR to quantify the expression of immune genes *abaecin, AmEater,AmPPO, defensin-1, hymenoptaecin* and *relish*, as well as reference genes *pros54* and *ß-actin* (Table S1). cDNA synthesis was completed using QuantiTect Reverse Transcription Kits (Qiagen) with 2 µg of RNA, following the manufacturer's protocol. qPCR was performed on 1-µl aliquots of each sample, in triplicate, in a total reaction volume of 10 µl, utilizing SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on Multiplate 96-well optical PCR plates (Bio-Rad). All analyses were run on CFX Connect Real-Time PCR Detection Systems (Bio-Rad), using previously optimized thermal protocols (Table S1).

### Migratory colonies

Pools of 30 whole bees, placed into 30 mL tubes (19-6358Z, Omni), were homogenized using a Bead Ruptor Elite (Omni). RNA was extracted using the Maxwell RSC SimplyRNA extraction kit (Promega) following the manufacturer's protocol. RNA quality and quantity was assessed using a NanoDrop One. cDNA synthesis and qPCR were conducted as described above. qPCR was used to quantify the expression of immune genes *defensin-1*, *abaecin*, *hymenoptaecin*, *AmPPO*, and *AmEater*; bacteria *Bartonella apis*, *Bifidobacterium asteroides*, *Lactobacillus* Firm-4 phylotype,

*Lactobacillus* Firm-5 phylotype, *Snodgrassella alvi*, and *UniBact*, a primer coding for a universal bacterial gene sequence; viruses Acute bee paralysis virus (ABPV), Black queen cell virus (BQCV), Chronic bee paralysis virus (CBPV), Deformed wing virus A (DWV-A), Deformed wing virus B (DWV-B), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), Lake Sinai virus 1 (LSV-1), and Lake Sinai virus 2 (LSV-2); genes associated with European foulbrood; as well as reference genes p*ros54* and *ß-actin* (Table S1).

## *Statistical analysis*

All statistical analyses were performed using R Statistical Software (v4.2.1; R Core Team 2022).

### Landscape analysis

We calculated percent cover of herbaceous and woody wetlands and forest and shrubs in the areas surrounding the stationary and migratory yards. Unlike water, grass and pasture, corn and soy, and other crops, these landscape types are likely to contain resin-producing plants (Orth et al. 2022, Ribeiro Pereira et al. 2009). Because the presence of resin resources in developed land varies depending on the type of development, this landscape type was excluded from analysis. We used a simple linear model to determine the correlation between the presence of landscapes likely rich in resin resources (percent cover) and propolis deposition score.

### Colony-level measures

We assessed the effect of box type on multiple colony-level response variables, including propolis deposition, number of frames of bees, total bee population (i.e., number of frames of bees + number of frames of brood), honey production, *Varroa* load, brood

disease, and survival after one year. For stationary colonies, we used ANOVA to determine the effect of box type (i.e., rough, propolis trap, control) on propolis deposition, frames of bees, honey production, and survivorship. For migratory colonies, some data were collected at multiple time points and/or across multiple yards (i.e., propolis collection, frames of bees, honey production). Thus, where possible, we generated mixed-effects models where fixed effects included box type, sample date, and the interaction between sample date and box type. Random effects included colony (when multiple data points were available for each colony) nested within yard. For both stationary and migratory colonies, when more than two treatments, yards, or time points were compared, we used post-hoc two-tailed t-tests with a Bonferroni adjustment to determine differences between groups.

**Gene expression**

Ct values were determined using the Bio-Rad CFX Maestro™. $\Delta$Ct was calculated for each target gene by subtracting the average Ct for reference genes *Pros54* and *ß-actin* from the target gene Ct. Samples with Ct values greater than 30 or less than 23.5 were excluded from analysis.

$$\Delta Ct = target\ gene\ Ct - \overline{x}\ (reference\ genes)\ Ct$$

Gene expression was calculated using the transformation $2^{-\Delta Ct}$ following Schmittgen and Livak (2008). Since this transformation resulted in a non-normal distribution of linear model residuals, data were log-transformed ($log(2^{-\Delta Ct})$) for all statistical analyses.

We fit Bayesian linear mixed-effects models to describe the relationship between propolis score and both the mean and standard deviation of gene expression for all colonies. In these distributional models, both the mean and standard deviation of gene expression were allowed to vary with propolis score. We fit random intercepts to each colony and to each date nested within yard. To make inferences about whether

differences in propolis score were associated with differences in mean gene expression, we examined the posterior distribution of the slope parameter of the mean. Similarly, to make inference about whether differences in propolis score were associated with differences in the variability of gene expression, we examined the posterior distribution of the slope parameter of the standard deviation. A negative slope parameter for the standard deviation indicates that as propolis score increases, variability in gene expression decreases; this can be interpreted as a stabilizing effect of propolis on gene expression. To obtain point estimates of these parameters we used the median of the posterior distributions, and to assess uncertainty in our estimates we computed 66%, 90%, and 95% quantile credible intervals of the posteriors.

This analysis was done using Stan software version 2.30 (Stan Development Team 2022) and the R packages cmdstanr (Gabry and Češnovar 2021), brms (Bürkner 2018), and bayestestR (Makowski et al. 2019).

## Results

### *Landscape analysis*

Landscapes surrounding year one migratory yards were dominated by grass and pasture (percent land use > 50%) (Fig. 3.3). Crops covered 19-38% of these landscapes, with corn and soy plantings representing 42-71% of total crop cover. The landscape surrounding the stationary yard had a greater presence of forest and shrubs (25%), herbaceous and woody wetlands (18%), water (18%) and developed land (13%), with crops representing only 12% of the total landscape. Our simple linear model indicated that propolis score was positively correlated ($r(3) = 0.94$, $p = 0.02$) with percent cover of herbaceous and woody wetlands and forest and shrubs, landscapes likely rich in resin resources.

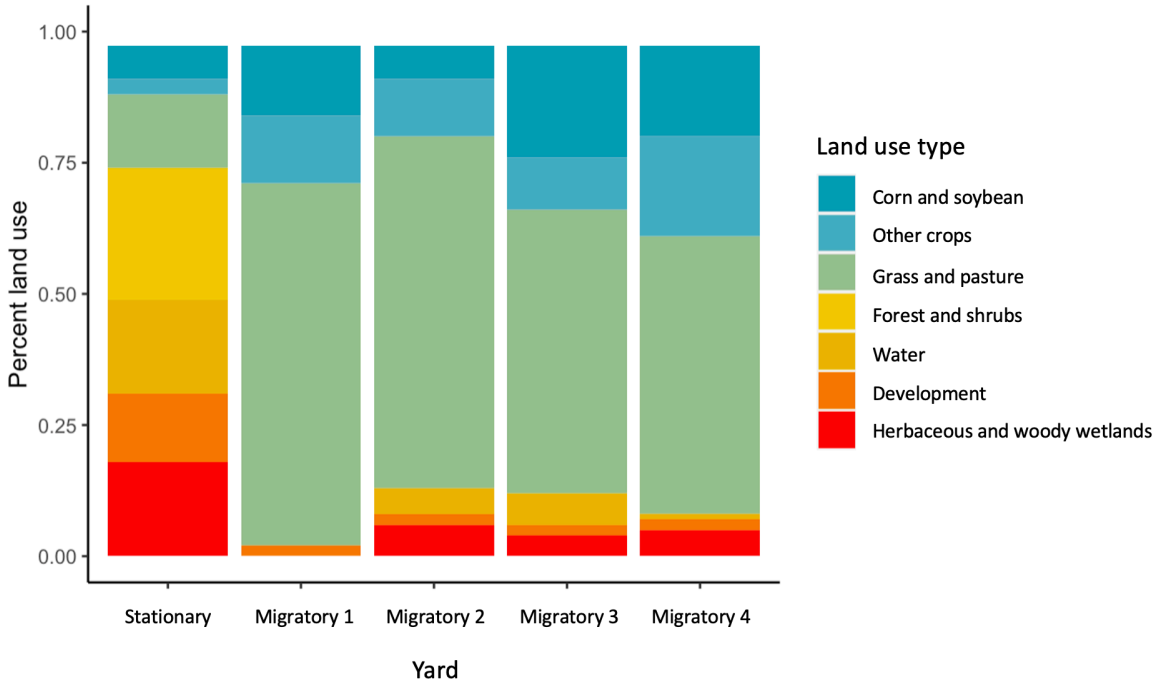


**Figure 3.3. Land use in landscapes surrounding stationary and migratory bee yards**. Land use for areas surrounding bee yards (radius = 2.5 km) was analyzed using the USDA-NASS Cropscape database's 2019 Cropland Data Layer. Landscapes surrounding migratory yards were dominated by grass and pasture. Landscape surrounding the stationary yard contained higher percentages of forest and shrubs, herbaceous and woody wetlands, water, and development. Propolis score was positively correlated ($r(3) = 0.94$, $p = 0.02$) with percent cover of herbaceous and woody wetlands and forest and shrubs, landscapes likely rich in resin resources.

## *Propolis deposition*

Bees deposited more propolis in rough boxes than in other box types (Fig. 3.4). In stationary colonies, just four months into colony development, propolis score averaged 7.5 (SE = 0.2) in rough boxes. This score was significantly higher than the 4.9 (SE = 0.2) average in propolis trap boxes, ($t(27) = 10.1$, $p < 0.001$) and the 1.7 (SE = 0. 1) average in control boxes ($t(27) = 21.9$, $p < 0.001$).

In migratory colonies, our mixed-effects model indicated that propolis deposition was significantly affected by both box type ($p < 0.0001$) and the interaction between box type and sample date ($p < 0.0001$). Bees deposited more propolis in rough boxes

compared to controls for all dates, and this difference grew more pronounced over time. In August of 2019, propolis score averaged 3.2 (SE = 0.2) in rough box colonies, which was significantly higher than the 2.0 (SE = 0.2) average in control colonies ($t(149) = 5.5$; $p < 0.0001$). By February of 2021, propolis scores had more than doubled to an average of 7.2 (SE = 0.4) in rough box colonies ($t(138.8) = 11.7$; $p < 0.0001$) but remained stagnant at 2.2 (SE = 0.3) in control colonies.

Propolis deposition was higher in rough box stationary colonies than in rough box migratory colonies in August of 2019 when all colonies were evaluated ($F(1,102) = 72.5$, $p < 0.0001$). Migratory rough box colonies took over a year to achieve the levels of propolis deposition that stationary colonies achieved in just four months.

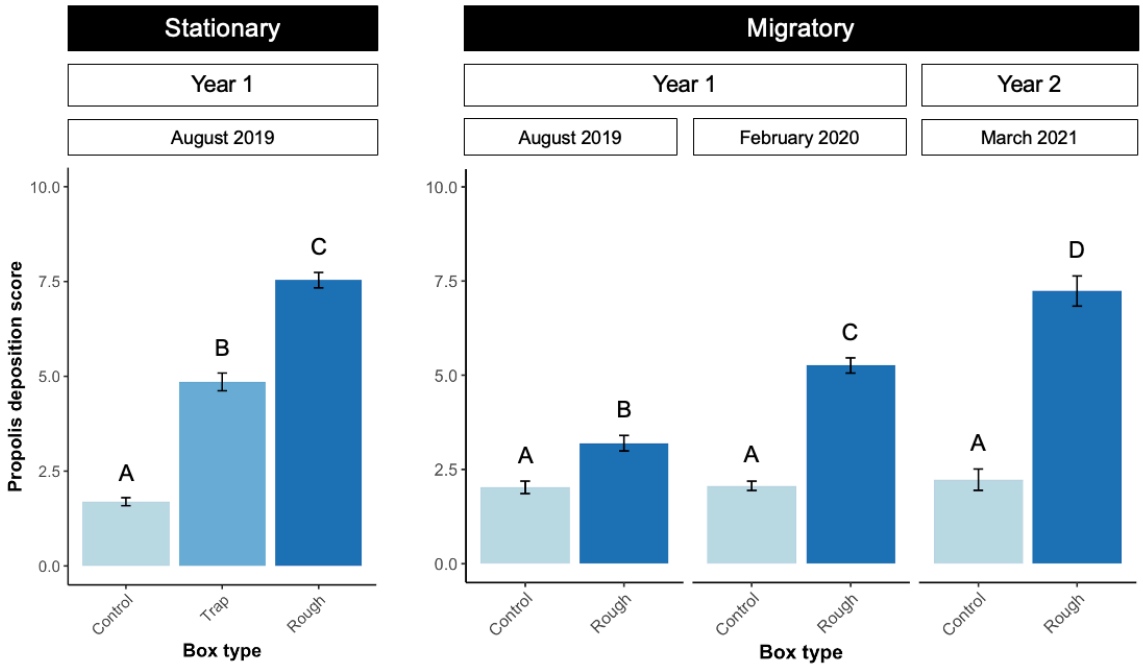


**Figure 3.4. Propolis deposition across box types in stationary and migratory contexts.** Propolis deposition on each interior brood chamber wall was scored on a scale from 1-10 where one is 0-10% wall coverage and 10 is 90-100% wall coverage. Scores were averaged to calculate each colony's "propolis score." Stationary colonies (n = 30) were evaluated in August of 2019, after four months of propolis deposition. Migratory colonies were evaluated in August of 2019 (n = 106), February of 2020 (n = 75), and March of 2021 (n = 27). Propolis score was higher in rough box colonies than in trap colonies and control

colonies. Propolis score increased over time in rough box migratory colonies. Mean propolis score ± standard error is shown for each treatment. Letters indicate significant differences between treatments, and, in the case of migratory colonies, differences between years ($p < 0.05$).

## *Colony size*

Frames of bees were counted for all colonies at all sampling dates; frames of brood were counted in migratory colonies at multiple time points during both years of the experiment. Where possible, we combined frames of brood and frames of bees to calculate "total bee population."

There were no significant differences in the number of frames of bees across treatment for the stationary colonies (Fig. 3.5). In the migratory operation, there were no differences across treatment in total bee population in August of 2019, but by the end of year one (February of 2020), the total bee population in rough box colonies was significantly larger than in control colonies, by a margin of nearly two frames of bees plus brood ($F(1,74) = 4.4$, $p = 0.04$). There were no statistically significant differences in total bee population across treatment in year two, though by the end of year two (February 2021), we observed a non-significant increase in total bee population in rough box colonies.

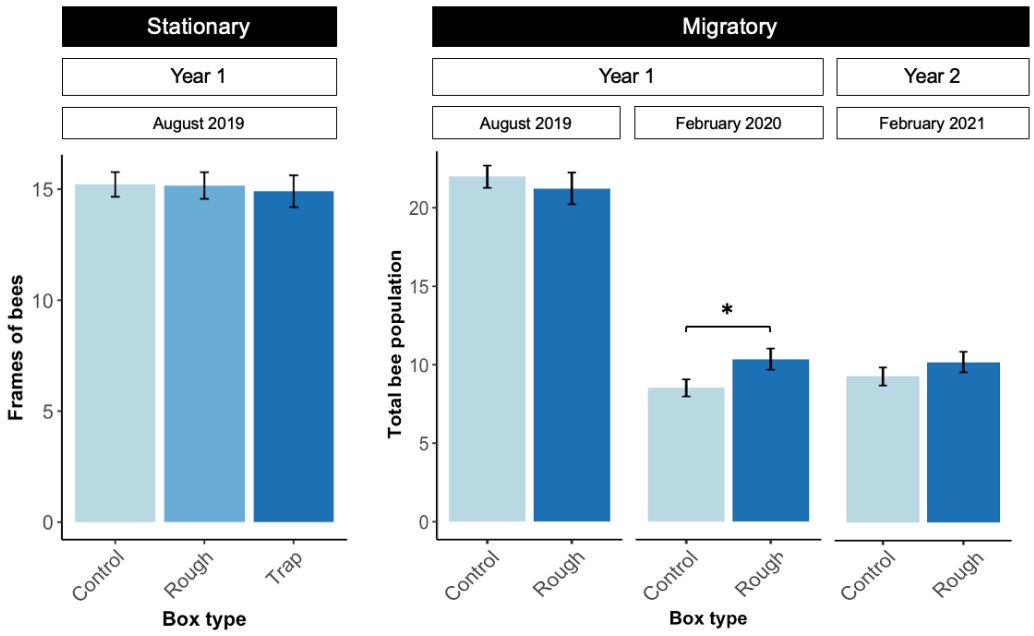


**Figure 3.5. Frames of bees and total bee population across box type in stationary and migratory contexts.** Frames of bees were quantified by counting the number of frames covered in bees in the first and second brood chambers. Total bee population was calculated by adding the number of frames of bees and the number of frames with brood present in the first and second brood chamber. Stationary colonies (n = 30) were evaluated in August of 2019. Migratory colonies were evaluated in August of 2019 (n = 110), February of 2020 (n = 76), and February of 2021 (n = 55). There was no difference between treatments in the number of frames of bees (stationary) or total bee population (migratory) in August of 2019 or February of 2021. Total bee population was significantly higher in rough box colonies (mean number of frames = 10.4, SE = 0.7) than in control colonies (mean number of frames = 8.5, SE = 0.5) at the end of year one ($F(1,74) = 4.4$, $p = 0.04$). Mean number of frames of bees/total bee population ± standard error are shown for each treatment. Frames of brood are added to frames of bees for migratory colonies. Asterisks indicate significant differences between treatments ($p < 0.05$).

## *Immune gene expression*

Propolis deposition had a seasonal effect on both the amount and variability of immune gene expression for multiple immune genes in stationary and migratory contexts (Fig. 3.6; Table S3.2).

For the August 2019 sample date in both stationary (n = 30) and migratory (n = 102) colonies, immune gene expression tended to decrease with increasing propolis score. In stationary colonies, our distribution model provided strong evidence for a negative correlation between propolis score and *defensin-1* expression. This model also provided some evidence that *relish*, *hymenoptaecin*, and *AmEater* expression decreased with increasing propolis score. However, propolis score was positively correlated with *AmPPO* expression.

In migratory colonies, our distribution model provided moderate evidence that *defensin-1* expression tended to decrease with increasing propolis score, and some evidence that *abaecin* expression tended to decrease with increasing propolis score.

Our distribution model also provided some evidence that immune gene expression stabilized as propolis score increased. Variation in *hymenoptaecin*, *AmEater*, and *abaecin* expression tended to decrease with increasing propolis score in stationary colonies, as did variation in *abaecin* expression in migratory colonies. In contrast, in migratory colonies, our distribution model suggested that *AmEater* expression tended to destabilize with increasing propolis.

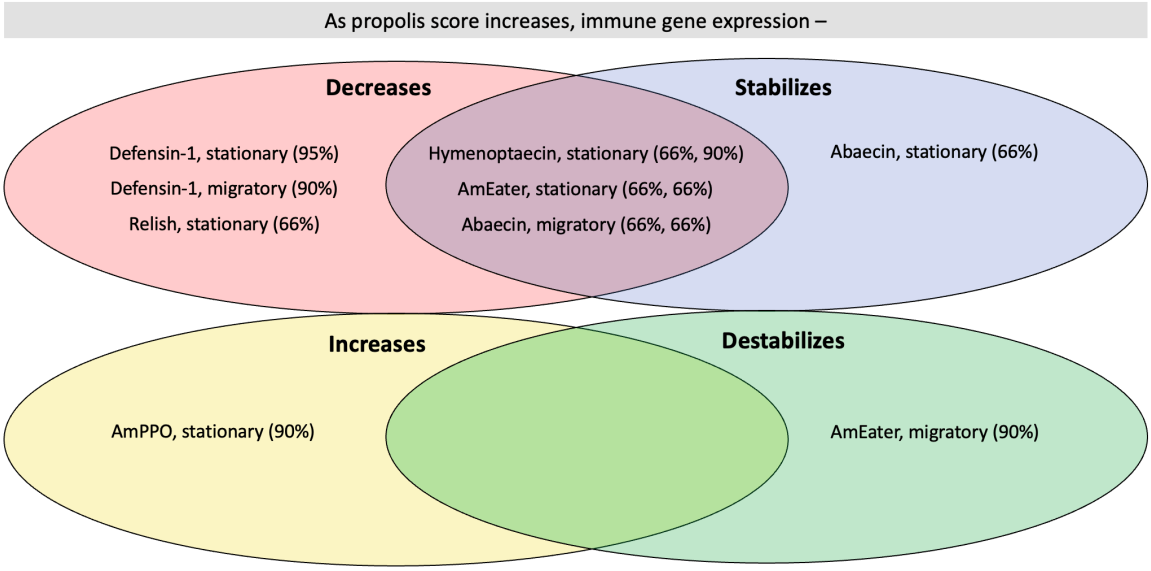

**Figure 3.6. Trends in immune gene expression with increasing propolis score for stationary and migratory operations, for August 2019 sampling date.** Gene expression in seven-day-old bees (stationary) and young bees collected from frames with sealed brood (migratory) was quantified using real-time PCR. Six immune genes (*abaecin, defensin-1, hymenoptaecin, relish, AmPPO,* and *AmEater*) were analyzed in stationary colonies (n = 30), and gene expression trends were analyzed at both the apiary and colony level. The same genes, with the exception of *relish*, were analyzed in migratory colonies (n = 102) at the apiary level. A distributional regression model was used to determine the probability that gene expression increases, decreases, stabilizes, or destabilizes with increasing propolis score (Table S2). The percentages listed refer to the quantile credible interval, as determined by our model, and reflect the widest possible credible interval supporting the indicated trend (not containing zero). When two percentages are listed for one gene (e.g., *hymenoptaecin*, stationary (66%, 90%)), the first number listed corresponds to the grouping on the left (e.g., decreases); the second corresponds to the grouping on the right (e.g., stabilizes).

When we compared gene expression in individual bees from stationary colonies, we found convincing evidence that variation in *relish* expression decreased with increasing propolis score at the colony level, and some evidence that variation in *defensin-1* expression decreased with increasing propolis score at the colony level (Fig. 3.7).

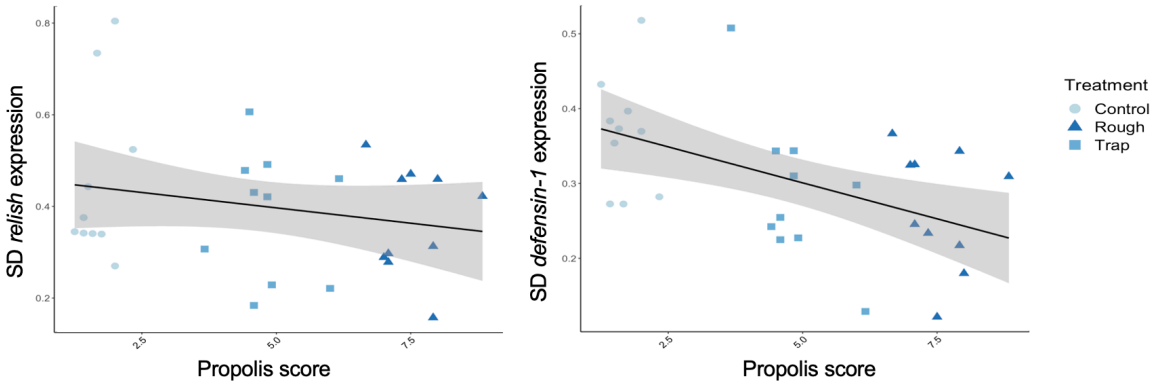


**Figure 3.7. Within-colony variation in immune gene expression across box types.** An average of seven bees per colony were collected to measure immune gene expression in stationary colonies using real-time PCR. Standard deviation in immune gene expression ($\log(2^{-\Delta Ct})$ was calculated for each colony, to determine whether variation in immune gene expression was correlated with propolis score. Standard deviation decreased with increasing propolis score for immune genes *relish* and *defensin-1*.

In migratory colonies, where bees were sampled for immune gene expression in August 2019, February 2020, and February 2021, the relationship between propolis score and immune gene expression differed among sample dates for some genes (Fig. 3.8, Table S3.2). *Defensin-1* expression tended to decrease with increasing propolis score in August 2019, but tended to increase with increasing propolis score in February 2020 and February 2021. For other genes, gene expression patterns were consistent among dates. Expression of *AmPPO* tended to increase and stabilize with increasing propolis score in February of 2020 and tended to increase in February of 2021. *AmEater* expression tended to destabilize with increasing propolis score in August 2019 and tended to increase and destabilize in February 2021. *Abaecin* expression tended to decrease and stabilize with increasing propolis score in August 2019, and tended to decrease in February 2021. There was no effect of propolis score on the expression of *hymenoptaecin* at any sampling date.

