Developing sustainable parasitic mite management strategies for beekeepers

by

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Abstract

The Western honey bee (*Apis mellifera*) is an important pollinator worldwide, contributing billions annually to the global economy through pollination services. Honey bees face a variety of different stressors that can cause colony losses. Colony losses have increased in recent decades and one of the leading causes of them in the Northern hemisphere is the parasitic *Varroa destructor* mite. The mite damages honey bee colonies by feeding on adult and developing honey bees while also vectoring various viruses. In the United States, this parasite is primarily controlled by synthetic acaricides; however, continued reliance on these synthetic acaricides, most recently amitraz, has led to increases in resistant *V. destructor* mites, thereby limiting potential treatment options. Compounding the issue of invasive parasitic mites for beekeepers is the appearance of a new threat, *Tropilaelaps mercedesae*. While still primarily limited to its native range in Southeastern Asia and Australasia, *T. mercedesae* has recently been confirmed in parts of Western Asia and Eastern Europe, regions previously thought to be unsuitable for the mite. *Tropilaelaps mercedesae* feeds on developing honey bees, like *V. destructor*, but not adults and can cause colony failures. In this dissertation, I conducted experiments exploring sustainable beekeeping practices while looking to fill knowledge gaps relating to *V. destructor* and *T. mercedesae*. I conducted two experiments that evaluated the effectiveness of fall *V. destructor* treatments in the Southeastern U.S., a critical time to manage *V. destructor* infestations. From these two experiments, I identified viable alternative treatments to amitraz and demonstrated that continuous treatment with this synthetic chemical increases the amitraz resistance allele frequency for *V. destructor*, albeit briefly. For *T. mercedesae*, I contributed to mapping the current global range of the mite and documented a potential dispersal method for the mite on adult honey bees exiting the colony. This suggests that the mite could

disperse through honey bee drifting or robbing behaviors. Lastly, I showed that *T. mercedesae* could be controlled in, although not eradicated from, colonies using a combination of a cultural and chemical control method. Overall, my work provides important information regarding sustainable mite management strategies to help beekeepers understand and control both *V. destructor* and *T. mercedesae*. Ultimately, my findings will help to limit future colony losses.

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Table of Contents

List of Tables

List of Figures

Main

Supplementary

Chapter 1

Introduction

The Western honey bee (*Apis mellifera*) is an economically impactful managed insect species, performing pollination services for approximately 58 different crops and contributing billions annually to the United States and global economy (Calderone, 2012; Jordan et al., 2021). Honey bees are now managed on nearly all habitable portions of the world where they are managed to produce honey and perform pollination services (vanEngelsdorp and Meixner, 2010); however, numerous stressors have led to increases in colony losses, thus increasing the costs for beekeepers and reducing the profitability of managing honey bees (Aurell et al., 2024; Bruckner et al., 2023; Lamas et al., 2024; vanEngelsdorp and Meixner, 2010). According to U.S. national colony loss surveys, documenting self-reported beekeeper data, the average annual colony losses have increased in recent years from 25% in 2011-12 to 39% in 2021-22 (Aurell et al., 2024; Spleen et al., 2013).

While there are many different stressors leading to increases in honey bee colony losses including poor nutrition, pathogens, and pesticide exposure (Decourtye et al., 2010; Goulson, 2013; Potts et al., 2010; Steinhauer et al., 2018), it is widely believed that introduced parasites, the *Varroa destructor* mite in particular, are the leading cause of colony losses worldwide (Aurell et al., 2024; Jack and Ellis, 2021; Morfin et al., 2024; Rosenkranz et al., 2010). Originally a parasite of the Asian honey bee *Apis cerana*, *V. destructor* was able to shift hosts, feeding on developing and adult *A. mellifera* (Rosenkranz et al., 2010; Traynor et al., 2020). Since shifting hosts, *V. destructor* has become synonymous with beekeeping and has spread to all areas where

A. mellifera are managed, causing significant colony losses despite different available beekeeping management strategies (Jack and Ellis, 2021; Rosenkranz et al., 2010).

While *V. destructor* is a primary cause of colony losses, a new parasitic mite threat, *Tropilaelaps mercedesae*, has increased in notoriety and concern for beekeepers. Originally a parasite of the giant honey bee *Apis dorsata*, *T. mercedesae* also successfully switched hosts to begin feeding on *A. mellifera*. Although it is still primarily found in Southeast Asia, *T. mercedesae* has recently been confirmed in Western Asia and Eastern Europe (Brandorf et al., 2024; De Guzman et al., 2017; Janashia et al., 2024). While not as globally ubiquitous as *V. destructor*, *T. mercedesae* presents another parasitic mite threat capable of overwhelming honey bee colonies, and its potential for future range expansion increases global concern (Chantawannakul et al., 2018, 2016).

Varroa destructor

Varroa destructor background

While there are multiple *Varroa* species, *V. destructor* is the species of most interest, as it is associated with honey bee colony losses worldwide. Since its introduction to the U.S. in the 1980s (Wenner and Bushing, 1996), *V. destructor* has become a self-reported leading cause of honey bee colony losses in the U.S. (Aurell et al., 2024; Bruckner et al., 2023). This has led to a need for research to better understand *V. destructor* mite biology and determine successful intervention strategies to reduce *V. destructor* infestation rates in colonies (Jack and Ellis, 2021).

Varroa destructor has two distinct phases within honey bee colonies, the reproductive and dispersal phases (Reams and Rangel, 2022; Traynor et al., 2020). The reproductive phase is initiated when a gravid, female *V. destructor* mite (also known as a "foundress" mite) enters the brood cell of a 5th instar honey bee larva which is about to be capped for pupation (Reams and

Rangel, 2022). Typically, a male offspring hatches from the first egg laid by foundress *V. destructor* and subsequent eggs hatch into females; the females develop and these offspring mate (Rosenkranz et al., 2010). During the honey bee's pupation, the foundress mite and her offspring feed on the pupating bee, primarily its hemolymph (Han et al., 2024). The average number of mature female daughters produced by a foundress mite is 0.7 – 1.45 per female, worker honey bee cell, compared to 1.6 – 2.5 per male, drone cell due to three days more spent under the capping during the male honey bee drone's pupation (Rosenkranz et al., 2010; Traynor et al., 2020). Upon emergence with the adult honey bee, *V. destructor* mites begin the dispersal phase. The dispersal phase is traditionally categorized by mature female mites attaching to adult honey bees, typically beneath the honey bee's ventral sclerites, where they feed on fat bodies (Ramsey et al., 2019). The dispersal phase seems particularly beneficial to newly mated daughter mites, as they reproduce more effectively after feeding on adult honey bees for five to seven days before entering the reproductive phase themselves (Häußermann et al., 2016). Research has shown that *V. destructor* mites prefer younger "nurse" honey bees over older "forager" honey bees for dispersal, likely due to their proximity to larval hosts (Cervo et al., 2014); however, this preference disappears as the *V. destructor* infestation rate on adult honey bees increases (Cervo et al., 2014). *Varroa destructor* mites have been known to survive on adult honey bees for an extended period of time and can frequently move between adult honey bees of different ages (Lamas et al., 2023; Rosenkranz et al., 2010). Overall, the number of mites in each phase is largely driven by *V. destructor* mite colony infestation levels, honey bee colony size, and availability of fifth instar larvae (Beetsma et al., 1999).

While the feeding of *V. destructor* on hemolymph and fat bodies weakens honey bees, this feeding also allows *V. destructor* to transmit a number of viruses thereby weakening entire

honey bee colonies (Reams and Rangel, 2022). *Varroa destructor* has a known association with deformed wing virus and acute bee paralysis virus, both viruses that reduce the lifespan and functionality of adult honey bees (De Miranda et al., 2010; Martin and Brettell, 2019; Rosenkranz et al., 2010; Traynor et al., 2020). The presence of viruses, especially at high titers, negatively affects colonies and their ability to withstand *V. destructor*, even at lower infestation rates, before collapsing. This increases the need for beekeeper intervention to keep *V. destructor* infestation rates low. Research shows that honey bee colonies are more susceptible to *V. destructor* infestations during the winter when brood rearing decreases or ceases, forcing *V. destructor* into their dispersal phase when they feed on fat bodies, necessary for survival of adult honey bees (Amdam et al., 2004; Jack et al., 2023; Morfin et al., 2024). Similarly, colony size dwindles in the winter which increases the mite to bee ratio, and thus mite pressure. Pending brood availability, *V. destructor* can increase yearly populations 12 to 800 times (Martin, 1998). Without some kind of beekeeper intervention to control *V. destructor* infestation rates, honey bee colonies often die within two years or less (Jack et al., 2023), imploring beekeepers to implement management strategies designed at limiting *V. destructor* infestation and the subsequent damage.

Varroa destructor control

While there are a number of different available *V. destructor* management strategies, most beekeepers, notably larger-scale, commercial beekeepers, primarily rely on chemical treatments to reduce *V. destructor* infestations (Haber et al., 2019; Jack and Ellis, 2021). This reliance on chemical treatments has led to significant resistance issues rendering synthetic chemical treatment options, like coumaphos and *tau*-fluvalinate, ineffective (Elzen et al., 2000; Gracia-Salinas et al., 2006; Maggi et al., 2009). Although highly effective for a number of years, amitraz, the most widely used synthetic acaricide by beekeepers, has recently seen increases in

resistance levels thereby minimizing its effectiveness (Hernández-Rodríguez et al., 2022; Hernández-Rodríguez et al., 2025; Marsky et al., 2024; Rinkevich, 2020; Rinkevich et al., 2023). There are different types of amitraz resistance in *V. destructor*, depending on the region where different *V. destructor* populations have developed mutations to convey the resistance (Hernández-Rodríguez et al., 2022; Hernández-Rodríguez et al., 2025; Marsky et al., 2024; Rinkevich et al., 2023). These mutations include amino acid changes to the mites β2 octopamine receptor which inhibits amitraz and its secondary metabolites from binding to kill the mites (Hernández-Rodríguez et al., 2022; Hernández-Rodríguez et al., 2025; Lee et al., 2025; Rinkevich et al., 2023) This illustrates that *V. destructor* is capable of developing resistance to amitraz in multiple ways. The continued reliance and application of amitraz positively selects for *V. destructor* resistant genotypes, thus increasing the overall prevalence of resistance. The presence of amitraz resistant *V. destructor* mites forces beekeepers to implement and adopt alternative integrated pest management strategies using different chemical treatments (Jack and Ellis, 2021).

Compounding the issue of effective treatments is the *V. destructor* life cycle. Since the mite spends the reproductive phase alongside the pupating honey bee, it is protected by a cell capping limiting a chemical treatment being able to come in contact with the mite (Jack and Ellis, 2021; Reams and Rangel, 2022; Rosenkranz et al., 2010). Thus, rapid acting treatments that rely on contact such as the organic treatments of hops β acids or oxalic acid are effective at killing *V. destructor* mites in the dispersal phase, but they have limited effects on *V. destructor* in the reproductive phase when they are protected by a cell capping (Berry et al., 2023, 2022; Gregorc et al., 2017; Kulhanek et al., 2023; Vandervalk et al., 2014). Meanwhile, formic acid, another organic treatment, volatilizes in the colony and penetrates the cell capping, killing

V. destructor in the brood (Van Engelsdorp et al., 2008). However, it has a limited temperature range and can cause queen death when used at high temperatures (NOD Apiary Products, 2017; Underwood and Currie, 2005). These treatment limitations combined with greater frequency of resistance make it more difficult to control *V. destructor* infestations and potentially lead to higher colony losses.

Beekeepers in the Southeast have an additional challenge of controlling *V. destructor* as the climatic conditions allow for an extended brood rearing season presenting a time where *V. destructor* mites are protected under a cell capping (Jack et al., 2023). To address this, I dedicated the first two chapters of my dissertation to evaluate different chemical *V. destructor* mite treatments in the Southeastern U.S. to find an effective alternative treatment to amitraz during the fall. This time point was chosen because this is a critical period where beekeepers need to lower *V. destructor* infestation rates for their colonies to survive winter (Amdam et al., 2004; Jack et al., 2023; Morfin et al., 2024).

Tropilaelaps mercedesae **mites**

Tropilaelaps mercedesae background

Tropilaelaps mercedesae is a parasitic mite species native to Asia and is of increasing concern for beekeepers since it began parasitizing *A. mellifera* upon the introduction of this honey bee species to Southeastern Asia (De Guzman et al., 2017; Delfinado and Baker, 1961). While four species of *Tropilaelaps* exist, only two, *T. mercedesae* and *T. clareae*, have shown the ability to feed on *A. mellifera,* switching from their original host, the giant honey bee *A. dorsata* (Chantawannakul et al., 2018). Furthermore, only *T. mercedesae* has significantly increased its geographical range, proving to be the primary species of concern for beekeepers (Chantawannakul et al., 2018; De Guzman et al., 2017; Ramsey, 2021).

Tropilaelaps mercedesae damages colonies by feeding on developing honey bee larvae and pupae, reducing their lifespan and weight at emergence (Han et al., 2024; Khongphinitbunjong et al., 2016). Additionally, *T. mercedesae* is capable of transmitting different viruses, although limited research has investigated the specific types of viruses (Khongphinitbunjong et al., 2015). Unlike *V. destructor*, *T. mercedesae* is incapable of feeding on adult honey bees and dies within six days without access to larvae or pupae, thereby limiting its survival to periods when colonies contain brood (Khongphinitbunjong et al., 2019; Pettis and Chaimanee, 2019; Rinderer et al., 1994; Woyke, 1994). While this inability to feed on adult honey bees restricts *T. mercedesae* survival when colonies do not have brood, it also results in a shorter dispersal phase where mated female mites can enter new cells to reproduce less than 48 hours after emerging (Woyke, 1987a), typically producing anywhere from 0.79 to 2.1 daughter offspring per honey bee worker cell (De Guzman et al. 2017). The limited time in the dispersal phase allows *T. mercedesae* to rapidly reproduce when brood is present and outcompete *V. destructor* in co-infested colonies (Buawangpong et al., 2015; Woyke, 1987b, 1987c). This rapid reproduction also allows *T. mercedesae* to quickly overwhelm colonies (Camphor et al., 2005; Tokach et al., 2024).

While there are publications documenting the range of *T. mercedesae*, there are major knowledge gaps concerning the current global range of (Chantawannakul et al., 2018, 2016; De Guzman et al., 2017). Due to the inability to feed on adult honey bees, it was originally believed that *T. mercedesae* would be primarily restricted to its native region, Southeastern Asia and Australasia, in the tropics (Chantawannakul et al., 2016; De Guzman et al., 2017). However, *T. mercedesae* has expanded its range and its presence has been confirmed in Western Asia and Eastern Europe, temperate regions believed to have periods with intermittent brood production

(Brandorf et al., 2024; Janashia et al., 2024; Mohamadzade Namin et al., 2024). Following these new confirmations and the rising concern by beekeepers, there have been numerous, previously undocumented, reports on new countries and regions where *T. mercedesae* has been found. For Chapter 4, I provide an updated account on where *T. mercedesae* has been confirmed. Using peer-reviewed literature, I looked to find publications where *T. mercedesae* was confirmed using morphological and DNA-based methods. Additionally, I monitored which *Apis* species, whether *A. mellifera* or *A. dorsata*, the mite was collected from. This helps provide the most recent known global range of *T. mercedesae* and informs beekeepers and policy makers of regions most at threat to see new introductions.

Clearly, *T. mercedesae* is able to expand its range, however it remains unclear how the mite disperses. It has been proposed that *T. mercedesae* disperses through beekeeper mediated management practices like movement of colonies or transfer of infested combs, in addition to dispersing on adult honey bees through drift or robbing behaviors, similar to *V. destructor* (De Guzman et al., 2017; Peck and Seeley, 2019; Rath et al., 1991); however, while it has been documented that *T. mercedesae* will phoretically attach to adult honey bees in both the lab and colony (Gill et al., 2024; Khongphinitbunjong et al., 2012; Pettis et al., 2013), there has never been confirmation of *T. mercedesae* exiting honey bee colonies on adult honey bees. This is an important distinction, as it relates to how *T. mercedesae* might naturally disperse to new colonies and how to prevent future spread of this parasite

Tropilaelaps mercedesae control

Most research on *T. mercedesae* management strategies has primarily focused on using acaricides adapted from use for *V. destructor*. Since *T. mercedesae* does not feed on adult honey

bees, they spend the majority of their time in the reproductive stage underneath a brood cell capping. Brood cell infestation can be 16 – 37 times higher than infestation on adult honey bees, depending on season (Woyke, 1987a). Like *V. destructor*, this means they are often protected from contact acaricides, limiting their effectiveness (Pettis et al., 2017). While certain contact acaricides, like flumethrin and fluvalinate, have shown moderate effectiveness against *T. mercedesae* (Camphor et al., 2005; Roberts et al., 2020), others such as amitraz and hops β-acids have failed to reduce *T. mercedesae* infestations (Pettis et al., 2017). This could mean that a lower dosage of the pyrethroids flumethrin and fluvalinate are needed to kill *T. mercedesae* mites comparatively to amitraz or hops β-acids. Meanwhile, formic acid, a treatment capable of penetrating the cell cappings, has shown a great ability to reduce *T. mercedesae* infestations, but its narrow temperature range limits the times when it is effective and allowed in the colony (NOD Apiary Products, 2017; Pettis et al., 2017; Raffique et al., 2012). While chemical treatments can differ in overall efficacy, a consistently successful management strategy involves inducing a brood break (Roberts et al., 2020; Woyke, 1984). Due to the inability to feed on adult honey bees, a brood break, a period where there is no larvae or pupae in the colony, kills almost all *T. mercedesae* through starvation. While brood breaks naturally occur in colder climates when temperatures drop and the queen ceases egg laying, brood breaks can also be induced artificially to control *T. mercedesae.* Common practices to induce a brood break in warmer climates where colonies always have moderate levels of brood are queen caging or queen replacement. Although brood breaks can reduce honey production (Kovačić et al., 2023), they seem to be the most effective strategy for reducing *T. mercedesae* infestations.

As *T. mercedesae* continues to expand its geographical range, there has been an increase in efforts to determine an effective eradication strategy in the event the mite is introduced to a

new country or region. Chapter 6 addresses this need by combining a cultural control of a brood break with a chemical control to eliminate practically all *T. mercedesae* mites in a honey bee colony. Although proven effective, brood breaks have not exhibited the 100% efficacy needed in an eradication program (Roberts et al., 2020; Woyke, 1984); therefore, I combined brood breaks with an additional chemical treatment to evaluate this management strategy for potential eradication programs upon introduction of *T. mercedesae* to a new region.

Overall, this work with *V. destructor* and *T. mercedesae* looks to address significant issues beekeepers are facing and fill knowledge gaps regarding effective mite management strategies. For *V. destructor*, this research was designed to find effective acaricide treatments that could be utilized as an alternative to amitraz. Additionally, I worked to understand how amitraz resistant *V. destructor* mites were impacted by changes in active ingredients of acaricides. Meanwhile, my *T. mercedesae* research focused on bridging the gaps in *T. mercedesae* research to provide a better understanding the emerging threat of this parasitic mite. Here, I looked to define the current *T. mercedesae* range, understand potential mechanisms of dispersal, and determine the viability of an eradication plan. These studies will hopefully inform beekeepers and help minimize colony losses in the future.

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Chapter 2

Managing amitraz resistant *Varroa destructor* mites in honey bee (*Apis mellifera*) colonies: Effects of winter treatment with extended-release oxalic acid

Abstract

The parasitic *Varroa destructor* mite is the premier cause of honey bee colony losses worldwide. To combat the mite in the United States, beekeepers frequently use amitraz. However, continued reliance on amitraz-based treatments has led to the emergence of resistance in *V. destructor* mite populations throughout the country. This experiment aimed to determine whether a treatment with a non-amitraz acaricide during winter could provide better efficacy on amitraz resistant mites compared to an amitraz based treatment. In coordination with a commercial beekeeper with known amitraz resistant mite populations, we tested two extended-release oxalic acid treatments – a commercial formulation (Aluen CAP®) and a homemade formulation (oxalic acid and glycerin in absorbent pads – OA SpillTech®) – as alternatives to amitraz applied as Apivar®. The OA Spilltech® treatment had significantly higher mite infestation rates on Day 67 compared to Aluen CAP® and Apivar®. On Day 97, both Aluen CAP® and OA Spilltech® treatments had higher *V. destructor* infestation rates compared to Apivar®. For colony strength, Aluen CAP® had a negative effect on frames of adult honey bees throughout the study and had numerically lower survival compared to the OA Spilltech® and Apivar® treatments.

Introduction

Honey bees (*Apis mellifera* L.) are an economically important insect responsible for contributing billions to the global economy because of their pollination services (Jordan et al.,

2021). The parasitic mite *Varroa destructor* (Anderson & Trueman) is a leading cause of colony loss, especially in the northern hemisphere, by feeding on developing and adult honey bees, and transmitting viruses (Aurell et al., 2024a; Han et al., 2024; Ramsey et al., 2019; Rosenkranz et al., 2010; Traynor et al., 2020).

The most common management for *V. destructor* includes applications of various chemical acaricides capable of killing the ectoparasitic mite with minimal damage to honey bees (Jack and Ellis, 2021; Rosenkranz et al., 2010). In the United States (U.S.), there are multiple active ingredients used in synthetic acaricides that are designed to combat *V. destructor* including *tau*-fluvalinate, coumaphos, and amitraz (Jack and Ellis, 2021). The use of all three of these active ingredients has resulted in the development of resistance in *V. destructor* populations, thus limiting their effectiveness (Elzen et al., 2000; Gracia-Salinas et al., 2006; Hernández-Rodríguez et al., 2025; Higes et al., 2020; Marsky et al., 2024; Rinkevich, 2020; Rinkevich et al., Unpublished; Vlogiannitis et al., 2021). Of the three synthetic acaricides, amitraz remains the most commonly-used by commercial beekeepers due to its effectiveness at reducing mite infestation rates (Gregorc et al., 2018; Haber et al., 2019; Jack et al., 2024, 2020); however, its continued reliance by beekeepers as the primary treatment for *V. destructor* has resulted in increasing both numbers of resistant populations and levels of resistance within those populations (Hernández-Rodríguez et al., 2022; Hernández-Rodríguez et al., 2025; Marsky et al., 2024; Rinkevich, 2020; Rinkevich et al., 2023; Rinkevich et al., Unpublished). There is a clear need for improved adoption of integrated pest management principles concerning rotation of active ingredients to limit selection of resistant mite populations.

