UNIVERSITY OF CALIFORNIA SAN DIEGO

The Role of Social Immunity in Feral Honey Bees (*Apis mellifera*) in Response to the Parasitic Mite (*Varroa destructor*)

A thesis submitted in partial satisfaction of the requirements

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in

Biology

by

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Professor Aspen Reese

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The thesis of Brandon T. Mukogawa is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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DEDICATION

To my family, who have always supported my aspirations. Thank you for placing your trust and faith in me to carve my own path.

To my friends, who have seen me at my worst. Thank you for all the laughs, tears, and memories.

To all the bees and mites who took part in this research. Thank you for your contribution, for your lives.

EPIGRAPH

Experiments are not about discovery,

But about listening and translating the knowledge of other beings.

Robin Wall Kimmerer

The bee is more honored than other animals,

Not because she labors,

But because she labors for others.

Saint John Chyrsostom

Knowing that you love the earth changes you,

Activates you to defend and protect and celebrate.

But when you feel that the earth loves you in return,

That feeling transforms the relationship from a one-way street into a sacred bond.

Robin Wall Kimmerer

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ABSTRACT OF THE THESIS

The Role of Social Immunity in Feral Honey Bees (*Apis mellifera*)

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By

Brandon T. Mukogawa

Master of Science in Biology

University of California San Diego, 2022

Professor James Nieh, Chair

Varroa destructor is a parasitic mite that threatens managed and feral *Apis mellifera* colonies worldwide. Managed honey bees are regularly treated with miticides to control for *Varroa*, but the use of these chemicals reduces bee fitness and leads to the evolution of miticide resistance in *V. destructor*. However, feral colonies, which tend to be more Africanized, may tolerate mites without chemical treatment. Some studies have shown Africanized colonies demonstrate increased hygienic behavior by removing more dead brood and by grooming more intensely, making them potentially more *Varroa*-resistant. Thus, comparing the behavior of feral and managed bees can reveal the potential role of social immunity in feral bee tolerance against *V.*

destructor. These findings can better inform traits of interest for bee breeding programs. Interestingly, no differences in mite infestation were observed despite that managed bees were treated with miticides at multiple times throughout the year. This result suggests that feral colonies have ways to reduce their mite levels. There were no observed differences in the social immunity of feral and managed honey bees as measured by their hygienic, self-grooming, or mite biting behavior. However, we provide the first evidence that both feral and managed honey bees bite off mite forelegs at higher rates than other legs; mite forelegs contain chemosensory organs that mites use to find brood cells to reproduce in. Such biting may therefore impair mite reproduction. Future studies should therefore focus on other mechanisms that evidently allow feral bees to resist *Varroa* infestation.

Introduction

Western honey bees (*Apis mellifera*) play a major ecological role as pollinators in natural landscapes and for agriculture. *Apis mellifera* is the most common pollinator within natural habitats, accounting for 13% of recorded flower visits worldwide (Hung et al. 2018). This species also provides over $17-18 million in pollinator services in the U.S. (Glenny et al. 2017). However, about one in three honey bee colonies have died annually since 2006 from various factors including pathogens, pesticide exposure, and nutritional deficiencies (Glenny et al. 2017). The most significant harm stems from *Varroa destructor*, a parasitic mite that feeds on honey bee fat reserves (Ramsey et al. 2019). The introduction of *Varroa* mites caused historic declines in both managed and feral honey bee colonies in North America (Genersch 2010, Kraus and Page 1995) . Mite populations can grow quickly and increase colony mortality if left uncontrolled (Rosenkratz et al. 2010). Managed colonies usually die in 2-3 years if not treated with miticides (Rosenkratz et al. 2010). In addition, *Varroa* mites spread pathogens that can increase honey bee mortality (Tehel et al. 2016). For example, deformed wing virus (DWV) is commonly vectored by *V. destructor* and suppresses honey bee immune systems, thereby increasing susceptibility to other stressors like pathogens and pesticides (Di Prisco et al. 2016; Yang and Cox-Foster 2005). *Varroa* mites are therefore a leading cause of honey bee colony declines, affecting *A. mellifera* globally (Jack and Ellis 2021; Tehel et al. 2016).