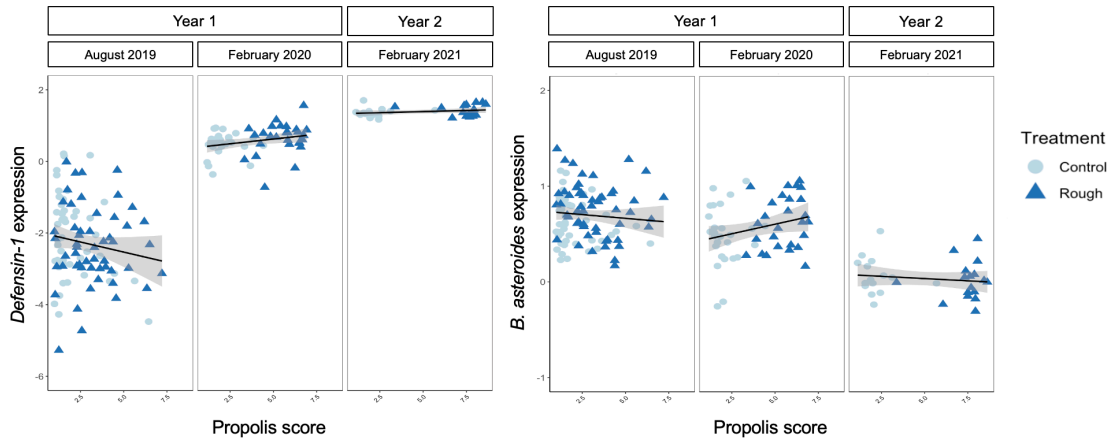


**Figure 3.8. Seasonal effects of propolis score on immune and relative bacterial gene expression in migratory colonies.** In some cases, genes whose expression tended to decrease and stabilize in August exhibited opposite trends in February. In migratory colonies, in August of 2019, expression of immune gene *defensin-1* (Median: -0.316, 90% QCI: [-0.6, -0.045]) and bacterial gene *B. asteroides* (Median: 0.065, 66% QCI: [0.017, 0.111]) tended to decrease with increasing propolis score, but in February of 2020 and 2021, *defensin-1* expression tended to increase with increasing propolis score (Median: 0.099, 90% QCI: [0.003, 0.199]; Median: 0.035, 66% QCI: [0.013, 0.053], respectively). *B. asteroides* expression tended to increase with increasing propolis score in February of 2020 (Median: 0.065, 66% QCI: [0.017, 0.111]).

## *Bacterial gene expression*

Our distribution models indicated that propolis deposition likely had a seasonal effect on bacterial gene expression in migratory colonies (n = 102; Fig. 3.9, Table S3). In August of 2019, *B. asteroides*, *UniBact*, and Firm-5 phylotype expression tended to decrease with increasing propolis score. In February, *B. asteroides* and *UniBact* expression demonstrated the opposite tendency, increasing with increasing propolis score in 2020 and 2021, respectively. The effect of propolis deposition on *Bartonella* expression was fairly consistent across seasons; *Bartonella* expression tended to increase and stabilize with increasing propolis score in August 2019, and continued to increase with increasing propolis score in February 2020. Expression of the Firm-4 phylotype decreased with increasing propolis score in February 2021. There was no effect of propolis deposition on the expression of *S. alvi* at any time point in this study.

## *Pests and pathogens*

The expression of *Melissococcus plutonius*, the causative agent for EFB, was significantly reduced in bees collected from rough box migratory colonies ($F(1,77) = 5.66$, $p = 0.02$, Fig. 3.9A). Although signs of European foulbrood (scores calculated based on number of symptomatic brood cells observed) were approximately 30% less severe in migratory rough box colonies than in migratory control colonies, these results were not significant ($F(1,67) = 2.8$, $p = 0.10$, Fig 3.9B). Similarly, *Varroa* infestation (number of mites/100 bees) was reduced by nearly one third in rough box colonies, though this difference was non-significant ($F(1,108) = 1.9$, $p = 0.18$, Fig 3.9C).

Our distributional models provided some evidence that viral load for multiple viruses tended decrease with increasing propolis deposition (Table S3). CBPV, IAPV, and LSV-1 decreased with increasing propolis deposition in August of 2019, and DWV decreased with increasing propolis deposition in both February of 2020 (DWV-A) and

February of 2021 (DWV-A and DWV-B). In contrast, BQCV tended to increase with increasing propolis score in August of 2019.

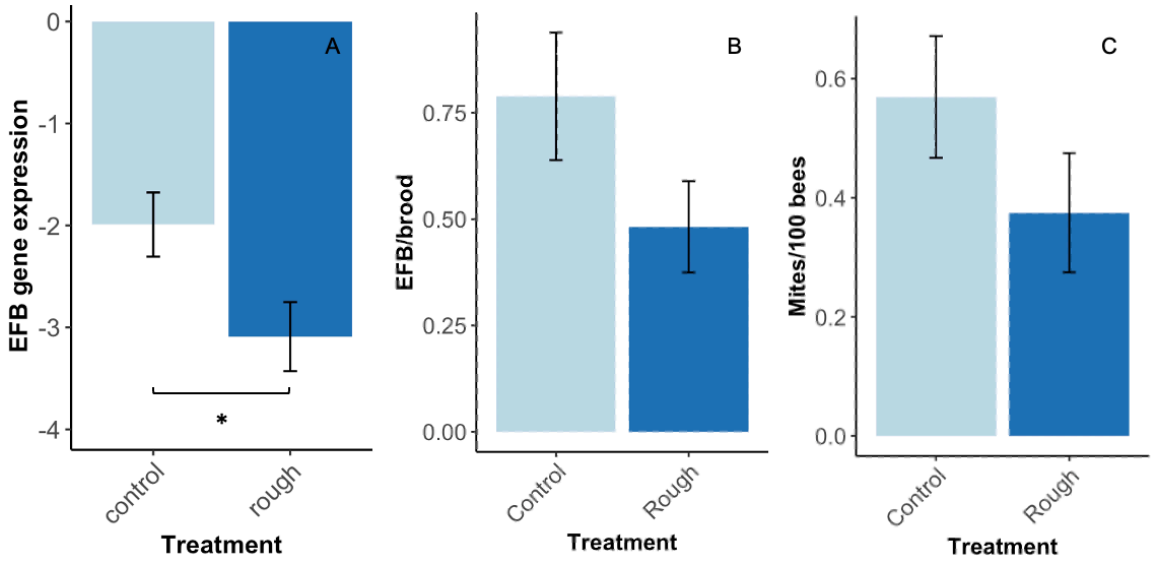


**Figure 3.9. Pathogen load in control and rough box migratory colonies.** (A) Detection of EFB via gene expression was significantly reduced in rough box colonies (n=79, $F(1,77) = 5.66$, $p = 0.02$) compared to control colonies in February 2020. Mean gene expression ($\log(2^{-\Delta Ct})$ ± standard error. (B) There was a marginal reduction in signs of EFB observed in March of 2020 at the colony level (n=69, $p = 0.10$). Mean EFB/brood ± standard error, where EFB score is divided by the number of frames with EFB present, and score is determined according to the following: 0 = no cells, 1 = less than 10 cells, 2 = 11-100 cells, 3 = more than 100 cells. (C) When colonies were sampled for *Varroa* mites in August of 2019 there were 35% fewer mites in rough box colonies than in control colonies, though this difference was not statistically significant (n=110, $p = 0.18$). Asterisks indicate significant differences between treatments ($p < 0.05$).

## *Survival*

Only 13% of stationary colonies survived year one, with no differences in survivorship across treatments ($F(2,34) = 0.37$, $p = 0.69$). High losses in the stationary yard were attributed to an issue with fall feeders, which prevented colonies from entering winter with sufficient food stores. In the migratory operation, only 58% of colonies (n = 231)

survived and were deemed suitable to be sent to California for almond pollination services at the end of year one, but there were no differences in survival between rough box and control colonies ($F(1,229) = 0.036$, $p = 0.85$).

### *Honey production*

In the stationary colonies, there were no differences in mean honey production across treatments (Fig. 3.10). In the migratory colonies, our mixed-effects model indicated that box type had a significant effect on honey production ($p = 0.001$), as did date ($p < 0.0001$), and the interaction between box type and date ($p = 0.01$). Rough box colonies produced less honey than control colonies by a margin of 33 pounds (38%) in year one ($t(215.8) = 3.2$, $p = 0.02$); these differences corresponded to colony size. Large rough box and control colonies (>15 frames) were fairly even in terms of honey production, but small control colonies (<15 frames) produced more honey than small rough box colonies by a margin of 45 pounds ($t(102) = 4.5$, $p = 0.0001$, Fig. 3.11). However, decreased honey production in small rough box colonies did not correspond to a significant increase in propolis deposition ($t(102) = 2.0$, $p = 0.19$). In year two, when colonies were started in boxes used in year one that were already propolized and when honey production was higher overall, there was no effect of box type on honey production.

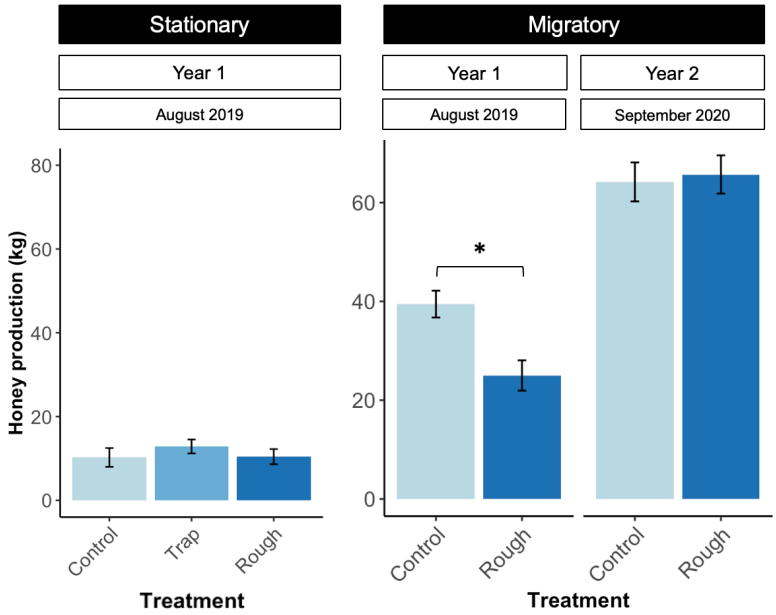


**Figure 3.10. Honey production across box type in stationary and migratory colonies.** Honey production did not differ between treatments in stationary colonies (n=30). In migratory colonies, in year one (August of 2019), honey production was lower in rough box colonies by a margin of 33 pounds (n=112, $t(215.8) = 3.2$, $p = 0.02$). By year two (September of 2020), there were no differences in honey production between treatments (n=104). Mean pounds of honey ± standard error is shown for each treatment. Asterisks indicate significant differences between treatments ($p < 0.05$).

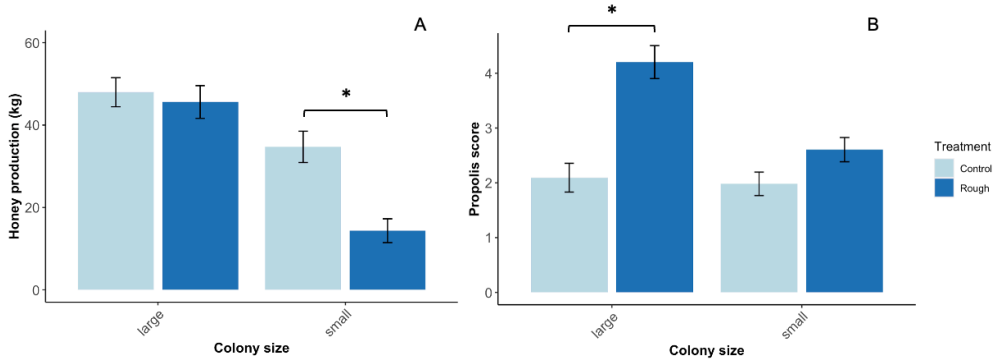


**Figure 3.11. Correspondence between colony size, honey production, and propolis collection in migratory rough box and control colonies.** Honey production (A) and propolis score (B) were quantified in August of 2019. Large rough box and control colonies (>15 frames) were fairly even in terms of honey production. Small control colonies (<15 frames), produced significantly more honey than small rough box

colonies ($t(102) = 4.5$, $p = 0.0001$). Large rough box colonies deposited significantly more propolis than large control colonies ($p < 0.0001$), but there was no difference in propolis deposition between small rough box colonies and small control colonies ($t(102) = 2.0$, $p = 0.19$). Asterisks indicate significant differences between treatments ($p < 0.05$).

## Discussion

Our study evaluated strategies that beekeepers can use to support bees' construction of a natural, health-supportive propolis envelope. To date, propolis envelope support strategies have largely been tested in research settings and over relatively short periods of time. Here, we compared propolis deposition and colony health in rough wood boxes, boxes outfitted with propolis traps, and standard smooth wood boxes in a stationary context over one year and in a migratory beekeeping operation over two years. Our results provide convincing evidence that rough wood boxes are an effective means to stimulate propolis collection and support colony health and homeostasis in both stationary and migratory beekeeping contexts.

### *Propolis deposition*

Rough boxes were highly effective in stimulating propolis collection, compared to control boxes and boxes outfitted with propolis traps. Stationary rough box colonies collected 50% more propolis than stationary colonies outfitted with propolis traps, demonstrating that rough boxes outperform this previously established method for supporting bee health (Bankova et al. 2019; Borba et al. 2015). This result is in contrast with findings from Hodges et al. (2018), where there were no differences in propolis deposition between rough box and propolis trap colonies. This discrepancy could be due to the fact that Hodges et al. (2018) used boxes roughened with a mechanized wire brush, creating a two-dimensional rough surface. Our rough boxes contained texturized grooves, a three-dimensional rough surface which likely allowed for higher levels of propolis deposition. Future use of rough box colonies should strive to imitate the combination of

rough wood textures, cracks, and crevices found in the hollow tree cavities where feral colonies nest.

In migratory colonies, bees deposited more propolis in rough boxes than in control boxes for all dates, and rough box propolis deposition increased over time, while control box propolis deposition remained stagnant. This suggests that, when provided with a stimulus, colonies continue to bring in resins to fully form and refresh the "propolis envelope." Notably, propolis build-up was slower in migratory rough box colonies than in stationary rough box colonies; it took migratory colonies nearly two years to come close to the amount of propolis that stationary colonies collected in just four months. Mountford-McAuley et al. (2021) note that, in addition to box type, there are multiple factors that affect propolis production, among them resource availability and genetics. In our study, the landscape surrounding the stationary yard was more diverse than the landscape surrounding the migratory yards, with a notable presence of forest and shrubs and herbaceous and woody wetlands. The percent cover associated with these plant communities was significantly correlated with propolis deposition scores. Previous research has established that areas of high plant biodiversity tend to provide more resin resources than areas of low plant diversity, corresponding to increased propolis production (Ribeiro Pereira et al. 2009). Since different plant resins are effective against different pathogens, the implications of landscape composition could extend beyond propolis score (Drescher et al. 2014). Future studies should examine the ways in which landscape factors shape the composition of the propolis envelope (in addition to the amount of area it covers) and affect honey bee health.

Genetic differences may have also contributed to the variation we observed in propolis score, both between migratory and stationary yards, and between colonies in the same box type, in the same yard. Propensity for propolis collection is a highly heritable trait (coefficient of heritability = 0.87, Garcia et al. 2013), and selection efforts can yield high-propolis colonies (Nicodemo et al. 2013). Different honey bee stocks were used in the stationary and migratory study, which, along with landscape differences, may have contributed to the variation in propolis deposition across contexts. Taken together, these findings suggest that, in order to fully realize the potential of the rough box, beekeepers

must take steps to integrate resin resources into the landscape and, where possible, select for bees with propolis-collecting genetics. Still, even with unselected bees and across landscapes with varying levels of diversity, rough wood boxes supported improvements in multiple measures of colony health.

## *Colony health*

The use of rough boxes mitigates some forms of pathogen pressure. Throughout year one, rough box migratory colonies experienced a marginal reduction in *Varroa* mite load. In our study, *Varroa* loads in migratory colonies were extremely low, with an average of approximately 0.5 mites per 100 bees. It is possible that these low overall numbers made it difficult to detect a significant contrast in mite infestation across treatments. Regardless, the marginal reduction in mite load that we did observe is consistent with recent findings from Pusceddu et al. (2019), who found that the application of field-realistic quantities of propolis to artificial brood cells resulted in a near 20% increase in *Varroa* mortality during brood rearing. Pusceddu et al. (2019) also observed an increase in resin foraging activity in *Varroa*-infested hives, pointing towards a possible example of social medication (Spivak et al. 2019). These effects have not been found to translate to reductions in mite loads at the colony level, possibly due to differences in the way researchers have attempted to simulate the propolis envelope. Previous studies have transplanted propolis harvested from one set of colonies to the tops of frames in a separate set of colonies to create a propolis-rich environment (Drescher et al. 2017) or used propolis traps to encourage the formation of a natural propolis envelope (Borba et al. 2015), a strategy we now know to be less effective than the use of rough boxes. It is possible that a robust, honey bee-made propolis envelope helps mitigate *Varroa* load in ways that a human-made, or propolis trap-induced propolis envelope does not.

Although propolis has recently been shown to inhibit the growth of *Melissococcus plutonius* – the causative agent of European foulbrood (EFB) – *in vitro* (Murray et al. BioRxiv 2022), to our knowledge, ours is the first study to observe a decrease in *M.*

*plutonius* detection in propolis-rich colonies. The significant reduction in *M. plutonius* gene expression that we observed in bees from rough box colonies corresponded to a marginal decrease in colony signs of EFB the following month. Future studies should investigate impacts of propolis-rich hive environments on this pathogen, taking into account both molecular methods and field observations in colonies experimentally challenged with EFB.

Propolis deposition also appeared to impact viral loads in migratory colonies. In August of 2019, viral loads for CBPV, IAPV and LSV-1 tended to decrease with increasing propolis deposition, and DWV tended to decrease in February of 2020 (DWV-A) and 2021 (DWV-A and DWV-B). Surprisingly, BQCV load tended to increase with increasing propolis deposition in August 2019. Previous studies have compared viral loads in bees from propolis-rich and propolis-poor environments and detected no differences (Borba et al. 2015, Pusceddu et al. 2021) or nuanced differences (Drescher et al. 2017). Notably, these studies compared viral loads across a propolis-rich/propolis-poor binary while our study examined viral loads along a propolis deposition gradient, which allowed us to take a closer look at the relationship between propolis deposition and viral load. In addition, the quantile credible interval metric we used in our analysis is more expansive (i.e., not limited to a strict p-value of 0.05), than metrics used in previous analyses, and thus picks up on broader trends. While a 66% QCI is far from decisive, the fact that viral load tended to decrease with increasing propolis score in six different instances suggests that propolis likely has some impact on honey bee viruses, or on the bees' ability to fight off viruses. This builds on previous work showing that propolis plays a constitutive role in social immunity, where it has a constant, background preventative effect against parasites and pathogens (Simone-Finstrom, 2017). The question of whether propolis functions as a therapeutic or induced defense against pathogens requires further exploration, particularly in regard to its potential as a treatment against bee disease.

Perhaps related to decreased pathogen pressure, the use of rough boxes corresponded to an increase in total bee population in migratory colonies. Although there were no significant differences in total bee populations between rough box and control

colonies early in year one, by February of 2020 – ten months into the colony life cycle – migratory rough box colonies were significantly larger than migratory control colonies, by a margin of nearly two frames of bees plus brood. While colony size cannot be considered a direct measure of colony health, this result is likely significant for beekeepers, particularly those who rent their colonies for crop pollination and must provide growers with a substantial foraging force in order to get paid (Toni et al. 2018). Interestingly, throughout year two of the migratory experiment, rough box and control colonies were similar in size though still trended larger. Borba et al. (2015) also observed colony-level differences between propolis-rich and propolis-poor colonies during one replicate year but not the other, possibly pointing to context-dependent fluctuations in external factors that support or detract from honey bee health.

The expression of multiple immune genes tended to decrease and stabilize with increasing propolis deposition in both stationary and migratory colonies in August of year one. Decreased immune gene expression in propolis-rich environments is consistent with the results of previous studies (Simone-Finstrom et al. 2009, Borba et al. 2015). As previously mentioned, in this study, because we quantified the amount of propolis inside the hive (rather than relying on a propolis-rich/propolis-poor binary), we were able to examine the ways in which variation in gene expression changes with respect to propolis score. This analysis revealed a stabilization effect: as propolis deposition increases, gene expression becomes less variable for multiple immune genes. If, following Dawkins (2013), a healthy population is characterized by greater uniformity in health-related metrics, then this stabilization effect likely benefits colony health, contributing to hive homeostasis. Indeed, Borba et al. (2015) speculated that the modulation of immune system activity might be the most important function of the propolis envelope. The stabilization of immune gene expression may also correspond to the stabilization of the microbiome in propolis-rich environments, which has been observed in previous studies (Dalenberg et al. 2020, Saelao et al. 2020). Full sequencing of the microbiome was beyond the scope of this study, but we did note that the expression of multiple bacterial genes was correlated with propolis score. Taken together, results of the current study and previous work indicate that propolis likely plays a strong role in maintaining not only

nest environment homeostasis but also social homeostasis, ultimately improving social resilience in the face of stressors (Ulgezen et al. 2021).

The effects of propolis score on gene expression were seasonal, consistent with previous work. In this study, propolis deposition had contrasting effects on the expression of immune genes *defensin-1* and *AmEater*, and bacterial gene *B. asteroides* in August and February of one or both years. Seasonal effects of propolis on immune gene expression were also recorded by Borba et al. (2015), who noted that the antimicrobial activity of the propolis envelope decreases over the winter, and tends to be low in the spring. By February of both our study years, the propolis envelope may not have been "fresh," and this could explain why certain trends in gene expression were diluted at this time of year. Alternatively, the February sample dates may have corresponded to increased immune gene expression due to greater colony stress. In February, colonies were in California for almond pollination. Migratory movement of colonies has been associated with increased viral load (Alger et al. 2018, Simone-Finstrom et al. 2022), increased oxidative stress, and decreased worker bee life span (Simone-Finstrom et al. 2016). It is possible that colonies were more exposed to pathogens during this period; the presence of EFB symptoms was notable at this time. Turcatto et al. (2018) determined that bees fed a propolis-rich diet exhibit increased expression of immune genes when challenged with *E. coli* injection, compared to bees not fed propolis. Thus, it is possible that the increased expression of certain immune genes in bees in propolis-rich rough box environments is reflective of a healthy response to increased environmental stressors. However, myriad interacting factors influence immune gene expression, so more data is needed to test the effect of environmental stressors on immune gene expression in the presence and absence of propolis. In our study, the collection of gene expression data, and colony health data in general, were unfortunately limited due to COVID-19 travel restrictions.

## *Honey production*

Honey production is an important metric for beekeepers operating in a commercial context; we tracked this metric to determine whether the use of rough boxes

impacts honey production in any way. There were no differences in honey production across box type in stationary colonies. However, in the migratory operation in year one, rough box colonies did produce less honey than control colonies by an average of 33 pounds per colony (a 38% decrease). This result is in contrast with findings from other studies, which have found a positive correlation (Manrique and Soares 2002, Nicodemo et al. 2013) or no correlation (Garcia et al. 2013) between honey production and propolis collection. These contrasting results may point towards the importance of additional factors, such as resource availability and colony size, in shaping nectar and resin foraging dynamics. In our study, migratory beekeepers described year one as "a bad honey year" overall. Indeed, year one honey production was about half that of year two. Notably, differences in honey production across box type were evident in small colonies, but not large colonies (i.e., small rough box colonies produced less honey than small control colonies, but there were no differences in honey production across large control and rough box colonies). It is possible that small rough box colonies produced less honey because foragers were occupied with resin collection, but since these colonies did not bring in significantly more propolis than small control colonies, there is no clear evidence indicating a nectar/resin tradeoff. Further research is required to more fully evaluate the conditions under which this type of tradeoff might emerge. However, in practical terms, a potential resin/nectar tradeoff might only be a short-term concern for beekeepers. Year one rough boxes were reused in year two, and in year two there were no differences in honey production across treatments. This might indicate that, once the propolis envelope is established, colonies invest fewer bees in resin foraging, and resin foraging does not detract from honey production. Moving forward, beekeepers may also weigh for themselves the benefits of a health-supportive propolis envelope against the cost of a possible, temporary dip in honey production. It is also possible that, since beekeepers require populous colonies to fulfill pollination contracts, the population boost that rough boxes provide could help balance this calculus.

## Conclusions

Our study demonstrates that using rough boxes to stimulate the construction of a propolis envelope represents an important opportunity to bolster honey bees' natural defenses. Compared to other interventions, using rough boxes to boost propolis collection could be considered an "easy win" because their implementation requires minimal disruption to beekeeping operations and offers measurable benefits to honey bee health in a cost-effective manner. However, the fact that propolis deposition was highly variable even within the rough box treatment suggests that, in addition to modifying box surface texture, further measures should be taken to facilitate the construction of a robust propolis envelope. Some of these measures include fortifying landscapes with resin-producing plants and selecting for bees that engage in resin-hoarding behaviors. Taken together, these actions should contribute substantially to the restoration of the propolis envelope as a natural defense for honey bees.