Oxalic acid has become an increasingly popular organic treatment option that beekeepers have begun using at increasing frequency as an alternative to synthetic acaricides; however, it is

often limited in effectiveness to times where there is limited or no capped brood (pupating honey bees) in the colony (Berry et al. 2023; Gregorc and Poklukar, 2003). This is because *V. destructor* has two phases in the colony, a reproductive phase where mites reproduce under a cell capping in the brood while honey bees pupate, and a dispersal phase where mites attach to adult honey bees, feeding on the honey bees while being transported around the colony (Ramsey et al., 2019; Reams and Rangel, 2022; Traynor et al., 2020). To maximize effectiveness, oxalic acid, most commonly applied through dribble or vapor, should be applied when colonies have limited capped brood since it only kills mites in the dispersal phase outside of the cell (Bacandritsos et al., 2007; Berry et al., 2023, 2022; Gregorc and Poklukar, 2003; Jack and Ellis, 2021; Rosenkranz et al., 2010). To account for this problem, a product registered in several countries called Aluen CAP® employs an extended-release approach to oxalic acid exposure (Maggi et al., 2016; Rodríguez Dehaibes et al., 2020); instead of being applied as a rapid acting contact treatment like conventional dribble and vapor applications, OA is gradually released from impregnated fiber strips throughout the colonies for more than 30 days to kill *V. destructor* previously protected under a cell capping as they emerge alongside a teneral honey bee (Kanelis et al., 2023; Maggi et al., 2016; Rodríguez Dehaibes et al., 2020). Many beekeepers have created and applied homemade versions of this treatment using glycerin and shop towels or sponges (Oliver, 2022). Research shows these homemade treatments, when applied with brood present, can reduce *V. destructor* infestation rates when compared to untreated colonies (Kanelis et al., 2023; Sabahi et al., 2020, 2017), but some studies show the treatment may be incapable of causing reduction in infestation rates (Bartlett et al., 2023; Plamondon et al., 2024), forcing beekeepers to perform an additional treatment of any acaricide to reduce infestation rates if they remain high.

To date, most research has only examined extended-release oxalic acid when brood is present, leaving knowledge gaps as to the success of this application method as a winter treatment when mites are predominately in the dispersal phase outside of cells and when more traditional rapid acting oxalic acid treatment methods of application like dribble and vapor are recommended. Therefore, this experiment investigated the effectiveness of two types of extended-release oxalic acid treatments (Aluen CAP® and OA Spilltech®), as well as one amitraz based product (Apivar®), on *V. destructor* in honey bee colonies during winter when limited brood was present. The experiment was conducted in partnership with a commercial beekeeper with moderate levels of amitraz resistance within their *V. destructor* populations (Rinkevich, Unpublished). The beekeeper customarily treated with amitraz during winter but was interested in adopting alternative treatments to ensure appropriate mite control. It was predicted that the two extended-release oxalic acid treatments (Aluen CAP® and OA Spilltech®) would perform better than Apivar® during this winter experiment as they would be better equipped to kill amitraz resistant *V. destructor* mites, especially during a low to no brood scenario within colonies.

Materials and Methods

Apiary locations

We conducted this experiment in coordination with a commercial beekeeper whose *A. mellifera* colonies were located in the Southeastern U.S. during the winter of 2022-2023. The apiaries were all in zone 9A of the 2023 USDA plant hardiness zone map ("USDA Plant Hardiness Zone Map," 2025), an area with limited brood production during portions of the winter. We previously sampled *V. destructor* from 20 of their colonies during the fall of 2021 to perform amitraz resistance tests. These tests were conducted by shaking adult honey bees from a

frame containing sealed brood into a plastic tub and scooping approximately 1/2 cup of honey bees into premade amitraz resistance cups as detailed in Rinkevich (2020). After the three-hour amitraz resistance test were collected from the trays. The cups were flipped and amitraz resistant mites were collected by performing the triple-rinse alcohol wash method as detailed in Aurell et al. (2024b). Honey bees from the cups were then counted to determine the exact mite per 100 bee infestation rates. In 2021, observed amitraz resistance of those 20 colonies was 19.1%; we sampled 20 colonies again in 2022 prior to the start of this experiment on a different subset of colonies from what we used for the experiment, and observed amitraz resistance was 60%. Ninety-four *A. mellifera* colonies housed in single deep, 10-frame Langstroth boxes were initially inspected across five apiary locations to identify they had a laying queen and were suitable for this experiment.

Varroa destructor treatments

Colonies were divided into treatment groups based on initial mite infestation rate and all treatments were applied five days after initial colony inspections in late November of 2022. All five apiaries received an equal number of colonies in each treatment group. All colonies in the yard received one of three treatments– Apivar®, Aluen CAP®, or OA SpillTech®. The Apivar® group received two strips impregnated with amitraz per colony placed within the brood chamber between frames 4-5 and 6-7. The Aluen CAP® group received three cellulose U-shaped strips each containing 10 grams of oxalic acid mixed with 20 mL of glycerin; strips were placed over frames 4, 5, and 6 within the colony. The OA SpillTech® group had oxalic acid strips made based on modified methods from Bartlett et al., (2023) and Oliver, (2022). To create the strips, SpillTech® Universal Maximum Absorbent Strips (1-3/4” x 7-1/2”) were used where 40 strips were placed in a pan. 605 grams of Apibioxal® oxalic acid were combined with 605 mL of

glycerin and heated to just under 77°C until all oxalic acid was dissolved. The solution was poured over the strips and strips were left untouched overnight to allow for absorption where each strip absorbed approximately 25 – 30 grams of the 1:1 solution. Four strips were applied per colony perpendicularly on top of the frames. Although treatments were labeled to only last 42 days, strips for all treatments were left in the colony through Day 97 as customary of normal beekeeping practices by the commercial beekeeper. Otherwise, registered treatments were applied as per label rate and instructions. The Apivar® group began with 32 colonies while the Aluen CAP® and OA SpillTech® groups both started with 31 colonies. Other than treatments, colonies received the same management customary of the commercial beekeeper's standard operating procedures.

Colony assessments

Colonies were initially assessed in late November 2022 to determine *V. destructor* infestation rate and frames of adult honey bees. The latter was determined by looking at the top and bottom of each hive box to determine how many frame spaces were occupied by honey bees giving a frames of adult honey bees estimate, which was then averaged between the two numbers (Guzman-Novoa et al., 2024). For the former, we performed initial amitraz resistance tests using methods previously described adapted from Rinkevich (2020). Colonies were again assessed for colony strength using the same methods as previously described, and mite infestation rate using only alcohol washes, in mid-January and mid-February, which represent Day 67 and Day 97 after treatments were applied. Colonies were removed from the study if they went queenless or died.

Statistics

Statistics were conducted using R 4.2.2 (R Core Team, 2022) and packages *lme4*, *emmeans*, *dplyr*, *DHARMa*, and *ggplot2* (Bates et al., 2015; Hartig, 2022; Lenth, 2023;

Wickham, 2016; Wickham et al., 2023*). Varroa destructor* infestation rate was analyzed using a generalized linear mixed model with negative binomial distribution, fixed effects of *treatment, day,* and *treatment by day* interaction, and a log offset to account for different numbers of honey bees within each sample. The model included a random effect of *hive* to account for repeated measurements (Hurlbert, 1984). Frames of adult honey bees were analyzed using a generalized linear mixed model with normal (Gaussian) distribution. Fixed effects were again *treatment, day,* and *treatment by day* interaction with a random effect of *hive*. Both models used the *joint_tests* function in the *emmeans* package to determine significant interactions between treatment and day (Lenth, 2023). If there was a significant interaction, the *emmeans* package was used to make pair-wise comparisons using Tukey HSD adjusted p-values with $\alpha < 0.05$. Colony survival was analyzed by taking colony data from the Day 97 inspection colonies. Surviving colonies were given a 1 while colonies that died during the experiment were given a 0. This data set was analyzed using a generalized linear model with binomial distribution and a fixed effect of *treatment*. The *emmeans* package was again used to make pair-wise comparisons using Tukey HSD adjusted p-values where the alpha was 0.05. All models were tested for fit by testing residuals, dispersion, and zero inflation using the *DHARMa* package to ensure the best model was selected (Hartig, 2022).

Results

Varroa destructor infestation rate

We observed a significant interaction between experiment day and treatment for *V. destructor* infestation rate ($\chi^2_2 = 18.40$, P = 0.001). Although we did not see any significant differences for *V. destructor* infestation rates at Day 0 (F ratio$_{2,\infty} = 0.031$, P = 0.97) (Figure 2.1; estimates and confidence intervals in Table 2.1), we observed significant differences among

treatments groups at Day 67 (F ratio$_{2,\infty}$ = 9.32, P < 0.001) and Day 97 (F ratio$_{2,\infty}$ = 6.07, P = 0.002). At Day 67, OA SpillTech® had an estimated infestation rate of 0.89%, which was significantly greater than 0.30% for Aluen CAP® (z-ratio$_{\infty}$ = -2.42, P = 0.041) and 0.13% for Apivar® (z-ratio$_{\infty}$ = -4.20, P < 0.001). Aluen CAP® and Apivar® groups were not significantly different (z-ratio$_{\infty}$ = -1.65, P = 0.23). At Day 97, OA SpillTech® had an estimated infestation rate of 0.43%, which was significantly greater than the Apivar® groups' 0.07% infestation rate (z-ratio$_{\infty}$ = -3.46, P = 0.002). Aluen CAP® had an infestation rate of 0.20% and did not significantly differ from either OA SpillTech® (z-ratio$_{\infty}$ = -1.61, P = 0.24) or Apivar® group (z-ratio$_{\infty}$ = -1.82, P = 0.16).

Only the Apivar® and Aluen CAP® treatment groups saw a significant decrease in *V. destructor* infestation rate during the experiment from Day 0 to Day 97 (F ratio$_{2,\infty}$ = 12.76, P < 0.001; F ratio$_{2,\infty}$ = 3.32, P = 0.04). Apivar® experienced a significant reduction from 0.59% at Day 0 to 0.13% at Day 67 and 0.07% at Day 97 (z-ratio$_{\infty}$ = 3.632, P < 0.001; z-ratio$_{\infty}$ = 4.484, P < 0.001). Conversely, OA SpillTech® did not experience any significant differences in *V. destructor* infestation rate throughout the experiment (F ratio$_{2,\infty}$ = 2.52, P = 0.08), whereas Aluen CAP® only experienced a significant difference in infestation rate between Days 0 and 97, reducing *V. destructor* infestation from 0.61% to 0.20% (z-ratio$_{\infty}$ = 2.54, P = 0.03).

Amitraz resistance assessments at Day 0 showed that experimental colonies had an initial average amitraz resistance of 38.53% against *V. destructor*. For each treatment group, average amitraz resistance at this assessment date were 38.53% for Apivar®, 36.3% for Aluen CAP®, and 40.7% for OA SpillTech®.

Table 2.1. Model estimates. Point estimates and 95% confidence intervals for *Varroa destructor* infestation rate, frames of adult honey bees (*Apis mellifera*), and colony survival through the end of the experiment among treatment groups on each experimental day.

Variable	Day	Treatment	Estimate [95% CL]
Varroa destructor infestation rate (mites per 100 honey bees)	0	Apivar®	0.59 [0.34 – 1.04]
		Aluen CAP®	0.61 [0.33 – 1.14]
		OA Spilltech®	0.55 [0.32 – 0.96]
	67	Apivar®	0.13 [0.06 – 0.27]
		Aluen CAP®	0.30 [0.14 – 0.62]
		OA Spilltech®	0.89 [0.52 – 1.54]
	97	Apivar®	0.07 [0.03 – 0.17]
		Aluen CAP®	0.20 [0.10 – 0.42]
		OA Spilltech®	0.43 [0.24 – 0.77]
Frames of adult honey bees	0	Apivar®	7.86 [7.25 – 8.47]
		Aluen CAP®	8.09 [7.40 – 8.77]
		OA Spilltech®	7.74 [7.13 – 8.35]
	67	Apivar®	5.15 [4.54 – 5.76]
		Aluen CAP®	2.72 [2.03 – 3.40]
		OA Spilltech®	4.24 [3.63 – 4.85]
	97	Apivar®	7.76 [7.15 – 8.37]
		Aluen CAP®	6.01 [5.33 – 6.69]
		OA Spilltech®	7.22 [6.62 – 7.83]
Colony survival through Day 97 (%)	97	Apivar®	0.97 [0.81 – 1.00]
		Aluen CAP®	0.74 [0.56 – 0.86]
		OA Spilltech®	0.97 [0.80 – 0.99]

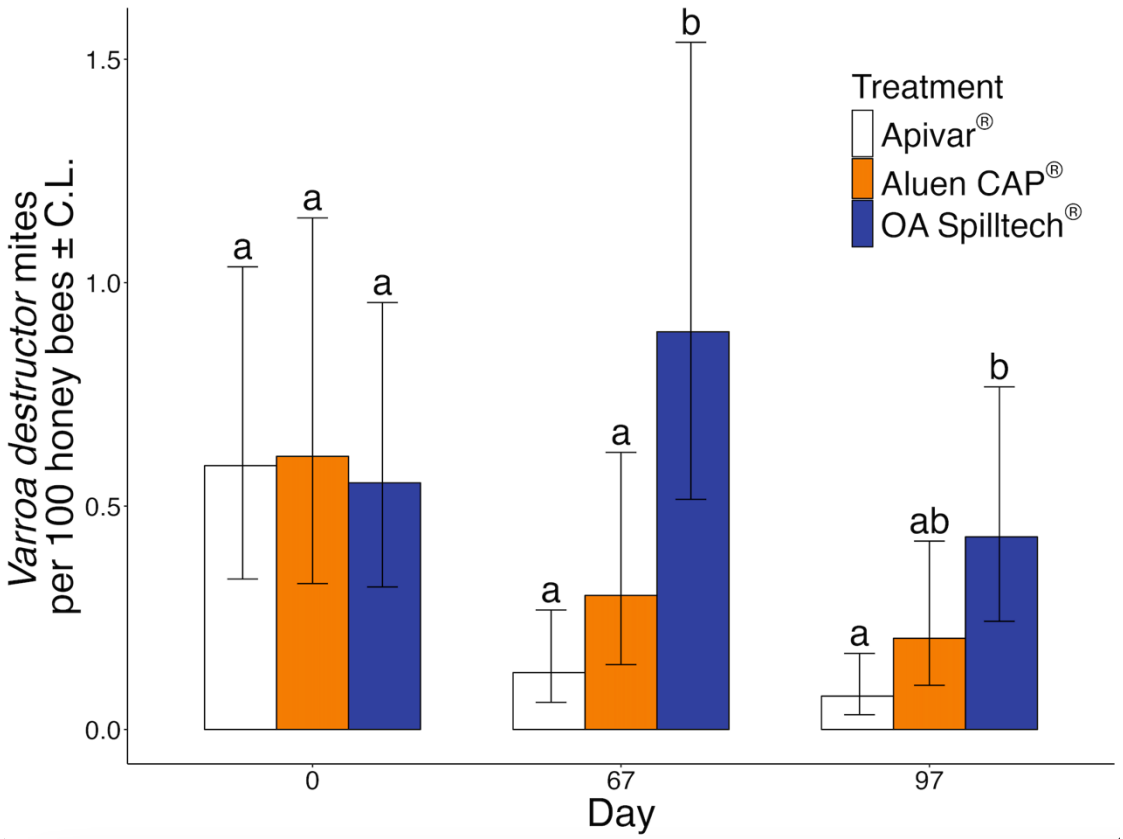

Figure 2.1. *Varroa destructor* infestation rate of adult *Apis mellifera*. Mean infestation rate of *V. destructor* per 100 adult honey bees by treatment group and experimental day. Model estimates and 95% confidence limits are shown. Different letters indicate significant differences between treatments for each individual sampling period based on statistical comparisons. ($P < 0.05$) (OA = oxalic acid).

Frames of adult honey bees

We observed a significant interaction between experiment day and treatment for frames of adult honey bees ($\chi^2_2 = 36.45$, $P < 0.001$). We did not observe any significant differences among treatment groups at Day 0 ($F\ ratio_{2,\infty} = 0.28$, $P = 0.76$), but did so at Day 67 ($F\ ratio_{2,\infty} = 13.76$, $P < 0.001$) and Day 97 (and $F\ ratio_{2,\infty} = 7.26$, $P < 0.001$) (Figure 2.2; Model estimates and confidence intervals in Table 2.1). At Day 67, Aluen CAP® had a model estimated 2.72 frames of adult honey bees, significantly less than the 5.15 and 4.24 frames of adult honey bees for Apivar® and OA SpillTech®, respectively ($t\text{-}ratio_{\infty} = 5.23$, $P < 0.001$; $t\text{-}ratio_{\infty} = -1.52$, $P = 0.003$). Aluen CAP® also had significantly fewer frames of adult honey bees at Day 97, with 6.01 compared to Apivar® and OA SpillTech®, which had model estimates of 7.76 and 7.22 frames of adult honey bees, respectively ($t\text{- }ratio_{\infty} = 1.75$, $P < 0.001$; $t\text{-}ratio_{\infty} = -2.61$, $P = 0.026$). Apivar® and OA SpillTech® did not significantly differ at Day 67 or Day 97 ($t\text{-}ratio_{\infty} = 2.071$ $P = 0.098$; $t\text{-}ratio_{\infty} = 1.22$, $P = 0.44$).

All treatment groups – Apivar®, Aluen CAP®, and OA SpillTech® – saw a significant decrease in frames of adult honey bees from Day 0 to Day 67 ($t\text{-}ratio_{\infty} = 8.74$, $P < 0.001$; $t\text{- }ratio_{\infty} = 15.39$, $P < 0.001$; $t\text{-}ratio_{\infty} = 3.50$, $P < 0.001$), respectively; however, Apivar® and OA Spilltech® had no significant differences between frames of adult honey bees at Day 0 and Day 97 ($t\text{-}ratio_{\infty} = 0.33$, $P = 0.94$; $t\text{-}ratio_{\infty} = 1.67$, $P = 0.22$, respectively); only Aluen CAP® saw a significant decrease in frames of adult honey bees from Day 0 to Day 97 ($t\text{-}ratio_{\infty} = 5.95$, $P < 0.001$).

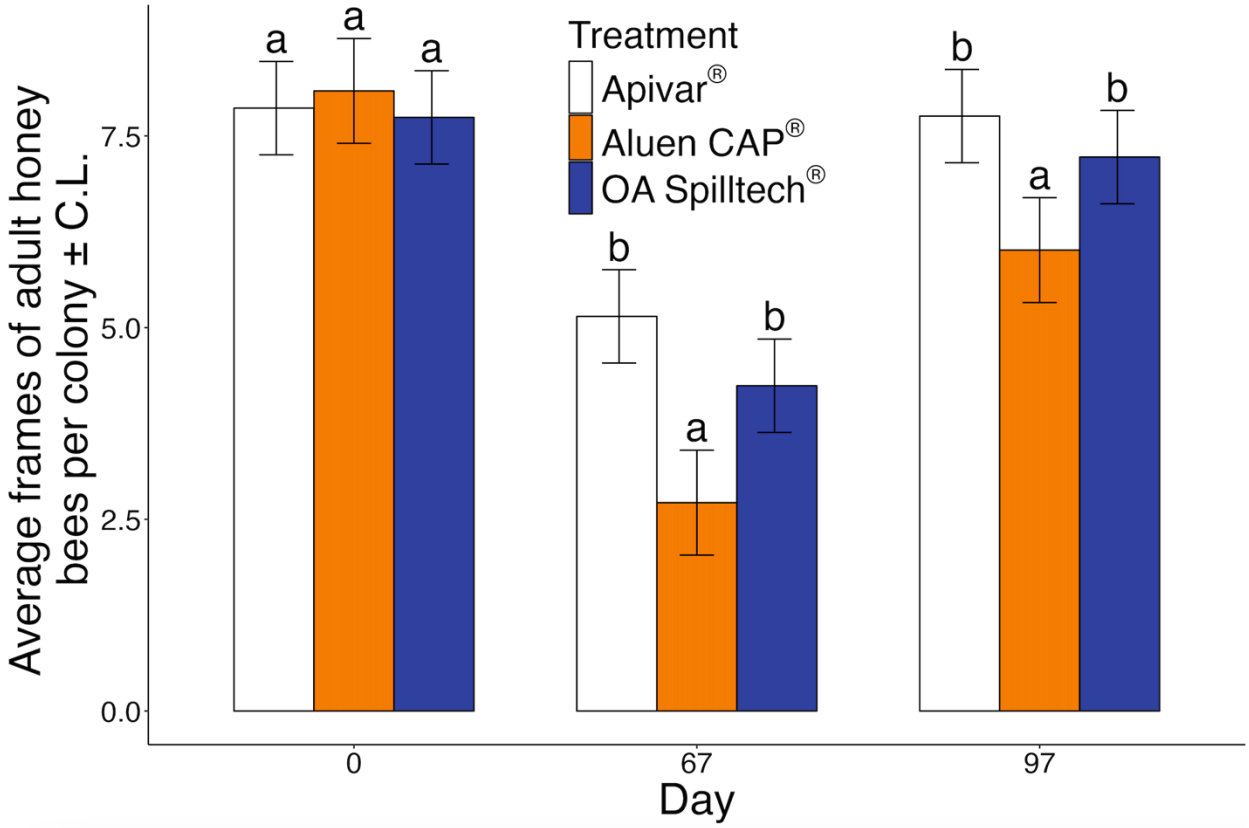

Figure 2.2. Mean frames of adult *Apis mellifera*. Mean adult honey bee population in frames of adult honey bees by treatment group and experimental day. Model estimates and 95% confidence limits are shown. Different letters indicate significant differences between treatments for each individual sampling period based on statistical comparisons. ($P < 0.05$) (OA = oxalic acid).

Colony survival

We did not observe any significant differences among treatments for colony survival (Figure 2.3; Estimates and confidence intervals in Table 2.1). The Aluen CAP® group had the lowest survival model estimate of 0.74, but that was not significantly different than Apivar® or OA SpillTech® groups (z-ratio$_\infty$ = 2.17, P = 0.076; z-ratio$_\infty$ = -2.14, P = 0.082).

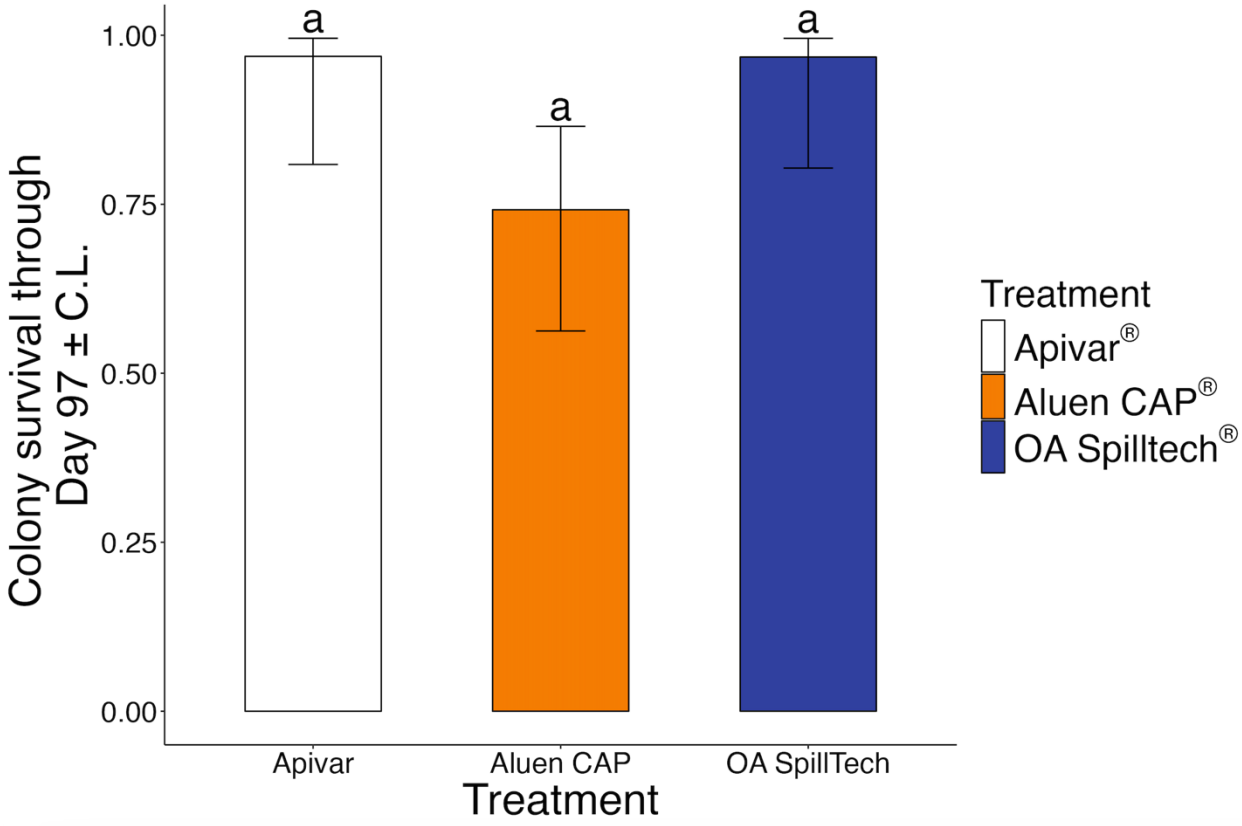

Figure 2.3. Colony survival. Mean *Apis mellifera* colony survival at the end of the study on Day 97 by treatment group. Model estimates and 95% confidence limits are shown. Different letters indicate significant differences between treatments for each individual sampling period based on statistical comparisons. ($P < 0.05$) (OA = oxalic acid).