To defend against mites and other pathogens, honey bees use social immunity: traits that provide immune defense at the colony level. These include behaviors defenses like hygienic behavior and grooming. In hygienic behavior, honey bee colonies remove dead and infected larvae from the colony (Simone-Finstrom 2017); these behaviors disrupt the reproductive life cycle of *Varroa* mites, which require larval hosts to develop into adults (Spivak 1996). Honey

bees also groom to remove mites. During self-grooming, individuals dislodge *Varroa* with vigorous movements on their own (Invernizzi et al. 2015; Aumeier 2001). In addition, honey bees can bite off mite legs during grooming to limit mite mobility and limit parasitization; colonies with higher rates of mite leg mutilation have lower mite infestations (Simone-Finstrom 2017, Mondragón et al. 2005).

These behavioral defenses are heritable and vary in intensity among different genetic lines (Aumeier 2001; Guerra Jr. et al. 2000; Guzman-Novoa et al. 1999). Multiple studies have shown that feral *A. mellifera* colonies are infested with *Varroa*, but manage to survive without receiving anti-*Varroa* treatments (Seeley 2017, Locke et al. 2016). However, the extent to which various social immunity behaviors contribute to feral honey bee resilience against *Varroa* is unclear.

One group of feral bees, Africanized bees, which consist of genetic admixtures between European hybrids and *A. mellifera scutellata*, is widespread and successful. They have spread from Central and South America into the U.S. as far as 34° N in California (Zarate et al. 2022). In fact, 70% of feral bees in San Diego carried African ancestry (Kono and Kohn 2015). Africanized honey bees remove a greater proportion of infested brood compared to Carnolian, European, and Italian subspecies (Aumeier 2001; Guerra Jr. et al. 2000; Guzman-Novoa et al. 1999). In laboratory self-grooming assays that placed *V. destructor* on honey bees, Africanized bees initiated self-grooming more rapidly and groomed with greater intensity than Carnolian bees (Aumeier 2001). Thus, better understanding the role of Africanization in mite tolerance within feral colonies is critical as these insights allow better *Varroa* control in managed settings.

By studying the social immunity of feral and managed bees, we aim to understand whether various behavioral defenses can successfully counter *V. destructor* because these mites

have developed a growing resistance to miticides. Chemical control has therefore become increasingly less effective by requiring increased chemical use and continual development of novel chemicals (DeGrandi-Hoffman et al. 2017; Giuffre et al. 2019). Additionally, commonly used miticides that contain *tau*-fluvinate and coumaphos decrease honey bee macronutrient levels, potential immune responses, and queen reproductive fitness (Reeves et al. 2018, Rangel and Tarpy 2015). Thus, finding miticide alternatives is imperative.

Identifying the extent to which social behaviors facilitate *Varroa* resistance in feral colonies may inform honey bee breeding practices. Currently, breeding programs artificially select for increased mite-biting and *Varroa*-sensitive hygienic (VSH) behaviors against mite-infested larvae. And although hygienic behavior and grooming in Africanized *A. mellifera* have been studied in Brazil, Central America, and Texas (Guzman-Novoa et al. 1999; Garcia et al. 2013; Aumier 2001; Invernizzi et al. 2015), few studies have examined these behaviors in southern California where Africanized feral bees are common (Kono and Kohn 2015). Thus, we compared the behavioral defenses of feral and managed *A. mellifera* colonies against *V. destructor* in San Diego: assessing hygienic behavior with pin-kill assays, self-grooming behavioral assays in the lab, and measuring mite mutilation in colonies.

Materials and Methods

1. Study Sites and Colonies

We conducted our study at two apiaries: the Biology Field Station (BFS) (32.885614137503346, -117.22997498068543) at the University of California San Diego containing only managed bees and the Elliott Chaparral Reserve (ECR) (32.89564249287754, -117.08667665732133) containing only feral bees captured during bee rescues in San Diego,

California. At the BFS, we used nineteen *A. mellifera ligustica* colonies obtained as nuclear colonies from bee breeders in northern California and requeened, as necessary, with European *A. mellifera ligustica* queens. The BFS colonies were managed using standard honey bee health protocols and were regularly treated with acaricides as needed when mite levels were above 3 mites/100 bees (Currie and Gatien 2006). Treatments were calibrated to the degree of mite infestation, beginning with thymol patties, escalating to formic acid, and culminating with tau-fluvalinate via Apistan strips at the highest infestation levels. BFS bees were also fed supplemental sucrose solution (50% v/v) and pollen patties (Ultra Bee High Protein Pollen Substitute, 58% crude protein, Mann-Lake Bee & Ag Supply Ltd) *ad libitum*.