Importantly, while facilitating the construction of a robust propolis envelope does support bee health, our findings also indicate that propolis is not a silver bullet. Despite clear benefits of propolis to multiple measures of colony health, we observed no differences in survivorship between box types. This result is not entirely surprising; the restoration of one aspect of social immunity should not be expected to completely counteract the effects of the multiple interacting stressors that threaten bee health within and beyond industrial agriculture systems. This does not diminish the promise of the propolis envelope as a health-supportive tool. Rather, it suggests that rough boxes represent one important intervention to implement in concert with other management, landscape, and systems-level efforts to support honey bee health.

## Chapter 4: Resin use by stingless bees: A review[8]

**Summary:** Bees, ants, and other insects harvest antimicrobial resins from plants and use this material for a variety of purposes, from nest construction to defense against predators and pathogens. Resin use is thought to have facilitated the evolution of sociality in stingless bees, and today, resin use remains fundamentally important for stingless bee colony function. Most species use resin to build brood comb, storage pots for honey and pollen, and various protective structures within the nest. Many also use resin to protect their nests from predators, fortifying nest entrances with a barrier of sticky resin droplets or applying resin directly to would-be invaders. For some species, the presence of resin inside the nest space can also influence the physical properties of the bees themselves, enriching the chemical composition of the outermost layer of their exoskeleton, and possibly shaping the communities of bacteria and fungi that are found on the bees, and in their nests. This article brings together studies from a variety of fields to illustrate the importance of resin use for stingless bee colony function and conservation, and to point towards areas of future research.

**Abstract:** Stingless bees (Meliponini) are highly social bees that are native to tropical and sub-tropical ecosystems. Resin use is vital to many aspects of stingless bee colony function. Stingless bees use resin to build essential nest structures, repel predators, and kill would-be invaders. Furthermore, resin-derived compounds have been found to enrich the cuticular chemical profiles of many stingless bee species, and resin may play an important role in shaping the microbial communities associated with stingless bees and their nests. Despite its importance for colony function, previous reviews of resin use by stingless bees are lacking. This topic grows increasingly urgent as changes in beekeeping and land use practices occur, potentially diminishing stingless bees' ability to incorporate resin into the nest environment. In this article, we review existing literature on resin use by stingless bees and discuss potential areas of future research.

[8] This chapter was co-authored with Marla Spivak, who contributed to editing and revisions, and was published in *Insects* on August 11, 2021.

## Introduction

Stingless bees (Meliponini) are highly social bees that are native to tropical and sub-tropical ecosystems. With approximately 550 species known to science, stingless bees comprise the largest and most diverse group of corbiculate bees (Euglossini, Apini, Bombini, and Meliponini). They represent approximately 70% of all eusocial bee species (Grüter 2020) and exhibit a dizzying diversity of morphologies, behaviors, and life histories. Their geographic distribution spans five continents, and their colonies can range in size from a few hundred to many thousands of individuals. Their nesting habits vary widely, with some species nesting in tree cavities, others nesting inside active termite or ant nests, and still others building subterranean nests up to three meters underground (Schwarz 1948). Humans have been in relationship with stingless bees for millennia through the practice of stingless beekeeping, or meliponiculture (Suryanarayanan and Beilin 2020, Cortopassi-Laurino et al. 2006, Reyes-González et al. 2020, Chan Mutul et al. 2019). In fact, stingless bee research often draws from the local ecological knowledge that stingless beekeepers from indigenous and rural communities have cultivated for generations. This includes information on the myriad medicinal uses for the resinous materials that beekeepers harvest from stingless bee nests (Chan Mutul et al. 2019, Cano-Contreras et al. 2013, Arnold et al. 2018, Popova et al. 2021).

Stingless bees collect the sticky resins that plants secrete and use this material for a variety of purposes. Most species use resin to build essential nest structures such as brood comb, storage pots for honey and pollen, and various protective structures (Schwarz 1948, Roubik 2006). For many species, resin is also an important part of nest defense; stingless bees use resin to build barriers, trap predators, and kill would-be invaders (Halcroft et al. 2011, Nunes et al. 2014, Greco et al. 2010, Duangphakdee et al. 2009, Drescher et al. 2014). For some species, the presence of resin inside the nest space can also influence the physical properties of the bees themselves and their microbial associates. Resin-derived compounds have been found to enrich the cuticular chemical profiles of many stingless bee species (Leonhardt 2017), and resin may play an important

role in shaping the microbial communities associated with stingless bees and their nests (Paludo et al. 2018, Menezes et al. 2015, Dalenberg et al. 2020, Saelao et al. 2020).

Despite the importance of resin use for stingless bee colony function, previous reviews of this topic are lacking. In part, our understanding of resin use is limited because less than half of all meliponine nests have been described by Western science (Roubik 2006). Of these, only a small number of species have been studied intensively, and few studies have focused specifically on resin use (except see studies by the Leonhardt group, cited below). The information we do have is difficult to generalize across species because stingless bees are highly diverse, and resin use is a particularly variable trait. Lastly, as is the case for living systems throughout the world, scientific literature represents only a limited portion of human knowledge of stingless bees. Though there have been numerous recent efforts to account for indigenous and local ecological knowledge of stingless bees (Reyes-González et al. 2020, Arnold et al. 2018, Gonzalez et al. 2018), Western science has historically excluded these knowledge systems, so a review of the existing literature is limited in scope.

In spite of these challenges, this review brings together research from disparate fields (e.g., natural history, chemical ecology, microbiology) to examine resin use in stingless bees. Taken together, these studies highlight the centrality of resin use to stingless bee colony function. In the following sections, we review existing literature on the role of resin in stingless bee nest construction and defense, discuss resin foraging and resin handling by stingless bees, and review studies on the effects of resin on bees' cuticular chemical profiles and their microbial associates. Finally, we point to gaps in knowledge that warrant further study.

## What is resin?

The chemistry, evolution, ecology, and ethnobotany of plant resins has been reviewed by Langenheim (2003). Plants secrete resin from buds, wounds, fruits, and flowers to defend themselves from herbivores and microorganisms, and, in some cases, to

attract pollinators and seed dispersers (Langenheim 2003, Leonhardt et al. 2014, Wallace and Lee 2010, Armbruster 1984). Resin can trap, immobilize, or deter predators, disinfect wound sites, and help to guard against the proliferation of endophytic fungi (Langenheim 2003). This versatile material is chemically complex. It consists of lipid-soluble mixtures of volatile and non-volatile phenolic compounds (e.g., flavonoids, aromatic acids, and benzopyranes) and terpenoids (e.g., mono-, di-, and sesquiterpenes) that possess a variety of anti-inflammatory, antifungal, antibacterial, and antiviral properties (Langenheim 2003). The specific chemical composition of resin varies between plant species, and can even vary between individuals of the same species (Leonhardt et al. 2010). Predators and pathogens are limited in their ability to evolve resistance to the complex and variable mixture of bioactive compounds that resin contains.

A wide variety of animals—from humans to coatis to wood ants to bees—harvest resin from plants and use this resource as a medicine, defense, and building material (Langenheim 2003, Armbruster 1984, Gompper et al. 1993, Simone et al. 2009, Roubik 1989). Many bee species use plant resins in nest construction; of these, the majority belong to the families Megachilidae and Apidae. In fact, with the exception of bumblebees (Bombini), almost all corbiculate bees harvest and make use of plant resins (Martins et al. 2014). Resin use by honey bees has received increasing attention in recent years (reviewed by Simone-Finstrom and Spivak (2010), Simone-Finstrom and Spivak (2017), and Mountford-McAuley (2021)). In stingless bees, resin use is even more extensive; many stingless bee species collect resin in copious amounts and use it to support multiple aspects of colony function.

## Resin use by stingless bees

### *Nest construction*

Nest construction strategies vary widely across stingless bee species, with different species building nests in different spaces. Some species build exposed nests

adhered to tree branches, others make use of existing cavities including hollow trees, termite nests, or electric light posts, and others build subterranean nests deep underground (Grüter 2020, Schwarz 1948, Boongird and Michener 2010). Nest construction materials range from fecal matter to soil to human-made products such as wet paint and adhesives (Roubik 2006). Though nest construction varies both across and within species (Rasmussen and Camargo 2008), almost all stingless bees use resin in some part of their nest.

Resin is an effective construction material for several reasons. Resins are malleable when secreted but harden over time, so they can be shaped to build durable structures (Langenheim 2003). They are also water insoluble, so they can be used to create waterproof nest spaces and water-tight storage pots (Roubik 1989, Berenbaum and Calla 2021). Lastly, the antimicrobial properties that resins possess may help regulate the microbial communities found inside stingless bee nests, preventing food spoilage and pathogen attack (Roubik 1989). In fact, the use of antimicrobial resins in nest construction may have been central to the evolution of sociality in stingless bees.

For many insects and other organisms, managing microbial communities is a key part of building and maintaining a successful nest. These efforts are particularly important in tropical environments, where conditions favor the proliferation of microbes, and in social insect societies, where the risk of disease transmission is increased due to large numbers of genetically similar individuals living in close proximity (Roubik 1989, Stow et al. 2007). Many insects use antimicrobial compounds to prevent the spoilage of food and the spread of pathogens. These compounds can be self-produced (e.g., many stinging insects apply antimicrobial venom to their cuticle and nests (Baracchi et al. 2011, Baracchi et al. 2017)), symbiont-produced (e.g., beneficial microbes secrete antimicrobial compounds that prevent the spoilage of food stores (Vasquez et al. 2012)), or environmentally acquired (e.g., bees, ants, and other insects bring foreign materials—such as antimicrobial resin—into their nests (Simone et al. 2009, Christe et al. 2003)). Since the use of foreign materials allows for new forms of nest construction, this evolutionary adaptation is thought to have facilitated a massive range expansion and diversification for bees (Litman 2011). Because resins help preserve food stores and

enable the construction and defense of resource-rich nest spaces, resin use, specifically, is thought to have facilitated the social evolution of stingless bees in tropical ecosystems (Roubik 1989) (p. 388) and the subsequent diversification of stingless bee species (Requier and Leonhardt 2020). A molecular phylogeny constructed by Rasmussen and Camargo (2008) supports this hypothesis, indicating that ancestral *Trigona* species likely used resin to build their nests. Today, resin use persists as a crucial component of nest construction for stingless bees.

Most stingless bees mix resin with wax to produce cerumen, which is the material they use to build brood combs, honey and pollen pots, and various other structures inside the nest (except see *Schwarzula* sp. (Camargo and Pedro 2002) and *Trigona australis* (Milborrow et al. 1987) (Fig. 4.1). Because it contains both wax and resin, cerumen has sometimes been equated with honey bee propolis (Massaro et al. 2011). However, cerumen serves as the primary construction material within the stingless bee hive, so it is actually closer in function to beeswax, though it differs from beeswax in several important ways. Rather than forming part of a permanent comb structure, cerumen is continuously reworked and recycled within the nest. The physical properties of cerumen can vary. This is likely due, at least in part, to the variable proportions of wax and resin the material contains. Cerumen can be soft, flexible, and light brown in color (possibly containing more wax and less resin) or rigid, brittle, and dark brown or black in color (possibly containing less wax and more resin) (Wille and Michener 1973). Although there has not yet been a comparative study of cerumen characteristics across species, Roubik (2006) noted that the cerumen produced by certain small stingless bees (e.g., genera *Hypotrigona, Trigonisca, Schwarzula,* and *Plebeia*) contains little to no resin, and is closer to pure wax. *Schwarzula* sp. appear to use no resin at all, instead farming scale insects within the nest cavity and mixing their wax with self-produced wax to form a cerumen equivalent (Camargo and Pedro 2002). At the other end of the spectrum, the resin content of cerumen in some species can surpass 40% (Schwarz 1948). The factors that influence the amount of resin that different stingless bee species incorporate in cerumen—and in other parts of the nest—are not yet understood. Blomquist et al. (1985)

suggested that excluding resin may help some species cope with the high temperatures in the spaces where they nest, but this hypothesis has yet to be confirmed.

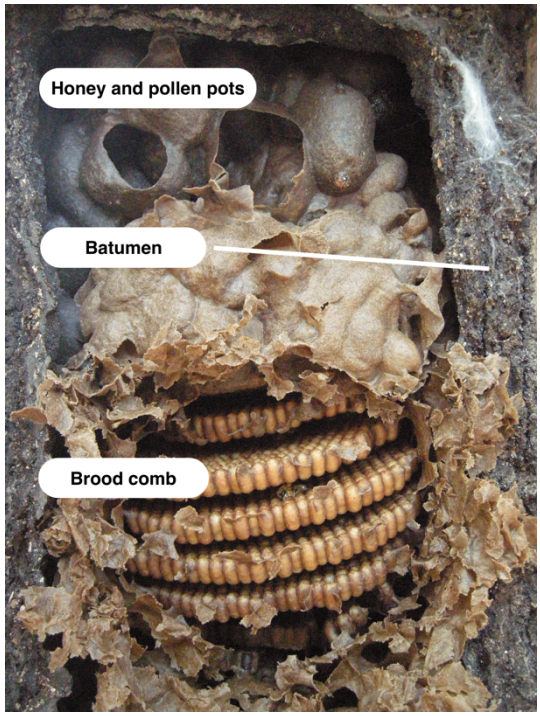


**Figure 4.1.** Nest structures such as brood comb and honey and pollen pots are made of cerumen, a mixture of wax and resin. The batumen is a wall-like structure that surrounds and protects many stingless bee nests; it is often made of resin. Photo by Miguel Angel Guzmán Díaz.

In addition to using resin to produce cerumen, stingless bees incorporate resin into the nest environment in the form of deposit-resins, propolis, and geopropolis (defined below), and in structures such as the nest entrance and batumen (Table 4.1). These terms are often conflated in the literature, with propolis being used as a catch-all to describe any resinous material inside the nest, aside from cerumen. However, it is useful to distinguish between these terms, since a single nest may contain multiple types of resin-rich materials and resin-based structures, each serving a different purpose.

| | |
|---|---|
| Cerumen | A mixture of wax and resin that stingless bees use to build brood combs, honey and pollen pots, and other nest structures |
| Deposit-resins | Caches of resin stored by some species on the floor or walls of their nests (also known as resin deposits or viscous propolis deposits) |
| Propolis | Resin mixed with small amounts of salivary gland secretions and wax and used to seal cracks and crevices throughout the nest |
| Geopropolis | Resin mixed with soil, silt, and/or sand particles |
| Batumen | A wall-like structure that often contains resin; many species build a batumen to separate the inner nest environment from the external world |
| Nest entrances | For some species, nest entrances consist of hardened resin tubes, which can extend both inside and outside the nest |

**Table 4.1.** Resin-rich materials and nest structures.

Deposit-resins, also referred to as resin deposits or viscous propolis deposits (Roubik 2006, dos Santos et al. 2009, dos Santos et al. 2010), are resin caches located on the nest floor or walls (Massaro et al. 2015). For some species, these caches serve as temporary storage where resins accumulate until they can be incorporated into other nest structures or used for defensive purposes (e.g., *Trigona (Trigona) p. pallens* (Roubik 1979); *Tetragonisca angustula* (Latreille) and *Plebeia* spp. (Roubik 1989, dos Santos 2010)). Unlike most resin, which hardens upon contact with air, deposit-resins remain viscous for a prolonged period of time. This property could have to do with the resin source; deposit-resins may contain a greater proportion of floral resins, which are slow to harden (Murphy and Breed 2008). Alternatively, or additionally, the prolonged viscosity of deposit-resins could result from chemical processing. While definitive research on this topic is lacking, a comparative analysis of the morphology of head salivary glands and intramandibular glands of bees of various ages suggests that *Plebeia emerina* workers modify deposit-resins using secretions, which might help to maintain their viscosity (dos Santos et al. 2010).

Propolis refers to the resins that stingless bees bring back to the nest and mix with small amounts of salivary gland secretions and, purportedly, wax (dos Santos et al. 2009). Numerous studies report that stingless bee propolis is more chemically diverse than honey bee propolis (reviewed by Popova et al. 2021). As with honey bees, many stingless bee species use propolis to seal cracks and crevices throughout the nest. For colonies managed in box hives, bees often seal cracks with a layer of propolis so thick that beekeepers must pry the lid from the hive body in order to access the nest (Fig. 4.2).

Though most studies state that propolis contains wax, the extent to which stingless bees incorporate wax in propolis is unclear. The amount of wax in *A. mellifera* propolis is known to be highly variable, and reports of wax content often lack precision (Salatino and Salatino 2021). In a detailed study of stingless bee resin handling, Gastauer et al. (2011) observed resin deposition in six bee species, but noted no mixing of resin with wax. It is possible that propolis produced by different species and for different purposes could contain variable amounts of wax, and in some cases no wax at all, but this has yet to be verified.

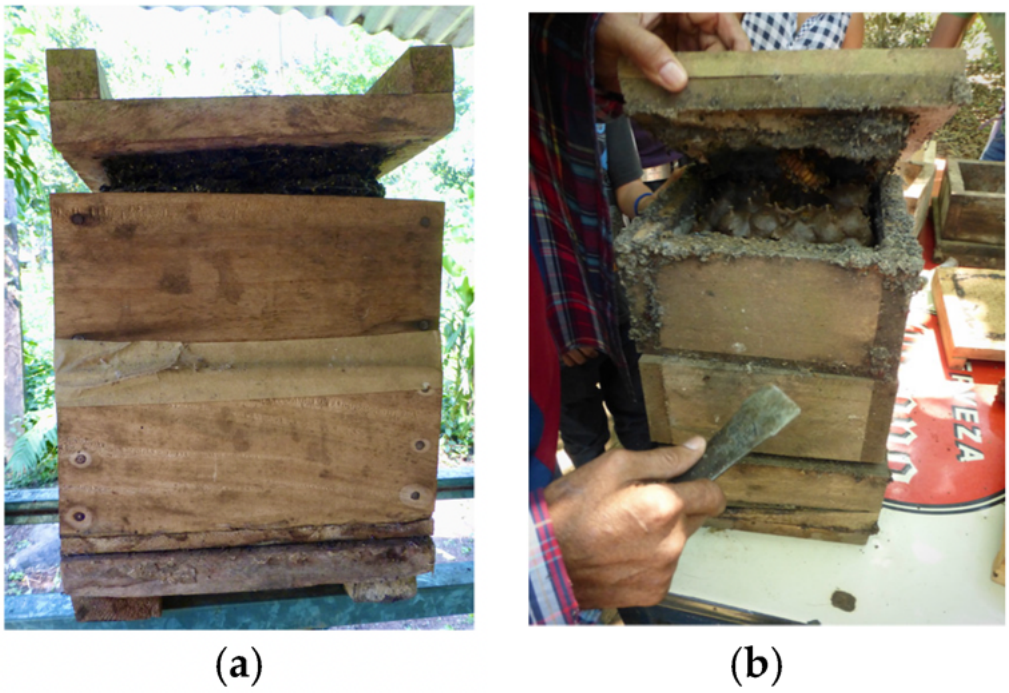


**Figure 4.2.** (a) Even in wooden box hives, many colonies seal cracks with a thick layer of propolis. (b) To access these colonies, beekeepers often use a hive tool to pry the lid from the hive body; excess propolis is sometimes lost in this process.

Some stingless bees use geopropolis in place of pure propolis. Geopropolis is a mixture of plant resins and soil. This mixture can consist of up to 90% soil, silt, and sand particles (Bonsucesso et al. 2018). It is less malleable than pure propolis, but serves a similar function inside the nest (Lavinas et al. 2019). Some studies use the terms propolis

and geopropolis interchangeably, or state that geopropolis is the propolis of stingless bees (Barth 2004), but these are actually distinct materials, differentiated by the presence or absence of soil (Popova 2021).

Propolis and geopropolis are often incorporated into other nest structures such as the nest entrance and batumen. Nest entrances commonly consist of hardened resin tubes, which can extend both inside and outside the nest. Internally, these convoluted maze-like structures are often designed to thwart enemy intruders (Roubik 2006). The batumen, also called the external involucrum (Wittmann 1989), is a wall-like structure that many stingless bee species build to separate the inner nest environment from the external world. This structure is usually made of resin and can also include mud, seeds, wood, feces, and other materials (Roubik 2006). Batumen construction is a variable trait among stingless bee species. Some species construct sturdy batumen walls measuring up to 10 cm in thickness (e.g., *Melipona* spp. (Roubik 2006)); others build no batumen at all (e.g., *Hypotrigona* and *Trigonisca* spp. (Wille and Michener 1973)). When present, the batumen can take many forms. In one comparative study of stingless bee nest architecture, Wille and Michener (1973) described several batumen types, and noted their presence or absence for 145 stingless bee species found in Costa Rica. According to this study, *exposed batumen* is a hard outer layer that surrounds and protects exposed or partially exposed nests. *Batumen plates* are sturdy plates that surround and protect nests within a cavity, allowing the bees to adjust the cavity size to suit the needs of the colony (e.g., genera *Melipona* and *Cephalotrigona*; *Meliponula bocandei*) (Roubik 2006). *Lining batumen* is a thin, continuous resinous lining, generally less than 2 mm in thickness, similar to the so-called propolis envelope that *A. mellifera* colonies use to coat the rough inner surfaces of the hollow tree cavities where they nest (Seeley 1976). *Laminate batumen* consists of multiple layered sheets. The channels found in laminate batumen allow bees to move between layers, and may also facilitate air flow and water evaporation (Roubik 2006, Wille and Michener 1973). In addition to providing a protective shield, these various types of batumen may serve to waterproof the nest cavity and help control fungal growth (Wille and Michener 1973).

## *Defense*

Bees contend with a variety of predators and parasites. Some examples include lizards, spiders, ants, wasps, assassin bugs, beetles, phorid flies, and parasitic stingless bees from the genus *Lestrimelitta* (Halcroft et al. 2011, Roubik 1989). Since stingless bees are unable to sting, they rely on a variety of other strategies to defend their nests. Defensive strategies vary across species and include such behaviors as hiding, building cryptic nests, biting, and burrowing in hair. Stingless bees also employ resin in a variety of ways to deter, trap, and kill predators and parasites. Here, we categorize resin-based defenses in two groups: (1) structural defenses, where bees build resinous structures or add fresh resin to existing structures to prevent invasion, and (2) direct defenses, where bees apply resin to the bodies of their enemies or to their own bodies to defend their nests.

### Structural defenses

Many stingless bee species (e.g., genera *Lepidotrigona, Scaura, Tetragona, Tetragonula,*and *Trigonisca*) fortify their nest entrances with a barrier of fresh resin droplets (Roubik 2006) (Fig. 4.3). This sticky material serves as a defense that is both mechanical and chemical in nature (reviewed by Leonhardt (2017). The terpenoid compounds commonly found in resin repel many predators (Roubik 2006, Duangphakdee et al. 2009, Langenheim 2003, Wang et al. 2018). The predators (largely ants) that do attempt to advance across the resin droplets often become trapped in the sticky material (Leonhardt and Blüthgen 2009), and are only able to breech the barrier when they use the bodies of other ants to bridge the so-called resin "moat" (Schwarz 1948, Bänziger et al. 2011, Alves 2018). Over time, the resin droplets harden, their adhesive and repellant properties likely diminish, and fresh stores must be applied (Leonhardt and Blüthgen 2009, Howard 1985). For some species (e.g., *Trigona cilipes, Tetragonilla collina*, and related species), the continuous application of fresh resin results in long, slender entrance tubes (Roubik 2006). In the case of one remarkable species, nest entrance resin produces

a dazzling architectural effect. The minute, tear-drinking stingless bee *Pariotrigona klossi* (Schwarz) builds a nest entrance consisting of dozens of tubelets that branch like coral. Each tubelet is adorned with strings of clear resin beads which together resemble the "quartz pendants of a chandelier" (Bänziger et al. 2011). For invading ants, this resinous terrain is difficult to navigate when hardened, likely impassable when fresh, and may also be visually disorienting, further deterring ant attack.