Discussion

Managing against *V. destructor* continues to be a vital part of colony management for beekeepers, especially prior to winter (Guzmán-Novoa et al., 2010; Van Dooremalen et al., 2012). Reliance on amitraz, as well as increases in amitraz resistance (Haber et al., 2019; Rinkevich, 2020; Rinkevich et al., 2023), result in beekeepers needing alternative treatment strategies to help manage *V. destructor* infestations while limiting use of amitraz. Our experiment showed that even when working with honey bee colonies containing moderate levels of amitraz resistant *V. destructor* mite populations, the amitraz based treatment group Apivar® was the only group to significantly reduce mite infestation rate over 67 and 97 days. The Aluen CAP® group significantly reduced *V. destructor* infestation rate from Day 0 to Day 97, but this group had reduced colony strength and survival compared to the other treatment groups. Meanwhile, the OA SpillTech® group had significantly higher mite infestation rates on Day 67 compared to the other two treatments and was the only treatment to not significantly reduce infestation rate over 97 days. Overall, more work needs to be done to determine effective, alternative *V. destructor* treatments for the Southeastern U.S., which experiences higher temperatures and increased capped brood later in the year, thus allowing *V. destructor* infestations to continue to grow and limits the available treatment opportunities for beekeepers.

For this experiment, *V. destructor* infestation rates started low, under the recommended threshold of treatment going into a dormant period of 1% infestation per 100 honey bees (Honey Bee Health Coalition, 2022). A negative control was not included in this experiment, due in part to partnership with a commercial beekeeper who was hesitant to leave colonies untreated in the same yard. Additionally, there was concern given the length of the experiment that untreated colonies could become “robber lure” colonies likely to die due to high mite infestation rates

resulting in re-infestation of treated colonies negating any impacts seen by those treatments (Jack et al., 2023; Peck and Seeley, 2019). Although we partnered with this beekeeper due to moderate levels of amitraz resistance within their operation, they still routinely treated with amitraz and were able to keep mite infestation rates low. The moderate levels of amitraz resistance seen, averaging at 38.53% resistance across all treatment groups at Day 0, also highlight that amitraz based products are likely still effective against *V. destructor*, but they should result in reduced efficacy compared to populations with no resistance (Rinkevich, Unpublished). Additionally, due to the low infestation rates, extra factors such as genetic drift could play an important factor in influencing resistance dynamics within *V. destructor* populations by changing resistance allele frequencies within populations (Papkou et al., 2016; White et al., 2021). Subsequently, significant influences of amitraz resistance may have been absent due to the limited sample size of mites (Papkou et al., 2016; White et al., 2021).

Our experiment illustrated slight differences between two extended-release oxalic acid options for beekeepers on *V. destructor* infestation rates. While homemade extended-release oxalic acid treatments, as represented by our OA SpillTech® group, have shown some success in certain experiments (Kanelis et al., 2023; Sabahi et al., 2020, 2017), our results were in line with recent publications in the Southeast region demonstrating homemade extended-release oxalic acid treatments were incapable of reducing mite infestation rates throughout the experiment (Bartlett et al., 2023; Jack et al., 2024; Plamondon et al., 2024). The other extended-release treatment group that can be purchased as ready-to-use product, Aluen CAP®, had a significantly lower *V. destructor* infestation rate on Day 67 compared to the homemade OA SpillTech® group. While Aluen CAP® significantly reduced infestation rates compared to the beginning of the experiment, it took until Day 97 to see infestation rates significantly reduced from Day 0. This is

over twice as long as the labeled treatment time and longer than previous experiments using the same treatment during the summer when more brood area was present (Maggi et al., 2016; Rodríguez Dehaibes et al., 2020). There is potential that having strips inserted into the colony between the frames touching the comb near the brood nest as done with the Aluen CAP® group allows the oxalic acid to more easily contact *V. destructor* mites and kill them, compared to placing the oxalic acid glycerin strips above top bars of brood frames, which was how the OA SpillTech® treatment group had their strips applied.

For honey bee colony strength, the Aluen CAP® group had significantly fewer frames of adult honey bees and numerically lower colony survival compared to the other treatment groups. All treatment groups saw a significant reduction in frames of adult honey bees from Day 0 to Day 67, but that was to be expected as colonies naturally see reductions in populations during winter in the location where the trial was performed. Previously, there has not been any evidence of extended-release oxalic acid having a negative impact on colony strength (Bartlett et al., 2023; Kanelis et al., 2023; Maggi et al., 2016; Plamondon et al., 2024; Rodríguez Dehaibes et al., 2020; Sabahi et al., 2020); however, this is one of the first experiments to test extended-release oxalic acid during winter when capped brood area is more limited. It is possible that the Aluen CAP® strips, which have some moisture on them due to the glycerin, being inserted into the brood nest prevented colonies from properly clustering as colony strength was not impacted in the other two treatment groups. Like the Apivar® and OA SpillTech® groups, the Aluen CAP® group appeared to rebound in frames of adult honey bees, but it still had significantly fewer frames of adult honey bees at Day 97 and lost numerically more colonies comparatively.

Results from this experiment show that the commercially available amitraz based Apivar® can still be used as a viable treatment to control for *V. destructor* before winter. The

commercially available, albeit not in the U.S., extended-release oxalic acid Aluen CAP® also showed an ability to reduce *V. destructor* infestation rates, but it came at the cost of a reduction in colony strength, especially frames of adult honey bees. The homemade extended-release OA SpillTech® group demonstrated good colony survival, but the treatment appears to be more complimentary as it maintains *V. destructor* infestation rates instead of reducing them. Overall, more research needs to be undertaken to understand the dynamics of amitraz resistant *V. destructor* populations and determine how long resistant traits stay in the population. Additionally, more experiments need to be conducted to determine alternative treatments to amitraz and how to eliminate amitraz resistant mite populations.

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Chapter 3

Late-season *Varroa destructor* treatment effectiveness in the U.S. southeast, and impact on amitraz resistant mites

Abstract

Varroa destructor is a leading cause of honey bee (*Apis mellifera*) colony losses worldwide. Although commercial beekeepers in the U.S. have access to multiple active ingredients with miticidal properties, amitraz has been among their most preferred for *V. destructor* treatment for more than a decade because of its high effectiveness and convenience of application. As a result, *V. destructor* resistance to amitraz has recently been documented. Here we evaluated U.S. registered *V. destructor* treatments with differing active ingredients on *V. destructor* infestation rates, as well as their impact on amitraz resistant *V. destructor*, to identify alternative treatment options for commercial beekeepers just prior to winter, a critical time of year to have low mite infestation. Treatment groups included an untreated control, Apivar® (active ingredient amitraz), FormicPro™ (a.i. formic acid), Hopguard® 3 (a.i. hops beta acids) and ApiBioxal® (a.i. oxalic acid) (OA) vapor. Treatments were implemented in late September and colonies were assessed 63 and 133 days later. We found that the Apivar®, FormicPro™ and HopGuard® treatment groups suppressed increases in *V. destructor* infestation rates but did not decrease *V. destructor* infestation rates throughout the study, whereas *V. destructor* infestation rates significantly increased in the untreated control and OA Vapor treatment groups. Queen loss was observed in one-third of FormicPro™ colonies. For colony strength, we saw only minor differences among treatment groups for weight and no differences in frames of capped honey bee brood and frames of adult honey bees. Genetic analysis showed that amitraz resistance allele frequency

significantly increased from Day 0 to Day 63 after the application of Apivar®, but this change disappeared by Day 133 after the overwintering period; non-amitraz-based treatments did not impact amitraz resistance allele frequency in *V. destructor* populations, which suggests that amitraz resistance may not have a large fitness cost, and there is no cross-resistance of amitraz to other active ingredients tested.

Introduction

Varroa destructor is a parasitic mite of the western honey bee (*Apis mellifera*) and is widely thought to be the leading cause of colony losses in the United States and other areas of the northern hemisphere (Rosenkranz et al. 2010; Aurell et al. 2024a). These mites feed on developing and adult honey bees while transmitting viruses that reduce honey bee colony productivity and survival (Highfield et al. 2009; Ramsey et al. 2019; Martin and Brettell 2019; Han et al. 2024). Failure to manage *V. destructor* populations by beekeepers often results in colony losses in less than one year (Kulhanek et al. 2021b; Jack et al. 2023). While multiple management strategies exist for *V. destructor* control, beekeepers, especially large-scale commercial beekeepers, primarily rely on chemical treatments to control mites (Haber et al. 2019; Jack and Ellis 2021). This reliance on chemical treatments has resulted in *V. destructor* resistance to some synthetic acaricides, thereby limiting the effectiveness of these treatments (Gracia-Salinas et al. 2006; Maggi et al. 2009; Rinkevich 2020; Higes et al. 2020; Vlogiannitis et al. 2021; Hernández-Rodríguez et al. 2022; Marsky et al. 2024; Hernández-Rodríguez et al. 2025).

Since amitraz was registered in the U.S., it has become one of the most widely used acaricides against *V. destructor* as it is capable of significantly reducing mite populations after a single treatment (Al Naggar et al. 2015; Rinkevich 2020; Jack et al. 2020, 2024; Aurell et al.

2024b); however, continued treatments of amitraz has resulted in development of amitraz resistant *V. destructor* mites (Rinkevich 2020; Hernández-Rodríguez et al. 2022; Rinkevich et al. 2023; Marsky et al. 2024; Hernández-Rodríguez et al. 2025). Complicating matters is that development of amitraz resistance is not consistently driven by one mutation, with amitraz resistance being associated with different mutations geographically around the world (Hernández-Rodríguez et al. 2022; Rinkevich et al. 2023; Marsky et al. 2024; Hernández-Rodríguez et al. 2025). In the U.S., amitraz resistance has been linked with a Y215H mutation in the β2 octopamine receptor (Octβ2R) (Rinkevich et al. 2023). It is believed that *V. destructor* mites possessing the homozygous recessive genotype for this mutation are amitraz resistant (Rinkevich et al. 2023), and further application of amitraz based products will preferentially select for this resistance genotype, resulting in increases in the amitraz allele frequency. Currently, there is little known about amitraz resistant *V. destructor* population dynamics, and if there is any potential fitness cost to having the resistance allele or the impacts on resistance allele frequency when *V. destructor* are treated with a different active ingredient. These knowledge gaps limit the understanding of how long amitraz resistant alleles may remain in the population if alternative management strategies are employed.

While other chemical *V. destructor* treatments with different active ingredients exist, many of them have limitations that have prevented widespread adoption and driven beekeepers' reliance on amitraz (Jack and Ellis 2021). *Varroa destructor* behavior and biology can be a limiting factor for treatments as mites have two stages, the dispersal and reproductive (Traynor et al. 2020; Reams and Rangel 2022). During the reproductive stage, foundress mites reproduce and feed on pupating honey bees while protected by the wax brood cell capping, thereby limiting effectiveness of contact treatments when brood is present (Gregorc and Planinc 2002, 2004;

Bacandritsos et al. 2007; Vandervalk et al. 2014; Jack and Ellis 2021; Berry et al. 2022, 2023). Rapid acting treatments that rely on contact with *V. destructor* to kill mites, such as oxalic acid and HopGuard® (active ingredient of hops β-acids), are most effective when limited brood is present allowing the acaricide to contact and kill *V. destructor* in the dispersal phase when *V. destructor* is present on adult honey bees (DeGrandi-Hoffman et al. 2014; Al Toufailia et al. 2015; Rademacher et al. 2015; Coffey and Breen 2016; Büchler et al. 2020; Reams and Rangel 2022; Kulhanek et al. 2023). Meanwhile, formic acid is the only acaricide capable of penetrating cell cappings, killing mites within brood cells during its reproductive phase (Van Engelsdorp et al. 2008; Traynor et al. 2020). However, FormicPro™ (active ingredient of formic acid) has a narrow temperature range and, when used at temperatures above this range, has shown to kill adult honey bees, especially workers and queens, thus limiting its potential use in regions with elevated temperatures most of the year (Underwood and Currie 2005; Giovenazzo and Dubreuil 2011; NOD Apiary Products 2017). In addition to the implications of colony condition regarding capped honey bee brood area and the temperature window, cost of treatment applications is an important factor for beekeepers. Treatments like FormicPro™ and HopGuard® are more expensive than using unregistered amitraz based treatments, thus less economical for beekeepers especially at a commercial scale (Haber et al. 2019).

This experiment was designed to test the effectiveness of various treatments with differing active ingredients for *V. destructor* in the southeastern region of the U.S. Due to climatic conditions, this region often has high temperatures and moderate amounts of brood later into the fall and early winter, thereby limiting treatment selection and effectiveness for colonies going into winter, a critical time for *V. destructor* treatments (Jack et al. 2023, 2024). Therefore, we tested the effectiveness of four different treatments (Apivar®, FormicPro™, HopGuard®, and

oxalic acid) to evaluate their effectiveness at reducing *V. destructor* infestation rates. Additionally, *V. destructor* mites were collected and genotyped to determine amitraz resistance allele frequency to determine how resistance allele frequencies are impacted by treatments with various active ingredients.

Materials and Methods

Experimental Design

The experiment used a total of 75 colonies in two apiaries, both located in Auburn, Alabama, USA. All colonies were queenright with Italian *Apis mellifera* queens at the beginning of the experiment and housed in two deep 10-frame Langstroth boxes. Colonies were initially assessed the week of 25 September 2023 for *V. destructor* infestation rate on adult honey bees, capped honey bee brood area, frames of adult honey bees, and colony weight. *Varroa destructor* infestation rate was determined by shaking adult honey bees from a frame that had open worker honey bee larvae into a plastic tub and scooping ~ 120 grams (1/2 cup) of honey bees into a 946 mL (32 oz) plastic deli container containing 35% isopropyl alcohol. Triple rinse mite washes were performed using methods described in Aurell et al. (2024b), and mites were collected and stored in a freezer at – 20°C. Adult honey bees were also collected after mite washes and counted to record *V. destructor* infestation rate as mites per 100 honey bees. Colony strength assessments of frames of capped honey bee brood and frames of adult honey bees were done using the methods described in Guzman-Novoa et al. (2024); all colonies were weighed to determine differences in accumulated resources like honey, nectar, and pollen by using a tripod pulley system to lift colonies on a spring scale. After initial colony inspections, colonies were assigned into one of five treatment groups based on *V. destructor* infestation rate through stratified randomization (Kernan et al. 1999; Altman 2001; Moher et al. 2010; Giacobino et al. 2014;

Molineri et al. 2017; Tokach et al. 2024). Colonies were organized by descending order of *V. destructor* infestation rate with five colonies per stratum and each treatment being represented once per strata. The proposed treatment assignments were reviewed and re-randomized several times to assist in the homogenization of other colony strength variables and to evenly distribute treatment groups between the two locations. Treatments were applied the week of 25 September 2023, and colonies were re-assessed for the same four metrics using previously described methods the week of 27 November 2023 and the week of 5 February 2024 (63 and 133 days after treatments were applied, respectively) These dates were selected to evaluate *V. destructor* infestation rates after the treatment and overwintering periods. In the Southeast, late November presents a time when brood production is reduced, allowing for the opportunity of an additional treatment, while early February constitutes a point when brood production begins to increase and spring management starts. Colonies were also monitored monthly to assess queen status. Colonies were removed from the study if they were queenless after two consecutive inspections or if they had less than one frame of adult honey bees by field assessment. Additionally, colonies were moved to a secondary location after Day 63 assessments if more than 45 *V. destructor* mites were found in the mite wash (~15% *V. destructor* per 100 honey bees) to prevent re-infestation of previously treated colonies (Dynes et al. 2019; Peck and Seeley 2019; Kulhanek et al. 2021a; Bartlett et al. 2024). Data collected up until the point of colony removal were kept in the analysis.

Varroa destructor treatments

This experiment included five different treatment groups with 15 colonies per group. The treatments included an untreated Control, Apivar® (active ingredient amitraz at 3.3%), FormicPro™ (active ingredient formic acid at 65%), HopGuard® 3 (active ingredient hops β-

acids at 16%), and an Apibioxal® oxalic acid vapor (OA Vapor) regimen (active ingredient oxalic acid at 99%). For the Apivar® group, two strips of Apivar® per deep body for a total of four strips per colony were placed in the brood nest and removed after 42 days. Prior to treatment with Apivar®, all colonies in this treatment group (n = 15) had an amitraz resistance test performed using methods previously described in Rinkevich (2020) to give a baseline understanding of amitraz resistance levels which would help explain the performance of Apivar®. The FormicPro™ group had two FormicPro™ pads placed in-between the deep boxes, and the pads were removed 14 days later. The HopGuard® group had two strips of HopGuard® 3 per deep body for a total of four strips per colony placed in the brood nest. These strips were removed after 14 days and another round of HopGuard® 3 was applied in the same manner which was also subsequently removed after another 14 days. The OA Vapor group received 2 grams of ApiBioxal® per brood box for a total of 4 grams per colony three times with treatments spaced 7 days apart. OA Vapor was administered using a ProVap® device (OxaVap). The 4 grams of ApiBioxal® were placed in the lid. The vaporizer was inverted, and the lid was secured before the vaporizer was flipped and inserted into the colony. The vaporizer was allowed to reach 230°C which took approximately two minutes before it was removed. Prior to application, all cracks in the hive were sealed with tape, and a rag was used to plug the entrance of the colony. The rag was removed ten minutes after the OA Vapor treatment had been applied. All treatments and dosages were applied as per label instruction.

Genotyping

All *V. destructor* from all assessments and treatment groups that were collected in alcohol washes were shipped to the USDA-ARS Honey Bee Breeding, Genetics, and Physiology Lab (HBBGPL), Baton Rouge, LA, where they were stored at -80°C until genotyping. Genotyping

was done to assess allele frequency of the Y215H mutation in the β2 octopamine receptor (Octβ2R) (Rinkevich et al. 2023) for each different treatment group individually per sampling day. A total of 1815 *V. destructor* were genotyped before treatment (September, n=800), after treatment (November, n=763), and after overwintering (February, n=252). Genomic DNA was extracted from individual *V. destructor* by homogenizing in a Bead Ruptor Elite Mill (OMNI-International) using the Maxwell RSC PureFood GMO and Authentication Kit (Promega) according to the manufacturer's directions. DNA concentration was measured on a NanoDrop (ThermoFisher). Genotyping for the Y215H mutation in the β2 octopamine receptor (Octβ2R) was performed using a TaqMan assay following the methods of Rinkevich et al. (2023) and Hernández-Rodríguez et al. (2023) which provided data regarding the presence or absence of the amino acid mutation for amitraz resistance.

Statistics

All statistics were conducted using R.2.2 (R Core Team 2022). Packages used included *lme4*, *dplyr*, *tidyverse*, *glmmTMB*, *emmeans*, *DHARMa*, and *ggplot2* (Wickham 2016; Brooks et al. 2017; Wickham et al. 2019, 2023; Hartig 2022; Lenth 2023). *Varroa destructor* infestation rate was analyzed using a generalized linear mixed model with negative binomial distribution, fixed effects of *Day*, *Treatment*, and a *Treatment* by *Day* interaction, a log offset to account for variation in the number of adult honey bees per sample, and a random effect of *Hive* to account for repeated measurements (Hurlbert 1984; Brooks et al. 2017). Colony weight was analyzed with a linear mixed model with normal (Gaussian) distribution, fixed effects of *Day*, *Treatment*, and a *Treatment by Day* interaction, and a random effect of *Hive*. Since both models had significant interactions between *Treatment* and *Day*, models were tested using the *joint-tests* function in the *emmeans* package to determine if there were significant differences between

treatment groups within day (Lenth 2023). Pairwise comparisons were then made using the *emmeans* package where Tukey-HSD adjusted p-values were used and significance was determined with an alpha of 0.05. Frames of adult honey bees and frames of capped honey bee brood were analyzed using a linear mixed model with normal distribution, fixed effects of *Day* and *Treatment*, and a random effect of *Hive*. An F-test of nested models indicated that the *Treatment* by *Day* interaction did not significantly improve model fit for either the frames of adult honey bees ($\chi^2_8 = 2.74$, $P = 0.95$) or the frames of capped honey bee brood models ($\chi^2_8 = 3.89$, $P = 0.87$). Consequently, descriptive statistics were used to describe differences among treatment groups within day. Additionally, scaled quantile residuals were used and QQ plots were visualized utilizing the *DHARMa* package to test for model fit and ensure an appropriate model distribution was selected (Hartig 2022).

Genotype data were summarized and condensed based on treatment group and genotype. Generalized linear mixed models with binomial distribution with a fixed effect of *Treatment by Day* interaction and a random effect of *Hive* were used to analyze differences among resistance allele genotype for homozygous resistant, heterozygous, and homozygous susceptible genotypes pending sampling day (Hurlbert 1984; Brooks et al. 2017). Since the FormicPro™ treatment group did not have any *V. destructor* mites with a heterozygous allele genotype on Day 133, the data were removed from the model, and the FormicPro™ treatment group was given an estimate of zero (Tokach et al. 2024). Pairwise comparisons were made using the *emmeans* package for each genotype, where Tukey-HSD adjusted p-values were used and significance was determined with an alpha of 0.05. All pairwise comparisons were done as specific model estimates were of interest for all allele frequencies in this study. The *DHARMa* package was again used to visualize

QQ plots utilizing quantile residuals to test for model fit and confirm the use of the appropriate model distribution (Hartig 2022).