Fifteen feral *A. mellifera* colonies were used at ECR and consist of unmanaged honey bees collected from southern California at different locations around San Diego. Like BFS colonies, each ECR colony was housed in a 10 frame Langstroth hive. Genomic admixture analyses confirmed that these bees had the same level of Africanized genes as feral bees sampled throughout San Diego (Zarate in prep.). None of the ECR colonies were treated with miticides or other chemicals, and they were also not fed sucrose solution or pollen patties.

However, all colonies at both sites had access to water in horse troughs, as per San Diego County apiary regulations. Because two colonies at each field site died out during the study, they were replaced with other colonies at their respective field sites to maintain a sample size as close to fifteen as possible at each location. We surveyed for mites and measured honey bee colony size roughly once per month at each field site.

2. Bee Colony Size Estimates

We based our monthly colony size measurements on standard methodology described by

Delaplane et al. (2013) for estimating *A. mellifera* colony strength. This method uses the Liebefeld method described in Dainat et al. (2019), but takes into account the fluctuating bee densities on each frame side, as opposed to using a standardized estimation of 1100 workers per deep Langstroth frame. We took photos of every frame side with bees with an iPhone (iPhone XR and iPhone 13). Using GIMP 2.10 software, 5x7 (height x width) grids were electronically overlaid on these pictures to subdivide frames into smaller cells (450 x 450 pixels) for easier measurement (Fig. 1). We selected a cell with a bee density representative of the majority of the cells in the photo and counted the number of individual bees in that specific cell. On average, this value was 30.57± 2.85 (STD) bees per 450 x 450 pixel cell. We then multiplied this number by the number of cells occupied by bees in the frame. This process was repeated for each frame side. We then summed our counts from all occupied frames to determine colony size. To validate our method, we also randomly selected some frames and counted all bees from photos and also applied our estimation method, as described above. These counts were in good agreement. To ensure consistency between researchers counting bees, we randomly selected frame photos and had both researchers count bees on the same photos during count training. We compared the counts made by trainer and trainee with linear regression, and trainees needed to obtain $R^2 \geq 0.85$ before they were allowed to collect count data used in our analyses.

3. Measuring Colony Mite Levels

To avoid disturbing feral colonies as much as possible, we avoided using alcohol washes and sugar shakes to measure mite levels. Instead, we placed sticky traps (Mann Lake #DC-081) under the frames of bee colonies. The bottom board was replaced with a wire mesh through which mites, but not bees, could fall through to a cardboard board were sprayed with a thin layer

of canola oil as an adhesive (Guzman-Novoa et al. 2012, Smith et al. 2020, Kovacic et al. 2018). Five days after trap placement, all adult female *V. destructor* mites (characterized by their brown coloration) (Smith et al. 2020; Kirrane et al. 2018) were removed from the canola oil with a small paintbrush and placed in 30 ml plastic cups (4 cm top diameter x 2.5 cm high x 3 cm base diameter) with a slightly moist size 0000 paintbrush.

4. Pin-Kill Assays

We measured hygienic behavior, the degree to which a colony will remove larvae, with a standard pin-kill assay. There is conflicting data on whether or not removing freeze-killed and pin-killed brood is comparable to removing mite-infested brood (Leclercq et al. 2017; Tehel et al. 2016). Spivak and Downey (1998) asserted freeze-kill assays are more effective measures of *Varroa*-sensitive hygiene than pin-kill assays since olfactory signals from pin-killed brood may not reflect those of *Varroa*-parasitized larvae (Mondet et al. 2014). However, Boecking et al. (2000) has shown a correlation between removal of pin-killed and *Varroa*-infested larvae and Shakeel et al. (2020) observed similar hygienic behavior in freeze-kill and pin-kill assays. Thus, we opted for the less intensive pin-kill assay.