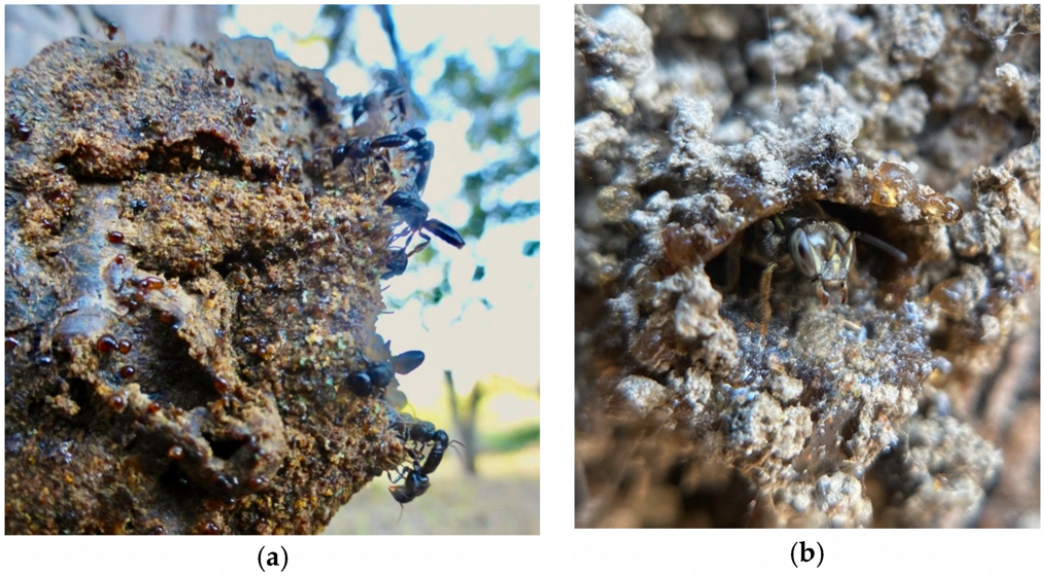


**Figure 4.3.** Many stingless bee species surround their nest entrances with (**a**) a barrier of resin droplets (**b**) or a continuous layer of resin to repel and trap would-be intruders. Photos by Héctor Morales Urbina.

Some stingless bees also use resin and cerumen pieces to barricade the nest entrance at night, or when disturbed (e.g., *Meliplebeia tanganyilcae medionigra* (Cockerell) and *Plebeiella lendliana* (Fries) (Roubik 2006, de Portugal Araujo 1963). Some species (e.g., *Melipona panamica, Melipona flavolineata*, and other *Melipona* species) even keep a designated resin ball on hand for this purpose (Nunes 2014). When the colony is under attack, the bees roll the hardened resin ball into place and use fresh resin to fasten it to the entrance to prevent invaders from breaching the nest. Over time, discarded resin balls accumulate near the internal entrances of these nests (Roubik 2006, Nunes et al. 2014).

Stingless bees that live in active termite or ant nests often surround their cavities with a full defensive resin barrier. This allows them to inhabit otherwise hostile environments. For instance, when some myrmecophilous stingless bees (e.g., *Trigona moorei*) initiate a nest, they begin by building a provisional batumen structure to establish ant-free spaces. They then expand this resinous shield as they burrow deeper into the ants' nest (Sakagami et al. 1989). A similar behavior can be seen in *Scaura latitarsus*; these bees form their nest by excavating a cavity in an active termite nest and lining that cavity with a continuous batumen shell (Roubik 1989).

**Direct defenses**

In addition to using resin in nest structures to prevent invasion, many species also apply resin directly to perceived threats. This behavior has been referred to as "resin daubing" (see detailed description of *Austroplebeia australis* nest defense by Halcroft et al. (2011), and it can lead to the immobilization or total mummification of predators (Greco et al. 2010). When certain species sense a threat, defending bees harvest resin and cerumen from deposit-resins and/or other parts of the nest, carrying these materials in their mandibles and corbiculae (Halcroft et al. 2011). They then attack would-be invaders outside the nest (e.g., plastering resin to human hair) (Schwarz 1948), or trap and mummify intruders within the nest (e.g., immobilizing parasitic fly pupae, ants, and various types of beetles) (Schwarz 1948, Greco 2010, Duangphakdee 2009, Bobadoye et al. 2018, Bordoni et al. 2020). Curiously, some stingless bee species also use "resin pellets" to kill virgin queens from their own colonies when these are in excess (Drumond et al. 1995).

Stingless bees do not just apply resin to the bodies of intruders; some species apply resin to their own bodies as well. Several stingless bee species have been observed leaving the nest with small amounts of both viscous and hardened resin in their corbiculae (Leonhardt et al. 2007, Harano et al. 2020). While soft, sticky resin can be used to entangle would-be invaders, the reason for carrying hardened resin is not entirely clear. In *Melipona subnitida*, Harano et al. (2020) observed that 11% of worker bees

leaving the nest carried resin in their corbiculae under normal (i.e., undisturbed) conditions. About half of these carried soft, sticky resin loads, while the other half carried dry, hardened resin. When the nest was disturbed, the number of worker bees leaving the nest with resin increased to 90%, with a majority (80%) of these carrying hardened resin. Both resin bearers and nectar foragers were paint-marked, and their movements were monitored. The short flight duration for resin bearers suggested that they were circling the nest, rather than relocating resources to an alternative nest site in response to predator attack. The authors speculated that, because of its repellant properties, hardened resin may serve as a type of armor, deterring would-be predators from eating the resin bearers (Harano et al. 2020). Alternatively, the resin bearers may sacrifice themselves for the benefit of the colony; after eating one unpalatable resin bearer, a predator might be dissuaded from further predation. This is not the first account of stingless bees carrying visible amounts of resin on their bodies for a purpose other than resin-daubing. The cuticle of *Tetragonula carbonaria* is covered with resin, so that the whole body is sticky; a thin layer of resin has been observed on the legs, head, and thorax of *Tetragonsica angustula*, and *Trigona (Tetragonula) melanocephala* nectar foragers have been observed leaving the nest with resin in their corbiculae (Leonhardt et al. 2007, Wenzel 2011, Leonhardt et al. 2015, Jones et al. 2012). However, the study conducted by Harano et al. (2020) provides the first detailed observation of bees carrying hardened resin on their bodies as part of an apparent mobilized defense, taking a piece of their nest with them for individual or collective protection.

Resin-based defenses can be triggered by both visual stimulation and chemical cues. In *Melipona flavolineata* (Friese), the head secretions and mandibular gland extract of the robber bee *Lestrimelitta limao* (Smith) elicited increased resin transport and the barricading of the nest entrance tube with hardened resin balls (Nunes et al. 2014). In *Tetragonilla collina*, resin foraging activity increased after nest entrances were damaged, and doubled after ant attack; worker bees used resin to elongate their entrance tubes and fortify them with a barrier of resin droplets (Leonhardt and Blüthgen 2009).

Resins from different plant species are effective against different predators and pathogens, and stingless bees may select resins based on their functional properties. This

means that access to diverse resin sources is important for stingless bee defense (Drescher et al. 2014). It is not yet clear whether stingless bees alter resin resource preferences in response to pressure from specific threats (i.e., collecting resins that are particularly effective in repelling small hive beetles in response to a small hive beetle attack). It is also unclear whether stingless bees use resin as a defense against bacterial or fungal pathogens. In *A. mellifera*, the presence of a propolis envelope has been found to decrease the severity of multiple brood diseases (Simone-Finstrom and Spivak 2017). Furthermore, colonies increase resin collection when challenged with *Ascosphaera apis*, the causative agent of the larval disease chalkbrood. This suggests that honey bee colonies use resin to self-medicate in response to certain pathogens (Simone-Finstrom and Spivak 2017, Simone-Finstrom and Spivak 2012). Similar behaviors may occur in stingless bees, and numerous studies indicate that stingless bee resin inhibits the growth of multiple microbes (reviewed by Bankova and Popova (2007)). However, with the exception of the bacterium *Lysinibacillus sphaericus*, which causes brood to degenerate, there are no known examples of pathogenic microbes in stingless bee colonies (Heard 2016; as cited in Leonhardt 2017). Consequently, the antimicrobial activity of resin is generally tested against human pathogens, so its effect on microbes associated with stingless bee colonies is unknown.

## *Resin foraging*

Although resin is essential to many aspects of stingless bee nest construction and defense, little is known about how stingless bees obtain this vital resource. Some information on resin foraging can be gleaned from studies on general foraging behavior (de Freitas et al. 2020, do Nascimento and Nascimento 2012, Silva and Gimenes 2014, Ferreira Junior et al. 2010), but there are few studies that examine resin foraging in stingless bees specifically (except see Wallace and Lee 2010, Leonhardt and Blüthgen 2009, Howard 1985).

Resin foragers make up <10% of the foraging force for many species (e.g., *Tetragonula minangkabau, Heterotrigona itama, Trigonella moorei, Melipona*

*bicolor bicolor, Trigona sapiens,* and *Trigona hockingsi*) (Wallace and Lee 2010, Inoue et al. 1985, Hilário et al. 2000), and are often outnumbered by pollen foragers (e.g., *Melipona bicolor schencki* (Gribodo), *Trigona iridipennis* (Smith), and *Melipona fasciculata* (Smith)) (de Freitas et al. 2020, Ferreira Junior et al. 2010, Layek and Karmakar 2018). However, some species collect copious amounts of resin, with resin foragers outnumbering pollen foragers (e.g., *Melipona asilvai* (do Nascimento and Nascimento 2012)). For *Tetragonula carbonaria*, resin foragers can account for up to 50% of the foraging force (Leonhardt et al. 2014). For *Tetragonilla collina*, up to 90% of foragers have been observed returning with resin, likely during periods of nest construction (Leonhardt and Blüthgen 2009). This is in stark contrast to *A. mellifera*, where resin foragers make up only 1–3% of the foraging force (Mountford-McAuley 2021).

A variety of environmental (e.g., temperature, light intensity, humidity, resource availability) and colony (e.g., population size, developmental stage) conditions influence resin foraging frequency at the colony level, and these factors have different effects on different species (Biesmeijer and Slaa 2004). For example, for some species (e.g., *Trigona iridipennis, Melipona asilvai, Melipona bicolor schencki,* and *Melipona colimana*) resin foraging activity fluctuates seasonally, but for other species (e.g., *Melipona fasciculata*), resin foraging is constant throughout the year (de Freitas et al. 2020, do Nascimento and Nascimento 2012, Ferreira Junior et al. 2010, Layek and Karmakar 2018, Macías-Macías et al. 2017). Seasonal changes in resin collection could be related to many variables, such as resource availability, fluctuating pathogen pressure, and colony developmental stage (Ferreira Junior 2010), but these are largely unexplored. For some species (e.g., *Melipona bicolor bicolor*), resin foraging increases with colony strength (as determined by comb diameter) (Hilário et al. 2000). For others, resin collection may be intense in the early stages of colony development and then taper off once the structural components of the nest are established (Leonhardt and Blüthgen 2009). For species that use resin-daubing or resin barriers as a form of defense, pathogen pressure can lead to increased resin foraging (Leonhardt and Blüthgen 2009). Finally, there is some evidence that certain species (e.g., *Plebeia emerina*) hoard resin stores,

possibly in preparation for periods of resin scarcity, or in preparation for increased predator or parasite pressure (dos Santos et al. 2009).

Resin collection is primarily carried out by worker bees (Bassindale 1955). Curiously, Boongird and Michener (2010) observed resin and pollen loads on the hind tibiae of male stingless bees from several species in Thailand (*Tetragonula fuscobalteata* (Cameron), *Tetragonula (Tetragonula) pagdeni* (Schwarz), *Tetragonula collina* (Smith), and *Heterotrigona (Tetrigona) apicalis* (Smith)). It is unclear whether and how resin-bearing males contribute to colony function, but since they were not seen depositing their loads in storage pots or on other nest structures, the authors concluded that male bees do not contribute significantly to resin foraging.

At an individual level, it is unclear what factors drive a forager to choose resin foraging over nectar or pollen foraging. In *Melipona beecheii*, Biesmeijer and Tóth (1998) found that half of observed foragers specialized in just one resource throughout their foraging career, and the other half alternated between pollen, nectar, resin, and mud (Biesmeijer and Toth 1998). This result is consistent with Inoue et al. (1985), who examined foraging behavior in three Sumatran stingless bee species (*Trigona (Tetragonula) minangkabau* (Sakagami and Inoue), *Trigona* (*Heterotrigona) itama* (Cockerell), and *Trigona (Trigonella) moorei* (Schwarz)) and found approximately 50% of foragers to be one-material specialists. In *A. mellifera*, foragers initiate resin collection when they detect a need for it inside the nest (e.g., by sensing a rough surface, crevice, or draft of cool air), and use the waggle dance to recruit additional resin foragers (Nakamura and Seeley 2006). It is unclear whether stingless bees respond to similar stimuli, and whether and how they recruit other bees to collect resin.

When foraging, stingless bees use both visual and olfactory cues to discover and distinguish between resin sources. Specifically, they home in on particular combinations of volatile mono- and sesquiterpenes (Leonhardt et al. 2010). This sensory capacity allows stingless bees to discover new resin sources quickly, sometimes locating artificially induced tree wounds within a matter of minutes (Howard 1985). When certain resin sources are highly preferred, as occurs in the seed-dispersal mutualism between the Eucalypt tree *Corymbia torelliana* and the stingless bee *T. carbonaria*, even minor

experimental modifications to a resin odor (i.e., changes in single mono- or sesquiterpenes) resulted in reduced visitation. This demonstrates that stingless bees are capable of learning complex scents and responding to multiple compounds within the resin bouquet, and may be more selective for resin sources than floral sources (Leonhardt 2017, Leonhardt et al. 2014).

After locating a resin source, stingless bees use their mandibles to gather resin from plant buds, leaves, flowers, or bark. They then use the tarsi and basitarsi on their front and middle legs to load this sticky material onto their corbiculae (Gastauer et al. 2011, Bassindale 1955) (see Supplementary Materials). They repeat this process until they have amassed a sizable resin load, which they carry back to the nest. Some stingless bees induce plants to secrete resin by biting plant tissues and collecting the resin that seeps from the resulting wound (Wille and Michener 1973, Reyes-González 2020). Howard (1985) reported that foragers of certain species can milk an active resin source for days or weeks at a time. In fact, since resin foraging can damage tissues, some stingless bees (e.g., *Trigona fuscipennis* and *Trigona nigerrima*) have been considered pests for agricultural crops (López-Guillón 2019). Many stingless bee species collect resin in groups and vigorously defend preferred resin resources. Some species have been observed fighting to the death over resin, stealing cerumen from other nests, or harvesting materials from abandoned nests (Leonhardt 2009, Howard 1985). Howard (1985) suggested that these behaviors indicate that resin is a precious resource for many stingless bee species, and that resin resource availability is likely a limiting factor for colony growth.

Stingless bees demonstrate clear preferences for some resin-producing plants, and neglect others (Drescher et al. 2014, Wallace and Lee 2010, Leonhardt and Blüthgen 2009). The factors that determine stingless bees' resin preferences are unknown. As discussed, bees may target certain plants based on the potency of the antimicrobial or repellent properties their resins possess (Drescher et al. 2014). Morphological parameters likely also dictate the resources that each species can access. Some minute species (e.g., *Trigona jatiformis*) seek out resin sources that are too small to be seen with the naked eye; the resin they collect is only identifiable once it has been accumulated in the

bees' corbiculae (Howard 1985). Larger bees likely neglect minute resin sources, but may be more likely to use their mandibles to induce plant injury to encourage resin flow. Smaller bees often take advantage of the resin sources tapped by larger species, either collecting alongside the larger bees, attempting to supplant them, or waiting until the larger bees have abandoned the resin source (Howard 1985). So far, these behaviors have been examined through the lens of competition, but the interdependence implicit in these interactions is both fascinating and noteworthy. The fact that certain bee species depend on other bee species for access to resin resources could have implications for stingless bee conservation.

## *Resin handling*

Once resin foragers return to the hive, they unload resin from their corbiculae on their own, or with the help of another worker (dos Santos et al. 2010, Gastauer et al. 2011, Bassindale 1955). The often brightly colored resin loads are mixed with wax to form cerumen, or incorporated into other nest structures. The terpenoid compounds in resin become oxidized over time, causing them to darken in color (Patricio et al. 2002). Unloading and processing resin is a laborious task; bees must be careful to manipulate this material without getting stuck. In one study, it took *Plebeia lucii* and *Frieseomelitta varia* foragers seven to thirteen minutes to unload resin back at the hive. Most of this time was spent removing resin residue from their tarsi (Gastauer et al. 2011).

How do stingless bees handle the sticky substance that they use to immobilize and kill their enemies without harming themselves? Stingless bee body parts do not appear to possess inherently anti-adhesive properties. Gastauer et al. (2013) used electron microscopy and adhesive force experiments to compare the mandibles of stingless bee *Tetragonisca angustula* and the trochanter of invader ant *Camponotus sericeiventris*. They determined that resin actually adheres more to the smooth bee mandible than it does to the scaled ant trochanter. This suggests that stingless bees must utilize a lubricating substance (e.g., secretions or nectar) to reduce adhesion of resin to mandibles.

Some studies suggest that the ability to produce lubricating substances and avoid adhesive hazards is associated with bee age and physiological development. In *T. angustula, Plebeia emerina,* and *Trigona (Hypotrigona) grihodoi,* resin handling is a task reserved for advanced-age workers (dos Santos et al. 2009, Bassindale 1955, Ferreira Grosso and Rolani Bego 2002). An examination of the head salivary and intramandibular gland morphology of *P. emerina* suggested that when workers reach a certain developmental stage, they begin to produce secretions that help maintain propolis viscosity and allow the bees to handle this material without getting stuck (dos Santos et al. 2009). The development of the head salivary and intramandibular glands late in life does not occur in all stingless bee species, and may occur only in bees that maintain viscous propolis stores or deposit resins within the hive.

There is some evidence for a genetic basis for "propolis preparation"—presumably resin handling—in *Melipona quadrifasciata* (Waldschmidt et al. 1997). In one study, young bees (1–5 days old) from ten different source colonies were tagged and introduced into three different observation hives, with each observation hive containing bees from all ten source colonies. Their activities were observed for 35 days. Resin foraging was similar across source colonies, but bees from certain source colonies were significantly more prone to participate in propolis preparation (Waldschmidt et al. 1997). This study was limited in that observation colonies were made up of workers from a single age cohort. Stingless bee workers demonstrate plasticity, with workers changing tasks based on the needs of the colony. In several species, resin handling occurs late in life, so the lack of older bees in observation hives may have influenced the resin handling behavior of the young bees in this experiment. If this is the case, the higher incidence of propolis preparation observed in bees from certain source colonies may indicate higher levels of plasticity, and not necessarily a genetic predisposition to resin handling, but this possibility warrants further investigation.

## *Resin shapes the cuticular chemical profile of some stingless bees*

For some stingless bee species, the presence of resin inside the nest space can also influence the physical properties of the bees themselves. The outer layer of the insect cuticle is made up of lipid compounds that serve a variety of functions. These compounds help protect insects from predators, desiccation, and abrasion, and they also play a role in nestmate recognition and other forms of communication (Lockey et al. 1988). The so-called cuticular chemical profile consists of compounds that are polar (e.g., alcohols, esters, ketones, aldehydes, and oxidized terpenes) and non-polar (e.g., n-alkanes, alkenes, and methyl-branched alkanes) (Leonhardt et al. 2009). These compounds can be self-produced or environmentally acquired (Leonhardt et al. 2015). Some stingless bee species acquire certain cuticular compounds (e.g., terpenoids, such as mono-, sesqui- and triterpenes) from resin (Patricio et al. 2002, Leonhardt et al. 2009, Leonhardt et al. 2011a, Leonhardt et al. 2010, Leonhardt et al. 2011b).

Among social insects, stingless bees are the only group known to enrich their cuticular chemical profile with resin-derived compounds (Leonhardt 2017, Leonhardt et al. 2015). To our knowledge, this has not been examined in *A. mellifera*. This trait appears to have emerged separately in multiple stingless bee lineages. It occurs in more evolutionarily derived genera and is generally absent from more basal genera (e.g., *Melipona* and *Plebeia*), with at least one exception (Martin et al. 2017, Leonhardt et al. 2013). Despite overlap in foraging behavior (i.e., different species often utilize many of the same resin sources) the uptake of resin-derived compounds results in species-specific terpenoid profiles that are consistent across diverse geographic regions (Howard 1985, Leonhardt et al. 2009, Leonhardt et al. 2011a, Leonhardt et al. 2013, Leonhardt 2011b). The overlap between the nest entrance chemical profile and the cuticular chemical profile of multiple stingless bee species suggests that these compounds are most likely derived from the resin present in the nest environment (Leonhardt et al. 2009, Leonhardt et al. 2011b). Leonhardt et al. (2011b) suggested that some kind of filter mechanism must enable the uptake of certain compounds while excluding others. Resin collected from the corbiculae of stingless bees is not chemically different from resin

collected by researchers directly at resin wounds. Thus, if resin is modified, this must occur within the hive (Leonhardt 2011b). Different stingless bee species may possess different enzymes or microbial associates that alter the incoming resins, resulting in the species-specific selective uptake of terpenoid compounds. Additionally, or alternatively, genetically determined species-specific differences in cuticular chemistry could determine which compounds 'bind' to the bee (Leonhardt 2011b). Further research is needed to understand how and why certain resin-derived compounds enrich the cuticular chemical profile of certain stingless bee species, and to further elucidate the implications this has for colony function.

Recent studies have demonstrated that a resin-enriched cuticular chemical profile can help protect bees from predators and may reduce interspecific aggression, facilitating nest aggregations. Resin confers repellant properties to the cuticle of some stingless bee species, adding to the effects of the genetically determined repellent compounds that the bees produce themselves (Leonhardt 2015). The repellent properties of cuticular terpenoids were observed in a study that compared two species—*Tetragonula carbonaria*, a bee that collects extensive amounts of resin, whose cuticular compounds are 50% resin-derived, and *Austroplebeia australis*, a bee that collects minimal resin, whose cuticular compounds are just 1% resin-derived. In behavioral assays, high-resin *T. carbonaria* bees repelled predator ants, but low-resin *A. australis* did not. Washing both bee species diminished the ants' preference, suggesting that repellant properties can be attributed to the resin-derived compounds found on the bees' cuticle (Leonhardt et al. 2015).

Resin-derived terpenes present in the cuticle might also help facilitate nest aggregations. These compounds may mask chemical differences between bee species, contributing to reduced interspecific aggression (Leonhardt et al. 2011). One study compared aggressive behaviors between bees from the same nest aggregation, different aggregations, and non-aggregated nests, and found that aggression was reduced between bees from associated colonies (Leonhardt et al. 2010). The authors hypothesized that the presence of resin-derived terpenoids, specifically sesquiterpenes, mediates reduced aggression. They experimentally manipulated the chemical profile of *Tetragonula*

*melanocephala*, a stingless bee whose cuticle lacks sesquiterpenes, and found that applying either pure sesquiterpenes or an extract derived from the sesquiterpene-rich cuticle of *Tetragonula collina* (an unusually peaceable bee) to non-nestmates resulted in decreased aggression. Based on these results, the authors suggested that sesquiterpenes may facilitate nesting aggregations in tropical environments, but more research is needed to determine the precise role that resin-derived compounds play in mediating complex inter- and intraspecies interactions.

While it is clear that resin-derived compounds contribute to the cuticular chemical profile of many stingless bee species, and this profile is thought to influence nestmate recognition (Nunes et al. 2011, Jungnickel et al. 2004), the impact of resin on nestmate recognition is less clear. When Jones et al. (2012) exposed *Tetragonisca angustula* workers to extracts made from nestmate and non-nestmate resin or wax, all treatments resulted in decreased acceptance rates, regardless of the material source. In the same study, Jones et al. (2012) transferred resin stores from donor colonies to recipient colonies to determine whether bees use in-hive resin stores as a reference for recognition cues. They observed decreased acceptance of nestmates in donor colonies after interference, but no change in non-nestmate acceptance by donors. They also observed increased acceptance of non-nestmates in recipient colonies, and general guard confusion. Based on these results, the authors concluded that wax and resin do not contribute to nestmate recognition in *T. angustula*. However, it is possible that the artificial transfer of resin-derived compounds (i.e., exposing bees to resin-enriched hexane extract rather than raw wax or resin) impacted these results. Similarly, conducting behavioral assays only a short time after transferring resin stores from donor colonies to recipient colonies could have led to increased defensive behavior, muddling the nestmate recognition findings. Further studies are needed to determine the potential relationship between nest materials and nestmate recognition in stingless bees.

## *Microbiota associated with stingless bees*

There is growing interest in sequencing and understanding the functional significance of the microbial communities associated with stingless bees and their nests (Kwong et al. 2017, Cerqueira et al. 2021, Leonhardt and Kaltenpoth 2014, Ngalimat et al. 2019, Ngalimat et al. 2020, de Sousa 2021). The antimicrobial activity of stingless bee resin has been studied extensively in a human health context (Popova et al. 2021, Campos et al. 2015, Al-Hatamleh et al. 2020, da Cunha et al. 2013, Franchin et al. 2012, Aparecida Sanches et al. 2017), and much stingless bee research mentions, in passing, that resin likely plays a role in shaping the microbiota inside the nest. However, despite the demonstrated importance of bacteria and fungi to stingless bee colony function (Paludo et al. 2018, Menezes et al. 2015, Machado 1971), and the assumed importance of resin in maintaining microbial balance (Popova et al. 2021, Roubik 1989), whether and how resin modulates the microbial communities associated with stingless bees and their nest spaces is understudied.