Results

Varroa destructor infestation rate

There was a significant *Day* and *Treatment* interaction for *V. destructor* infestation rate (χ^2_8 = 27.47, $P < 0.001$). On Day 0, we did not observe any significant differences between treatment groups (F ratio$_{4,\infty}$ = 0.02, $P = 0.99$) (Figure 3.1), where *V. destructor* infestation rates were estimated at 2.47%, 2.43%, 2.53%, 2.51%, and 2.32% for Control, Apivar®, FormicPro™ , HopGuard®, and OA Vapor treatment groups, respectively. There were significant differences between treatment groups on Day 63 (F ratio$_{4,\infty}$ = 5.85, $P < 0.001$) – the Control group had an estimated *V. destructor* infestation rate of 9.20%, which was significantly higher than the 2.95% infestation estimated for the Apivar® group (z-ratio$_\infty$ = 3.29, $P = 0.009$), 2.58% for the FormicPro™ group (z-ratio$_\infty$ = 3.33, P = 0.008), and 2.74% for the HopGuard® group (z-ratio$_\infty$ = 3.30, $P = 0.008$). The OA Vapor group had a *V. destructor* infestation rate on Day 63 of 7.80%, which was also significantly higher than the Apivar®, FormicPro™ , and HopGuard® groups (z-ratio$_\infty$ = -2.78, $P = 0.044$; z-ratio$_\infty$ = -2.87, $P = 0.033$; z-ratio$_\infty$ = -2.82, $P = 0.038$, respectively). No other significant differences in *V. destructor* infestation rate existed between treatment groups on Day 63. As on Day 0, we did not find any significant differences among treatment groups on Day 133 (F ratio$_{4,\infty}$ = 0.75, P = 0.56) – *V. destructor* infestation rates were estimated at 2.53%, 1.63 %, 1.28 %, 1.32 %, and 1.38 % for Control, Apivar®, FormicPro™ , HopGuard®, and OA Vapor treatment groups, respectively (Figure 3.1).

We did not observe any significant changes over time of *V. destructor* infestation rate across experimental days for the Apivar® (F ratio$_{2,\infty}$ = 2.56, $P = 0.077$), FormicPro™ (F ratio$_{2,\infty}$ =

2.42, $P = 0.089$), or HopGuard® (F ratio$_{2,\infty}$ = 2.78, $P = 0.062$) treatment groups. For the Control and OA Vapor treatment groups we found significant differences for *V. destructor* infestation rate across experimental days (F ratio$_{2,\infty}$ = 16.8845, $P < 0.001$; F ratio$_{2,\infty}$ = 19.45, $P < 0.001$). The Control treatment groups had significantly higher *V. destructor* infestation rates on Day 63 compared to Day 0 or Day 133 (z-ratio$_{\infty}$ = -4.88., $P < 0.001$; z-ratio$_{\infty}$ = -5.39., $P < 0.001$). Similarly, the OA Vapor treatment group had significantly higher *V. destructor* infestation rates on Day 63 compared to Day 0 or Day 133 (z-ratio$_{\infty}$ = 3.89, $P < 0.001$; z-ratio$_{\infty}$ = 5.33, $P < 0.001$).

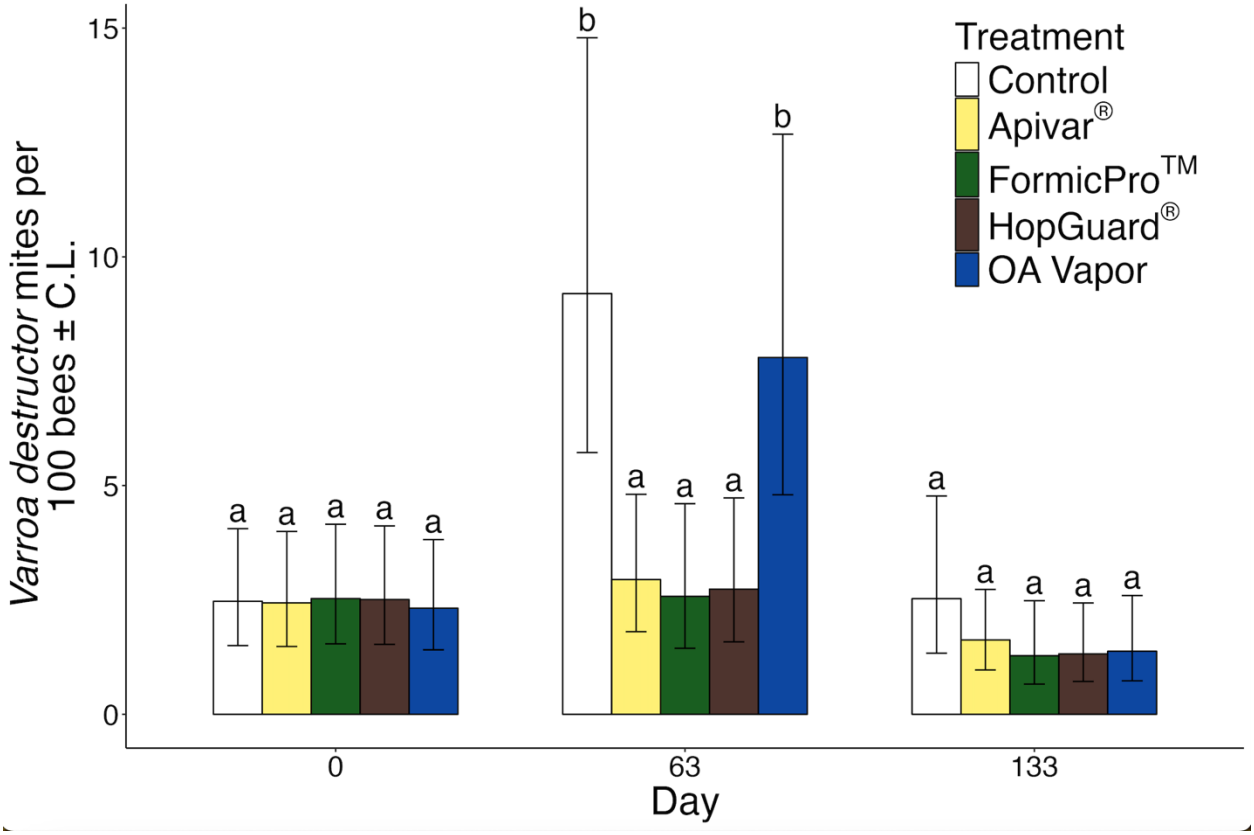

Figure 3.1. *Varroa destructor* infestation rate on adult honey bees (*Apis mellifera*). Mean *V. destructor* infestation rate of adult honey bees among treatment groups on each experimental day. Model estimates are shown with 95% confidence limits. Statistical comparisons where different letters indicate significant differences between treatments for each individual sampling period. ($P < 0.05$) (OA = oxalic acid).

Colony strength metrics

There was no significant *Day* by *Treatment* interaction on frames of adult honey bees (χ^2_8 = 2.74, P = 0.95) or frames of capped honey bee brood (χ^2_8 =3.89, P = 0.87). For both frames of adult honey bees and frames of capped honey bee brood, there was a significant effect of *Day* (χ^2_2 = 130.47, P < 0.001; χ^2_2 = 774.49, P < 0.001), but no significant effect of *Treatment* throughout the study (χ^2_4 = 0.65, P = 0.96; χ^2_4 = 2.47, P = 0.65) (Figures 3.2 and 3.3). There was a significant *Day* by *Treatment* interaction on colony weight (χ^2_8 = 36.74, P < 0.001) (Figure 3.4). There was no significant difference between treatment groups for colony weight on Day 0 (F ratio$_{4,179}$ = 0.69, P = 0.60), where average colony weights ranged from 39.6-42.2 kg. There was a significant difference between treatment groups on Day 63 (F ratio$_{4,179}$ = 4.46, P = 0.002). On Day 63, colonies in the FormicPro™ treatment group had an estimated weight of 37.1 kg, which was significantly less than the Control (t-ratio$_{179}$ = 2.85., P = 0.039), Apivar® (t-ratio$_{179}$ = 3.49., P = 0.006), and OA Vapor (t-ratio$_{179}$ = -2.89., P = 0.035) treatment groups, which had model estimates of 42.1, 43.2, and 42.2 kgs, respectively. The FormicPro™ treatment group did not significantly differ on Day 63 from the HopGuard® treatment group (t-ratio$_{179}$ = -0.91., P = 0.89), which had a model estimate of 38.7 kg, and there were no other significant differences between treatment groups for colony weight on Day 63. On Day 133, there were no significant differences between treatment groups (F ratio$_{4,179}$ = 1.29, P = 0.27), as colony weights ranged from 36.0 – 39.2 kg.

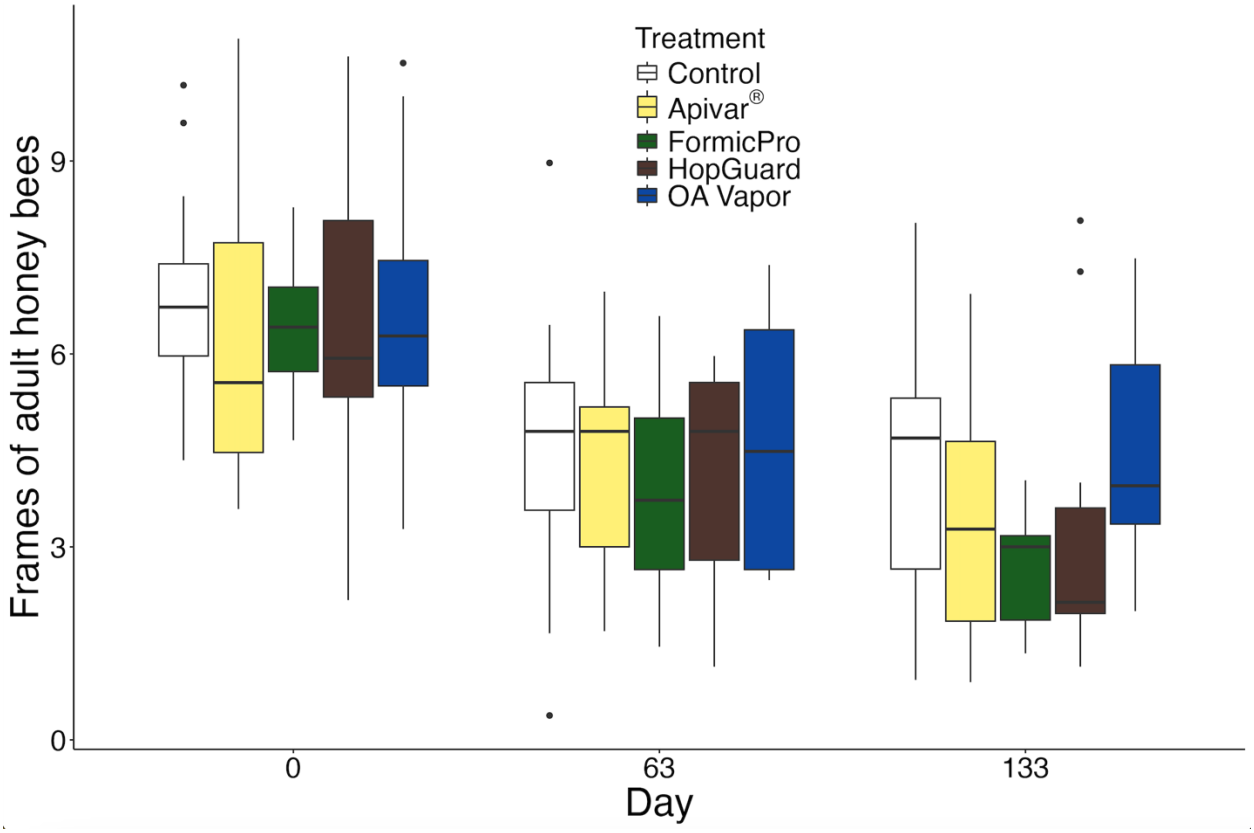

Figure 3.2. Frames of adult honey bees (*Apis mellifera*). Boxplot showing frames of adult honey bees among treatment groups on each experimental day. No significant differences were observed between treatments for any individual sampling period. ($P < 0.05$) (OA = oxalic acid).

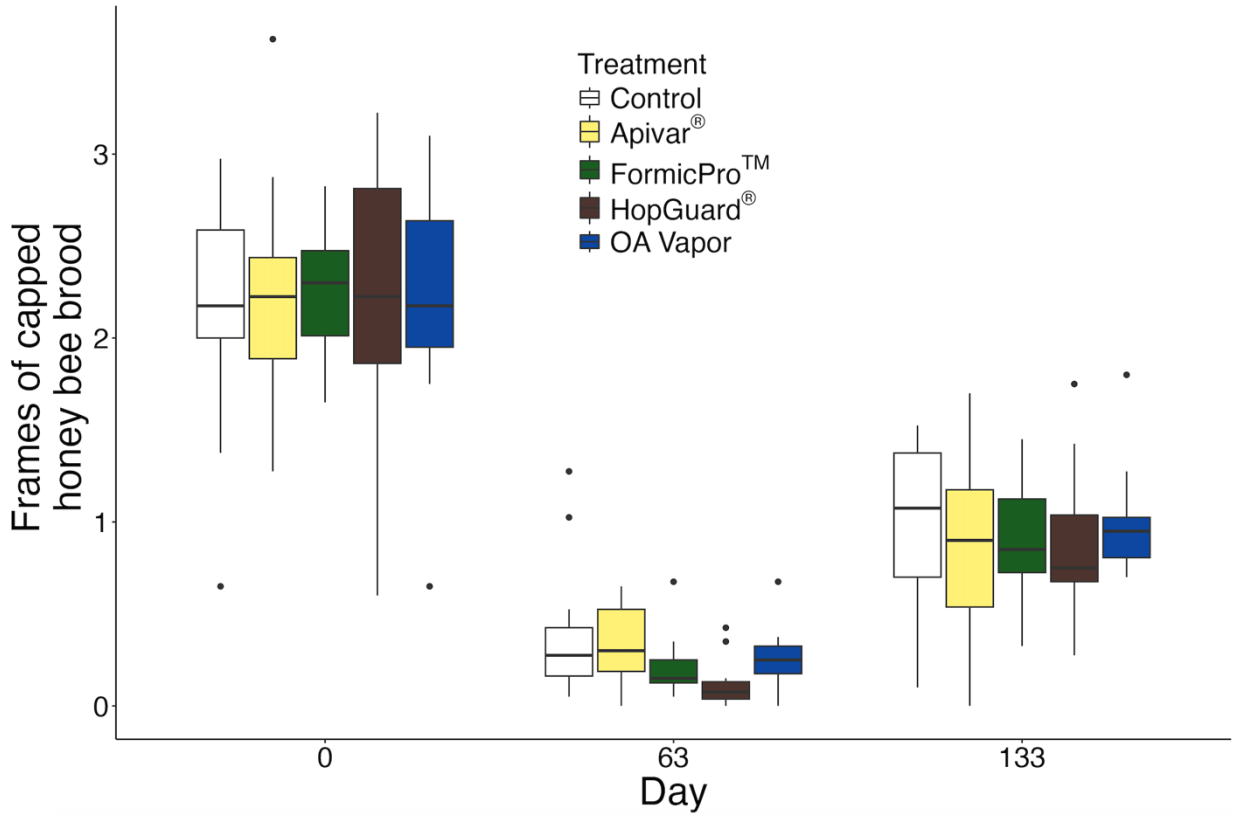

Figure 3.3. Frames of capped honey bee (*Apis mellifera*) brood. Boxplot showing frames of capped honey bee brood among treatment groups on each experimental day. No significant differences were observed between treatments for any individual sampling period. ($P < 0.05$) (OA = oxalic acid).

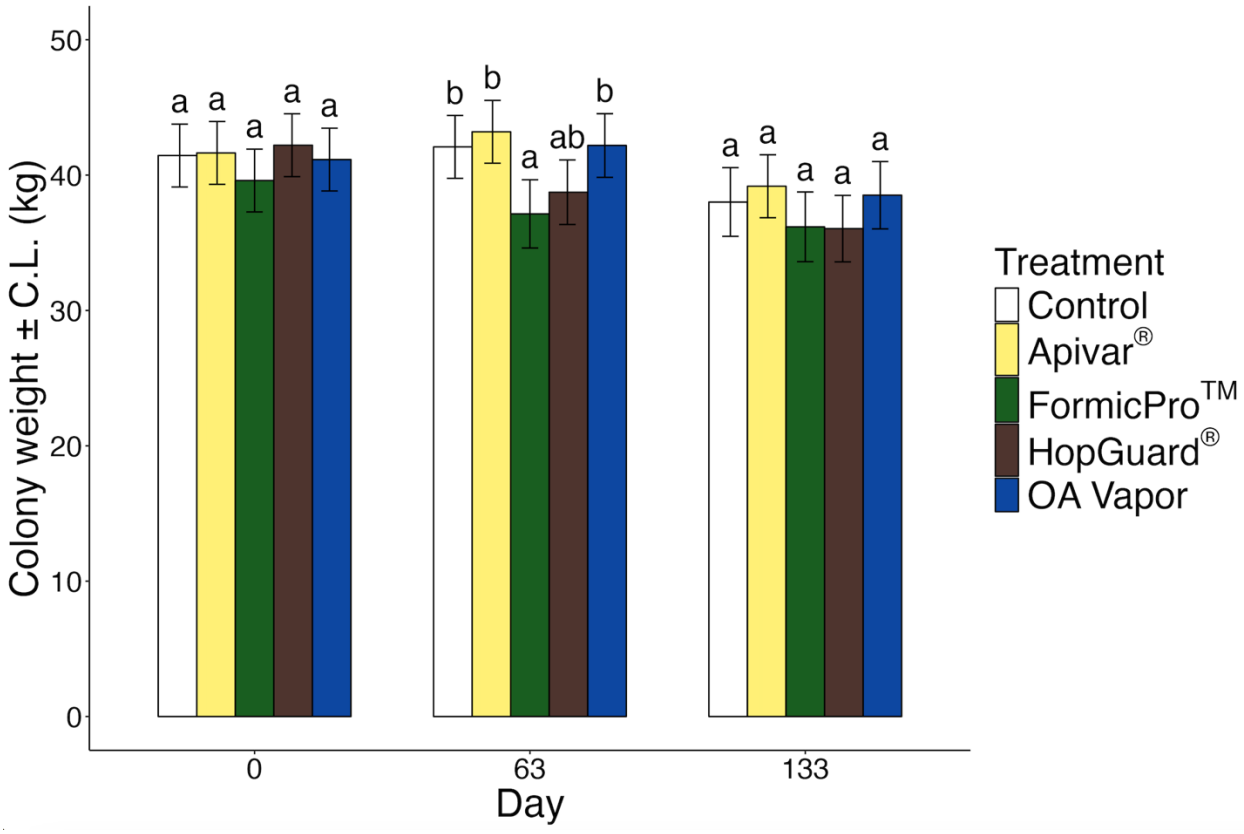

Figure 3.4. *Apis mellifera* colony weight. Mean colony weight among treatment groups on each experimental day. Model estimates are shown with 95% confidence limits. Statistical comparisons where different letters indicate significant differences between treatments for each individual sampling period. ($P < 0.05$) (OA = oxalic acid).

Final colony status

The final colony status on Day 133 and reason for removal from the trial varied among treatment groups with the Apivar® treatment group being the only treatment group whereby all 15 colonies survived throughout the experiment (Figure 3.5). The Control treatment group had six colonies removed on Day 63 because *V. destructor* mite counts exceeded the 45 *V. destructor* mites in the alcohol wash. This represented our aforementioned limit where colonies were removed to minimize drift and potential re-infestation of treated colonies. The FormicPro™ treatment group had five colonies removed from the experiment due to queen failure, all prior to Day 63 in the six weeks after initial FormicPro™ application. This treatment group also had one colony removed due to small colony size between Days 63 and 133. The HopGuard® treatment group had a total of four colonies removed from the study. This included, on Day 63, one colony removed due to a high *V. destructor* count in the alcohol wash, one for queen failure, one for weak colony size, and one colony due to a beekeeping error where a queen was collected in an alcohol wash. The OA Vapor treatment group had four colonies removed from the trial on Day 63 for *V. destructor* counts that exceeded the predetermined threshold of 45 mites in the alcohol wash. One colony was also removed due to queen failure prior to Day 63. All colonies removed due to high *V. destructor* counts in the alcohol wash were moved to a secondary location and were inspected on Day 133; however, none of these colonies survived. Final colony numbers on Day 133 remaining in the experiment were 15 for the Apivar® treatment group, 9 in the untreated control, 9 in the FormicPro™ treatment group, 11 in the HopGuard® treatment group, and 10 in the OA Vapor treatment group.

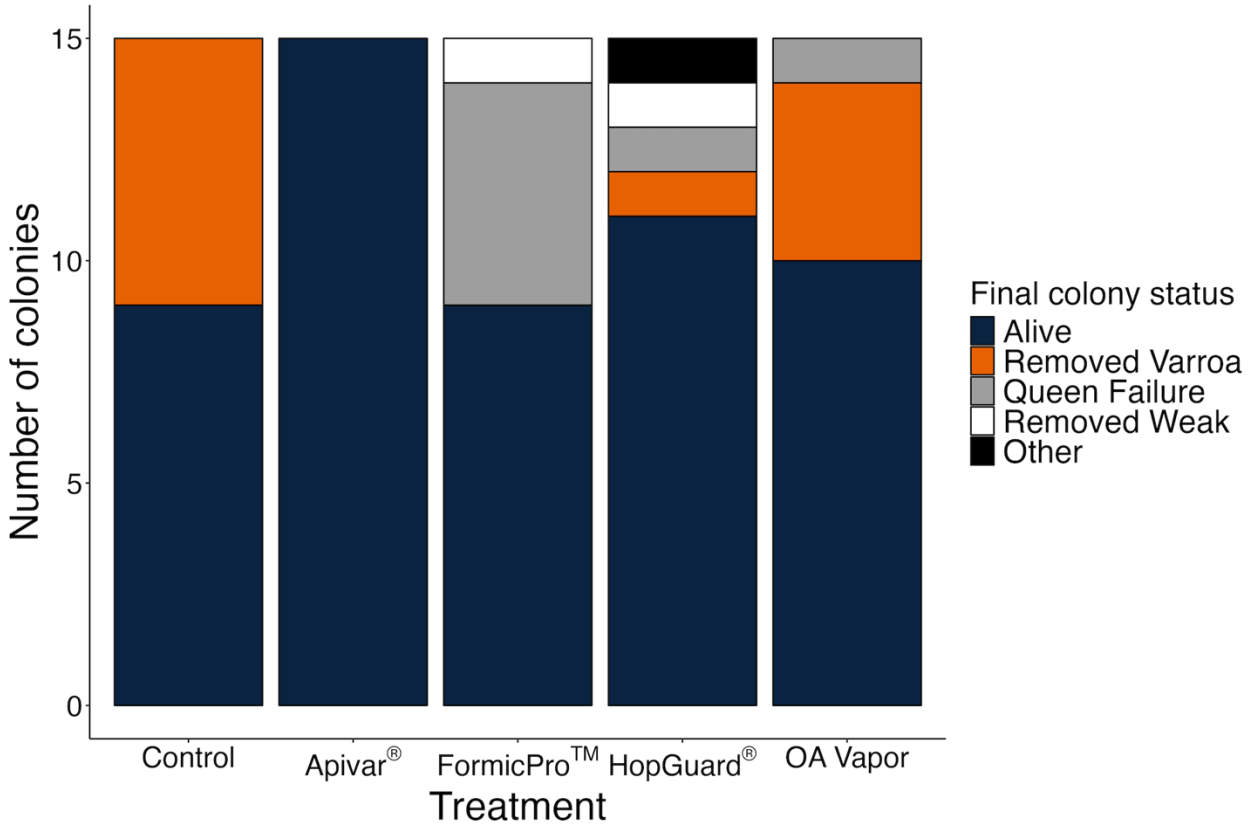

Figure 3.5. Final honey bee (*Apis mellifera*) colony status. Condition of colony on Day 133 across different treatment groups. All treatment groups started with 15 colonies. Colonies removed due to high *V. destructor* infestation had > 45 *V. destructor* mites in the wash representing approximately >15% *V. destructor* infestation rate of adult bees. Colonies removed due to being weak had <1 frame of adult honey bees during an inspection. (OA = oxalic acid).