Only colonies with a substantial amount of capped brood (at least two full frame sides) were used to ensure that colony fitness was not substantially disrupted. A circular indentation was made in the capped brood with a 119 mm diameter metal cylinder (111.2 cm^2, mean 260.62 capped brood cells) to mark the assay area. Two indentations were made: with one per treatment. In the pin-kill treatment, all capped brood cells within the circular indentation were perforated with a #2 insect pin (Shakeel et al. 2020) to kill developing larvae. In the control treatment, the circular indentation was left without perforation of capped brood. Photos were taken directly before perforation and 24 hours later to measure the number of pin-killed brood removed by the

colony from the assay area (Fig. 2). To ensure that the natural emergence of adult honey bees was not mistaken for hygienic behavior, data were excluded when there were visual cues of emergence or when control treatments had a removal rate higher than 15%.

5.1 Self-grooming Behavioral Trials: Collecting Bee and Mite Specimen

Worker bees were collected with individual snap vials (2 cm x 5 cm) from the surface of combs within each colony. To obtain worker bees at specific ages, it is necessary to paint or mark them as they emerge. However, such marking could affect their self-grooming behavior, and we therefore did not use worker bees of a specific age. Our workers likely represent a broad range of ages in both BFS and ECR colonies. We were, however, able to exclude newly emerged bees (easily characterized by their appearance) (Smith 2012). Collected workers were stored in snap cap vials individually in dark 30℃, 50% relative humidity (RH) incubators until self-grooming trials (LeDoux et al. 2000).

Varroa destructor mites were collected via the sugar shake method in which bees were dusted with powdered sugar and shaken through a white tulle fabric (as a filter) to dislodge mites. Mites were collected from powdered sugar debris and placed in a 250 mL glass beaker coated with a Fluon perimeter at the top to prevent mite escape. Beakers with mites were wrapped in perforated plastic wrap and incubated in the dark at 30℃ and 50% RH in preparation for self-grooming assays. Before self-grooming trials, a fine tip 0000 paint brush was used to wipe off remaining powdered sugar off the mites. Only mites that were in good condition, determined by their ability to climb onto the paint brush were used for trials. We used one mite per bee per trial. Mites were not reused.

5.2 Measuring Self-grooming through Behavioral Assays

Self-grooming bioassays were conducted at room temperature (21°C) in plastic 60 mm x 15 mm Petri dishes lined with press-in beeswax comb foundation. These Petri dishes were vertically orientated to simulate natural colony comb orientation. A Sony HD Video Camera Recorder (HXR-NX70U) was mounted onto a tripod so that it was level with the petri dishes. A lamp with a 5 Watt LED bulb (3000K color temperature) illuminated the bees (Fig. 3). Only bees that were healthy and able to travel vertically up their vial were included as possible participants for these behavioral trials. Once two bees from the same colony were chosen, they were chilled in an ice bucket until their activity slowed (about 1 min) to facilitate transfer into the test dishes and placement of irritants. Any bees that did not quickly recover from this chilling after being placed in a dish were excluded.

Durations of self-grooming behaviors were video recorded for 5 min and a single observer classified these behaviors based upon Invernizzi et al. (2015). The observer was blind to whether or not the bees used came from BFS or ECR colonies. Behavior was categorized as "Weak Cleaning" (legs stroking the head, thorax, or abdomen or metathoracic legs) or "Intense Grooming" (legs stroking multiple body parts simultaneously). For Weak Cleaning, we also noted the body part being groomed. We also scored "Attempting to Fly" when a bee buzzed its wings in an attempt to fly and scored "Mite Removal" when bees successfully displaced the mite off their body. The time elapsed before this successful mite removal was also recorded. Different behavioral events were delineated by workers stopping grooming for at least one second or switching to groom a different body part. Lastly, we measured "Grooming Latency," defined as the time elapsed before the first self-grooming behavior (Kirrane et al. 2018). If bees did not

display any self-grooming during the 5-minute trial, grooming latency was recorded as 300 seconds (the entire trial duration).