Recent studies provide some insight into these complex interactions. In *A. mellifera* colonies, researchers have found that the presence of a propolis envelope stabilizes the microbial communities found in bees' guts and on the cuticle of their mouthparts. The propolis envelope is thought to support the proliferation of putatively beneficial bacterial associates, and reduce the expression of pathogenic or opportunistic microbes (Dalenberg et al. 2020, Saelao et al. 2020). However, the role of resin in shaping the microbiota associated with stingless bees (e.g., cuticular, gut, whole-bee, and nest microbiomes) is less clear.

One recent study compared the bacterial communities associated with the interior nest surfaces of four stingless bee species (*Frieseomelitta varia, Melipona quadrifasciata*, *Tetragonisca angustula,* and *Trigona spinipes*) (de Sousa 2021). Differences in these bacterial communities were attributed, in part, to the diverse materials that each species uses in nest construction (e.g., clay, resin, wax, and feces). Unfortunately, this study did not include a detailed characterization of nest architecture for the species in question, and it is unclear which surfaces were swabbed for bacteria.

Nevertheless, this study points toward the importance of understanding the complex microbial ecosystems that exist within the stingless bee nest, and the broader role of nest construction materials, such as resin, in shaping those ecosystems.

Leonhardt and Kaltenpoth (2014) used sequencing to characterize the microbiota associated with three sympatric Australian stingless bee species, two that incorporate large quantities of resin in their nest (*Tetragonula carbonaria* and *Trigona hockingsii*), and one that uses almost no resin (*Austroplebeia australis*). DNA was extracted from six worker bees from ten different colonies, and whole-bee microbiomes were compared. Species-specific differences in microbial communities were observed. However, more species must be sampled to determine whether these changes can be attributed to the presence or absence of resin. Moreover, since many additional species-specific factors (e.g., genotype, diet, external environment, and nest construction materials) can influence microbial communities (de Sousa 2021, Bahrndorff 2016), within species comparisons (i.e., comparing the microbial communities associated with high-resin colonies vs. low-resin colonies, as occurred in *A. mellifera* studies) may be instructive.

Another study examined the rate of mold growth on the bodies of some of the same high-resin (*T. carbonaria*) and low-resin (*A. australis*) bees, to determine whether a resin-rich environment confers antimicrobial properties to the stingless bee cuticle (Leonhardt et al. 2015). In this study, the rate of mold growth was not found to differ between species. However, it is possible that resin-poor *A. australis* has evolved compensatory physiological traits (e.g., increased secretion of self-produced antimicrobials) to replace resin resources, as proposed by Roubik (1989). If this is the case, the cuticle of both the resin-rich and resin-poor species should possess antimicrobial compounds that inhibit the growth of mold, with the difference being the source (self-produced versus environmentally acquired). Further studies are needed to elucidate the impact of resin on the bacteria and fungi naturally present on the stingless bee cuticle and within the stingless bee nest, and to determine how the presence of resin relates to self-produced antimicrobial compounds.

Perhaps the most compelling example of the importance of microbial associates to colony function is the mutualism between the stingless bee *Scaptotrigona depilis* and a

fungus of the genus *Zygosaccharomyces* (Paludo et al. 2018, Menezes et al. 2015). This fungus exists in a dormant state in the cerumen of *S. depilis*colonies. When it comes into contact with the liquid larval food found inside the brood cells, it enters a growth phase, extending visible white mycelia from the brood cell wall towards the larval food supply. Originally thought to be pathogenic, these mycelia actually produce steroid precursors that *S. depilis* larvae require for pupation (Paludo et al. 2018). Since resin is a key ingredient in cerumen and since it inhibits the growth of some but not all microbes, it is likely that the presence of resin helps support the growth of *Zygosaccharomyces*. This mutualism is just one visible example of countless probable stingless bee-microbe associations that could prove essential to colony function.

Though further research is needed, this evidence, alongside recent discoveries demonstrating that propolis helps shape the gut and mouthpart microbiomes of *A. mellifera*, suggests that resin may help stabilize and/or support microbial communities that could prove essential to stingless bee colony function.

## Future studies

Existing research demonstrates that resin use is vital to stingless bee colony function. Resin is essential to nest construction and defense, and many species invest substantial effort in resin foraging and handling. Resin-derived compounds influence the cuticular chemical profiles of many stingless bee species, and resin likely shapes the microbial communities associated with stingless bees and their nests. Further research is needed in each of these individual areas, and at their intersections.

What are the causes and consequences of different levels of resin use by different species, and by different colonies of the same species? Some stingless bees invest vast amounts of energy in resin collection, some collect only the minimum necessary to build nest structures, and there is at least one example of a stingless bee that does not use resin at all (Camargo and Pedro 2002). Differences in resin use can occur even when many major variables (e.g., species, location, and hive structure) remain constant. Roubik (2006) attributed individual variation in nest architecture—including the thickness of the

resinous batumen surrounding the nest—to three possible causes: (1) nest age, (2) bee genetics, and (3) micro-environment (e.g., predators, parasites, symbionts, rain, wind, and sun). Further studies are needed to evaluate the effects of each of these factors on resin use, and to determine how differences in resin use impact colony function, cuticular chemical profile, and the microbial communities associated with stingless bees. For example, if resin contributes significantly to colony function, do low-resin species or low-resin colonies compensate physiologically for the lack of resin in their space (e.g., through increased antimicrobial secretions or increased diversification of self-produced cuticular chemical compounds) (Roubik et al. 1989)? More broadly, might examining tradeoffs between the secretion of antimicrobial compounds and the collection of antimicrobial materials help inform our understanding of the evolution of social insects? For example, is it possible that intensive resin use emerged in stingless bees following the loss of the stinging apparatus, and the antimicrobial venom that may have accompanied it (Baracchi and Tragust 2017)? Mixing secreted and collected materials for nest construction is common in invertebrates, but the selective pressures that favor secretion versus collection are poorly understood, and have not been examined in stingless bees (Hansell and Ruxton 2013).

How does the presence and prevalence of resin in the nest space impact other aspects of the stingless bee nest ecosystem and colony function? Since resin is present throughout the nest in the form of cerumen and is in direct contact with both brood and food stores, it is possible that resin-derived compounds may leech into stingless bee honey and pollen, enriching these food sources with phytochemicals (Leonhardt 2017). Honey produced by *A. mellifera* has been found to contain phytochemicals that likely originate from propolis (Berenbaum and Calla 2021). Does resin also contribute phytochemicals to stingless bee honey? Does the amount of resin that bees incorporate in the nest environment affect the quantity or type of resin-derived compounds found in the honey? If resin-derived phytochemicals are an important part of the stingless bee diet, then how might certain beekeeping practices (e.g., introducing sugar syrup or *A. mellifera* honey in periods of dearth, or removing excess resin stores from a colony to facilitate colony management) impact stingless bee health?

As previously mentioned, the microbiota associated with stingless bees and their nests is a vast and fascinating area, the advancement of which could inform questions relating to the chemical ecology of stingless bees, among other aspects of colony function. For example, do the resin-derived compounds that comprise the cuticular chemical profiles of some stingless bee species impact their cuticular microbiome? How does this affect the ability of the cuticular chemical profile to repel predators, reduce aggression, etc.? Does the cuticular microbiome, in turn, influence the cuticular chemical profile? Does resin help shape the microbiota associated with stingless bees and their nests? How does this impact colony function?

There are interesting points of overlap—and important differences—in resin use by honey bees and stingless bees. These points of comparison could inform future research in both study systems. For instance, does resin use constitute a social immunity mechanism in stingless bees (Simone et al. 2009)? Does resin use inhibit the growth of microbial pathogens within the stingless bee nest space? Does the presence of resin help modulate the stingless bee immune system, as occurs in *A. mellifera*? If so, how does immune expression compare in high-resin and low-resin species? Future stingless bee research could draw from recent honey bee research (Simone-Finstrom and Spivak 2010, Simone-Finstrom and Spivak 2017) to investigate these questions. In turn, honey bee research could draw from stingless bee research to investigate, for example, whether *A. mellifera* foragers use olfactory cues to locate resin sources, as occurs in stingless bees, and whether the presence of a propolis envelope influences the cuticular chemical profile of *A. mellifera*.

Underlying all of these questions is the need for more research on the natural history of stingless bees. Many of the predominant natural history studies in this field date back over half a century and cover a relatively small number of species. While informative, these studies cannot be considered representative because stingless bees are so diverse, and resin use is such a variable trait. More research is needed to add further breadth to the foundational studies that figure so strongly into current conceptions of resin use in stingless bees. In this pursuit, and in other areas of future research, there is an important opportunity to partner with and follow the leadership of the stingless beekeepers,

indigenous communities, and stewards of local ecological knowledge that have been in relationship with stingless bees for generations.

## *Conclusions*

Understanding the role of resin use in stingless bee colony function grows increasingly urgent as changes in beekeeping and land use practices occur, potentially diminishing stingless bees' ability to incorporate resin into their nest environment (Suryanarayanan and Beilin 2020, Cortopassi-Laurino et al. 2006, Drescher et al. 2014). In recent decades, the massification of beekeeping operations and the transportation of stingless bee colonies to monocrop fields for pollination services has expanded (Slaa et al. 2006), and—among other deleterious effects—these changes could limit bees' access to diverse resin sources, potentially inhibiting nest construction and defense and influencing their cuticular chemical profiles (Kämper et al. 2019) and microbial associates (Hall et al. 2021). Bees are already known to substitute resin for human-made products such as wet paint, adhesives, and asphalt (Roubik 2006, Alqarni et al. 2015). As the role of resin likely extends beyond its adhesive properties, the extent to which such substitutions—potentially driven by resin resource scarcity—impact stingless bee colony function in the long term is cause for concern.

A deeper understanding of the importance of resin use for stingless bee colony function could lend support to the conservation of resin-rich non-floral resources that might otherwise be overlooked (Requier and Leonhardt 2020). Since bees depend on diverse resin sources to carry out a variety of functions, targeted conservation efforts could bolster stingless bees' ability to defend against pathogens, parasites, and predators, and support colony health in ways we cannot yet anticipate (Drescher et al. 2014). In this context, it is crucial to review and expand upon the many varied studies of resin use in stingless bees so we can understand and appreciate its importance for colony function and stingless bee health.

# Chapter 5: Beekeeping practices impact resin collection and foraging dynamics in stingless bee *Scaptotrigona mexicana* Guérin [9]

## Abstract

Meliponiculture, or stingless beekeeping, is a practice that dates back millennia. In recent years, meliponiculture, has grown increasingly popular, and a growing number of beekeepers are keeping stingless bee colonies in standardized, smooth wood boxes. While these boxes do provide ready access to the inner workings of the brood nest and allow beekeepers to attend to forage shortages and pest pressure, they may inadvertently result in reduced resin collection. A large number of social and solitary bees use resin for a variety of purposes, including to protect their nests from predators and pathogens. Though resin is ubiquitous in most stingless bee nests, the role of resin in stingless bee social immunity is unknown. Our study sought (A) to examine the impacts of box type and hive placement on foraging dynamics in *Scaptotrigona mexicana,* with a particular focus on resin collection, and (B) to determine whether the amount or manipulation of propolis stores inside a nest space impacts resin foraging and colony growth. We monitored resin collection, colony development, and drift over the course of one year in smooth-interior wood boxes, rough-interior wood boxes with vertical cuts designed to mimic the cracks and crevices found in a hollow tree cavity, and smooth, thin boxes designed to test the hypothesis that bees use propolis to insulate against temperature change. We also added or removed propolis stores from a second set of colonies and monitored the effect of propolis manipulation on resin foraging and colony development over the course of one year. We found that the use of rough-interior wood boxes leads to increased resin collection, but we did not detect an effect of increased resin collection on colony weight. We also found that both propolis manipulation in general, and propolis

[9] The studies described in this chapter were conducted in collaboration with Miguel Guzmán Díaz, Erik de Jesus Solórzano-Gordillo, Estefhanía López-Roblero, Héctor Morales Urbina, Rémy Benoît Vandame, and Marla Spivak.

removal specifically led to increased resin collection, a finding that could have important implications for beekeepers looking to sustainably harvest propolis for medicinal or commercial use.

## Introduction

Stingless bees (Apidae: Meliponini) comprise a diverse group of social, honey-producing bees native to tropical ecosystems (Roubik 2023). The practice of keeping, tending to, or stewarding stingless bee colonies is called meliponiculture (Chan Mutul et al. 2019). Indigenous and land-based communities have practiced meliponiculture for generations; in Mexico this practice dates back over 2,000 years (Paris et al. 2018, Reyes-González 2020). For many years, colonization drove such stark declines in meliponiculture that scholars once feared this rich biocultural legacy would be lost forever (Quezada-Euán et al. 2001, Villanueva-G et al. 2008). However, recent years have seen a resurgence in stingless beekeeping (Quezada-Euán et al. 2022). Today, meliponiculture is characterized by a wide variety of motivations (e.g., cultural, conservation, commercial) and management practices (Chan Mutul et al. 2019, Aldasoro Maya et al. 2023).

In traditional[10] meliponiculture, beekeepers often keep their bees in hollow log or clay pot hives and maintain minimally invasive management practices. These beekeepers typically open their hives a few times each year to harvest honey, pollen, or other hive products, or to move brood comb and food stores to a new hive to create a new colony. Though many beekeepers continue to practice traditional meliponiculture today, in recent decades, a growing number of beekeepers in Mexico and throughout tropical regions have begun to keep their bees in wood box hives, sometimes called “rational” or “technified” hives (Noguiera-Neto 1997, Sommeijer 1999). These multi-part boxes provide beekeepers ready access to the internal workings of the hive, and facilitate frequent, hands-on management interventions. They are designed to enhance colony

[10]Following Chan et al. (2019), here, the word traditional does not refer to something old or antiquated. Rather, it signals a practice that is rooted in the past and is passed from generation to generation.

productivity, facilitate pest control, and allow beekeepers to provide bees with supplemental food when forage resources are scarce (González-Acereto et al. 2006, Quezada-Euán et al. 2001, Soto-Leyva et al. 2009, Villanueva-G et al. 2008). However, while increased nest access may offer some beekeeping benefits, these smooth wood boxes – and the management practices that often accompany their use – could impede certain aspects of stingless bee behavior, such as resin collection.

Plants produce sticky, bioactive resins to mitigate predator and pathogen threats (Langenheim 2003). Honey bees (*Apis mellifera*), stingless bees and numerous other bee species collect these resins and bring them back to their nest spaces (Shanahan and Spivak 2022, Chui et al. 2021). Resin is ubiquitous in stingless bee nests. Stingless bees mix resin with self-produced wax to form propolis (low wax) and cerumen (higher wax). Propolis is often used to seal cracks and crevices, among other functions. Cerumen is used to build brood comb, honey pots, pollen pots, and other nest structures (Roubik 2006). For many stingless bee species, resin and resin-rich materials also support nest defense. Some species surround their nest entrances with sticky resin droplets to keep invaders out, others use resin balls to barricade their nest entrances, and others attack invaders with sticky resin globs (reviewed by Shanahan and Spivak 2022). Finally, since resin possesses antimicrobial properties, its presence in stingless bee nests is also thought to influence the microbiota associated with stingless bees, their resources, and their nest spaces (Roubik 2023).

In addition to contributing to nest structure and defense and likely shaping stingless bee microbial associates, resin may also contribute to stingless bee health. Numerous studies show that, in honey bees, the resins that bees mix with wax and incorporate into their nests in the form of propolis are an important component of social immunity and support colony health in a variety of ways (reviewed by Simone-Finstrom and Spivak 2017). Propolis has been found to modulate honey bee immune function (Borba et al. 2015) and mitigate pathogen threats (Simone-Finstrom and Spivak 2012, Pusceddu et al. 2021). The benefits of stingless bee propolis to human health have been studied extensively (reviewed by Zullkiflee et al. 2022), and stingless bee propolis samples have been found to possesses antimicrobial, antioxidant, antiviral,

antimutagenic, and cytotoxic properties (Popova et al. 2021). However, the role of propolis in supporting stingless bee health is poorly understood. This is concerning because, although there have so far been few reports of pathogens impacting stingless bees (Roubik 2023), mounting environmental stressors and increased trade of stingless bee colonies could facilitate stingless bee pathogen spread in the future (Quezada-Euán et al. 2022, Reyes-González et al. 2020, Villanueva-G et al. 2005). Further, management practices used in both honey bee and stingless bee-keeping contexts are known to diminish propolis use by honey bees, and could impact stingless bees in a similar way.

When honey bees nest in hollow tree cavities, the cracks and crevices found in those cavities stimulate bees to build a robust propolis envelope (Nakamura and Seeley 2006). Unfortunately, this important social immunity behavior is rarely supported in managed contexts. The smooth-interior wooden boxes commonly used in beekeeping contain few cracks and crevices. As a result, managed honey bees have few places to put their propolis, so most beekeepers' colonies are propolis-poor. Moreover, since propolis has long been perceived as a sticky inconvenience, the propolis that honey bees do manage to accumulate is sometimes scraped off of hive surfaces and discarded by beekeepers. In recent years, beekeepers and researchers have come to understand the importance of propolis to honey bee health and are now taking steps bring tree cavity textures into honey bee hive design to restore the propolis envelope (see Chapter 3; Hodges et al. 2018). Whether similar patterns[11] play out in stingless beekeeping is an open question. With the recent rapid spread of stingless beekeeping, stingless bees are increasingly kept in smooth wood boxes, or in hives made from materials that do not necessarily align with stingless bee biology (Quezada-Euán et al. 2022). Moreover, beekeepers do sometimes remove the thick layer of propolis that bees use to fuse these boxes closed, either to "clean" the box or to harvest the propolis for medicinal or commercial use. Might the use of smooth-interior wood boxes impact resin collection in

[11] Honey bees and stingless bees differ from each other in many important ways. Therefore, we must use caution when comparing these species/groups. Nevertheless, as honey bee beekeeping practices are increasingly adopted in stingless beekeeping contexts, understanding the impact of beekeeping practices on honey bees can help stingless beekeepers learn from the challenges their counterparts have faced.

stingless bees, as occurs in honey bees? Could propolis removal – through harvesting or "cleaning" – impact stingless bee colony health or development? Despite its centrality to stingless bee colony function, there are few studies examining resin use by stingless bees, and the impact of these practices is unknown.

Our study sought (A) to examine the impacts of box type and hive placement on foraging dynamics in *Scaptotrigona mexicana,* with a particular focus on resin collection, and (B) to determine whether the amount or manipulation of propolis stores inside a nest space impacts resin foraging and colony size. This study consisted of four components. (1) We monitored foraging behavior and colony development over the course of one year in standard, smooth wood boxes (control); boxes with vertical cuts designed to imitate the cracks and crevices found in a hollow tree cavity ("rough boxes"); and thin boxes to test the hypothesis that bees use propolis to insulate against temperature change ("thin boxes"). (2) We added or removed propolis stores from a second set of colonies, and monitored the effects of propolis manipulation on resin foraging and colony development over the course of one year. (3) Finally, we compared foraging and colony development in colonies located in the middle of hive rows, versus at the ends of hive rows, to determine whether drift was occurring between colonies.

## Methods

### *Colony set up*

Experiments were conducted from December 2019 – March 2022 using *S. mexicana* colonies managed by El Colegio de la Frontera Sur (ECOSUR) in three locations in the Soconuzco region of Chiapas, Mexico: near the towns of Cacahoatán (14.9958182, -92.1671288), and Tuxtla Chico (14.9374337, -92.1671288), and at the ECOSUR campus in Tapachula (14.888490, -92.277810). *Scaptotrigona mexicana* is a medium-sized (5.0-5.3 mm) stingless bee that forms colonies consisting of over 7,000 individuals (Ayala 1999, Arzaluz Gutierrez et al. 2002). *S. mexicana* colonies are commonly found in both

wild and managed contexts. Their distribution extends from Mexico to Costa Rica (Hurtado-Burillo et al. 2016).

Fifty *S. mexicana* colonies consisting of four brood combs, a laying queen, worker bees, and a small amount of empty honey and pollen pots were transferred to new hive boxes in December of 2019 and housed in two *palapas*, or open-air roofed structures. The first *palapa* contained thirty colonies used in the box type experiment. These were established in rough box (n = 10), thin box (n = 10), and standard, smooth wood box (n = 10; control) hives (Fig. 1). Hive design for all boxes was based on the Portugal-Araujo model (Guzmán-Díaz et al. 2011). Interior measurements for all hive components (brood chamber, extensions, supers) measured 16 x 16 x 10cm. Rough and control box walls were 2cm thick. Thin boxes walls were 1cm thick. Rough box grooves were 0.5 cm deep and 0.2 cm wide; each wall contained 12 grooves.

The second *palapa* contained twenty colonies used in the propolis manipulation experiment. These were established in standard, smooth wood hives, assigned to propolis-added (n = 10) or propolis-removed (n = 10) treatment groups. Drift was evaluated in the box type experiment colonies. In January of 2021, all colonies were evaluated to determine propolis deposition levels (scale of 0-100; see supplementary materials). Three propolis-rich (propolis score > 57) and three propolis-poor (propolis score < 38; see below) colonies were selected for each round of the mold-growth experiment. Colonies from round one (March 2021) were also used in round three (September 2021) to test for a dry season/rainy season effect.

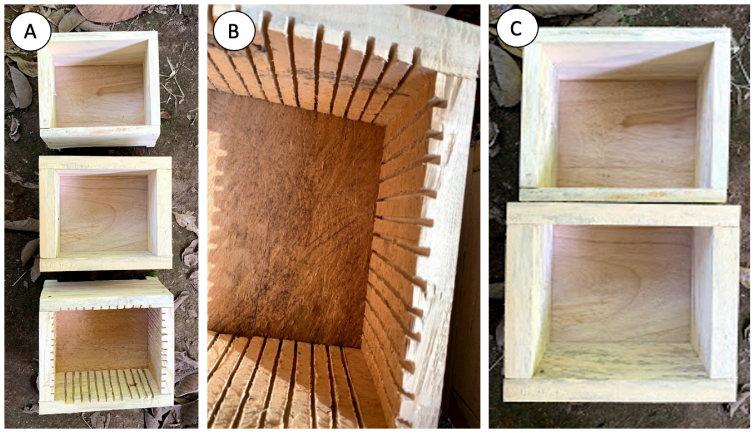


**Figure 5.1.** Thirty colonies were established in three hive types (A): rough box (B), thin box (C, top), and standard, smooth wood control (C, bottom) boxes. Interior measurements for all hives were 16 x 16 x 10cm. Rough and control box walls were 2cm thick. Thin boxes walls were 1cm thick. Rough boxes contained 12 grooves on each interior wall, measuring 0.5 cm deep and 0.2 cm wide.

## *Colony management*

Colonies were given routine management consisting of biweekly hive checks where queen status (queenright/queenless) and food stores were evaluated and colonies were monitored for the presence of phorid flies, a common stingless bee parasitoid and one of the principle drivers of colony loss. During the first month of colony establishment, colony food stores were supplemented with approximately 10g of honey per colony per week, a common beekeeping practice used to support small or weak stingless beekeeping colonies. Supplementary honey was poured into the empty cerumen honey pots that had been transferred to the hives at time of establishment. When phorid flies were present, a mesh net was used to trap and catch flies on the outer surface of the hive. Colonies that required a significant management intervention (e.g., supplementary brood combs to address queenlessness or boost a waning population) during the course of the year were excluded from analysis following intervention. In August of 2020 – ten months into colony development – phorid flies were invading colonies with substantial honey stores.

These stores were removed, and the weight of the honey harvested was added back to the total colony weight during analysis, so as not to penalize productive colonies.

## Drift experiment

### *Foraging behavior*

Foraging behavior and colony weight were monitored as in the "box type" colonies, described above. In March of 2020, when size discrepancies between colonies located at the ends of rows ("end colonies") and colonies located in the middle of rows ("middle colonies") were apparent, we painted colorful symbols on the front surfaces of each colony, to help bees locate their colonies of origin (Fig. 2).