Amitraz resistance genotype

Pre-treatment field resistance levels from the Day 0 amitraz resistance field tests showed an average of 32.80% amitraz resistance in the Apivar® treatment group, illustrating that colonies in this treatment group had amitraz resistant *V. destructor* mites before treatments. There were no statistical differences in frequency of genotype classes (i.e., susceptible (SS), heterozygous (SR), or resistant (RR)) across the three sampling periods for the Control (F $ratio_{2,\infty}$ = 1.62, P = 0.20; F $ratio_{2,\infty}$ = 1.37, P = 0.25; F $ratio_{2,\infty}$ = 0.54, P = 0.58), FormicPro™ (F $ratio_{2,\infty}$ = 0.089, P = 0.92; F $ratio_{2,\infty}$ = 0.24, P = 0.78; F $ratio_{2,\infty}$ = 0.38, P = 0.68), HopGuard® (F $ratio_{2,\infty}$ = 2.30, P = *0.10;* F $ratio_{2,\infty}$ = 0.27, P = 0.76; F $ratio_{2,\infty}$ = 1.60, P = 0.20), or OA Vapor (F $ratio_{2,\infty}$ = 0.67*,* P = 0.51; F $ratio_{2,\infty}$ = 0.12, P = 0.89; F $ratio_{2,\infty}$ = 0.44, P = 0.65) treatment groups (Figure 3.6). There was a significant difference of genotype class frequencies across sampling days for the Apivar® treatment group (F $ratio_{2,\infty}$ = 17.20, $P < 0.001$; F $ratio_{2,\infty}$ = 18.43, $P < 0.001$) (Figure 3.7). The amitraz susceptible genotype (homozygous susceptible (SS)) frequency declined to an estimated 0.22 on Day 63 after treatment compared to 0.52 on Day 0 (z-$ratio_{\infty}$ = -5.83, $P < 0.001$) or 0.46 on Day 133 (z-$ratio_{\infty}$ = 3.83, $P < 0.001$). There was no difference in the amitraz susceptible genotype frequency between Day 0 and Day 133 (z-$ratio_{\infty}$ = -0.83, P = 0.68). Meanwhile, the homozygous amitraz resistant genotype (RR) frequency significantly increased from 0.39 on Day 0 (z-$ratio_{\infty}$ = 6.05, $P < 0.001$) to an estimated 0.71 on Day 63 after treatment then decreased significantly to 0.47 on Day 133 (z-$ratio_{\infty}$ = -3.58, P = 0.001). Again, there was no difference in amitraz resistant genotype frequency between Day 0 and Day 133 (z-$ratio_{\infty}$ = 1.18, P = 0.46). There was no observed difference in heterozygous genotype (SR) frequency for the Apivar® treatment group across sampling days (F $ratio_{2,\infty}$ = 0.26, P = 0.77) (Figure 3.7).

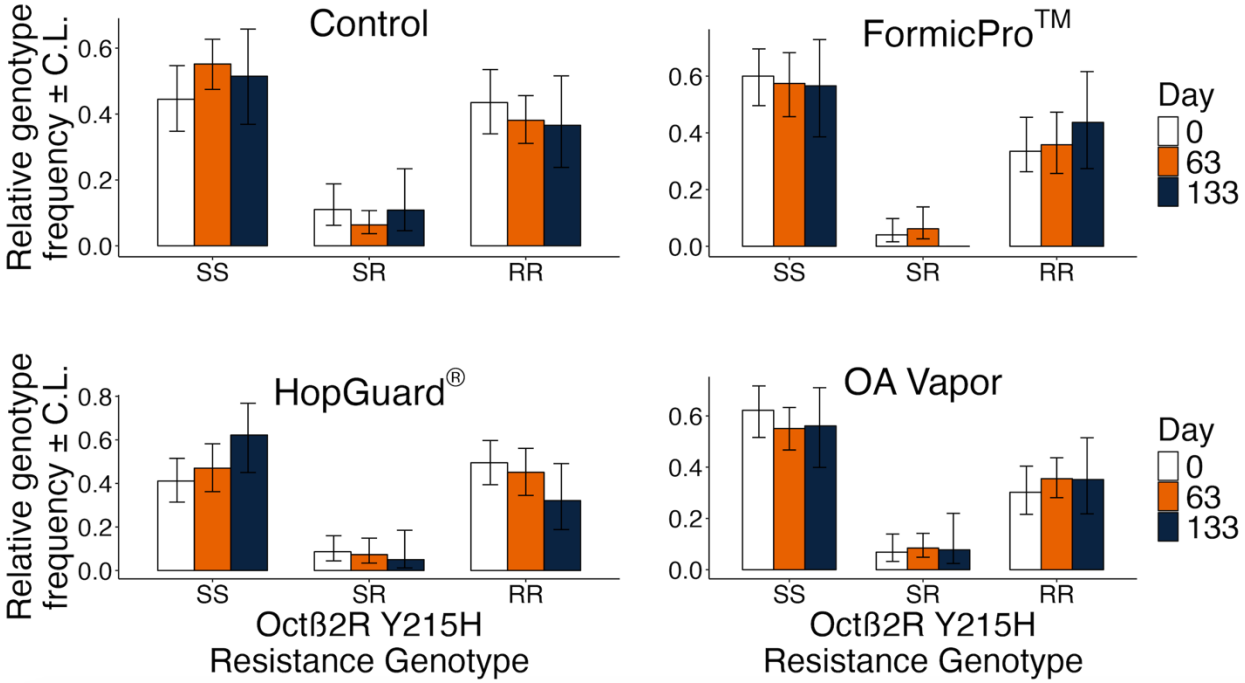

Figure 3.6. Amitraz resistance allele frequency in *Varroa destructor* mites from honey bee (*Apis mellifera*) colonies treated with nothing, FormicPro™ (active ingredient formic acid), HopGuard® (active ingredient hops β-acids), or Oxalic Acid Vapor (active ingredient oxalic acid). Relative amitraz allele frequency for the Octß2R Y215H resistance genotype for different treatment groups across experimental days. (SS = homozygous susceptible, SR = heterozygous, RR = homozygous resistant). Model estimates are shown. No significant differences were observed among resistance genotypes classes for any treatment across the three sampling periods. ($P < 0.05$) (OA = oxalic acid).

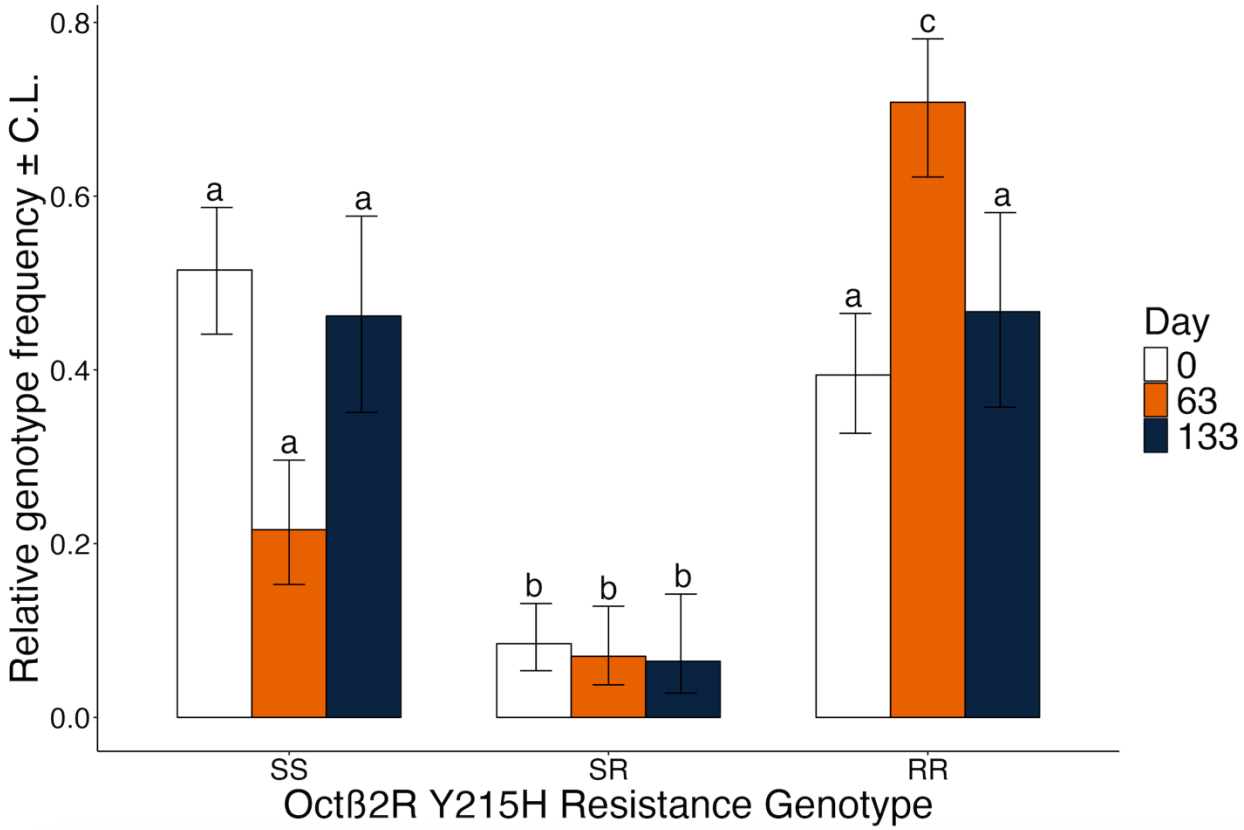

Figure 3.7. Amitraz resistance allele frequency in *Varroa destructor* mites from honey bee (*Apis mellifera*) colonies treated with Apivar®. Relative amitraz allele frequency for the Octß2R Y215H resistance genotype for the Apivar® (active ingredient amitraz) treatment groups across experimental days. (SS = homozygous susceptible, SR = heterozygous, RR = homozygous resistant). Model estimates are shown. Statistical comparisons where different letters indicate significant differences between treatments for each individual sampling period. ($P < 0.05$).

Discussion

Control of *V. destructor* before winter is a critical timepoint, integral to limiting honey bee colony losses over winter (Guzmán-Novoa et al. 2010; Van Dooremalen et al. 2012). Continued increases in amitraz resistance (Rinkevich 2020; Rinkevich et al. 2023, Rinkevich et al. Unpublished) and narrow treatment application windows, especially in the Southeastern U.S., limit the treatment options for beekeepers. This experiment demonstrated that Apivar®, FormicPro™ , and HopGuard® could all be potential viable treatments for use in the fall to limit *V. destructor* population growth in the Southeastern US and maintain infestation rates around 2 – 3% per 100 honey bees. Contrarily, OA Vapor at 2 g per brood box increased *V. destructor* infestation rate with brood present even when three applications were used. This experiment demonstrated that treatments of Apivar® can further increase the amitraz resistance allele frequency for *V. destructor* populations, albeit briefly, while treatments with other active ingredients do not. These results show that further research needs to be conducted to find effective treatments at reducing *V. destructor* infestation rates in the fall and understand how different treatments impact amitraz resistance allele frequency dynamics in *V. destructor*.

No treatment group significantly reduced *V. destructor* infestation rate at post-treatment sampling times, as infestation rates either remained consistent near 3% infestation per 100 honey bees or increased at the midpoint sampling. While the limited amount of capped brood area could have artificially increased *V. destructor* infestation rates on adult honey bees on Day 63 since less brood represents more mites on adult honey bees (Beetsma et al. 1999), the lack of *V. destructor* infestation rate decline after 63 days could increase the need for an additional treatment as colonies reduce brood production and begin preparing for winter (Vetharaniam 2012; Jack et al. 2023). Additionally, the absence of difference between treatment groups in *V. destructor* rates on

Day 133 is likely due to survivorship bias, as colonies in the OA Vapor (4 colonies) and Control (6 colonies) treatment groups had colonies fail due to high mite infestation rates with over 45 *V. destructor* mites in the mite wash on Day 63; this limited the sample solely to surviving colonies. For this reason, we believe that the Day 63 results are a better guide to understanding the true effects of treatment on *V. destructor* infestation.

These results provide further evidence that the Y215H mutation in the Octβ2R is associated with amitraz resistance, and that Apivar® application selects for amitraz resistant individuals thereby increasing resistance in populations. Originally, we hypothesized that Apivar® would be the treatment most likely to reduce *V. destructor* infestation rate to below treatment thresholds due to the moderate level of amitraz resistance (32.8%). However, we observed a slight numerical increase in *V. destructor* infestation rate that was not significant along with a significant increase in the resistant genotype frequency. While the *V. destructor* infestation rate was significantly lower after treatment with Apivar® colonies compared to the Control, the *V. destructor* infestation rate of these colonies slightly increased from the pretreatment *V. destructor* infestation rate after treatment with Apivar® (Pretreatment = 2.43% vs Posttreatment = 2.95%). This is consistent with other research that shows *V. destructor* infestation rate tends to maintain, rather than increase or decrease, at the level of amitraz resistance seen pre-treatment in this experiment (32.8%) (Rinkevich unpublished). It is important to recognize that maintaining, instead of decreasing, *V. destructor* infestation rates after treatment can be considered a treatment failure since amitraz-susceptible *V. destructor* populations should significantly decline after Apivar® application (Elzen et al., 2000; Jack et al., 2024; Rinkevich unpublished). Furthermore, this research shows that while populations may maintain in quantitative terms, they change in a qualitative sense in that the frequency of resistance

genotypes significantly increases, thus increasing the probability of a subsequent treatment failure.

Observing that Apivar® treatment affected resistance allele frequency supports previous findings that the Y215H mutation in the Octβ2R is a cause of target site resistance consistent with other classic examples, such as the myriads of *kdr*-type mutations in the sodium channel that provides resistance to pyrethroids (Williamson et al. 1996; Rinkevich et al. 2013) or truncated nicotinic acetylcholine receptors that provide spinosad resistance (Rinkevich et al. 2010). Consistent with previous results (Rinkevich et al. 2023), these findings reinforce the utility of genotyping assays for the Y215H mutation as a valuable tool for resistance monitoring; however, other mutations in Octβ2R exist in European and Asian populations (Hernández-Rodríguez et al. 2022; Hernández-Rodríguez et al. 2025; Lee et al., 2025), so frequent genomic reevaluations should be done to evaluate if other resistance alleles become important in the population, as has happened in *kdr*-resistant house flies (Kasai et al. 2017) and Colorado potato beetles (Rinkevich et al. 2012).

At first glance, the decline in the amitraz resistant genotypes and the resistant allele frequency during the overwintering period from Day 63 to Day 133 in the Apivar® treatment group can be taken as an indication that the Y215H mutation may impose a fitness cost on *V. destructor*. If this was a real fitness cost, this trend of reduced resistant genotypes and resistant allele frequencies over time should have been observed in all colonies regardless of treatment group. However, the data showed that this was only observed in the Apivar® treatment group. This opens the question as to why overwintering amitraz resistant allele frequency on Day 133 declined in the Apivar® treatment group to pretreatment levels after initially increasing by Day 63 following an Apivar® application. Previous parasite resistance literature shows that small

population size can increase the impact of genetic drift and result in the loss of resistance alleles from populations (White et al. 2021). There is the potential that *V. destructor* infestation rates were low enough that the resistance genotype was not maintained in the population from Day 63 to Day 133 due to random selection of genes; furthermore, the resistance allele frequency could have also been impacted through reinfestation and drift of *V. destructor* mites on adult honey bees from neighboring colonies (Dynes et al. 2019; Peck and Seeley 2019; Kulhanek et al. 2021a). Since colonies of all treatment groups were housed in the same two yards, movement of *V. destructor* mites among colonies could have homogenized the overall gene pool of *V. destructor* populations in the two yards.

The lack of change in the frequency of the resistant genotype in other treatment groups suggests that the Y215H mutation does not provide cross resistance to the other tested treatments, nor does it make individuals with this mutation more susceptible to other treatments. Therefore, *V. destructor* populations displaying amitraz resistance via the Y215H mutation in Octβ2R can be managed by application of other acaricides. Data from Day 63 of this study showed that FormicPro™ and HopGuard® were similarly effective as Apivar® in managing amitraz resistant *V. destructor* populations. Additionally, at higher levels of amitraz resistance, it is possible that the effect of these alternative treatments would be superior to an amitraz based treatment.

In this experiment, none of the treatments significantly reduced *V. destructor* infestation. To ensure overwintering colony success *V. destructor* infestation rates must be reduced prior to overwintering to prevent mites from feeding on fat bodies while in the dispersal phase, an important component in honey bee winter survival (Ramsey et al. 2019; Brejcha et al. 2023; Morfin et al. 2024). Without the initial 32.8% amitraz resistance presence observed, we would

have expected to see the Apivar® treatment group significantly reduce *V. destructor* infestation rates to near zero levels, likely showing higher effectiveness than the FormicPro™ and HopGuard® treatment groups as well (Rinkevich 2020; Jack et al. 2020). Consequently, this experiment demonstrated that regardless of treatment used, additional follow-up treatments may be necessary to reduce *V. destructor* infestations in the Southeastern US, particularly if beekeeping operations have some level of amitraz resistance present. These results also validated the importance of post-treatment monitoring to ensure that the treatment was effective in reducing or keeping *V. destructor* infestation rates manageable. Oxalic acid is a common treatment used late in the season to control *V. destructor* in the dispersal phase (Gregorc and Planinc 2001, 2004; Al Toufailia et al. 2015, 2018); however, this experiment illustrates the importance of avoiding treating with OA when colonies have brood present since the OA treatment group had significantly higher *V. destructor* infestation rates compared to other treatment groups and did not differ from the control on Day 63. This is reinforced by the observation that at Day 63, during the week of 20 November 2023, the colonies in this study still maintained some amount of brood, thus extending the reproductive period for *V. destructor* later in the year. Additionally, while both the HopGuard® and FormicPro™ treatment groups were able to prevent increases in *V. destructor* infestation rates, both treatment groups had multiple colonies removed before the end of the study. The HopGuard® treatment group had some colony loss, but there was no single major cause of loss; furthermore, we exhibited better survival than a previous overwintering study utilizing HopGuard® (Kulhanek et al. 2023). Contrarily, even with the treatment being applied within the label temperature range, the FormicPro™ treatment group had a consistent problem with five of the 15 colonies losing their queens before Day 63. This illustrates that FormicPro™ remains a difficult treatment to employ in the Southeast due to its

brief application window resulting in potential queen losses as evidenced in this experiment, despite adequate temperatures during the application period as per label instructions (Underwood and Currie 2005; Giovenazzo and Dubreuil 2011). We also saw reduced colony weight on Day 63 in the FormicPro™ treatment group, but that was not associated with any significant differences in frames of capped honey bee brood or frames of adult honey bees.

This experiment demonstrates that Apivar® is effective against *V. destructor*, but in operations with moderate to high levels amitraz resistance, beekeepers are unlikely to see successful reduction of *V. destructor* infestation rates. Similarly, this experiment again showed that repeated applications of OA Vapor at the current label rate is an insufficient treatment when capped brood is present (Berry et al. 2022, 2023). Meanwhile, FormicPro™ may be best utilized in the spring when beekeepers can more easily recover from potential queen loss. Comparatively, in conditions such as these, HopGuard® may be appropriate to maintain *V. destructor* infestation levels, but not to reduce them. As amitraz resistance continues to rise beekeepers need to optimize alternative *V. destructor* treatment methods to reduce reliance on amitraz and the subsequent increase in amitraz resistant *V. destructor* populations. Overall, more research needs to be done to investigate the longevity of resistance genotypes in *V. destructor* populations to assist beekeepers in managing colonies with these resistance pressures.

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Chapter 4

Global range of *Tropilaelaps mercedesae*

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Abstract

Tropilaelaps mercedesae is a parasitic mite of global concern because of its impacts on the colonies of the western honey bee *Apis mellifera*. In recent years, *T. mercedesae* has been detected in new countries and continents, thus increasing the urgency to clarify its range, as well as understand drivers for its expansion. By analyzing peer-reviewed scientific literature, we provide a comprehensive list of countries where a *T. mercedesae* specimen was collected in association with *A. mellifera* colonies, as well as in association with the colonies of its suspected original host, the giant honey bee *Apis dorsata. T. mercedesae* species confirmations were included here only if the reporting literature employed molecular or morphological methods appropriate for identification.

Introduction

Believed to originally be a parasite of the giant honey bee *Apis dorsata* in south and southeast Asia, the *Tropilaelaps mercedesae* mite began parasitizing the western honey bee *Apis mellifera* upon the introduction of *A. mellifera* to Asia, with the first recording occurring in 1963 (Chantawannakul et al., 2016; De Guzman et al., 2017; Delfinado, 1963). Since then, *T. mercedesae* has become a significant *A. mellifera* parasite in the region, causing severe economic loss to beekeepers (Buawangpong et al., 2015; Chantawannakul et al., 2018).

Tropilaelaps mercedesae damages *A. mellifera* colonies by feeding on developing individuals (brood) (Han et al., 2024). It can rapidly reproduce when brood is present going from less than 1% capped brood infestation in the colony to almost 15% capped brood infestation in just 60 days (Tokach et al., 2024), but mites are incapable of feeding on adult honey bees; this is believed to limit its survival to periods in the colony when brood is present (Khongphinitbunjong et al., 2019; Pettis and Chaimanee, 2019). Feeding by *T. mercedesae* reduces the weight of adult *A. mellifera* upon emergence, as well as its lifespan (Khongphinitbunjong et al., 2016); the mite also vectors several viruses that can also negatively impact *A. mellifera* health (Khongphinitbunjong et al., 2016, 2015).

Due to its dependence on brood, the range of *T. mercedesae* was suspected to be limited to tropical and sub-tropical areas that experience nearly year-round brood production (Woyke, 1984); however, *T. mercedesae* was recently confirmed in *A. mellifera* colonies in areas with colder climates that apparently have distinguishable periods of brood dearth (Brandorf et al., 2024; Janashia et al., 2024; Mohamadzade Namin et al., 2024). Currently, it is not well known how *T. mercedesae* persists in these new environments or how it spreads. Recent research illustrated that *T. mercedesae* can be found on adult *A. mellifera* exiting the colony, opening the possibility of dispersal through honey bee robbing or drifting behaviors (Tokach et al., 2025). Additionally, it is plausible that the mite is spread via beekeeper-mediated methods such as transfer of infested frames and combs to uninfested colonies, or long-distance colony movements. Regardless, *T. mercedesae* has been confirmed in multiple new countries such as Georgia, Uzbekistan, and the Krasnodar region of Russia in the recent years (Brandorf et al., 2024; Janashia et al., 2024; Mohamadzade Namin et al., 2024).