Two bees were recorded in their individual Petri dishes side-by-side in each trial. Morfin et al. (2020) showed that a wheat flour irritant is a suitable replacement for *Varroa destructor* and provides greater flexibility for the experimenter. Experimental groups had an irritant (wheat flour or *V. destructor* mite) placed on the individual's thorax with a fine 0000 paint brush. We used 20 mg of wheat flour (Arrowhead Mills Organic All Purpose Flour, Unbleached) as a replacement for live *Varroa* mites when it was not possible to collect mites based upon the flour assay developed by Morfin et al. (2020). Control group bees had no irritants applied, but were touched on their thorax with a clean, fine 0000 paint brush.

After trials were completed, the beeswax foundation was removed and the Petri dishes were washed and disinfected in a Liquinox and water solution. After drying, new press-in wax foundation was placed in the clean Petri dishes for additional trials.

6. Mite Biting Microscopy Analysis

Varroa destructor mites were collected with a fine 0000 paint brush over a five-day period from sticky traps under colonies (the same ones used to measure mite colony levels). Mites were then frozen at -20℃ until analysis. For each colony, all collected mites (or the first 50 mites collected, whichever came first) were viewed under a dissecting scope (Nikon C-PS) illuminated with a Dyna Lite 150 Fiber Optic light to measure leg damage. The number of legs removed from each mite, leg location, and the proportion of each leg removed was recorded. Leg damage was recorded in increments of thirds (0, 0.33, 0.66, or 1) to estimate the proportion of leg bitten per leg per mite. Immature *V. destructor* (recognized by their yellow color) and empty

dorsal shields were excluded from the analysis (Smith et al. 2020; Kirrane et al. 2018), allowing us to control for mite age and eliminate other potential causes for mite leg damage such as ant predation (Boecking and Spivak 1999).

7. Statistics

JMP Pro v16.0.0 software was used for all statistical analyses. All data reported are reported as mean ± one standard error. In all analyses, colony was a repeated measure, a random variable nested within field site. To analyze all our data, we used Repeated Measures Mixed Models (REML algorithm). To analyze colony size data over time, we tested the effect of field site and day of year (fixed effects) on colony size. Separately, we tested the effects of field site, day of year, and colony size (all fixed effects) on colony mite infestation rates. Mite infestation rates were log transformed based upon inspection of model residuals.

To analyze our pin-kill assay data, we used field site, treatment, irritant nested within treatment, assay day of year, mite infestation rate, and colony size (all fixed effects) on larval removal rates from pin-kill assays. To simplify our model and because our goal was to test for differences in the responses of ECR and BFS bees, we analyzed the effects of the control treatment (which resulted in essentially no removal behavior) separately from the experimental treatment.

To analyze our self-grooming data, we ran separate models to test the effects of field site, irritant, and treatment (all fixed effects) on the following durations: durations of weak cleaning, intense cleaning, attempting to fly, time to mite removal, total time grooming, and grooming latency. All duration data were log transformed based upon inspection of model residuals. Tukey Honestly Significant Difference (HSD) tests were used to make corrected all pairwise

comparisons between irritant treatments: no irritant, live mite, and wheat flour.

To analyze our mite damage data, we used field site, and assay day of year as fixed effects. Mite biting was measured through the total proportion of legs bitten per mite (a value ranging from 0 = no legs bitten to 8 = all legs bitten in units of 0.33, corresponding to 24 different levels) and percentage of damaged mites, which was calculated as proportion of damaged mites to toal number of mites surveyed under the dissecting microscope (Mondragon et al. 2005, Smith et al. 2021). Mite biting data w loereg transformed based upon inspection of model residuals. To test if mite biting influences mite infestation rates, we also analyzed the effect of field site, day of year, percentage of damaged mites, and number of legs bitten per mite (all fixed effects) on mite infestation levels per colony.

Finally, to determine if some mite legs tend to be more damaged than others, we tested the effects of field site and Leg ID (both fixed effects) on the proportion of leg bitten on the individual mite level. Mite was a random effect and the repeated measure nested within colony. A Tukey (HSD) test was used to make corrected all pairwise comparisons for damage among legs.