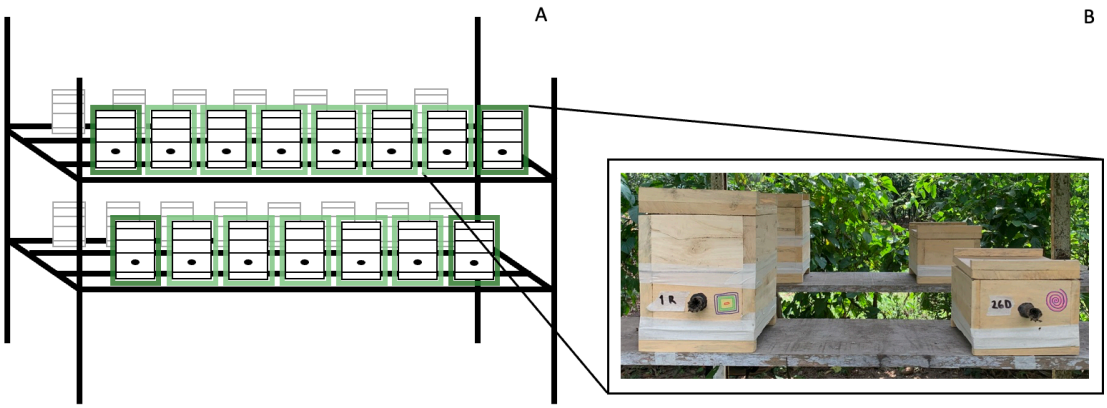


**Figure 5.2. Position of colonies in *meliponario* and markings made to mitigate drift.** Fifty *S. mexicana* colonies were kept together on two separate shelf structures in a *meliponario*, or stingless bee yard (A) under *palapas*, or open-air, roofed structures. Colonies were spaced 50 cm apart. Four months into colony development, colonies positioned at the ends of rows (dark green) had grown visibly larger than colonies positioned in the middle of rows (light green). In effort to mitigate possible drift between colonies, Colorful symbols were painted near the entrances on the fronts of the colonies in March of 2020, three months into colony development (B). Foraging behavior and colony weights were analyzed to determine whether drift was occurring, and whether the addition of colorful symbols impacted drift in any way.

### *Data analysis*

We constructed generalized linear models with negative binomial and Tweedie distributions to determine the impact of colony position on total number of returning foragers and colony weight, respectively. Negative binomial and Tweedie distributions were used to account for zero-inflated and skewed data distributions. Data visualizations suggested that for both box type and propolis manipulation colonies, colony weight differed between end and middle colonies from the beginning of the experiment until September 2020, so we analyzed weight differences prior to and following September 2020. Fixed effects included colony position (middle vs. end), sampling date, and the interaction between colony position and sampling date. Random effects included colony.

## Box type experiment

### *Foraging observations*

Foraging was monitored in *S. mexicana* colonies four times per week (twice/day for two days) in control, thin and rough box colonies, for one year. Nest entrances of colonies were plugged with mesh to prevent returning foragers from entering their hives. After ten minutes, bees at the nest entrance were collected using a net, and foragers bearing pollen, resin, or neither pollen nor resin were counted. Foragers that landed at the nest entrance immediately after netting were also counted.

### *Colony-level measurements*

Control, thin, and rough box colonies were evaluated on a regular basis over the course of one year. Evaluation metrics included colony weight (tared weight of each hive component), the percent of each hive body occupied by brood comb or food reserves, the diameter and height of the brood comb, the number of involucrum sheets surrounding the

brood comb, the number of resin balls visible on the surface of the involucrum, the length and diameter of the colony entrance, and the strength of the population on a scale from 1-3 (1 = weak, 2 = medium, 3 = strong). Propolis deposition was also evaluated; the floor, walls, corners, and underside of the colony lid were examined for the presence of propolis (Fig. 3). Each surface was scored on a scale from 1-10, where 1 = 0-10% coverage and 10 = 90-100% coverage. Over time, the floor and walls of lower boxes were obscured by the construction of brood combs, honey pots, pollen pots, and involucrum. When these surfaces were obscured, we used the score from the last feasible observation to calculate propolis coverage in those areas.

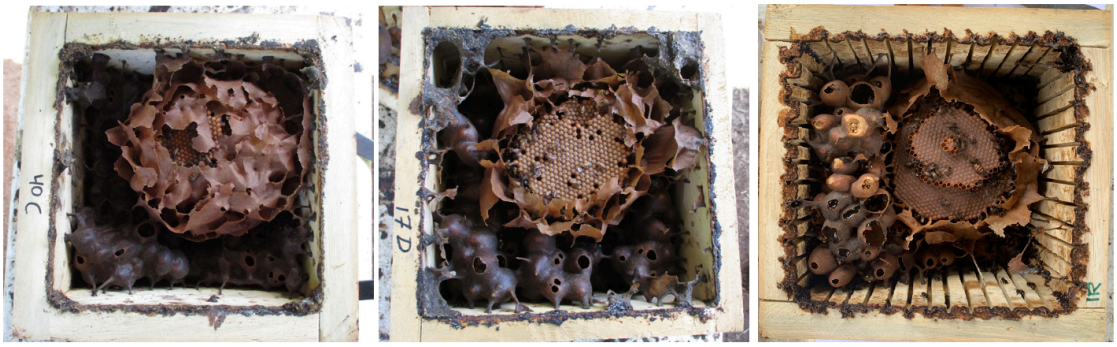

**Figure 5.3. Propolis deposition in control, thin, and rough box colonies.** Propolis deposition in control (n = 7), thin (n = 8), and rough box (n = 7) *S. mexicana* colonies was scored on a biweekly basis. Hive walls, floor, lid, and corners were examined for the presence of propolis; each surface received a score on a scale from 0-5, where 0 is no propolis and 5 is 100% coverage. Scores for each surface were added together to create a cumulative colony propolis score**.**

Opening hives exposes colonies to phorid fly invasion; this threat is particularly acute during the rainy season. As a result, evaluations were conducted on a biweekly basis during the dry season (roughly November-April), and on a monthly basis during the rainy season (roughly May-October). External metrics (colony weight and colony entrance measurements) were recorded every two weeks during the rainy season since these measurements require only minimal colony disturbance.

### *Data analysis*

Since colony position significantly impacted number of returning foragers and colony weight, end colonies were excluded from box type analysis. Because resin collection is positively correlated with colony size (Hilário et al. 2000), the number of resin foragers was divided by total brood area (brood comb radius$^2$ x brood comb height) to create a resin collection metric relative to colony size. We generated a generalized linear model to determine the impact of box type on resin collection. Our model used a Tweedie distribution to account for the disproportionate number of zeros in our data. Fixed effects included box type and sampling date. Because the Durbin-Watson test indicated positive temporal autocorrelation, we blocked the data by colony and used an autoregressive (AR1) autocorrelation structure. We also calculated estimated marginal means to determine differences in the mean number of resin foragers/brood area across box type over the course of the year. Finally, we generated a generalized linear model with a Poisson distribution to determine the effect of box type on propolis deposition. Fixed effects included box type, sampling date, and colony weight. Colony was included as a random effect.

## Propolis manipulation experiment

### *Propolis manipulation*

Propolis was manipulated once per month for three months during the dry season (January, February, March), and once during the rainy season (September). Propolis manipulation was suspended after September due to phorid fly pressure. For propolis-removed colonies, propolis was scraped from the underside of hive lids, where bees had deposited it in the crack between the box and the lid. 0.1-3.8 grams of propolis were removed per colony, leaving enough propolis behind so that colonies could fully reseal their hives following intervention. For propolis-added colonies, 10g of propolis (a

combination of propolis collected from propolis-removed colonies, and propolis collected prior to the start of the experiment) were divided into 1g portions and stuck to the underside of the lid. Smooth wood box colonies from the box type experiment were used as a control. Control colonies were opened and evaluated with the same frequency and timing as propolis-added and propolis-removed colonies, but propolis was neither removed from nor added to controls.

### *Foraging observations*

Foraging was recorded one day prior to propolis manipulation, and 1-2 days following propolis manipulation. Foragers were collected and counted using the same protocol used in the box type experiment.

### *Colony-level measurements*

Propolis-added and propolis-removed hives were evaluated on a biweekly-monthly basis using the same protocol used in the box type experiment.

### *Data analysis*

Since colony position significantly impacted the number of returning foragers and colony weight, end colonies were excluded from propolis manipulation analysis. We used paired and unpaired Wilcoxon rank-sum tests to compare resin collection/brood area between treatment groups, within sampling round (unpaired), and within treatment groups, between sampling round (paired). We also generated a generalized linear model with a Tweedie distribution to determine the impact of resin manipulation on weight. Fixed effects included intervention (e.g., propolis-added, propolis-removed, control), and sampling date. Because the Durbin-Watson test indicated positive temporal

autocorrelation, we blocked the data by colony and used an autoregressive (AR1) autocorrelation structure.

## Results

### *Foraging behavior*

Foraging behavior was monitored in thirty *S. mexicana* colonies over the course of one year (Fig. 4). From January to July of 2020, the majority of foragers returned to the nest carrying neither resin nor pollen and were likely mostly nectar or water foragers. Nectar/water foraging decreased sharply in June of 2020. From October of 2020 through January of 2021, the number of nectar/water foragers was similar to the number of pollen and resin foragers, averaging around 20 foragers per resource. Peaks in pollen foraging in January and March of 2020 corresponded with the coffee bloom. Later in the season, increases in pollen foraging appeared to directly precede surges in brood rearing.

Resin collection steadily increased over the course of the study year. Notably, numbers of resin, pollen, and nectar/water foragers observed were nearly equal in October of 2020, at the beginning of the dry season. Resin foraging exceeded pollen and nectar foraging in late December of 2020.

Colony weight tended to increase over time, though it plateaued during the first few months of the rainy season (May of 2020 – July of 2020) and then increased sharply in August of 2020. This increase may have corresponded with an increase in brood rearing, rather than an increase in resource intake.

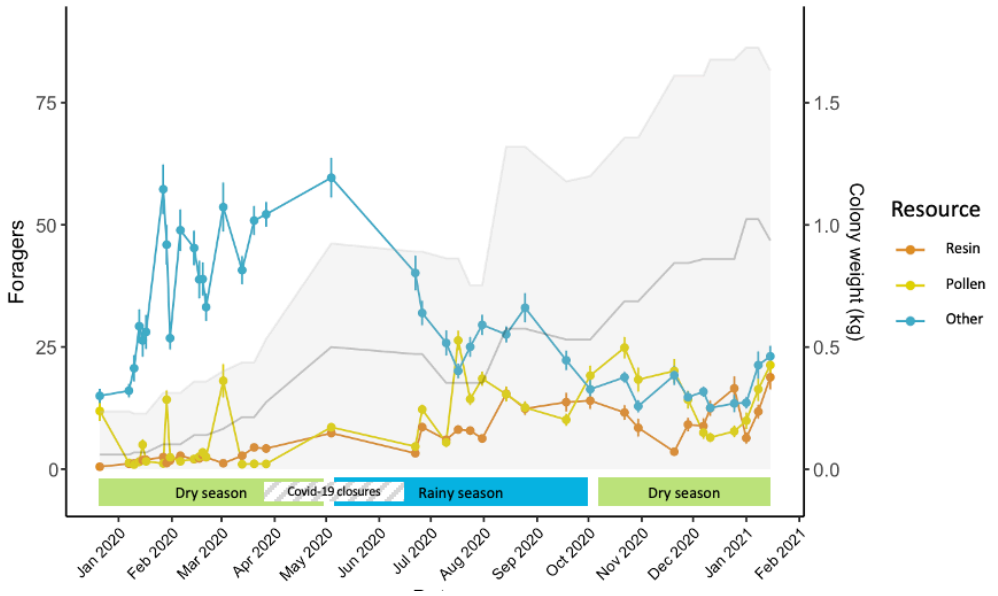


**Figure 5.4. Foraging behavior, colony weight, and weight of brood comb from December of 2019 to February of 2021.** Thirty colonies consisting of four brood combs, a laying queen, worker bees, and a small amount of honey and pollen resources were transferred to new hive boxes in January of 2020. Foraging was monitored four times per week for one year. Nest entrances of colonies were plugged with mesh to prevent returning bees from entering their colonies. After ten minutes, bees at the nest entrance were collected using a net, and foragers bearing pollen, resin, or neither pollen nor resin were counted. Foragers that landed at the nest entrance immediately after netting were also counted. Colony weight and percent of the hive box occupied by brood comb were also monitored 1-2 times per month. Resin collection was on par with pollen collection for much of the year. From January-March of 2020, field observations indicated that peaks in pollen foraging corresponded with the coffee bloom. Increase in colony weight beginning in August of 2020 seem to result from an uptick in brood-rearing. Mean number of foragers ± standard error is shown for each resource, for each sampling date. Mean colony weight is shown in light grey; mean brood comb weight (total weight x percent hive occupied by brood comb) is shown in dark grey.

## *Drift*

Colony position significantly impacted total number of returning foragers throughout the year ($\chi^2 = 13.5$, df = 1, p = 0.0002). It also impacted colony weight prior to September 2020 (F(1, 26) = 19.6, p = 0.0002); end colonies had more foragers and were heavier than

middle colonies (Fig. 5). Differences between middle and end colony weight did diminish over time. After September of 2020, colony position did not impact colony weight ($F(1, 26) = 0.1$, $p = 0.75$).

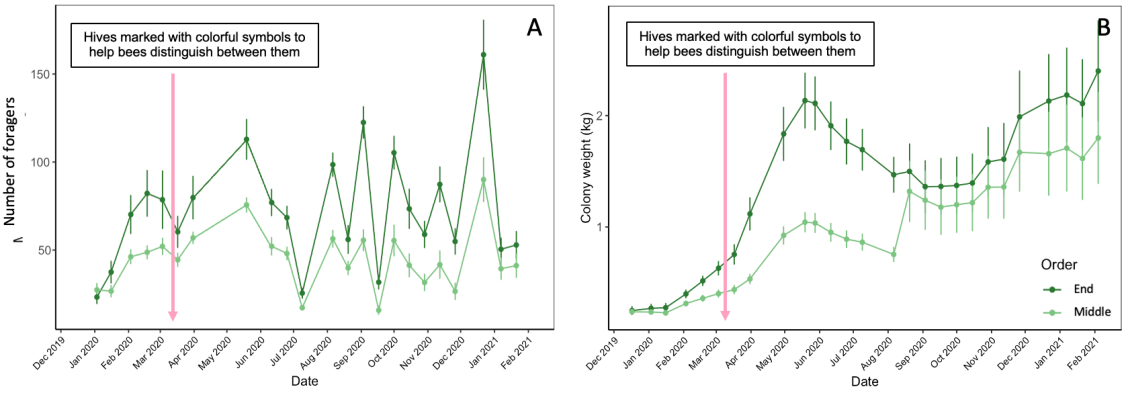


**Figure 5.5. Mean number of foragers and mean colony weight for colonies positioned in the middle or at the end of *meliponario* rows.** Foraging was monitored in *S. mexicana* colonies four times per week for one year. Nest entrances from colonies located at the end of rows (n = 8) and colonies located in the middle of rows (n = 22) were plugged with mesh to prevent returning foragers from entering their colonies. After ten minutes, bees at the nest entrance were collected using a net, and foragers were counted. Foragers that landed at the nest entrance immediately after netting were also counted. Our mixed-effects models indicated colony position significantly impacted number of returning foragers ($\chi^2 = 13.5$, df = 1, $p = 0.0002$), with end colonies having more foragers. Prior to September of 2020, colony position significantly impacted colony weight $F(1, 26) = 19.6$, $p = 0.0002$). After September of 2020, colony position did not impact colony weight ($F(1, 26) = 0.1$, $p = 0.75$). Colonies were marked with colorful symbols in March 2020 to mitigate drift, but this did not seem to have an effect. Similarities in colony weight following September 2020 may have been due to the increase in brood rearing that occurred at this time. Mean number of foragers (A) and mean colony weight (B) ± standard error are shown for end and middle colonies, for each sampling date.

## *Effect of box type on resin foraging and propolis deposition*

There were significant effects of box type on number of resin foragers/brood area ($\chi^2 = 11.9$, df = 2, $p = 0.003$) and propolis deposition ($\chi^2 = 10.5$, df = 2, $p = 0.005$) (Figs. 6 and 7). Mean number of resin foragers/brood area was higher in rough box colonies compared to control colonies by a margin of 0.011 foragers/$cm^3$ of brood comb, corresponding to a

57% increase (Z = -2.9, p = 0.0098). For a colony with a medium-sized brood comb measuring 162 $cm^3$ this would translate to a difference of 1.8 foragers/colony for each 20-minute observation period. If resin foraging occurs over the course of eight hours per day, this would translate to an additional 43 resin foragers/day in rough box colonies, compared to control colonies. Increased resin collection by rough box colonies was reflected in propolis deposition score results; mean propolis deposition score was higher in rough box colonies than in control colonies, by a near-significant margin of 1.4 points (Z = -2.3, p = 0.06) (Fig. 7).

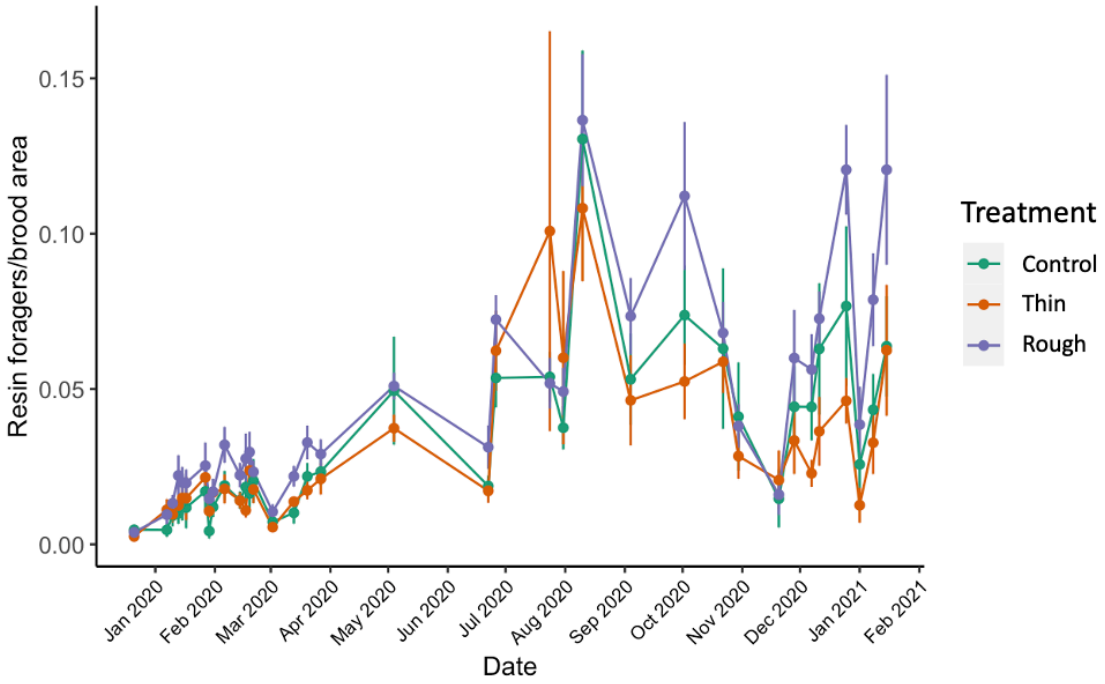


**Figure 5.6. Resin foragers/brood area for control, rough box, and thin box colonies from December 2019 to February 2021.** Resin foraging and nest size were monitored in *S. mexicana* colonies over the course of one year. Resin foraging was monitored twice per day, two days per week for one year. Nest entrances in control (n = 7), and thin (n = 8) and rough box (n = 7) colonies were plugged with mesh to prevent returning foragers from entering their colonies. After ten minutes, bees at the nest entrance were collected using a net, and foragers bearing resin, pollen, neither resource were counted. Foragers that landed at the nest entrance immediately after netting were also counted. Diameter and height of the brood comb were measured on a biweekly basis to calculate brood area. Mean number of resin foragers was divided by brood area at nearest evaluation date. Our generalized linear model showed a significant effect

of box type on number of resin foragers/brood area ($\chi^2 = 11.9$, df = 2, p = 0.003); mean resin collection/brood area was significantly higher in rough box colonies compared to control colonies (Z = -2.9, p = 0.0098), by a margin of 0.011 foragers/cm$^3$ of brood comb. Mean number of foragers/brood area ± standard error are shown for control, rough, and thin box colonies, for each sampling date.

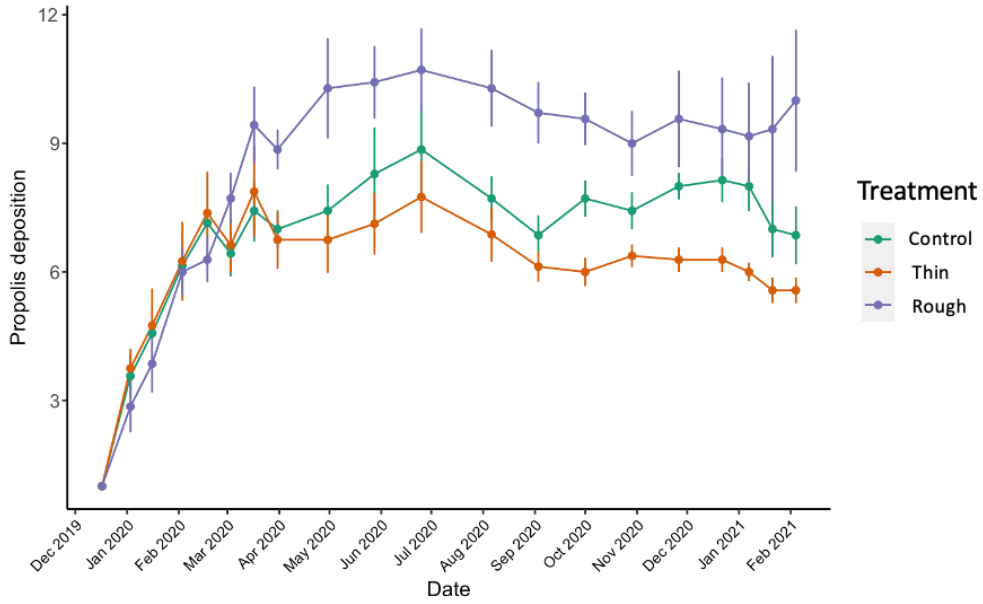


**Figure 5.7. Propolis deposition in control, thin, and rough box colonies from December of 2019 to February of 2021.** Control (n = 7), thin (n = 8), and rough box (n = 7) *S. mexicana* colonies were evaluated on a biweekly basis for propolis deposition, among other metrics. Our generalized linear model indicated a significant effect of box type on propolis deposition score ($\chi^2 = 10.5$, df = 2, p = 0.005); mean propolis deposition score was higher in rough box colonies than in control colonies, by a near-significant margin of 1.4 points (Z = -2.3, p = 0.06).

## *Effect of propolis manipulation on resin foraging*

Manipulating propolis stores affected resin collection (number of resin foragers/brood area). There were no differences in resin collection between treatments during round one (January), but during rounds two and three, resin-removed and resin-added colonies

exhibited significantly higher resin collection than control colonies, both before and after disruption (Fig. 8, Table 1).

The amount of propolis present inside the colony also appeared to influence resin collection. During round two, prior to disruption, resin collection was significantly higher in propolis-added colonies compared to propolis-removed colonies prior (Fig. 8, Table 1). In addition, during rounds two and three, resin collection increased in propolis-removed colonies immediately following propolis removal (Fig. 8, Table 2), but stayed the same in control and propolis-added colonies.