There are differing reports of where *T. mercedesae* has been confirmed, especially regarding countries like Bhutan, Iran, and Kenya (Chantawannakul et al., 2018, 2016; De Guzman et al., 2017). Furthermore, there is limited information regarding country-specific presence of the mite on the World Organisation for Animal Health's World Animal Health Information System portal ("WAHIS," 2025). This lack of information illustrates the need to communicate the current range of *T. mercedesae* with a standardized approach. Here, we aim to provide a comprehensive list of countries in which *T. mercedesae* was collected in association with colonies of *A. dorsata* and *A. mellifera*, its original and introduced hosts, respectively.

Materials and Methods

To identify *T. mercedesae* presence we used peer-reviewed literature that included morphological or molecular confirmation of *T. mercedesae*. Since there is conflicting information about current distribution of *T. mercedesae*, we chose to only use peer-reviewed publications as they are among the most credible sources of information due to their expert review process. We also only acknowledged *T. mercedesae* when clear morphological or molecular methods were used to determine species identification. Morphological identification was confirmed using standards determined in Anderson and Morgan (2007). Meanwhile, molecular confirmation was done using DNA testing. Together, these decisions mitigate the risk of reporting false positives but may result in a conserved country-specific range of the mite.

Peer-reviewed publications were compiled by first checking references from previous *T. mercedesae* review articles that stated global range (Chantawannakul et al., 2018, 2016; De Guzman et al., 2017). Secondly, keywords "*Tropilaelaps*" and "*Tropilaelaps mercedesae*" along with names of countries surrounding areas with confirmed *T. mercedesae* presence were searched on Google Scholar to find additional publications. Lastly, we exchanged information with Asian

honey bee specialists to assist in confirming undocumented reports and minimizing the risk of missing any potential publications.

Results

We confirmed the presence of *T. mercedesae* associated with *A. mellifera* in 13 countries (Figure 4.1, Table 4.1). Additionally, it was found in three countries in association with *A. dorsata*, and one country with an unknown host species association. All detections were confirmed through DNA molecular methods; furthermore, all detections, except Palawan Island of the Philippines, were confirmed through formal morphological identification as well.

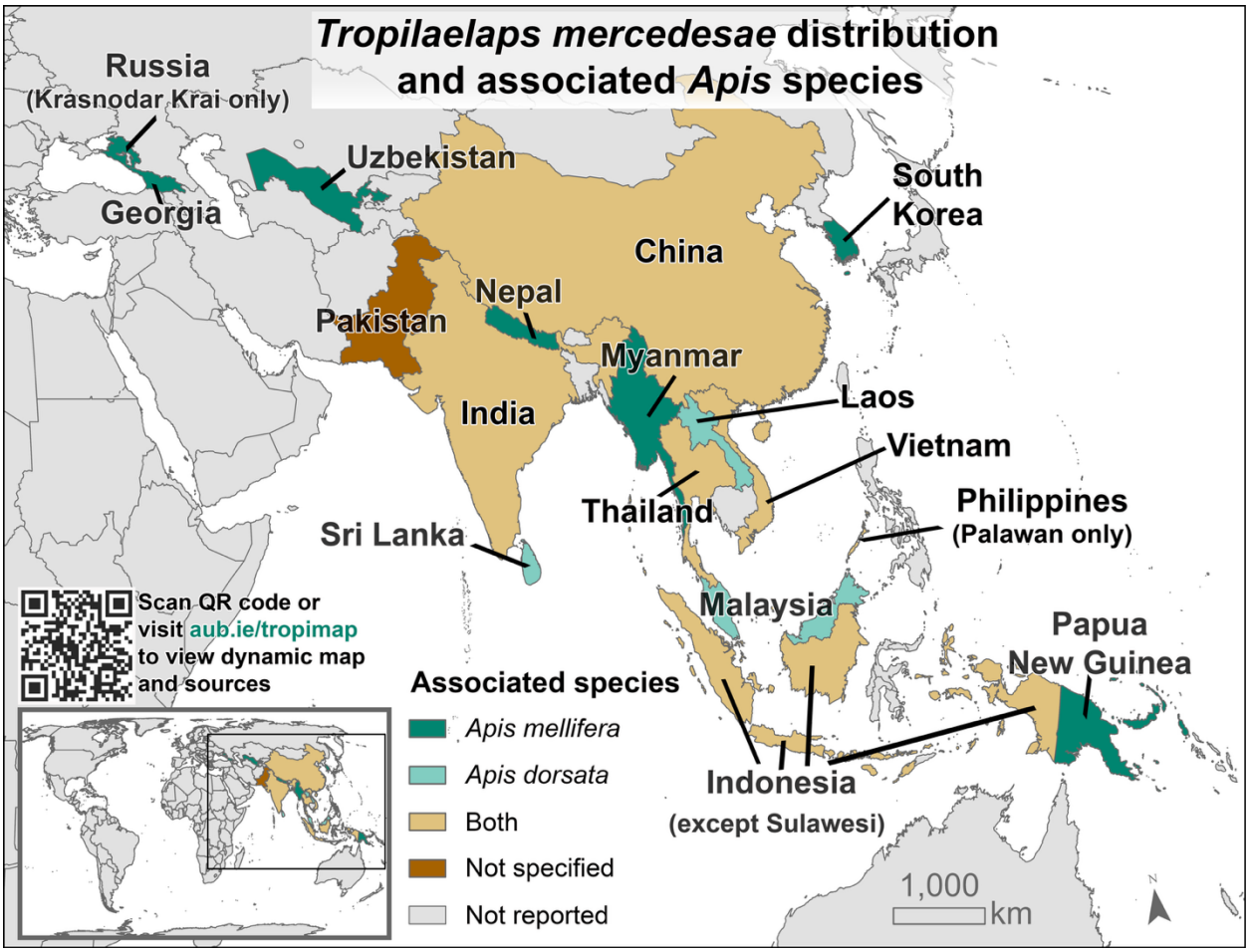

Figure 4.1. Current locations of confirmed presence of *Tropilaelaps mercedesae* and the respective *Apis* honey bee host species it was associated with, as confirmed by molecular (DNA) or morphological methods. Country boundaries and regions were compiled using the *10m Cultural Vectors* ("Natural Earth " 2025).The map is displayed using the *Equal Earth* projection ("Equal Earth Wall Map - Projection," 2025).

Table 4.1. Countries with presence of *Tropilaelaps mercedesae* and the respective *Apis* honey bee host species it was associated with, as confirmed by molecular (DNA) or morphological methods denoted by 'X'. Reference refers to the publication in which the presence was confirmed.

Confirmed presence of *Tropilaelaps mercedesae* **Associated *Apis* species**				**Method of confirmation**		
Country	***Apis mellifera***	***Apis dorsata***	**Unknown**	**DNA**	**Morphological**	**Reference**
China	X	X		X	X	Anderson & Morgan (2007)
Georgia	X			X	X	Janashia et al. (2024)
India	X	X		X	X	Anderson & Morgan (2007)
Indonesia (except Sulawesi Island)	X	X		X	X	Anderson & Morgan (2007)
Laos		X		X	X	Anderson & Morgan (2007)
Malaysia		X		X	X	Anderson & Morgan (2007)
Myanmar	X			X	X	Anderson & Morgan (2007)
Nepal	X			X	X	Mohamadzade Namin et al. (2024)
Pakistan			X	X	X	Del Cont et al. (2021)
Papa New Guinea	X			X	X	Anderson & Morgan (2007)
Philippines (Palawan Island only)	X	X		X	X[1]	De Guzman et al. (2020); Anderson & Morgan (2007)
Russia (Krasnodar Krai only)	X	X		X		Brandorf et al. (2024)
South Korea	X			X	X	Anderson & Morgan (2007); Mohamadzade Namin et al. (2024)
Sri Lanka		X		X	X	Anderson & Morgan (2007)
Thailand	X	X		X	X	Anderson & Morgan (2007)
Uzbekistan	X			X	X	Mohamadzade Namin et al. (2024)
Vietnam	X	X		X	X	Anderson & Morgan (2007)

[1]The *A. dorsata* species association was confirmed through morphological identification in Anderson & Morgan (2007), but the *A. mellifera* species association was not morphologically confirmed in De Guzman et al. (2020).

Discussion

Through this work, we determined the current global range of *T. mercedesae* and the *Apis* species it was associated with during collection. The standardized approach used, assists in mitigating undocumented reports and increases our understanding of the known current range. Interestingly, we showed that *T. mercedesae* has expanded its geographical range outside of the range of *A. dorsata* (Hepburn and Radloff, 2011), one of its original hosts. For example, it was recently observed in *A. mellifera* colonies in Georgia, the Krasnodar region of Russia, Uzbekistan, and previously in South Korea; all these countries or regions are outside the geographic range of *A. dorsata* (Brandorf et al., 2024; Hepburn and Radloff, 2011; Janashia et al., 2024; Mohamadzade Namin et al., 2024), and suggests that *T. mercedesae* can survive solely in *A. mellifera* colonies. In response, there has been an increase in efforts to study monitoring methods capable of rapidly detecting *T. mercedesae* incursions into new regions (Gill et al., 2024). If *T. mercedesae* continues to expand into Europe, this could have significant impacts on the European and later global beekeeping industry. Already, the discovery of *T. mercedesae* in nearby countries led to the suspension of *A. mellifera* package and queen imports from Ukraine to Canada in 2024 (Suminder, 2024). Beekeepers in countries with new *T. mercedesae* detections could see similar measures being taken, thereby limiting potential revenue streams while also dealing with probable colony loss from *T. mercedesae* damages (De Guzman et al., 2017).

Intriguingly, there are multiple geopolitical gaps in detection that currently exist for the range of *T. mercedesae*; for example, lack of observations of the mite in Cambodia or

Bangladesh (Figure 4.1). This is most likely driven by a lack of data collection and appropriate identification in these areas, rather than absence of *T. mercedesae*. While it is probable that countries with confirmed detection of *T. mercedesae* in association with *A. dorsata* also have experienced the mite switching its host to *A. mellifera*, absence of any procured specimens of *T. mercedesae* from these *A. mellifera* colonies limits the ability to confirm this. Of the countries without *T. mercedesae* bordering a country with confirmed presence of *T. mercedesae*, we are aware of only Turkey that has published a report investigating *A. mellifera* colonies for *T. mercedesae* during 2018 – 2019; this was two years before beekeepers in the Krasnodar region of Russia and five years before beekeepers in Georgia began reporting *T. mercedesae* infestation in their colonies (Brandorf et al., 2024; Gürler et al., 2024; Janashia et al., 2024). Meanwhile, multiple experimental trials on *Tropilaelaps* mites in *A. mellifera* colonies in Afghanistan and Pakistan are published (Camphor et al., 2005; Woyke, 1984); however, no mites were molecularly or morphologically identified.

It is important for beekeepers to understand the current global range of the parasitic *T. mercedesae* mite. This understanding of its current range provided in this publication allows for more targeted sampling to occur and greater awareness in determining to which regions the mite may expand . Additionally, this work shows where current gaps in known regions are, and it exemplifies the need for consistent *T. mercedesae* detection reporting, thereby minimizing the occurrences of undocumented reports and reducing the time between first detection and confirmation. Overall, more research is needed, and more specimens need to be collected to confirm presence of *T. mercedesae* in these various countries to fill gaps on the current geographical range of *T. mercedesae*. Visit https://www.honeybeepests.org/tropi to see a dynamic

map that visualizes an up-to-date range of *T. mercedesae* in association with *A. mellifera* and *A. dorsata* colonies.

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Chapter 5

Observation of *Tropilaelaps mercedesae* (Mesostigmata: Laelapidae) on Western honey bees (*Apis mellifera*) exiting colonies

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Abstract

Tropilaelaps mercedesae (Delfinado and Baker) is an emerging parasitic mite that can severely impact the Western honey bee (*Apis mellifera* L.). While *T. mercedesae* has been reported to be expanding its geographical range, the routes of inter-colony dispersal between *A. mellifera* colonies are still largely unknown. In this study, we used funnel traps to collect foraging honey bees exiting their colonies before performing an alcohol wash to collect any phoretic *T. mercedesae* mites. We found *T. mercedesae* on exiting adult honey bees; however, they were only detected when a colony had an elevated *T. mercedesae* brood infestation. We show that *T. mercedesae* can exit colonies through phoresy on adult *A. mellifera* which demonstrates the potential of these mites to be spread through natural movement of *A. mellifera* honey bees among colonies.

Introduction

Tropilaelaps mercedesae (Delfinado and Baker) is a damaging parasitic mite of the Western honey bee (*Apis mellifera* L.) that has recently been detected in new regions and thus poses a threat to apiculture globally (De Guzman et al., 2017; Brandorf et al., 2024 May 16; Janashia et

al., 2024 Oct 18; Mohamadzade Namin et al., 2024). Once established in a colony, this parasitic mite feeds on *A. mellifera* larvae and pupae while vectoring different honey bee viruses (Khongphinitbunjong et al., 2016; De Guzman et al., 2017; Chantawannakul et al., 2018). Continued spread of *T. mercedesae* threatens *A. mellifera* globally, as this mite rapidly reproduces and causes significant colony losses when left unmanaged (Chantawannakul et al., 2016; Chantawannakul et al., 2018).

As part of understanding its spread, and responding appropriately to new detections, it is important to understand *T. mercedesae's* inter-colony movement. It is believed that *T. mercedesae* disperses in a similar manner to *Varroa destructor*, another damaging honey bee parasitic mite with almost global distribution (Rosenkranz et al., 2010; De Guzman et al., 2017). *Varroa destructor* disperses between colonies when mites attach to free-flying adult honey bees that drift into neighboring colonies, or when mites attach to adult honey bees robbing a colony for its resources (Dynes et al., 2019; Peck and Seeley, 2019). Unlike *V. destructor* which feeds on adult honey bees, *T. mercedesae* has only been shown to feed on larvae and pupae within the colony (Rinderer et al., 1994; Woyke, 1994a; Ramsey et al., 2019). This should limit its need, and perhaps its capacity, to attach to adult honey bees. Therefore, its ability to disperse through phoresy should also be limited. While phoretic *T. mercedesae* mites have been observed on adults of the giant honey bee *Apis dorsata* that were away from their nest, mites have never been observed on *A. mellifera* outside of the colony (Laigo and Morse, 1968; Burgett et al., 1990).

Overall, there is little known about *T. mercedesae* dispersal, but some previous results suggest the possibility of dispersal through phoresy. *Tropilaelaps mercedesae* have shown the ability to attach to adult honey bees in different locations when in lab conditions without the presence of brood, and they have been found in low levels on adult honey bees in colonies

(Khongphinitbunjong et al., 2012; Pettis et al., 2013). Only one study has investigated *T. mercedesae* dispersal, but the method of dispersal was not determined. In the study, a single *A. mellifera* colony was continuously monitored and treated for *T. mercedesae* for five months while being surrounded by untreated colonies (Rath et al., 1991). While mites were found in the treated colony on the bottom board, it was only assumed mites were being spread through drift of honey bees from the other untreated colonies (Rath et al., 1991). This study aims to prove that *T. mercedesae* can be found on adult *A. mellifera* exiting the colony, thereby demonstrating that dispersal through drift or robbing behavior is possible.

Materials and Methods

Location and Colonies

Research was conducted at Chiang Mai University in Chiang Mai, Thailand. *Apis mellifera* colonies were acquired from a local beekeeper and were kept in single deep Langstroth boxes all containing eight frames. Data were collected at two different time points: July – August in 2023, and January – February in 2024.

Data Collection

To collect exiting adult honey bees, we fitted honey bee colonies with modified funnel traps (Medrzycki, 2013) (Figure S5.1). For data collection, traps were deployed in consecutive ten-minute intervals. Initial adult honey bees exiting colonies were collected for ten minutes. The trap was then re-deployed for an additional ten minutes. This two-part deployment was used to reduce congestion in the traps, and thus the number of honey bees that flew into the trap but retreated back into the colony. Once honey bees were collected, they were washed in 35% isopropyl alcohol using a triple rinse method to collect mites (Aurell et al., 2024), and all honey bees were counted to determine the number of honey bees collected and mite infestation per 100

bees. A subset of mites found were inspected under a dissecting microscope to determine sex. Colonies were also monitored for *T. mercedesae* infestation by uncapping 50 worker brood cells per side of two separate brood frames and inspecting a total of 200 cells for presence of *T. mercedesae* (Pettis et al., 2013; Pettis et al., 2017).

Mites on exiting *A. mellifera* at low colony infestation rates

In July and August of 2023, six colonies were used for experimental trapping and fitted with funnel traps (Table 5.1). Traps were deployed for the full 20 minutes nine times over the span of 30 days. Colonies were also monitored three times, once per week, for *T. mercedesae* brood infestation. Final trap deployments occurred two weeks after the final *T. mercedesae* brood infestation inspection.

Mites on exiting *A. mellifera* at high colony infestation rates

In January of 2024, 30 colonies were initially inspected for *T. mercedesae* brood infestation using the brood infestation inspection methods previously described. The colonies with the three highest observed brood infestation rates were then fitted with traps. From January – February 2024, traps were deployed seven times over eight days. After five collections, one colony began showing signs of population collapse due to extensive *T. mercedesae* infestation (Colony 7). In response, its trap was placed on a different colony showing moderate levels of infestation for the remaining collections (Colony 12) (Table 5.2). Final trap deployments occurred two weeks after initial *T. mercedesae* brood infestation inspection.

Results

Mites on exiting *A. mellifera* at low colony infestation rates

We did not detect any *T. mercedesae* on exiting *A. mellifera* at low colony infestation rates where *T. mercedesae* brood infestation ranged from 0 – 2.5% (infested cells per 100 cells) with

the overall average infestation for all colonies across the three inspections being 0.75% (Table 5.1). A total of 7,250 adult honey bees were collected in traps and alcohol washed. Zero mites were found from any trap deployment (Table 5.1). Colony C had its trap fail resulting in only four data collections and a lower overall number of honey bees collected and washed (Table 5.1).

Table 5.1. *Tropilaelaps mercedesae* funnel trap collections at low colony infestation rates. Total number of adult *A. mellifera* bees trapped exiting their colony and total number of *T. mercedesae* mites collected in alcohol wash across nine trap collections. Brood infestation rates are shown as a percentage (infested cells per 100 opened cells). Sampling occurred in July – August 2023.

Colony	Bees washed	*T. mercedesae* mites collected	Mites per 100 bees (%)	20/7/2023 brood infestation (%)	26/7/2023 brood infestation (%)	2/8/2023 brood infestation (%)	Average brood infestation (%)
A	2002	0	0	0.5	0.5	0	0.33
B	1276	0	0	0	0.5	7	2.50
C	443	0	0	1.5	0.5	0.5	0.83
D	1169	0	0	0.5	0	0	0.17
E	1060	0	0	1.0	0.5	0.5	0.67
F	1300	0	0	0	0	0	0
Total	7250	0	0	0.58	0.33	1.33	0.75

Mites on exiting *A. mellifera* at high colony infestation rates

Tropilaelaps mercedesae were recovered on exiting *A. mellifera* when colonies had high infestation rates. *Tropilaelaps mercedesae* brood infestation ranged from 11.5 – 37% (infested cells per 100 cells) and averaged 25.5% (Table 5.2). During the second round of data collection, a total of 2,974 honey bees were collected and washed. 18 mites were found on exiting honey bees, with a range of 1 – 14 total mites found per colony over the course of the data collection (Table 5.2). Average infestation of exiting honey bees was 0.74% (mites per 100 bees; Table 5.2). Of the 18 mites collected, nine were inspected to determine sex, and all nine mites were determined to be female.

Table 5.2. *Tropilaelaps mercedesae* funnel trap collections at high colony infestation rates. Total number of adult *A. mellifera* bees trapped exiting their colony and total number of *T. mercedesae* mites collected in alcohol wash across seven trap collections. Brood infestation rates are shown as a percentage (infested cells per 100 opened cells). Sampling occurred in January - February 2024.

Colony	Bees washed	*T. mercedesae* mites collected	Mites per 100 bees (%)	20/1/2024 brood infestation (%)
7	155	1	0.65	37
11	521	14	2.69	26.5
12	572	1	0.17	11.5
23	1176	2	0.17	27
Total	2424	18	0.74	25.5

Discussion

This study shows *T. mercedesae* on adult *A. mellifera* exiting their colonies thereby demonstrating the plausibility that these mites are capable of dispersing through movement of honey bees among colonies. Although beekeeper mediated dispersal is likely very important to the observed spread of mites, this study proves there is also a viable threat of spread of *T. mercedesae* through inter-colony dispersal via phoresy (De Guzman et al., 2017). This confirmed route of dispersal is important due to the rapid need for response in any region first detecting *T. mercedesae* – as it suggests nearby apiaries could become infested even without beekeeper movement of material (infested brood frames, colonies) to those apiaries.

This research suggests that high brood infestations may drive the presence of *T. mercedesae* on departing honey bees. When infestation rates were low throughout the 2023 collections, no mites were recovered on exiting honey bees showing infestation rates likely need to reach a certain threshold before mites are likely to attach to adult honey bees. Unfortunately, we lack a

sufficient range of *T. mercedesae* infestation in this study, limiting our ability to determine if there is a threshold at which mites begin to exit colonies on adult honey bees. *Varroa destructor* research has demonstrated that when *V. destructor* colony infestation rates are high or brood area is low, cell invasion frequency decreases, leading to more *V. destructor* on adult honey bees (Beetsma et al., 1999; Cervo et al., 2014). While *T. mercedesae* does not feed on adults, reduced brood area due to high infestation and subsequent competition for limited brood cells could lead to *T. mercedesae* attaching to adult honey bees, thus promoting potential dispersal. Although *T. mercedesae* infestation is a plausible driver of mite presence on departing honey bees, this does not eliminate the possibility of alternative causes that could promote *T. mercedesae* dispersal. With limited colonies utilized and honey bees collected, further investigation should be done to determine all factors that may induce *T. mercedesae* dispersal. Previously, the only other *T. mercedesae* dispersal study also proposed that mites dispersed from high to low infestation colonies, but no mites were actually observed dispersing and only increased mite drop on sticky boards in colonies previously treated with an acaricide was actually observed (Rath et al., 1991).

The capability of *T. mercedesae* to attach to honey bees has long been known (Woyke, 1984; Rinderer et al., 1994). When the opportunity to infest brood has been removed in lab settings, *T. mercedesae* has shown the ability to attach to adults on a variety of different sites with the majority of attachments seen in the petiole region of *Apis mellifera* (Rinderer et al., 1994; Khongphinitbunjong et al., 2012); however, to determine the exact threshold of when these behaviors begin to occur more frequently, additional work needs to be done investigating phoretic rates and dispersal of *T. mercedesae* at differing colony sizes and brood infestation rates.