Results

1. **Colony Sizes & Mite Infestation Rates**

Honey bee colony sizes and mite infestations data were collected from April 2021 to April 2022 from 19 BFS colonies and 15 ECR colonies. Colonies at both field sites were similar in size (no effect of field site: $F_{1,28} = 0.02,\ p = 0.88$). There was a slight potential change in sizes over time such that colonies were largest in April and May before declining into the Fall and increasing in February, but this was not a significant effect ($F_{1,100} = 3.75,\ p = 0.056$).

There were no differences in mite infestation rates between feral and managed colonies ($F_{1,17} = 0.44,\ p = 0.52$). However, infestation rates did change over time: peaking in April and decreasing in the Fall ($F_{1,45} = 7.36,\ p \prec 0.0094$, Fig.4). Additionally, there was a slight, but insignificant trend of mite infestation negatively correlating with colony size ($F_{1,106} = 3.49,\ p = 0.06$).

2. Pin-Kill Assays

We measured hygienic behavior from February-October 2021 and conducted 25 pin kill assays with 14 managed colonies and 23 pin-kill assays with eight feral colonies. As expected, pin-kill treatments significantly increased larval removal rate compared to that observed in control treatments ($F_{1,80} = 1801.78,\ p \prec 0.0001$) (Fig. 5). However, feral and managed colonies had similar levels of hygienic behavior and did not respond to changing mite infestation levels by varying their hygienic behavior (field site: $F_{1,11} = 0.23,\ p = 0.64$, Fig. 5, Table 1). Hygienic behavior increased over the year ($F_{1,33} = 27.17,\ p \prec 0.0001$, Fig. 10) and very slightly increased with colony size ($F_{1,28.3} = 7.67,\ p = 0.0098$) by approximately 1% per 1000 bees in the colony (slope significantly different from 0, t_{28} = 2.77, p=0.0098, Fig. 7).

3. Self-Grooming Behavioral Trials

We tested 161 bees from 10 managed colonies and seven feral colonies between September 2021- April 2022 (Table 2). Overall, bees with mite or flour irritants began to groom more quickly and for longer than did bee controls ($F_{1,240} \geq 26.66,\ p \leq 0.0001$). Individuals with flour and mites responded similarly ($F_{1,105} \leq 1.57,\ p \geq 0.21$), except that bees with flour

attempted to fly for longer durations than those with mites ($F_{1,141} = 55.73,\ p < 0.0001$).

There were no significant differences between the self-grooming of feral and managed bees ($F_{1,10} \leq 1.02,\ p \geq 0.34$, Fig. 8, Table 3). Individuals from both sites groomed with similar intensities and for similar durations. Feral and managed bees also had similar success in removing mites during the self-grooming assays. Of the 76 honey bees exposed to *Varroa destructor*, four individuals (10.6%) from the BFS and five individuals (13.2%) from the ECR successfully removed their mites. One individual from ECR removed a mite twice after the mite remounted on the host. Comparing mite removal between field sites and across time, there were no significant differences in the frequency of mite removal or time to removal ($F_{1,2} \leq 1.58,\ p \geq 0.25$, Table 4).

4. Mite Biting Behavior

To assess mite biting behavior, we inspected 4,161 *V. destructor* mites collected from honey bee mite traps (n = 1643 mites from 20 BFS colonies and n = 2518 mites from 15 ECR colonies) between January 2021 and April 2022. Overall, there were similar trends in mites from feral and managed colonies. The number of legs bitten per mite and the percentage of damaged mites per colony did not differ by site ($F_{1,24} \leq 0.02,\ p \geq 0.90$, Fig. 9). While the percentage of damaged mites did not differ significantly over time ($F_{1,168} = 0.66,\ p = 0.42$), the number of legs bitten per mite increased across the year ($F_{1,3870} = 5.84,\ p < 0.02$, Fig. 10). However, this increase was minimal with a predicted increase of 0.0075 more legs are bitten per mite per month. However, when comparing the proportion of each leg bitten, mites from both feral and

managed colonies had a greater proportion of their chemosensory forelegs bitten ($F_{1,28889} = 103.69,\ p < 0.0001$, Fig. 11; labeled Legs 4 and 5 in Fig. 12).