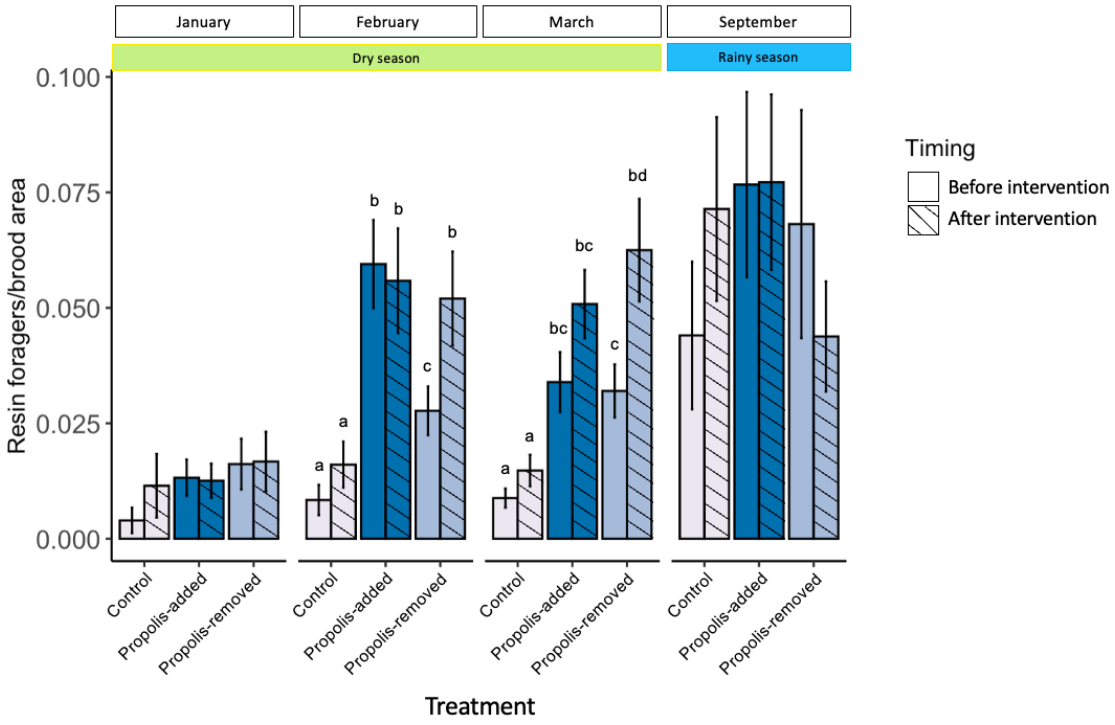


**Figure 5.8. Resin foraging in propolis-added, propolis-removed, and control colonies before and after intervention.** Propolis was added (n = 8; 10g/colony), removed (n = 8; 0.1-3.8g/colony), or not manipulated (n = 7) in 23 *S. mexicana* colonies once per month for the first three months of colony development (which coincided with the dry season), and once nine months into colony development, during the rainy season. Resin foraging was monitored one day before and 1-2 days after propolis manipulation. Wilcoxon rank sum tests (Tables 1 and 2) indicated that there were no differences in resin collection between treatments during round one (January), but by rounds two (February) and three (March), propolis-removed and propolis-added colonies demonstrated significantly increased resin collection compared to control colonies, both before and after disruption. In addition, resin collection was

significantly higher in propolis-added colonies compared to propolis-removed colonies before disruption. Finally, resin collection increased in propolis-removed colonies following propolis removal in rounds two and three. Mean number of resin foragers/brood area ± standard error is shown for each treatment, for each sampling date. Letters indicate significant differences in number of resin foragers.

| Round | Treatment 1 | Treatment 2 | Timing | p-value | Significance |
|---|---|---|---|---|---|
| 1 | Control | Propolis-added | 1 | 0.0840 | |
| | Control | Propolis-removed | 1 | 0.0810 | |
| | Propolis-added | Propolis-removed | 1 | 0.9665 | |
| | Control | Propolis-added | 2 | 0.9665 | |
| | Control | Propolis-removed | 2 | 0.4266 | |
| | Propolis-added | Propolis-removed | 2 | 0.8816 | |
| 2 | Control | Propolis-added | 1 | 0.0001 | * |
| | Control | Propolis-removed | 1 | 0.0150 | * |
| | Propolis-added | Propolis-removed | 1 | 0.0106 | * |
| | Control | Propolis-added | 2 | 0.0225 | * |
| | Control | Propolis-removed | 2 | 0.0052 | * |
| | Propolis-added | Propolis-removed | 2 | 0.8198 | |
| 3 | Control | Propolis-added | 1 | 0.0003 | * |
| | Control | Propolis-removed | 1 | 0.0033 | * |
| | Propolis-added | Propolis-removed | 1 | 0.9698 | |
| | Control | Propolis-added | 2 | 0.0004 | * |
| | Control | Propolis-removed | 2 | 0.0006 | * |
| | Propolis-added | Propolis-removed | 2 | 0.8504 | |
| 4 | Control | Propolis-added | 1 | 0.1851 | |
| | Control | Propolis-removed | 1 | 0.4445 | |
| | Propolis-added | Propolis-removed | 1 | 0.5017 | |
| | Control | Propolis-added | 2 | 0.6993 | |
| | Control | Propolis-removed | 2 | 0.4299 | |
| | Propolis-added | Propolis-removed | 2 | 0.1861 | |

**Table 5.1. Comparison of resin collection (number of resin foragers/brood area) between treatments (control, propolis-added, propolis removed), within sampling date.** Unpaired Wilcoxon rank sum tests were conducted to compare resin collection both before intervention and after intervention (Timing), for each round.

| Round | Treatment | p-value | Significance |
|---|---|---|---|
| 1 | Control | 0.4185 | |
| 1 | Propolis-added | 0.8295 | |
| 1 | Propolis-removed | 1.0000 | |
| 2 | Control | 0.2334 | |
| 2 | Propolis-added | 0.7536 | |
| 2 | Propolis-removed | 0.0357 | * |
| 3 | Control | 0.2293 | |
| 3 | Propolis-added | 0.0830 | |
| 3 | Propolis-removed | 0.0463 | * |
| 4 | Control | 0.1073 | |
| 4 | Propolis-added | 0.7268 | |
| 4 | Propolis-removed | 0.4469 | |

**Table 5.2. Comparison of resin collection (number of resin foragers/brood area) within treatments (control, propolis-added, propolis removed), between sampling dates.** Paired Wilcoxon rank sum tests were conducted to compare resin collection before and after intervention for each treatment (control, propolis-added, propolis-removed), for each round.

## *Effect of propolis on colony weight*

Propolis did not significantly impact colony weight in either the box type or the propolis manipulation experiments. Tared colony weights were calculated by subtracting the weight of each of the box components from the total weight of the hive. In the box type experiment, our generalized linear model showed a significant effect of date ($\chi^2 = 665.0$, df = 1, p = 0.003, p < 0.0001) and the interaction between date and box type ($\chi^2 = 118.0$, df = 2, p = 0.003, p < 0.0001) on colony weight (Fig. 9A). However, the effect of box type seemed to be primarily driven by thin box colonies, which were consistently lower weight than rough boxes and controls. A comparison of estimated marginal means indicated that control colonies started out marginally heavier than rough box colonies (Z = 2.19, p = 0.07), but weights evened out as colony development progressed. After one year, rough box colonies had gained more weight than control colonies by a margin of 0.5 kg (25%), but this difference was not significant ($F(2, 19) = 2.6$, p = 0.10; Fig. 9B).

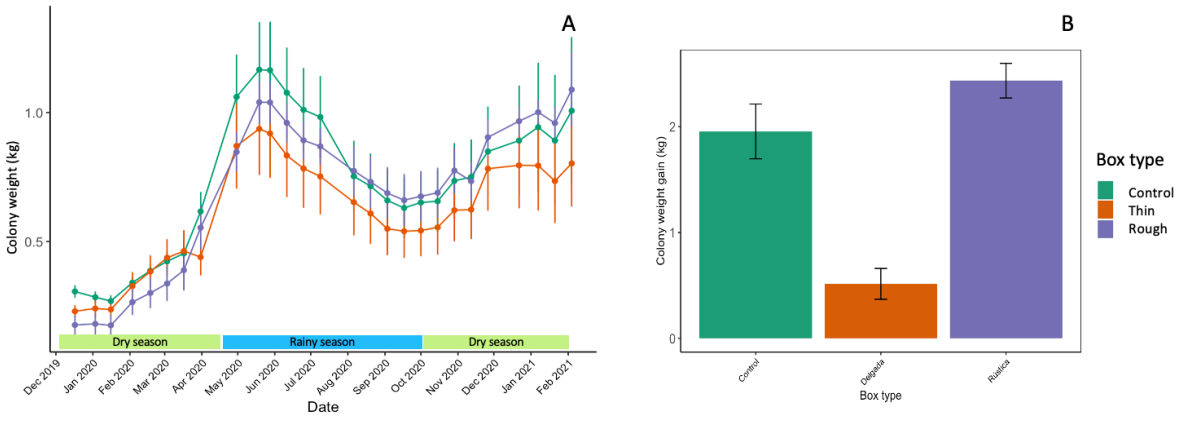


**Figure 5.9. Colony tare weight in control, thin, and rough box colonies from December of 2019 to February of 2021.** Control (n = 7), thin (n = 8), and rough box (n = 7) *S. mexicana* colonies were weighed on a biweekly basis over the course of one year. At the end of the year, colony weight gain was calculated by subtracting initial colony weight from final colony weight. Our generalized linear model showed a significant effect of date ($\chi^2 = 665.0$, df = 1, p = 0.003, p < 0.0001) and the interaction between date and box type ($\chi^2 = 118.0$, df = 2, p = 0.003, p < 0.0001) on colony weight (A), but the significance of box type seems to be primarily driven by thin box colonies, which were consistently lower weight than rough boxes and controls. After one year (B), rough box colonies had gained more weight than control colonies by a margin of 0.5 kg (25%), but this difference was not significant ($F(2, 19) = 2.6$, p = 0.10). Mean colony weight ± standard error is shown for each box type, for each sampling date.

In the propolis manipulation experiment, a comparison of estimated marginal means indicated there were no significant differences in colony weight between treatments (t(370) = 1.1, p = 0.29), though propolis-added and control colonies trended larger than propolis-removed colonies by an average of 0.36 kg (37%) and 0.12 kg (12%), respectively (Fig. 10).

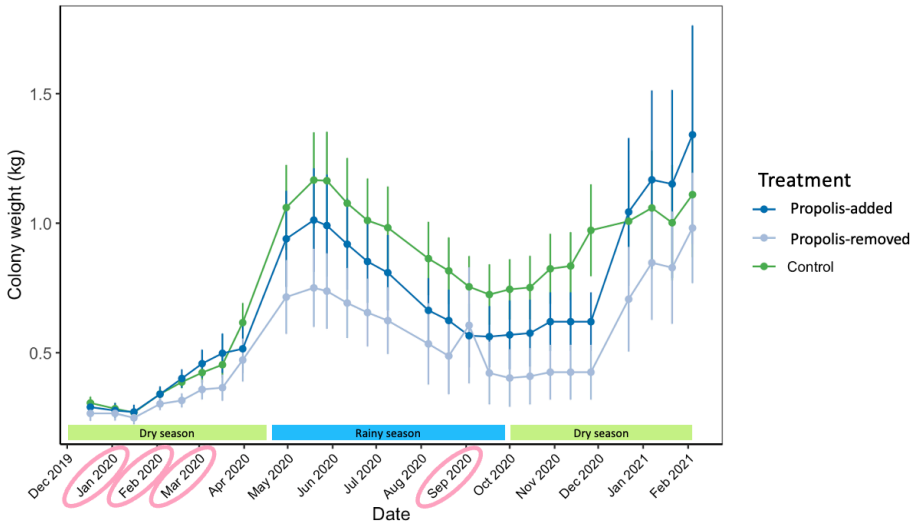


**Figure 5.10. Colony tare weight in propolis-added, propolis-removed, and control colonies from December of 2019 to February of 2021.** Propolis was added (n = 8; 10g/colony), removed (n = 8; 0.1-3.8g/colony), or not manipulated (n = 7) in 23 *S. mexicana* colonies once per month for the first three months of colony development (which coincided with the dry season), and once nine months into colony development, during the rainy season. Colony weight was measured on a biweekly basis. There were no significant differences in colony weights across treatment, though propolis-added colonies trended larger than propolis-removed colonies beginning in April of 2020. Mean colony weight ± standard error is shown for each treatment, for each sampling date. Months where propolis was added/removed are circled in pink.

## Discussion

Stingless bee colonies are increasingly transitioned from hollow log hives to smooth wood boxes for management purposes. Propolis is often removed from these hives, either for medicinal use or to facilitate management. The impact of box type and of the removal of propolis from colonies on colony growth and foraging dynamics has not been studied. We found that *S. mexicana* colonies kept in rough boxes collected more resin than colonies in smooth control boxes. We also found that adding or removing propolis stores inside a nest space led to increased resin foraging; removing propolis also led to a non-significant decrease in colony size.

## *Foraging behavior*

The number of pollen and resin foragers observed were fairly similar throughout the study year, demonstrating that resin is an important resource for *S. mexicana* colonies, on par with food resources. These findings roughly align with findings from a study that examined foraging behavior in three Sumatran stingless bees, *Trigona minangkabau, T. moorei* and *T. itama*: 10-20% of foragers returned to the colony with pollen loads, and close to 10% returned with resin loads (Inoue et al. 1985). In contrast, resin foragers constituted less than 5% of the foraging force in *Melipona bicolor bicolor,* with more than twice as much pollen foragers as resin foragers observed (Hilário et al. 2000). For other species, resin foragers outnumbered pollen foragers (e.g., *Melipona asilvai*, do Nascimento and Nascimento 2012), accounting for up to 50% (e.g., *Tetragonula carbonaria*, Leonhardt et al. 2014), or even 90% of the foraging force (e.g., *Tetragonilla collina*, Leonhardt and Blüthgen 2009). Extreme variation in resin foraging behavior amongst stingless bee species points towards the need for further species-specific, longitudinal studies to understand baseline foraging behavior.

Resin foraging tended to increase throughout the study year, even during the rainy season when nectar foraging began to drop. Presumably, the demand for resin to build honey pots during this time would have been low. Incoming resin may have been allocated to colony defense at this time, or to shoring up resin reserves in preparation for dry season resource flows, or for some other purpose. A precipitous increase in resin collection in August of 2020 occurred when phorid fly pressure was particularly high. Stingless bees are known to increase foraging in response to pressure from predators (Leonhardt and Blüthgen 2009), so this behavior may have been part of a defensive response.

Biesmeijer and Slaa (2004) note that multiple extrinsic and intrinsic stimuli influence foraging decisions in stingless bees, among them resource availability and colony development, but the effects of interactions between these factors are unknown. Does a dearth in pollen availability lead to spikes in resin foraging, with foragers taking advantage of “free time” to build up resin stores for nest construction or defense? Do

bees prioritize resin foraging over pollen and nectar foraging when they run out of places to store their food? If so, would full food pots serve as a stimulus for resin foraging?

### *Drift*

We observed significantly increased foraging and weight gain in end colonies compared to middle colonies, suggesting that drift was occurring. Drift has been reported in multiple stingless bee species (Oliveira et al. 2021, Stephens et al. 2016). In *Melipona fasiculata,* 64% of foragers were found to exhibit drifting behavior, and foragers from middle colonies drifted more than foragers from end colonies (Oliveira et al. 2021). If *S. mexicana* exhibits similar behavior, our colonies would have received drifting foragers from neighboring colonies but would have been less likely to send drifting foragers to their neighbors. In our study, marking colonies with colorful symbols in March of 2020 did not seem to diminish end colony advantage. These results are consistent with Oliveira et al. (2021) who found that, paradoxically, marking colonies with colorful symbols actually increased drift. Our end colonies' weight advantage did diminish beginning in September 2020, but this was likely due increased brood rearing in August of 2020. By this point, brood comb would have accounted for a greater portion of colony weight, and the influence of imbalances in incoming forage resources would be reduced.

Drift has implications both for the interpretations of the results of our experiment and for stingless bee management and overall colony health. If drift rates in *S. mexicana* are similar to those in *M. fasiculata*, this may diminish our ability to observe the effects of internal colony stimuli (e.g., box type) on resin collection. Further, it is possible that our experimental design inadvertently encouraged drift behavior. Nest entrances were plugged for 10 minutes while returning foragers accumulated at the front of the colony. It is possible that some foragers, returning to find their colony entrance obstructed, opted to enter a neighboring colony. If this is the case, because middle colonies were positioned between colonies of differing box type, we could expect our measurements to underestimate the effect of box type on resin collection.

Our colonies were spaced 50cm apart, following standard stingless bee management recommendations. We kept 50 colonies in two *palapas* at a distance of approximately 100 meters from each other. This colony density is not abnormal for stingless bee yards, but it far surpasses the density of wild colonies, which has been measured at 0.014-16 hives/ha (Eltz et al., 2002; Silva and Ramalho, 2016). Should stingless bee pathogen pressure intensify, frequent instances of drift in managed colonies could have serious implications for colony health. Drifting honey bee workers are known to transmit parasites and pathogens between colonies (Bordier et al., 2017; Nolan and Delaplane, 2017); the same could be true of stingless bees. Therefore, measures beyond minimum spacing and marking colonies with colorful symbols should be taken to minimize drift.

## *Effect of box type on resin foraging and propolis deposition*

Colonies in rough box colonies collected more resin than colonies in smooth box (control) colonies. This could indicate that transitioning stingless bee colonies from hollow log hives to smooth wood boxes diminishes resin collection behaviors. However, these results should be interpreted with some caution. Our experiment attempted to apply rough, tree-cavity textures to *S. mexicana* hive boxes. This strategy did allow us to closely monitor resin collection, propolis deposition, and colony development. However, a square wooden box with even grooves spaced at regular intervals is far from identical to a tree cavity. Thus, although our rough boxes did stimulate increased resin collection, we cannot with certainty say that hollow tree cavities would have a similar effect. Future studies should seek to characterize in detail the natural cavities where stingless bees nest, the textures that stimulate resin collection, the amount of propolis colonies apply to cavity walls, and the effect this propolis has on colony health and/or function.

Despite significant differences in resin collection, rough box colonies deposited only marginally more propolis on hive walls. It is possible that rough boxes provided increased stimulus for resin collection but that incoming resin was used for a variety of purposes, including brood comb construction, or honey and pollen pot construction.

Resin collection in thin box colonies did not differ from controls. While we cannot rule out the possibility that strong thin boxes colonies might increase resin collection to manage temperature change or adjust cavity size (Pérez-Sato et al. 2021), our thin box colonies did not exhibit this behavior. Throughout the experiment, our thin box colonies were smaller and lighter weight than their rough box and control counterparts. It is possible that the thin boxes we used were too thin, exposing colonies to more temperature change than they could tolerate, especially when they were small (Pérez-Sato et al. 2021). While testing thin box colonies could make sense in an experimental context, if beekeepers are looking to use ecological principles to inform colony design, it might make more sense to build thicker boxes, rather than thinner ones. Traditional hollow log hives often measure over 10cm in thickness. For *Melipona colimana*, Macías-Macías et al. (2016) recommend a minimum wall thickness of 13 cm, based on observations of wild colonies, which nest in tree cavities whose walls range from 10-30cm in thickness. Not all stingless bee colonies nest in trees with such thick walls; *M. beecheii* have been found to nest in trees with a wall thickness of just 5 cm (van Veen and Arce 1999). This observation might not entirely reflect preference, however. As deforestation limits the availability of natural nesting sites (Ramírez et al. 2013), stingless bees may be forced to build nests in smaller, thinner trees.

## *Effect of propolis manipulation on resin foraging*

Propolis manipulation appeared to impact resin foraging in a number of ways. There were no differences in resin collection across treatment groups in January, either before or after disruption (adding or removing propolis). However, in February and March resin collection was higher in propolis-added and propolis-removed colonies compared to controls, even prior to manipulation. This may indicate that disruption elicits an extended immune response, causing propolis-added and propolis-removed colonies to increase resin collection even weeks after disruption. Month-old January colonies may not have had the population resources to initiate this response; it may have come later, as colonies grew. On the other hand, findings from the box type experiment indicated significant

variability in resin collection, even within treatments, a result consistent with findings from honey bee studies (Garcia et al. 2013, Nicodemo et al. 2013). Therefore, it is also possible that colonies randomly selected for the propolis-added and propolis-removed treatment groups happened to be more inclined to collect resin than control colonies, and that this difference only showed up in February, after colonies were more established in their new hives.

The amount of propolis present inside the colony also appeared to influence resin collection. During round two, baseline resin collection was significantly higher in propolis-added colonies compared to propolis-removed colonies. In addition, during rounds two and three, resin collection increased in propolis-removed colonies immediately following propolis removal, but stayed the same in control and propolis-added colonies, indicating that colonies were not just responding to disruption. In a possible example of stigmergy, individual bees may have been assessing and addressing the amount of propolis present inside the colony (Biesmeijer and Slaa 2004, Heylighen et al. 2016).

We did not observe differences in resin collection during round four of the experiment (September). At this point in the rainy season, resin collection had increased across the board. This may have occurred in response to phorid fly pressure, or to amass sufficient construction materials to prepare for the coming dry season, or for another reason that we failed to perceive. Regardless of the cause, increased overall resin collection may have diluted the effect of disruption at this time.

### *Effect of propolis on colony size*

We originally sought to determine whether propolis contributes to stingless bee social immunity, but our ability to compare health metrics across colonies was limited, so we compared colony size instead. Colony size is a limited proxy for colony health, but may still be instructive in some ways.

We found no significant differences in colony size across box type, despite significantly increased resin collection in rough box colonies. At minimum, this non-

significance suggests that investing additional energy in resin foraging and using propolis for purposes other than nest construction and defense (i.e., to fill rough box grooves) did not seem to detract from rough box colony growth. This is notable because resin collection is a labor-intensive task. Inoue et al. (1985) observed that resin foragers of three Sumatran stingless bee species spent an average of 22.5 minutes on each resin foraging flight, compared to 13.4 minutes on pollen foraging flights and 8 minutes on nectar foraging flights. This means that one resin forager represents nearly twice the energy investment that a pollen forager does, and nearly three times the energy investment of a nectar forager. Investing in 43 additional resin foragers per day (as was the case for rough box colonies) would equate to sacrificing approximately 72 pollen loads, or 120 nectar loads. Admittedly, these numbers represent a cross-species extrapolation, and detailed natural history work would be required to determine the energetic "cost" of resin collection in *S. mexicana.* Nevertheless, the fact that increasing resin collection to fill in cracks in colony walls did not result in a fitness cost, at least in terms of colony weight, could indicate that peripheral propolis offers colonies some kind of fitness advantage.

In the propolis manipulation experiment, although there were no significant differencesin colony weight between treatments, propolis-added colonies trended 37% larger than propolis-removed colonies, and control colonies trended 12% larger than propolis-removed colonies. While not statistically significant, these differences but could be of interest to beekeepers, particularly those looking to sustainably harvest propolis from their colonies for medicinal or commercial purposes.

Our failure to detect a significant effect of propolis on stingless bee colony size is in contrast with honey bee studies, where propolis-rich colonies grew larger than propolis-poor colonies (Chapter 3; Borba et al. 2015). The mechanism through which propolis supports honey bee colony growth is unknown, but propolis does mitigate multiple pathogen threats, and it is possible that propolis supports honey bee colony growth by reducing the presence of pathogens that would be otherwise limiting (Chapter 3). The earliest peer-reviewed reports of stingless bee disease date back less than a decade, and stingless bee pathogens remain relatively uncommon today (Roubik 2023).

None of the colonies we worked with presented any signs of disease. It is possible that propolis would support improved colony health, and, by extension, increased colony size, if stingless bee colonies presented a disease state similar to that of honey bees. Roubik (2023) cautioned that "the industrial production of stingless bee colonies leads to unanticipated results," and Quezada-Euán et al. (2022) observed that the growing popularity of stingless beekeeping exposes stingless bees to a variety of threats, including pathogen spillover and rapid disease spread. If stingless beekeeping follows in the footsteps of honey beekeeping, the role of propolis in combatting stingless bee pathogens could, unfortunately, become clear.

## Conclusions

In summary, we observed increased resin collection by colonies housed in boxes texturized to mimic the rough inner surface of hollow tree cavities. While this may indicate that transitioning colonies to smooth wood boxes could negatively impact resin collection behavior, at this point, it is not clear whether increased resin collection confers a fitness advantage to *S. mexicana* colonies. We also found evidence of drift, with colonies positioned at the ends of rows boasting a larger foraging force and higher colony weight that middle colonies. Taken together, these results support recent calls to continue to look for ways to support stingless bee biology, even in managed contexts (Quezada-Euán et al. 2022). Fortunately, our findings suggest that minor modifications to management practices could have impactful results. If future studies reveal that resin supports stingless bee colony health in important ways, then modifying the surface texture of hive boxes could help bolster this behavior.