Interestingly, all inspected mites that were collected were female. Females typically live ten times longer than males (50 days compared to 5) (De Guzman et al., 2017). For successful inter-

colony dispersal of *T. mercedesae* to occur, either a single gravid female or both male and female mites must disperse to the same colony. While female mites are able to successfully mate outside of the cell, the shortened male lifespan may limit its dispersal (Woyke, 1994b); therefore, the most realistic form of effective dispersal relies on a female successfully mating, before establishing and reproducing in a new colony. Regardless, more samples need to be collected to determine if male mites also attach to exiting honey bees.

We did not observe any robbing events on colonies installed with traps, allowing this study to focus on determining the potential dispersal of mites through drift as seen with *V. destructor* (Dynes et al., 2019); however, *V. destructor* also commonly disperses when strong colonies rob resources from weaker colonies that can be failing due to elevated infestation levels (Peck and Seeley, 2019; Kulhanek et al., 2021). It is reasonable to believe that colonies failing due to *T. mercedesae* infestation are likely to be weaker and have less available brood for mites to feed on increasing the likelihood that mites will attach to robber honey bees if presented the opportunity.

This study confirms that *T. mercedesae* can be found on *A. mellifera* adults exiting the colony. To gain a broader understanding of *T. mercedesae* dispersal and make educated assumptions on how quickly transmission by phoresy could occur after initial infestation, more research needs to be conducted to determine if there is a threshold at which mites begin to leave the colony attached to adult honey bees. Additionally, dispersal may be accelerated by honey bee robbing behaviors. Overall, it is clear that *T. mercedesae* can spread between colonies through phoresy on adult *A. mellifera*.

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Chapter 6

Managing the parasitic honey bee mite *Tropilaelaps mercedesae* through combined cultural and chemical control methods

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Abstract

The western honey bee (*Apis mellifera*) is severely impacted by the parasitic *Tropilaelaps mercedesae* mite, which has the capacity to outcompete *Varroa destructor* mites (the current leading cause of colony losses) and more rapidly overwhelm colonies. While *T. mercedesae* is native to Asia, it has recently expanded its geographic range and has the potential to devastate beekeeping worldwide if introduced to new regions. Our research exploited the dependence of *T. mercedesae* on developing honey bees (brood) by combining a cultural technique (brood break) with U.S. registered chemical products (oxalic acid or formic acid) to manage *T. mercedesae* infestation. To evaluate this approach, we compared four treatment groups: 1) Brood Break; 2) Brood Break + Formic Acid (FormicPro®); 3) Brood Break + Oxalic Acid dribble (Api-Bioxal®); and 4) untreated Control. We found that the *Tropilaelaps* infestation rate of worker brood in Control colonies rose from 0.4% to 15.25% over 60 days, whereas all other treatment groups had infestation rates under 0.11% on Day 60. Mite drop assessments showed similar results, whereby Control colonies had 15.48 mites drop per 24 hours on day 60 compared to less than 0.2 mites for any other treatment group. Evaluation of colony strength revealed that Brood Break + Formic Acid colonies had slightly reduced adult honey bee populations. No

treatment eliminated all mites, so additional measures may be needed to eradicate *T. mercedesae* if detected in countries that do not currently have *T. mercedesae*.

Introduction

Parasitic mites have devastated the global honey bee (*Apis mellifera*) industry for decades and are a significant cause of colony losses worldwide[1–4]. *Tropilaelaps mercedesae* is a parasitic mite that feeds on developing honey bees (brood) and can rapidly cause colony failure[2,3]. With a historic distribution restricted to Asia, the *T. mercedesae* has recently expanded its geographic range into Central Asia and further west than it has been discovered before[3,5]. The parasitic mite *Varroa destructor* is currently the leading cause of colony losses in the U.S. and is widely considered to be a leading cause of colony failures worldwide[1,4]; however, *T. mercedesae* has been shown to outcompete *V. destructor* in colonies co-infested with both species by reproducing more successfully than its *V. destructor* counterparts[6].

T. mercedesae has two distinct phases, the reproductive and dispersal phase[2,3]. During the reproductive phase, mites enter late-stage honey bee larval brood cells that are about to be capped for pupation[7]. During honey bee pupation *T. mercedesae* females lay eggs which mature and later emerge as adult mites with the adult bee[8,9]. *T. mercedesae* weakens colonies by feeding on the hemolymph of developing honey bees throughout their reproductive phase, causing reduced honey bee emergence weight and overall lifespan, in addition to vectoring diseases[10,11].

Beekeepers predominately manage *Varroa* with chemical controls to treat infestations, but due to their biology, *T. mercedesae* has proven challenging to manage in this way[3,12]. Unfortunately for beekeepers, during their reproductive phase, parasitic mites are protected underneath wax brood cell cappings, thereby limiting most treatments that only kill mites in the

dispersal phase[13]. *T. mercedesae* has a very limited dispersal phase as it does not have a post-mating feeding requirement and can infest cells to lay eggs in under two days after first emergence[14,15]. Comparatively, *V. destructor* requires about five days post mating before spermatozoa are ready to fertilize female eggs; during this time *V. destructor* primarily feeds on adult honey bees, thus increasing the likelihood the mite will come in contact with a lethal dose of a treatment[16]. This difference not only allows *T. mercedesae* to reproduce at an accelerated rate[15], but also makes it difficult to control through chemicals alone. This shortened dispersal phase limits opportunities for *T. mercedesae* to come in contact with extended-release chemical treatments resulting in reductions in efficacy. Acaricides that act through contact to effectively inhibit *V. destructor* population growth such as amitraz, hops beta acids, or flumethrin are largely ineffective against *T. mercedesae*[17,18]. Formic acid is the only chemical to show consistent and effective control of *T. mercedesae* due to its ability to penetrate cell cappings and kill mites concealed within brood cells[17].

T. mercedesae is incapable of feeding on adult honey bees, making it vulnerable to beekeeper interventions that create periods without larvae or pupae in the colony[14,19–21]. Without access to honey bee larvae of least 3.5 days of age, *T. mercedesae* mites die from starvation in three days or less[14,21]. While *T. mercedesae* is harder to control under normal colony circumstances when brood is present, beekeepers may be able to exploit this pest's biology and reliance on brood by inducing a brood break[2]. A brood break where all larvae and pupae are eliminated from the colony has been proven to be an effective treatment against *T. mercedesae* even without any chemical intervention[18,22].

Caging queens to induce a brood break is a proven method to increase treatment efficacies for both *T. mercedesae* and *V. destructor* by forcing both mite species into the dispersal

stage in which they are not protected underneath cell cappings[18,23–25]. In addition to providing a potential treatment window for *T. mercedesae*, caging the queen can eliminate any appropriate-age brood in the colony for *T. mercedesae* to feed on, in effect starving the mites. In a colony devoid of capped brood or larvae, a newly laid honey bee egg would take 6.5 days until *T. mercedesae* was able to be feed on it, much greater than the three days the mites can survive without feeding[14,21]. Additionally, *T. mercedesae* would not be able to reproduce in a capped brood cell until nine days after the initial egg was laid. Chemical controls against *V. destructor* have demonstrated increased effectiveness when combined with a cultural control such as a brood break[23–25]; however, no investigation has examined if the addition of a chemical application to a brood break will result in increased treatment success against *T. mercedesae*. Therefore, this experiment investigated the effects of combining the cultural control of a brood break with a chemical treatment of either formic acid (FA) or oxalic acid (OA) on *T. mercedesae* infestation and impacts of those treatments on western honey bee colonies.

Results

Effect of treatment on *T. mercedesae* worker brood infestation

We observed a significant interaction between treatment and experiment day for *T. mercedesae* worker brood infestation ($\chi^2_2 = 65.74$, $P < 0.001$). On Day 0, we did not see any significant differences in *T. mercedesae* infestation rates (F $ratio_{3,\infty} = 0.139$, $P = 0.94$) (Fig. 6.1; estimates and confidence intervals in Table 6.1). On Day 60, *T. mercedesae* worker brood infestation significantly differed among treatment groups (F $ratio_{2,\infty} = 42.46$, $P < 0.0001$). An estimated 15.3% of capped worker cells were infested by *T. mercedesae* in Control colonies, which was significantly greater than the 0.1% infestation rate estimated for both Brood Break and Brood Break + OA colonies (z-$ratio_{\infty} = -6.90$, $P < 0.0001$; and z-$ratio_{\infty} = -7.35$, $P < 0.0001$)

(Table 6.1). Brood Break and Brood Break + OA treatment groups did not significantly differ from each other on Day 60 (z-ratio$_\infty$ = -0.14, P = 0.999). No mites were found in worker brood in any Brood Break + FA colonies, so the treatment group was given an estimate of zero.

Table 6.1. Model estimates. Point estimates and 95% confidence intervals for *Tropilaelaps mercedesae* infestation of *Apis mellifera* worker brood, *Tropilaelaps mercedesae* sticky board drop, adult honey bee population, and capped worker brood area among treatment groups on each experimental day.

Variable	Day	Treatment	Estimate [95% CL]
T. mercedesae worker brood infestation (%)	0	Brood Break	0.45 [0.19 – 1.04]
	0	Brood Break + FA	0.30 [0.12 – 0.76]
	0	Brood Break + OA	0.38 [0.17 – 0.87]
	0	Control	0.40 [0.17 – 0.94]
	60	Brood Break	0.10 [0.027 – 0.37]
	60	Brood Break + FA	0 [N/A]
	60	Brood Break + OA	0.11 [0.034 – 0.37]
	60	Control	15.25 [7.98 – 29.15]
T. mercedesae sticky board drop (mites per 24 hours)	1	Brood Break	0.17 [0.017 – 1.72]
	1	Brood Break + FA	0.16 [0.015 – 1.66]
	1	Brood Break + OA	0.061 [0.0049 – 0.77]
	1	Control	0.35 [0.036 – 3.33]
	12	Brood Break	3.95 [1.39 – 11.24]
	12	Brood Break + FA	2.15 [0.74 – 6.28]
	12	Brood Break + OA	2.18 [0.80 – 5.96]
	12	Control	0.60 [0.17 – 2.12]
	25	Brood Break	0.34 [0.081 – 1.44]
	25	Brood Break + FA	0.67 [0.16 – 2.82]
	25	Brood Break + OA	0.24 [0.055 – 1.02]
	25	Control	0.63 [0.17 – 2.43]
	50	Brood Break	0.020 [0.0025 – 0.16]
	50	Brood Break + FA	0 [N/A]
	50	Brood Break + OA	0.018 [0.0024 – 0.14]
	50	Control	13.22 [7.04 – 24.82]
	60	Brood Break	0.13 [0.045 – 0.40]
	60	Brood Break + FA	0.19 [0.069 – 0.52]
	60	Brood Break + OA	0.018 [0.0023 – 0.15]
	60	Control	15.48 [7.64 – 31.39]
Adult honey bee population (frames)	0	Brood Break	6.35 [5.99 – 6.70]
	0	Brood Break + FA	6.29 [5.92 – 6.66]
	0	Brood Break + OA	6.12 [5.78 – 6.47]
	0	Control	6.85 [6.49 – 7.20]

	24	Brood Break	7.15 [6.80 – 7.51]
	24	Brood Break + FA	7.17 [6.79 – 7.54]
	24	Brood Break + OA	6.84 [6.50 – 7.19]
	24	Control	7.08 [6.72 – 7.43]
	60	Brood Break	6.38 [6.03 – 6.74]
	60	Brood Break + FA	5.29 [4.92 – 5.66]
	60	Brood Break + OA	6.16 [5.81 – 6.50]
	60	Control	6.42 [6.07 – 6.78]
Capped brood area (frames)	0	Brood Break	1.73 [1.55 – 1.91]
	0	Brood Break + FA	1.99 [1.80 – 2.18]
	0	Brood Break + OA	2.05 [1.88 – 2.23]
	0	Control	2.08 [1.90 – 2.26]
	24	Brood Break	0 [N/A]
	24	Brood Break + FA	0 [N/A]
	24	Brood Break + OA	0 [N/A]
	24	Control	1.83 [1.65 – 2.01]
	60	Brood Break	1.08 [0.90 – 1.27]
	60	Brood Break + FA	1.04 [0.90 – 1.27]
	60	Brood Break + OA	0.92 [0.75 – 1.10]
	60	Control	1.17 [0.99 – 1.35]

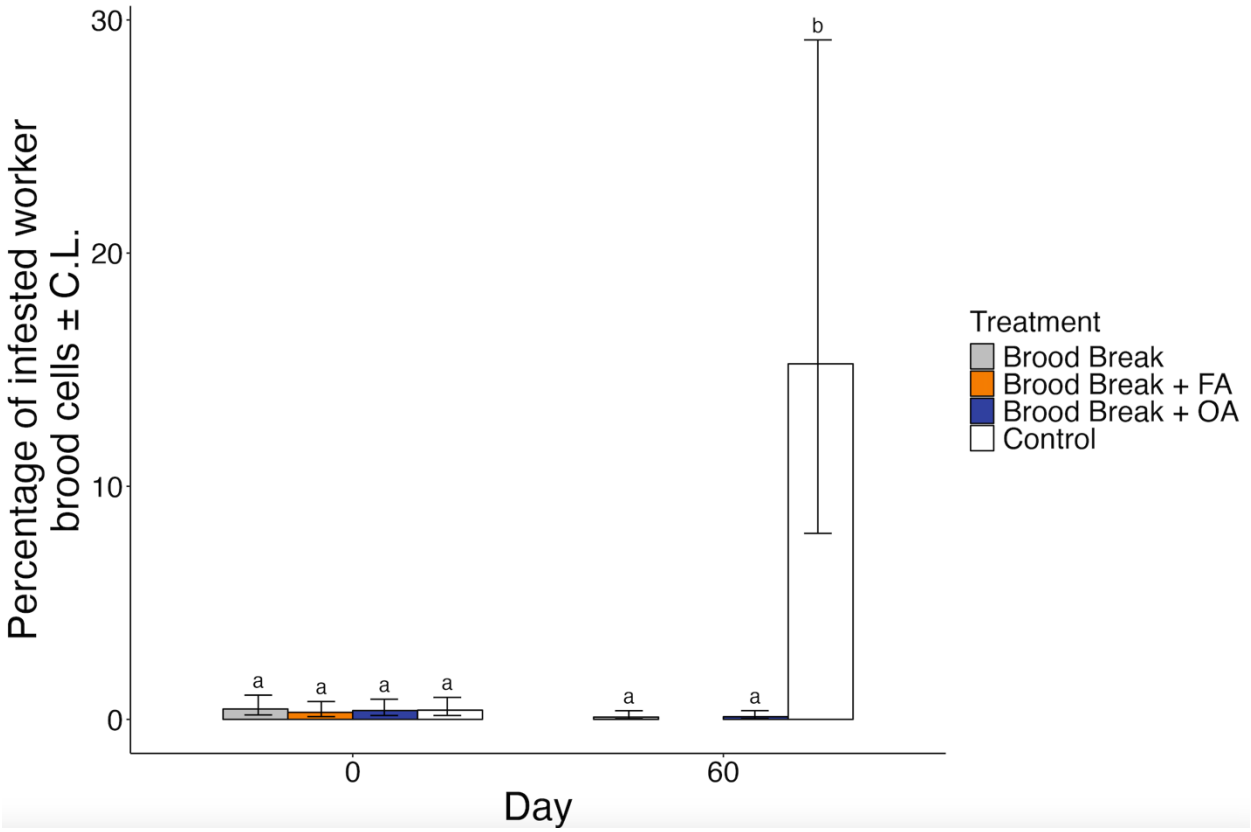

Figure 6.1. *Tropilaelaps mercedesae* infestation of *Apis mellifera* worker brood. Percentage of capped worker brood cells infested among treatment groups on each experimental day. Model estimates are shown. Different letters indicate significant differences.

Effects of treatment on *T. mercedesae* sticky board drop

We observed a significant interaction between treatment and experiment day for *T. mercedesae* drop onto sticky boards ($\chi^2_6 = 123.81$, $P < 0.0001$). We did not see any significant difference among treatment groups for Day 1 (F $ratio_{3,\infty} = 0.34$, $P = 0.80$) or Day 25 (F $ratio_{3,\infty} = 0.48$, $P = 0.70$). While we saw a numerical increase in mite drop for all colonies receiving a brood break on Day 12, no significant difference was seen among treatment groups (F $ratio_{3,\infty} = 1.77$, $P = 0.15$). We did observe a significant difference among treatment groups on Day 50 (F $ratio_{2,\infty} = 32.51$, $P < 0.0001$) (Table 6.1). On Day 50, Control colonies had an estimated daily mite drop of 13.22 mites, which was significantly greater than the estimated daily mite drop of 0.02 mites for Brood Break and Brood Break + OA colonies (z-$ratio_\infty = -5.88$, $P < 0.0001$; and z-$ratio_\infty = -6.00$, $P < 0.0001$, respectively) (Fig. 6.2, Table 6.1). Brood Break and Brood Break + OA colonies did not significantly differ (z-$ratio_\infty = 0.056$, $P = 0.99$). Similar to *T. mercedesae* infestation of worker brood, no mites were found on sticky boards on Day 50 in any Brood Break + FA colonies, so the treatment group was again given an estimate of zero. On day 60, we saw a significant difference among treatment groups (F $ratio_{3,\infty} = 31.28$, $P < 0.0001$), whereby Control colonies had significantly more mites (15.5 mites) on sticky boards compared to Brood Break (0.13 mites, z-$ratio_\infty = -7.19$, $P < 0.0001$), Brood Break + OA (0.018 mites, z-$ratio_\infty = -6.00$, $P < 0.0001$), and Brood Break + FA (0.19 mites, z-$ratio_\infty = -7.04$, $P < 0.0001$) colonies. No significant difference was observed among the brood break treatment groups on Day 60.

As assessed by sticky board counts, the mite infestations of Control colonies increased dramatically over the 60-day trial period. Based on a comparison of point estimates, the Control colonies had ending mite levels that were 44.2 times as high on Day 60 as they were on Day 1 (Table 6.1). In contrast, the Brood Break treatment group mite levels were 0.8 times as high,

Brood Break + OA mite levels were 0.3 times as high, and Brood Break + FA mite levels were 1.2 times as high on Day 60 compared to Day 1.

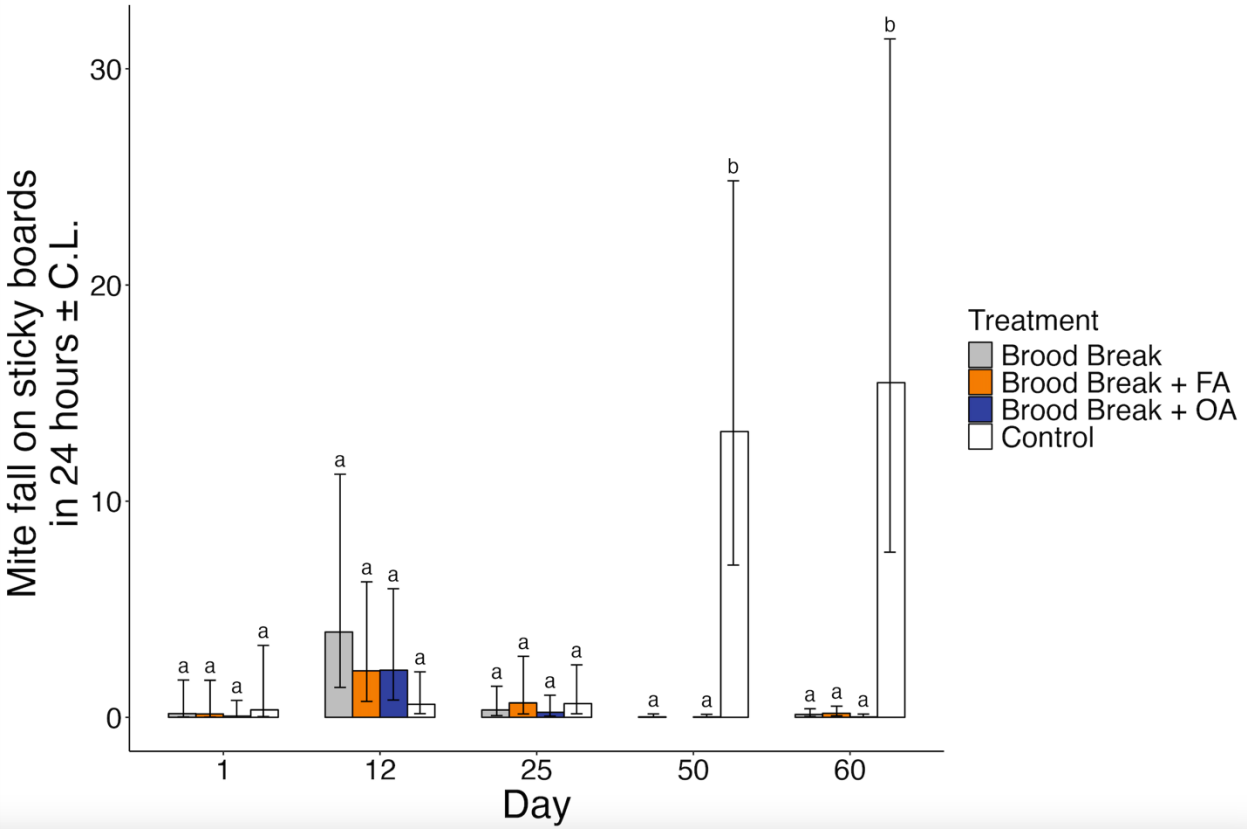

Figure 6.2. *Tropilaelaps mercedesae* mite drop. Estimated *T. mercedesae* mite drop per 24 hours in *Apis mellifera* colonies among treatment groups on each experimental day. Model estimates are shown. Different letters indicate significant differences.

Effects of treatment on adult honey bee population

We found a significant interaction between treatment and experiment day on adult honey bee population ($\chi^2_6 = 26.47$, P=0.00018). On Day 0 for adult bee population, we found a significant difference among treatment groups (F ratio$_{3,141}$ = 3.019, P = 0.032). Control colonies had significantly larger populations than Brood Break + OA colonies (t-ratio$_{141}$ = -2.89, P = 0.023). However, no other treatment groups significantly differed from each other on Day 0 (Fig. 6.3; Table 6.1). On Day 24, no significant difference was observed among treatment groups for adult population (F ratio$_{3,141}$ = 0.72, P = 0.54). However, we observed a significant difference among treatment groups on Day 60 (F ratio$_{3,141}$ = 8.049, P = 0.0001). Brood Break + FA colonies had significantly lower populations compared to Brood Break (t-ratio$_{141}$ = 4.18, P = 0.0003), Brood Break + OA colonies (t-ratio$_{141}$ = -3.38, P = 0.0051) and Control colonies (t-ratio$_{141}$ = -4.33, P = 0.0002). No other treatment groups significantly differed on Day 60.