Discussion

Summary

There were no observed differences between feral or managed *Apis mellifera* colonies in their behavioral defenses or in overall *Varroa* mite levels. However, we find this interesting because all managed colonies were repeatedly treated against mites each year and fed with sugar solution throughout most of the year to keep them in good condition, whereas feral colonies received no treatments or feeding at all. Our results suggest that feral colonies have mechanisms different from the social behavior defenses that we explored (hygienic removal behavior, self-grooming, or mite biting) to reduce mite loads. We did find that both feral and managed honey bees evidently preferentially bite off mite chemosensory forelegs at higher rates than other mite legs, suggesting a mechanism by which *A. mellifera* can reduce the ability of mites to find and parasitize brood cells.

Colony Size

Feral and managed honey bees had similar colony sizes and did not vary significantly over the year, although there may have been a slight increase over time (Fig. 7). Feral Africanized colonies tend to be smaller than managed ones and can be constrained by smaller nest cavities (Winston 1992). However, we housed all feral and managed colonies uniformly in 10 frame Langstroth hives, which may have led to both feral and managed colonies to achieve the same sizes. Colonies tend to be larger in temperate locations like San Diego, because of the

need to increase honey storage necessary for overwintering (Winston 1992) and seasonal fluctuations in colony size may therefore not be as dramatic. Colony size is often a proxy for health and fitness (Cavigli et al. 2016, Glenny et al. 2017) and thus the similarity in colony sizes across the year suggests that both feral and managed colonies were similarly healthy, although this should be confirmed in future studies. Geffre et al. (2021) measured the rates of viral infection throughout the year, including colonies in our study, and found no differences in viral loads between feral and managed colonies.

Mite Infestation

Feral and managed bees had similar mite infestation rates, matching the results of Mondragon et al. (2005), who observed similar mite infestation rates amongst feral, hygienic European x African hybrids, and commercial European x African hybrids. Some studies have suggested that Africanized bees colonies have shorter worker development times, which decreases mite fertility and thus increases their *Varroa*-resistance (Mondragon et al. 2005, Calderon et al. 2010). However, our feral and managed colonies were of similar size and therefore may have had similar levels of brood.

In our colonies, infestation rates slightly decreased from Spring to Fall (Fig. 4). This pattern appears atypical compared to the more commonly observed: increasing in late summer and fall (Safofski et al. 1990, Francis et al. 2013). But, Mondragon et al. (2005) similarly found mite infestation levels decreased two-fold from February to June and August, attributing this decline to lower brood attractiveness for mites (Vandame et al. 2000).

Pin-Kill Assays

Hygienic behavior did not vary between field sites (Fig. 5). This is surprising given that one study found that Africanized bees removed a greater proportion of brood artificially infested with *V. destructor* in Mexico and Brazil (Guzman-Novoa et al. 1999). However, our feral bees had a lower percentage of *A. mellifera scutellata* genes, and were therefore less Africanized, than those sampled in Central and South America. Zarate et al. (2022) found that managed bees had a mean of 22.96% ± 10.46% (STD) African ancestry while feral colonies (including those used in our study) contained 41.89% ± 2.68% (STD) African ancestry (Zarate in prep.). The percentage of African ancestry nuclear genes in our feral colonies was therefore higher than that found in managed bees, but was lower than the 76-89% African ancestry of colonies sampled in Mexico and South America (Zarate et al. 2022).

In our study, hygienic behavior tended to increase over the year (Fig. 6), perhaps as a result of a buildup of DWV titers. Studies have shown that colonies preferentially removed larvae with higher DWV titers, which may build up over time (Schoning et al. 2012, Mondet et al. 2014, Hedtke et al. 2011). Hygienic behavior also minimally increased with colony size. Larger colonies may have more workers to perform the various tasks required in hygienic behavior (Simone-Finstrom et al. 2017).