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# Appendix 1

## Chapter 1 Supplemental Information

| | | |
|---|---|---|
| **Objectives:** (1) to evaluate the top ten most cited honey bee health articles from the past decade in terms of framing (broad vs. narrow), and to characterize the future directions that these articles discuss | | |
| **Methods:** The search terms "honey bee" and "health" were input in the Web of Science search engine, to identify the ten most cited honey bee health articles published in the past decade (i.e., since January 2011) | | |
| **Evaluation criteria** | | |
| **Framing** | **Component 1** | Honey bee health is primary focus of the article |
| | **Component 2** | Article states that honey bee health is deteriorating and/or that colony loss is occuring at alarming rates |
| | **Component 3** | Article states that honey bees are important pollinators in agricultural systems and/or are important to agricultural production and/or global food security |
| | **Component 4** | Article states that intensive or industrial agriculture systems contribute to colony loss |
| | **Include (Y/N)** | All articles with a primary focus on honey bee health were included for analysis. Articles focusing broadly on bee health, pollinator health, or other themes are excluded. |
| | **Scope** | **Narrow framing:** Article discusses the problem of colony loss and the implications this has for agricultural production in the introduction section without acknowledging the ways in which intensive or industrial agriculture practices/systems contribute to colony loss.<br><br>**Broad framing:** Article connects the problem of colony loss and/or the multiple interacting stressors to intensive and/or industrial agriculture, in the introduction section.<br><br>**Other:** Article does not discuss colony loss and/or agriculture in the introduction section. |
| **Conclusions** | **Future directions** | Future research or actions that authors refer to in the conclusion section of the paper; some authors did not discuss future directions |
| | **Research vs. action** | Future directions were characterized as research, action, or research & action |
| | **Stated action** | Proposed actions were summarized |
| | **Action focus** | Actions were evaluated based on their area of focus (i.e., honey bee management or agroecosystem change) |

**Table S1.1.** Objective, methods, and evaluation criteria used in framing analysis of top ten most cited honey bee health articles from the past decade

| Article | | | | | | | Framing | | | | | | Conclusions | | | | Notes |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Criteria | Number | Author | Year | Citations as of Aug30, 2021 | Title | Author affiliation countries | Comp. 1 | Comp. 2 | Comp. 3 | Comp. 4 | Include | Scope | Future directions | Research vs. Action | Proposed action | Action focus | |
| Web of Science: "honey bee" AND "health", published after Jan 1, 2011 | 1 | Blacquiere et al. | 2012 | 553 | Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment | The Netherlands, Belgium | N | Y | Y | Y | N | N/A | N/A | N/A | | | Honey bee health is not the primary focus of this article. There is a broader focus on pollinator health. |
| | 2 | Engel et al. | 2012 | 385 | Functional diversity within the simple gut microbiota of the honey bee | U.S.A. | Y | Y | Y | N | Y | Narrow | Future research on the functional roles of bacteria related to colony health, nutrition, and pathogen defense | Research | | | |
| | 3 | Di Pasquale et al. | 2013 | 337 | Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter? | France | Y | Y | Y | Y | Y | Broad | "Pollinating areas of bees are currently changing due to intensification of agriculture and landscape alteration, and bees are often confronted with decreasing availability and diversity of resources in time and space... Therefore, maintaining and/or developing floral resources within agro-ecosystems is needed to prevent the negative impact of human activity and sustain the bee population." | Action | Maintaining/developing floral resources in agroecosystems | Agroecosystems | |
| | 4 | Vasquez et al. | 2012 | 269 | Symbiosis as Major Modulators of Insect Health: Lactic Acid Bacteria and Honeybees | Sweden, U.K., Germany | N | N | N | N | N | N/A | N/A | N/A | | | Honey bee health is not the primary focus of this article. Honey bees are not mentioned until fourth paragraph of the introduction section. |
| | 5 | Pettis et al. | 2013 | 262 | Crop Pollination Exposes Honey Bees to Pesticides Which Alters Their Susceptibility to the Gut Pathogen Nosema ceranae | U.S.A. | Y | Y | Y | N | Y | Narrow | Future research on "sub-lethal effects of fungicides and other chemicals that bees placed in an agricultural setting are exposed to" | Research | | | This article does mention "crop pollination" in its title, and it does evaluate the effect of pesticide exposure on pathogen susceptibility in honey bees, but it does not connect pesticide use to intensive/industrial agriculture, or implicate the ecosystems in colony loss. Instead, it lists habitat destruction, pesticide use, pathogens, climate change, honey bee diets, parasites, and disease as the causes of colony loss. |
| | 6 | Evans & Schwarz | 2011 | 220 | Bees brought to their knees: microbes affecting honey bee health | U.S.A. | Y | Y | Y | N | Y | Narrow | Future research "to understand and better manage (honey bee) interactions with microbes, chemicals, and other threats." | Research & Action | Managing honey bees' interaction with threats | Honey bees | |
| | 7 | Dainat et al. | 2012 | 207 | Predictive Markers of Honey Bee Colony Collapse | Switzerland, U.S.A, South Africa | Y | Y | Y | N | Y | Narrow | "This study provides evidence that Varroa destructor is a key player for winter colony losses and highlights the urgent need for efficient treatments against this parasite... [this work will] improve our understanding of bee losses, standardize methods for biomarkers of disease and finally to mitigate causes of bee declines" | Research & Action | Treating for Varroa | Honey bees | |
| | 8 | Tsvetkov et al. | 2017 | 205 | Chronic exposure to neonicotinoids reduces honey bee health near corn crops | Canada | Y | Y | N | Y | Y | Broad | None specified | | | | This article does not use the terms industrial or intensive agriculture in its framing, but it does provide a detailed description of large-scale corn production, and resulting pesticide exposure for honey bees, in the introduction section. |
| Web of Science: "honey bee" AND "health", published after Jan 1, 2011 | 9 | Sanchez-Bayo et al. | 2016 | 204 | Are bee diseases linked to pesticides? - A brief review | Australia, U.K., Italy, Japan, France | N | Y | N | Y | N | N/A | N/A | N/A | | | Honey bee health is not the primary focus of this article. There is a broader focus on pollinator health. |
| | 10 | Brandt et al. | 2016 | 191 | The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (Apis mellifera L.) | Germany | Y | Y | Y | N | Y | Narrow | "Our findings add a significant piece of information to the ongoing discussion of the role of neonicotinoid insecticides in colony losses. The results we report clearly indicate the need for more detailed laboratory and long-term field studies, aiming to assess how insecticides interfere with pathogen propagation and disease susceptibility." | Research | | | The multiple interacting stressors are mentioned (eg., diet quantity, quality, and diversity; pesticide exposure), but there is no mention of industrial, intensive, or any form of agriculture. |
| | 11 | Zhu et al. | 2014 | 191 | Four Common Pesticides, Their Mixtures and a Formulation Solvent in the Hive Environment Have High Oral Toxicity to Honey Bee Larvae | U.S.A. | Y | N | N | N | Y | Other | "The scope of pesticide risk assessment for non-target honey bees should be expanded from the present emphasis on acute toxicity of individual pesticides to a priority for assessment of chronic and mixture toxicities that incorporate fungicides, other pesticide pollutants and their 'inert' ingredients." | Research & Action | Expand scope of risk assessment | Agroecosystems | Does not include any kind of framing related to agriculture or colony loss in introduction section. Dives right in to pesticide impacts. |
| | 12 | Motta et al. | 2018 | 176 | Glyphosate perturbs the gut microbiota of honey bees | U.S.A. | Y | Y | Y | N | Y | Narrow | None specified | | | | This article discusses harmful impacts of glyphosate, but does not connect glyphosate or herbicide use to intensive or industrial agricultural production. Without a critical framing, glyphosate use comes across as a necessary, natural, and/or inevitable part of agricultural production. "The broad-spectrum herbicide glyphosate [N-(phosphono- methyl)glycine] has long been the primary weed management system, and its use is growing in connection with crops genetically engineered to be resistant to glyphosate." |
| | 13 | Lee et al. | 2015 | 158 | A national survey of managed honey bee 2013–2014 annual colony losses in the USA | U.S.A. | Y | Y | Y | N | Y | Narrow | Future research "This study highlights the benefits of performing multiyear surveys to better understand yearly trends." | Research | | | |

**Conclusions:** The search terms "honey bee" and "health" were input in the Web of Science search engine, to identify the ten most cited honey bee health articles published in the past decade (i.e., since January 2011). Of these, only articles focusing on honey bee health were included for analysis. Seven articles discussed the problem of colony loss and the implications this has for agricultural production in the introduction section without acknowledging the ways in which intensive or industrial agriculture contribute to colony loss. One article did not discuss colony loss or agricultural production in the introduction at all, and instead focused on pesticide toxicity. The two articles that did acknowledge the negative impacts of industrial agriculture on honey bee health were written by authors based at institutions outside of the U.S. at time of publication.

Articles with a narrow framing (i.e., articles whose framing failed to connect colony loss & the multiple interacting stressors to intensive or industrial agriculture) tended to conclude by recommending further research on the same topic, or related topics (6/7 articles). When narrowly framed articles did recommend action (2/7 articles), proposed actions focused on managing honey bee health, and did not address broader agroecosystem changes

**Table S1.2.** Framing analysis of top ten most cited honey bee health articles from the past decade

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Dainat, B., Evans, J. D., Chen, Y. P., Gauthier, L. & Neumann. P. (2012). Predictive markers of honey bee colony collapse, 7.

Engel, P., Martinson, V. G. & Moran, N. A. (2012). Functional diversity within the simple gut microbiota of the honey bee. *PNAS, 109,* 11002–11007.

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Motta, E. V. S., Raymann, K. & Moran, N. A. (2018). Glyphosate perturbs the gut microbiota of honey bees. *Proceedings of the National Academy of Sciencies U. S. A., 115,* 10305–10310.

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Sánchez-Bayo, F., Goulson, D., Pennacchio, F., Nazzi, F., Goka, K. & Desneux, N. (2016). Are bee diseases linked to pesticides ? — A brief review. *Environ. Int. 89–90,* 7–11.

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## Chapter 3 Supplemental Information

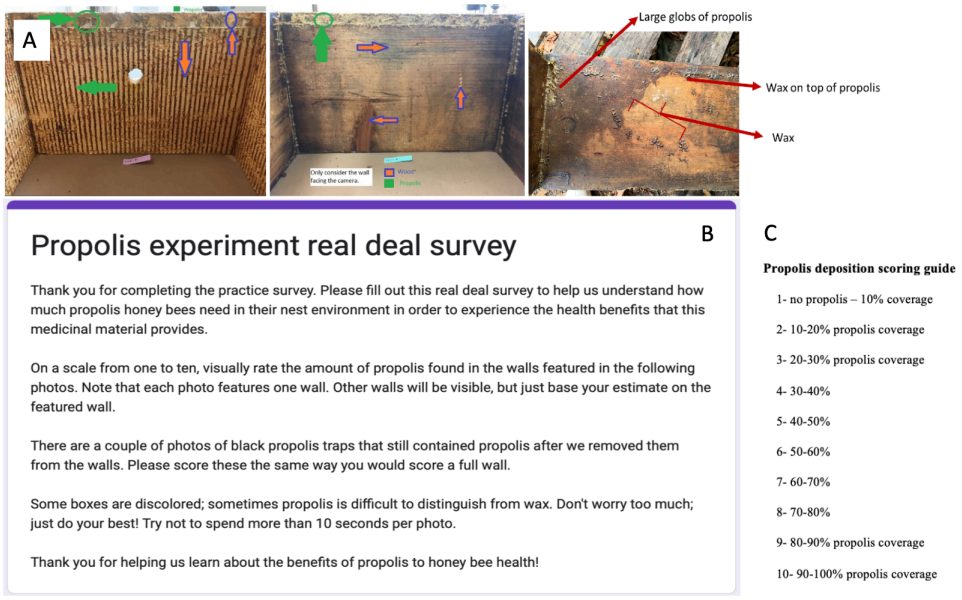


**Figure S3.1. Propolis scoring methods**. Four volunteers were provided reference photos (A) explaining what propolis looks like and differentiating wax from propolis. Volunteers were instructed to score photos on a scale from 1-10, based on % coverage of propolis, not on background coloration of the box or comb where it attached (B). Volunteers then used a Google form to fill out a practice survey, which allowed them to view and score ten sample photos. Finally, volunteers completed a full survey, scoring each wall of each box (C). Scores from all four walls, and from all four volunteers were averaged to create to create a composite "propolis score" for each colony.

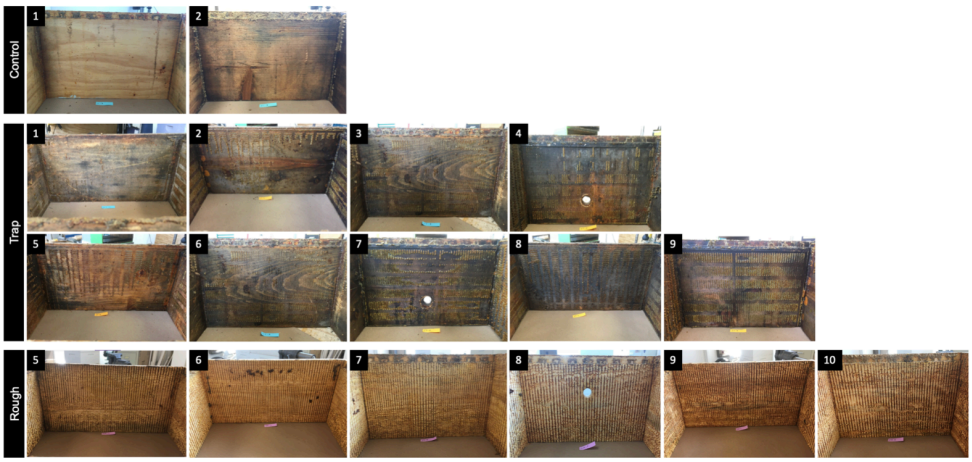


**Figure S3.2. Sample of propolis scoring results.** Control, trap, and rough box hive bodies were evaluated by four volunteers. The black text box at the upper left corner of each photo indicates the score assigned to that photo, according to one volunteer.

| Target | Fwd | Rev | Annealing Temp (°C)*# | Reference |
|---|---|---|---|---|
| *Defensin - 1* | TCA TGG CTG CAC CTG TTG AGG A | AGA CAG TTA GCA GCG CAA GCA C | 50.5 | [7] |
| *Abaecin* | CAG CAT TCG CAT ACG TAC CA | GAC CAG GAA ACG TTG GAA AC | 59 | [7] |
| *Hymenoptaecin* | ATA TCC CGA CTC GTT TCC GA | TCC CAA ACT CGA ATC CTG CA | 59 | [7] |
| *AmPPO* | AGA TGG CAT GCA TTT GTT GA | CCA CGC TCG TCT TCT TTA GG | 52.5 | [7] |
| *AmEater* | CAT TTG CCA ACC TGT TTG T | ATC CAT TGG TGC AAT TTG G | 59 | [7], EGFlikeA |
| *Relish* | GCA GTG TTG AAG GAG CTG AA | CCA ATT CTG AAA AGC GTC CA | 50.5 | [7] |
| | | | | |
| *Bartonella apis* | GTG GGA ATC TAC CTA TTT CTA CG | AAC GCG GGC TCA TCT ATC TC | 61.2 | [18] |
| *Bifidobacterium asteroides* | ATG CAA GTC GAA CGG GAT CC | CAT CCC ATR CCG GTA AAC CC | 60 | [18] |
| *Lactobacillus Firm-4* | AGT CGA GCG CGG GAA GTC A | AGC CGT CTT TCA ACC AGC ACT | 60 | [18] |
| *Lactobacillus Firm-5* | GCA ACC TGC CCT WTA GCT TG | GCC CAT CCT KTA GTG ACA GC | 60 | [18] |
| *Snodgrassella alvi* | CTT AGA GAT AGG AGA GTG CCT T | AAC TTA ATG ATG GCA ACT AAT GAC AA | 60 | [18] |
| *UniBact* | AGG ATT AGA TAC CCT GGT AGT CC | YCG TAC TCC CCA GGC GG | 60 | [18] |
| | | | | |
| EFB | TGT TGT TAG AGA AGA ATA GGG GAA | CGT GGC TTT CTG GTT AGA | 49.5 | |
| ABPV | ACC GAC AAA GGG TAT GAT GC | CTT GAG TTT GCG GTG TTC CT | 53.5 | [5] |
| BQCV | TCG CAG AGT TCC AAA TAC CG | TAT CAT CTC CCG CAC CAA CC | 59 | [2] |
| CBPV | CGC AAG TAC GCC TTG ATA AAG AAC | ACT ACT AGA AAC TCG TCG CTT CG | 52.5 | [14] |
| DWV-A | GAG ATT GAA GCG CAT GAA CA | TGA ATT CAG TGT CGC CCA TA | 53.5 | [3] |
| DWV-B | CTG TAG TTA AGC GGT TAT TAG AA | GGT GCT TCT GGA ATA GCG GAA | 59 | [15] |
| IAPV | CCA TGC CTG GCG ATT CAC | CTG AAT AAT ACT GTG CGT ATC | 52.5 | [4] |
| KBV | TGA ACG TCG ACC TAT TGA AAA A | TCG ATT TTC CAT CAA ATG AGC | 51.5 | [5] |
| LSV-1 | AGA GGT TGC ACG GCA GCA TG | GGG ACG CAG CAC GAT GCT CA | 59 | [19] |
| LSV-2 | CGT GCT GAG GCC ACG GTT GT | GCG GTG TCG ATC TCG CGG AC | 59 | [19] |
| | | | | |
| *pros54* | TCG AAC CAA GAT GGT ACT GGA A | TTG TTG TGC TTG CAG TCG TG | 55 | [17] |
| *β-actin* | AGG AAT GGA AGC TTG CGG TA | AAT TTT CAT GGT GGA TGG TGC | 52.5 | [16] |

**Table S3.1. Primers used to quantify the expression of immune, bacterial, and viral genes, as well as genes associated with European foulbrood and two reference genes.**

*All PCR reactions were conducted on a Bio-rad CFX Connect using SsoAdvanced Universal SYBR Green Supermix following the manufacter's recommended reaction mix for 10µl total volume. With the exception of DWV-A and BCQV where primers were included at a ratio of 1:2 (forward:reverse), all primers were included in the mix at a 1:1 ratio.

#All reactions were done using the following thermal protocol, varying only at the annealing temperature: 95°C for 5 minutes, 40 cycles of [95°C for 5 seconds, (Annealing temp) for 10 seconds, 72°C for 10 seconds], melting curve analysis

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| Operation | Sample date | Analysis level | Gene | Expression | QCI | Stabilization | QCI |
|---|---|---|---|---|---|---|---|
| Stationary | August 2019 | Apiary | *abaecin* | | | stabilized | Median: -0.090, **66%** QCI: [-0.146, -0.037] |
| | | | *defensin-1* | decreased | Median: -0.105, **95%** QCI: [-0.194, -0.012] | | |
| | | | *hymenoptaecin* | decreased | Median: -0.219, **66%** QCI: [-0.35, -0.088] | stabilized | Median: -0.092, **90%** QCI: [-0.184, -0.003] |
| | | | *AmPPO* | increased | Median: 0.068, **90%** QCI: [0.01, 0.124] | | |
| | | | *relish* | decreased | Median: -0.037, **66%** QCI: [-0.06, -0.014] | | |
| | | | *AmEater* | decreased | Median: -0.049. **66%** QCI: [-0.094, -0.002] | stabilized | Median: -0.097, **66%** QCI: [-0.16, -0.033] |
| Stationary | August 2019 | Colony | *abaecin* | | | | |
| | | | *defensin-1* | | | stabilized | Median: -0.013, **66%** QCI: [-0.023, -0.002] |
| | | | *hymenoptaecin* | | | | |
| | | | *AmPPO* | | | | |
| | | | *relish* | | | stabilized | Median: -0.019, **99%** QCI: [-0.036, -0.002] |
| | | | *AmEater* | | | | |
| Migratory | August 2019 | Apiary | *abaecin* | decreased | Median: -0.071, **66%** QCI: [-0.123, -0.021] | stabilized | Median: -0.072, **66%** QCI: [-0.116, -0.029 |
| | | | *defensin-1* | decreased | Median: -0.316, **90%** QCI: [-0.6, -0.045] | | |
| | | | *hymenoptaecin* | | | | |
| | | | *AmPPO* | | | | |
| | | | *AmEater* | | | destabilized | Median: 0.097, **90%** QCI: [0.006, 0.187] |
| Migratory | February 2020 | Apiary | *abaecin* | | | | |
| | | | *defensin-1* | increased | Median: 0.099, **90%** QCI: [0.003, 0.199] | | |
| | | | *hymenoptaecin* | | | | |
| | | | *AmPPO* | increased | Median: 0.164, **66%** QCI: [0.049, 0.277] | destabilized | Median: 0.141, **99%** QCI: [0.022, 0.256] |
| | | | *AmEater* | | | | |
| Migratory | February 2021 | Apiary | *abaecin* | decreased | Median: -0.066, **90%** QCI: [-0.129, -0.004] | | |
| | | | *defensin-1* | increased | Median: 0.035, **66%** QCI: [0.013, 0.053] | | |
| | | | *hymenoptaecin* | | | | |
| | | | *AmPPO* | increased | Median: 0.037, **90%** QCI: [0.024, 0.059] | | |
| | | | *AmEater* | increased | Median: 0.034, **95%** QCI: [0.001, 0.066] | destabilized | Median: 0.066, **66%** QCI: [0.02, 0.111] |

**Table S3.2. Distribution analysis results characterizing trends in immune gene expression with increasing propolis score for stationary and migratory operations across three sample dates.** Gene expression in seven-day-old bees (stationary) and young bees collected from frames with sealed brood (migratory) was quantified using real-time PCR. Six immune genes were analyzed in stationary colonies (n = 30), and gene expression trends were analyzed at both the apiary and colony level. The same genes, with the exception of *relish*, were analyzed in migratory colonies (n = 102) at the apiary level. A distributional regression model was used to determine the probability that gene expression increases, decreases, stabilizes, or destabilizes with increasing propolis score. The QCI listed refers to the quantile credible interval, as determined by our model, and reflects the widest possible credible interval supporting the indicated trend (not containing zero). Blank lines indicate no trend detected.

| Operation | Sampling date | Gene | Expression | QCI | Stabilization | QCI |
|---|---|---|---|---|---|---|
| Migratory | August 2019 | *B. asteroides* | decreased | Median: -0.043, **66%** QCI: [-0.084, -0.004] | | |
| | | *Bartonella* | increased | Median: 0.088, **66%** QCI: [0.028, 0.15] | stabilized | Median: -0.161, **99%** QCI: [-0.281, -0.035] |
| | | *Firm-4* | | | | |
| | | *Firm-5* | decreased | Median: -0.041, **66%** QCI: [-0.075, -0.008] | | |
| | | *S. alvi* | | | | |
| | | *UniBact* | decreased | Median: -0.055, **66%** QCI: [-0.09, -0.018] | | |
| Migratory | February 2020 | *B. asteroides* | increased | Median: 0.065, **66%** QCI: [0.017, 0.111] | | |
| | | *Bartonella* | increased | Median: 0.307, **95%** QCI: [0.048, 0.591] | | |
| | | *Firm-4* | | | | |
| | | *Firm-5* | | | | |
| | | *S. alvi* | | | | |
| | | *UniBact* | | | | |
| Migratory | February 2021 | *B. asteroides* | | | | |
| | | *Bartonella* | | | | |
| | | *Firm-4* | decreased | Median: -0.034, **66%** QCI: [-0.061, -0.008] | | |
| | | *Firm-5* | | | | |
| | | *S. alvi* | | | | |
| | | *UniBact* | increased | Median: 0.071, **99%** QCI: [0.006, 0.131] | | |

**Table S3.3. Distribution analysis results characterizing trends in bacterial gene expression with increasing propolis score for migratory operations across three sample dates.** Gene expression in young bees collected from frames with sealed brood from migratory colonies (n = 102) was quantified using real-time PCR. Six bacterial genes were analyzed at the apiary level. A distributional regression model was used to determine the probability that gene expression increases, decreases, stabilizes, or destabilizes with increasing propolis score. The QCI listed refers to the quantile credible interval, as determined by our model, and reflects the widest possible credible interval supporting the indicated trend (not containing zero). Blank lines indicate no trend detected.

| Operation | Sampling date | Virus | Expression | QCI |
|---|---|---|---|---|
| Migratory | August 2019 | *ABPV* | | |
| | | *BQCV* | increases | Median: 0.102, **66%** QCI: [0.013, 0.192] |
| | | *CBPV* | decreases | Median: -0.051, **66%** QCI: [-0.09, -0.013) |
| | | *DWV-A* | | |
| | | *DWV-B* | | |
| | | *IAPV* | decreases | Median: -0.151, **66%** QCI: [-0.275, -0.032] |
| | | *LSV1* | decreases | Median: -0.157, **66%** QCI: [-0.292, -0.022] |
| Migratory | February 2020 | *BQCV* | | |
| | | *CBPV* | | |
| | | *DWV-A* | decreases | Median: -0.125, **66%** QCI: [-0.227, -0.027] |
| | | *DWV-B* | | |
| | | *IAPV* | | |
| | | *LSV1* | | |
| | | *LSV2* | | |
| | | *KBV* | | |
| Migratory | February 2021 | *BQCV* | | |
| | | *CBPV* | | |
| | | *DWV-A* | decreases | Median: -0.114, **66%** QCI: [-0.186, -0.042] |
| | | *DWV-B* | decreases | Median: -0.074, **66%** QCI: [-0.127, -0.019] |
| | | *IAPV* | | |
| | | *LSV1* | | |
| | | *LSV2* | | |
| | | *KBV* | | |

**Table S3.4. Distribution analysis results characterizing trends in viral load with increasing propolis score for migratory operations across three sample dates.** Gene expression in young bees collected from frames with sealed brood from migratory colonies (n = 102) was quantified using real-time PCR. Nine viruses were analyzed at the apiary level. A distributional regression model was used to determine the probability that viral load increases, decreases, stabilizes, or destabilizes with increasing propolis score. The QCI listed refers to the quantile credible interval, as determined by our model, and reflects the widest possible credible interval supporting the indicated trend (not containing zero). Blank lines indicate no trend detected. Viruses with few positive reads were excluded from analysis.