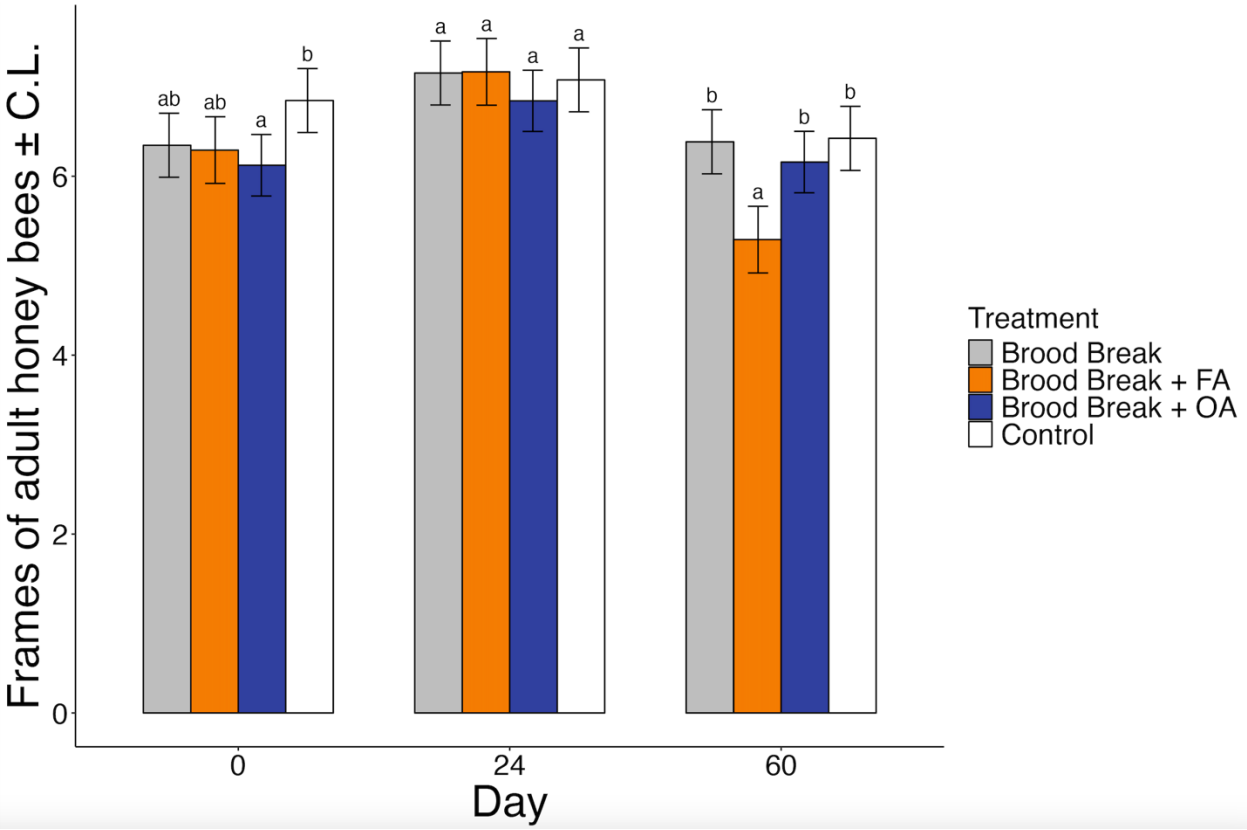

Figure 6.3. Adult honey bee population. Frames of adult honey bees (*Apis mellifera*) among treatment groups on each experimental day. Model estimates are shown. Different letters indicate significant differences.

Effects of treatment on capped worker brood area

We found a significant interaction between treatment and experiment day on capped worker brood area (χ^2_3 = 8.54.47, P=0.036). No difference was observed among treatment groups for capped worker brood area on Day 0 or Day 60; however, on Day 60, capped worker brood areas were lower in all treatment groups at the end of the experiment compared to Day 0 (Fig. 6.4;). Because there was no capped brood in colonies on Day 24 for any colonies that received a brood break, these treatment groups were given an estimate of 0 as Control colonies were the only ones with brood on Day 24.

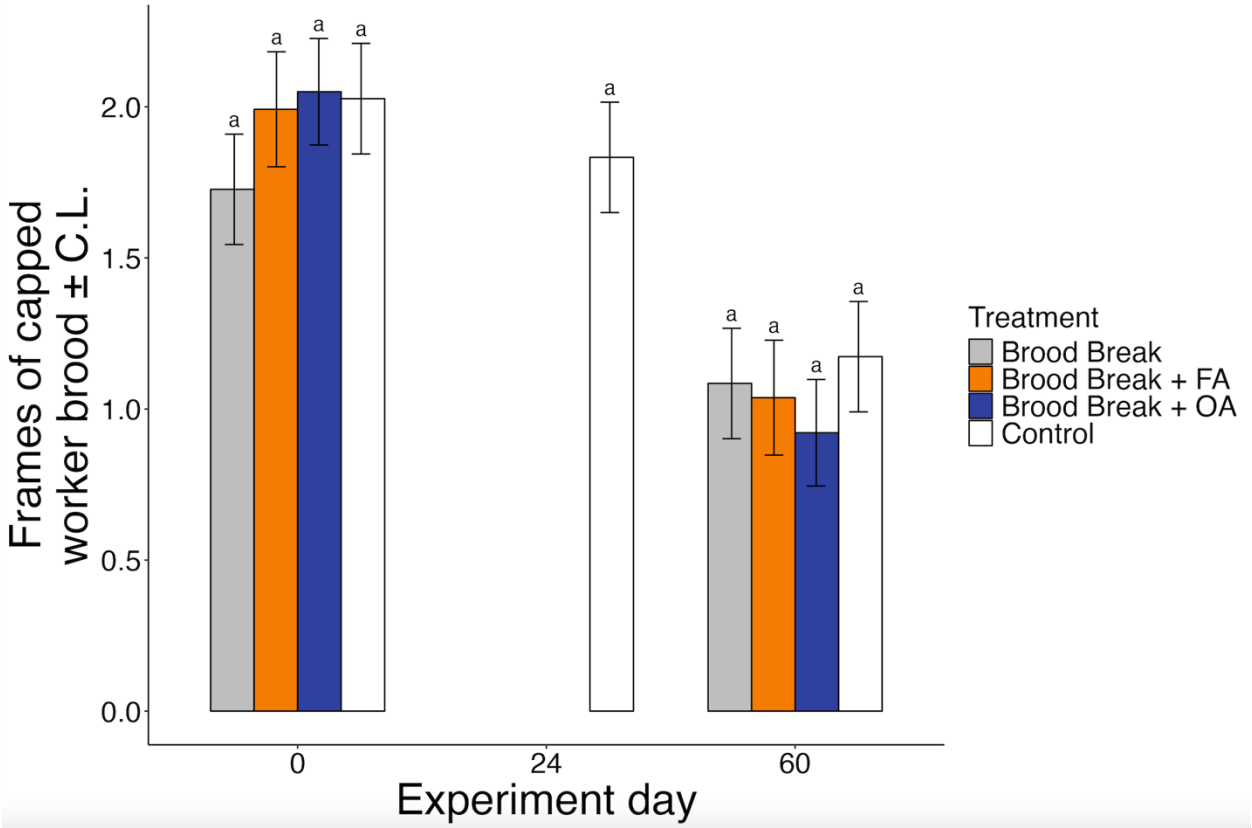

Figure 6.4. Capped worker brood area. Capped honey bee (*Apis mellifera*) worker brood area among treatment groups on each experimental day. Model estimates are shown. Different letters indicate significant differences.

Discussion

Tropilaelaps mercedesae is an ever-increasing threat as the species' geographic range continues to expand[5]. Viable management plans are necessary to control these parasitic mites as they have the ability to rapidly reproduce, leading to accelerated colony failure through their feeding on developing brood and virus transmission [11,14,15]. Our experiment demonstrated that brood breaks remain an extremely effective tool for *T. mercedesae* management, but that the addition of a chemical treatment to a brood break may not substantially improve efficacy or completely eliminate *T. mercedesae*.

While creating a brood break appears to be a viable method for reducing a *T. mercedesae* population in western honey bee colonies, similar to previous studies, but complete eradication could not be achieved [18,22]. The additional chemical treatment provided a marginal, but not significant, reduction in *T. mercedesae* worker brood infestation, as mites were still found within colonies on Day 60 in all colonies given a brood break, regardless of if an additional chemical application was applied. Neither the short-acting oxalic acid treatment nor the prolonged formic acid treatment were able to eradicate all mites from colonies by Day 60.

Interestingly, no *T. mercedesae* mites were found on sticky boards on Day 50 or in capped worker brood cells on Day 60 in colonies experiencing a brood break and formic acid treatment. This aligns with previous results in that formic acid has repeatedly shown to be the most promising chemical treatment[17,18]. However, these colonies did have marginally higher mite estimates on sticky boards for Day 60 compared to either other treatment group that experienced a brood break. The absence of detected mites on Day 50, but detection of mites on Day 60, could either be explained by some persistence of *T. mercedesae* mites despite the cultural and chemical control, or by some level of mite movement on adult bees between

colonies after treatment, resulting in re-infestation of these colonies experiencing a brood break and formic acid treatment[26]. While *T. mercedesae* dispersal is still relatively unknown, there has been some evidence to suggest mites can drift phoretically between colonies[9]. *Tropilaelaps mercedesae* can phoretically attach to adult honey bees for short periods, which could have enabled mites to enter these colonies by Day 60[7,19,20]. To investigate if combining a brood break with formic acid does eliminate all *T. mercedesae,* another study needs to be conducted where treatments are given to the entire yard, isolating the treated colonies from nearby untreated colonies.

While the colonies experiencing a brood break and oxalic acid treatment had very similar worker brood infestation levels to those colonies experiencing a brood break and formic acid treatment, mites were found at some level in all post-treatment assessments including those on Day 50. This suggests that a brood break plus application of formic acid may appear to be the best alternative to destroying colonies near to *T. mercedesae* infested colonies if detected in a new region. However, that may be dependent on weather conditions during a first detection, since FormicPro® and other products with formic acid as their active ingredient are strongly influenced by ambient temperature, which may limit its usefulness during certain time of year or in regions[27]. When used at high temperatures or in high concentrations, formic acid can cause death of adult honey bees[27,28]. Despite only one strip of FormicPro® being applied for 10 days due to lack of capped brood and temperatures that consistently reached over 30ºC, a reduction of adult honey bee population was seen in the colonies experiencing a brood break and formic acid treatment on Day 60 illustrating the potential detrimental effects that colonies could experience if this mitigation technique were employed after a *T. mercedesae* discovery in a region with high temperatures. In contrast, capped worker brood area did not differ among treatment groups on

Day 60; this suggests that formic acid may only have short-term detrimental effects on colony health[29,30].

Though previously documented, one noteworthy observation was the rapid increase in *T. mercedesae* population when left untreated in Control colonies[2]. Similar to previous studies, adult honey bee population and capped worker brood area were not significantly different between treatment groups at the end of the study[17,18]; however, it appeared capped worker brood for Control colonies was trending down compared to the treated colonies which were recovering from the brood break. This trend was likely due to damage from the mites and would likely continue until the colony was completely overcome with *T. mercedesae* as it took just 60 days for Control colonies to see a dramatic increase in mite infestation as measured both by infestation of worker brood and by mite drop. Despite the number of offspring produced per mite varying depending on location and field or laboratory conditions[3,6,7,15,19], this experiment showed that *T. mercedesae* infestation can reach concerning levels in as little as three brood cycles, which necessitates rapid detection and eradication plans.

Brood breaks are clearly an effective backbone of a strategy to control *T. mercedesae*, which is necessary due to the mite's ability to rapidly impact colony functions[2,10,18]. Although we observed a slight reduction in adult honey bee population on Day 60 in colonies experiencing a brood break and formic acid treatment, we did not see any other differences in capped worker brood area or adult honey bee population for colonies receiving a brood break showing these management plans represent a safe and effective treatment option for *T. mercedesae*. Optimization of this management practice in combination with additional treatments should be investigated to provide a potential solution for what management practices should be employed to eliminate *T. mercedesae* in countries where it may be introduced.

Methods

Experimental location

The experiment was conducted at Chiang Mai University in Chiang Mai, Thailand using *Apis mellifera* colonies purchased from a local beekeeper who had not treated them for one month prior to the start of the experiment in June of 2023. Colonies had eight frames and were kept in single modified Langstroth boxes (42 x 42 x 25 cm) with two entrances (8.5 x 1 cm), and a ventilation screen (31 x 7 cm) on the back; they were moved to campus where they were arranged in groups of up to four, with entrances pointing in opposite directions in an attempt to minimize drift of bees between colonies[31].

Pre-assessments

Colonies were first assessed for adult population, capped worker brood area, and *T. mercedesae* infestation. Adult population was assessed using a modification of the Bee Informed Partnership guide[32] by looking down into the hive and estimating the number of frames that adult worker honey bees occupied. Capped worker brood area was measured by estimating the percent of each frame covered with capped worker brood[33]. To measure *T. mercedesae* infestation, two brood frames per colony were removed, 200 worker cells (50 cells on either side of each frame) were opened with forceps, the prepupae or pupae were removed, and the brood and cell were visually inspected to determine presence or absence of *T. mercedesae* per cell[17].

Treatment assignments

To minimize *T. mercedesae* drift among colonies of different treatment groups, all colonies in a group were assigned to receive the same treatment. Based on the *T. mercedesae* infestation rate of brood during initial colony assessments, the groups were sorted into strata. Within strata, treatments were randomly assigned; the draft treatment assignment was reviewed

and re-randomized several times, with the goal that treatments should be spatially interspersed and that treatment groups should be homogenous for the following variables in order of decreasing priority: *T. mercedesae* infestation rate of worker brood, capped worker brood area, and adult honey bee population[34]. Eighteen groups each consisting of 2-4 colonies for a total of 52 colonies were used for this experiment. Treatment groups included a Control, which did not receive a brood break or chemical treatment, (4 groups; 12 colonies), Brood Break (4 groups; 13 colonies), Brood Break + Formic Acid (FA) (5 groups; 12 colonies), and Brood Break + Oxalic Acid (OA) (5 groups; 14 colonies).

To administer the experimental treatments, on Day 0, queens in all colonies receiving brood break were caught and placed in QCC cages (80 x 33 x 12 mm; Thorne Company, Lincolnshire, U.K.). A section of comb was removed from the bottom middle of a frame, the queen cage was then attached, and the frame was placed in fourth frame position within the hive. On Day 24, all queens in colonies receiving a brood break were released from their cages and cages were removed from the colonies. During this time, Brood Break + OA colonies were given a 40 mL volume of 2.9% oxalic acid dribble which had been mixed in a 1:1 w/v sugar water solution as per Api-Bioxal® label instructions. Meanwhile, Brood Break + FA colonies were given one strip of FormicPro® for 10 days, a half dosage based on label instructions. The half dosage was chosen because the lack of brood reduced the need to infiltrate cell cappings, and the experiment was conducted in high temperatures[at or slightly above the 30°C label recommendation[27].

Subsequent measurements

To assess *T. mercedesae* infestations, sticky boards and dissection of capped worker brood were used. Since colonies did not have a screened bottom board, sticky boards of tar paper

covered in petroleum jelly were inserted into colonies with a plastic meshed screen placed over the top to deter honey bees from removing mites. Sticky boards were removed and assessed on Day 1, 12, 25, 50, and 60; on the first three occasions they were inserted and removed 24 hours later[18], and on the last two occasions, they were removed after 72 hours in the colony, a more common practice for *V. destructor* monitoring[25,35–37]. On Day 60, *T. mercedesae* infestation of capped worker brood was assessed using the same methods as the pre-assessment.

To continue tracking the effects on honey bee colonies after the initial assessments, the adult honey bee population and capped worker brood area were assessed on Day 24 and 60 using the pre-assessment methods. Colonies were removed from the trial if they lost their queen to avoid compromising the experiment through an unintended brood break.

Statistics

Statistics and plotting were conducted using R 4.2.2[38] and the packages *lme4*, *emmeans*, *dplyr*, and *ggplot2*[39–42]. Frames of honey bees and frames of brood were analyzed using a linear mixed model (Gaussian distribution) with fixed effects of *treatment*, *day*, and a *treatment* × *day* interaction; random effects of hive nested within group were included to avoid temporal pseudoreplication[34]. For the capped worker brood model, all colonies receiving a brood break had their data excluded for Day 24 to allow the model to function properly and the treatment groups were given an estimate of zero. *Tropilaelaps mercedesae* cell infestation and on sticky boards were analyzed using generalized linear mixed models (negative binomial distribution), with fixed effects of *treatment*, *day*, *treatment* × *day* interaction, and random effects of hive nested within group. In the case of Day 50 for the sticky board model and Day 60 for the brood cell infestation model, because no mites were found in the brood break + FA colonies, these data were also removed from the model and the treatment group was again given an estimate of zero.

The sticky board model also had a dispersion factor for *day* included. For *T. mercedesae* cell infestation, all model output values were divided in half to show number of infested cells per 100 cells (i.e., percent infestation). Lastly, *T. mercedesae* on sticky boards was analyzed after multiplying Day 1, 12, and 25 results by three to adjust for differing lengths of sticky board collections. Model estimates were then divided by three to identify mite drop in 24 hours. All models were tested for fit by testing residuals, dispersion, and zero inflation. Additionally, if there was a significant interaction between treatment and day, we used the *joint_tests* function in the *emmeans* package to test if there were differences between treatment groups within each day[42]. When there were, pair-wise comparisons were made with the *emmeans* package using Tukey HSD adjusted *P* values, with an alpha of 0.05.

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Chapter 7

Conclusion

This dissertation focused on developing parasitic mite management strategies that are sustainable for Western honey bee (*Apis mellifera*) beekeepers. A portion of the research experiments included looking to find a suitable treatment alternative to amitraz for beekeepers to reduce *Varroa destructor* infestations before fall in the Southeastern U.S. Other experiments were designed to fill knowledge gaps and inform beekeepers of the newly emerging parasitic mite threat *Tropilaelaps mercedesae*.

Varroa destructor remains a consistent leading cause of colony losses in the northern hemisphere (Aurell et al., 2024; Bruckner et al., 2023; Rosenkranz et al., 2010). Compounding this issue is the ever-increasing presence of amitraz resistant *V. destructor* mites, which is the most commonly applied synthetic acaricide amitraz (Elzen et al., 2000; Hernández-Rodríguez et al., 2025; Maggi et al., 2009; Rinkevich, 2020; Rinkevich et al., 2023). In one experiment (Chapter 2), I demonstrated Apivar®, an amitraz based product, was still an effective treatment prior to winter against *V. destructor* when working with a commercial beekeeper with low Varroa infestation and low amitraz resistance levels. I also found that a registered product, albeit not currently registered in the U.S., Aluen CAP® was also capable of reducing *V. destructor* infestation rates, but it coincided with a negative impact on colony strength. On the other hand, a homemade extended-release OA treatment had no negative impacts on colony strength, but it was not capable of reducing *V. destructor* infestation rates.

My other *V. destructor* experiment evaluating registered, organic *V. destructor* treatments (Chapter 3) showed varying results when looking for an effective alternative to amitraz. I found that treatments of Apivar®, FormicPro™, and HopGuard® maintained infestation rates under 3%

V. destructor infestation but were incapable of reducing them. Apivar® did not reduce Varroa infestation due to moderate levels of amitraz resistance. Meanwhile, an untreated control and OA Vapor regimen saw *V. destructor* infestation rates significantly increase over 63 days. This experiment also explored impacts of different treatments on amitraz resistance allele frequencies. In only the Apivar® treatment group, I saw a significant increase in amitraz resistance allele frequency after an application of a treatment; however, this increase disappeared after the overwintering period from Day 63 to Day 133. Since I did not see this decrease after the overwintering period in any other treatment group, it is unlikely there was a fitness cost associated with resistance. Additionally, these findings show that there is unlikely to be cross-resistance of amitraz resistant *V. destructor* mites to other treatments with alternative modes of action.

Transitioning to *T. mercedesae*, it is critical that we determine the current range of the mite as there are currently differing accounts in various publications as to countries with a confirmed mite presence (Chantawannakul et al., 2018, 2016; De Guzman et al., 2017). Working through literature, I found *T. mercedesae* was confirmed in 17 countries with 13 countries having the mite found in association with *A. mellifera* (Chapter 4). Utilizing peer reviewed literature, these detections were confirmed by morphological and / or molecular (DNA) means. The resulting map illustrates the most current *T. mercedesae* range and illustrates the current gaps in sample collection and what regions have the greatest threat of potential introduction.

The dispersal method for *T. mercedesae* is still largely unknown, an important aspect of biology to know as *T. mercedesae* continues to be detected in new regions (Brandorf et al., 2024; Janashia et al., 2024; Mohamadzade Namin et al., 2024). Utilizing funnel traps, I conducted an experiment aimed at confirming that *T. mercedesae* can be found on adult honey bees exiting the

colony thus demonstrating that dispersal through honey bee drift or robbing behaviors is possible (Chapter 5). Although I did not find *T. mercedesae* exiting on adults when colonies had low brood infestation levels, I found several mites exiting on adult honey bees at higher brood infestation levels. This confirms that *T. mercedesae* dispersal is not limited to beekeeper mediated means and is possible from colony to colony. These findings also open the possibility of a response where *T. mercedesae* will more readily attach to adult honey bees as a potential means of transportation like researchers have documented with *V. destructor* (Beetsma et al., 1999; Cervo et al., 2014).

If *T. mercedesae* is introduced to a new country or region, the goal would be to first eradicate it. My final experiment looked at evaluating if a combination of a cultural and chemical control would be capable of eliminating all *T. mercedesae* from a colony. Utilizing a known effective cultural treatment of a brood break to kill *T. mercedesae* through starvation since they are unable to feed on adult honey bees (Khongphinitbunjong et al., 2019; Pettis and Chaimanee, 2019; Rinderer et al., 1994; Roberts et al., 2020; Woyke, 1985), I found that just a brood break can keep *T. mercedesae* infestation rates low. I also found that the additional chemical treatment of OA or formic acid provided a marginal but not significant reduction in *T. mercedesae* infestation rates. Although none of the treatment groups eliminated all *T. mercedesae* from the colony, they still all kept mite infestation rates dramatically lower than the control which saw a significant increase in *T. mercedesae* brood infestation rate and number of mites on sticky boards in just 60 days. This not only demonstrated the rapid ability *T. mercedesae* could reproduce, but, since all the colonies were housed in the same location, it opened the possibility of some colonies being reinfested (Tokach et al., 2025). Future experiments should look if eradication is possible if all colonies in the yard are treated.

In conclusion, honey bees still face many different stressors, namely parasitic mites with *V. destructor* already a leading cause of colony losses in the Northern hemisphere and *T. mercedesae* threatening to continue to expand their geographical range. With amitraz resistance continuing to rise, we need more effective and sustainable *V. destructor* strategies and treatments available for beekeepers. Furthermore, we need to continue to research and understand how amitraz resistant *V. destructor* dynamics are impacted by using alternative treatments to determine if amitraz will remain a viable treatment in the future. For *T. mercedesae*, there is a further need for more monitoring and sample collections to determine where it has already spread. Additionally, we need to continue to fill knowledge gaps regarding understanding of dispersal methods and effective treatments in the event the mite continues to spread into new areas. Overall, I hope that this research can be impactful to inform beekeepers of alternative *V. destructor* treatments and assist them in developing a better understanding of *T. mercedesae*.

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Appendix A

Supplementary Material for Chapter 5

Figure S5.1. Example of a Thai colony fitted with the modified funnel trap used to trap and collect foraging *Apis mellifera* adults exiting the colony.