We found that hygienic behavior did not correlate with mite infestation levels at either field site. This finding may suggest pin-kill assays do not accurately measure *Varroa*-sensitive hygiene, with different olfactory cues emitted from pin-killed larvae (Mondet et al. 2014). Freeze-kill assays and artificial infestations could be performed to further assess this result (Spivak and Downey 1998, Leclercq et al. 2017; Tehel et al. 2016). Palacio et al. (2005) found that hygienic colonies removed 99% of brood while non-hygienic colonies removed 53%, while

our feral bees removed about 76% of pin-killed brood and managed bees removed about 80% (Table 1). Because there were no differences in the removal of pin-killed larvae between feral and managed colonies, hygienic behavior cannot explain how feral honey bees tolerate *V. destructor* without miticide treatment.

Self-grooming Assays

Our self-grooming assays similarly found no differences between feral and managed bee self-grooming to either mites or our mite substitute, the flour irritant across all metrics (Fig. 8). Similarly, Kruitwagen et al. (2017) found that naturally mite-resistant colonies groomed similarly or less intensely than managed colonies treated with miticides in grooming assays with starch irritants. They suggested that mite-resistant colonies used other traits such as reduced mite fertility or higher hygienic behavior instead of grooming (Kruitwagen et al. 2017). Other studies have shown that Africanized bees self-groom more than European bees (Morfin et al. 2020, Guzman-Novoa et al. 2012). However, these studies were conducted in Central and South America, which have honey bees with higher African ancestry than those found in San Diego (Zarate et al. 2022). Therefore, self-grooming is not likely a driving factor allowing feral honey bees to tolerate *V. destructor* without miticides.

Mite Biting

In addition, there were no differences in mite biting intensity between feral and managed bees. Our results support findings that levels of mite damage do not differ between feral versus European-African hybrids (Mondragon et al. 2005) and between untreated versus miticide-treated colonies (Kruitwagen et al. 2017). However, Guzman-Novoa et al. (1999)

showed that Africanized bees can have increased rates of mite mutilation compared to European counterparts (Guzman-Novoa et al. 1999). Again, the lower level of Africanized genes in feral bees in San Diego may account for these differences. In addition, climatic factors like temperature and humidity can influence mite grooming and mutilation (Pritchard 2016) and the Guzman-Novoa et al. (1999) study was conducted in Mexico, which has a significantly different climate from San Diego. Guzman-Novoa et al. (2012) counted mite falls and found a negative correlation between percentages of damaged mites and mite infestation rates. We did not find such a significant correlation. However, Guzman-Novoa et al. (2012) used bees that were artificially bred for low *Varroa* growth.

Analyzing damaged mites collected underneath colonies is not a perfect measure of honey bee grooming because mites may naturally fall as they die when honey bee workers emerge and from predation by ants and wax moths (Pritchard 2016). Even so, mite fall is a commonly used measure and there is an established genetic basis for grooming behaviors, with associated genes like *neurexin* (Pritchard 2016, Russo et al. 2020, Arechavaleta-Velasco et al. 2012).

We found an interesting trend of mite chemosensory forelegs being bitten off at higher proportions compared to other mite legs (Fig. 11), the first such trend reported. These legs house the tarsal pit organs, which crucially allow the mite to find hosts. Nganso et al. (2020) covered these pit organs and forelegs with nail polish and found significant decreases in the ability of *V. destructor* to orient towards honey bee hosts, navigate to preferred feeding sites on the host (over the fat bodies), and to successfully reproduce as compared to controls. Thus, the tendency for worker bees to bite a higher proportion of these forelegs may be an anti-*Varroa* defense. However, it is possible that bees may not necessarily be targeting these forelegs and instead,

have greater access to bite thm when *Varroa* walk and extend their forelegs to detect odors (Nganso et al. 2020).

Summary

We found that three social immunity behaviors: hygienic behavior, self-grooming, and mite biting, are unlikely drivers of feral *Apis mellifera* resistance to *Varroa destructor* in San Diego. Therefore, these feral bees likely use other strategies. Increased swarming or absconding, which always induces a period of broodlessness, may disrupt *Varroa* development as they require larval hosts (Simone-Finstrom 2017). In a survival comparison, colonies in smaller nests that swarmed had decreased mite infestation levels and lower deformed wing virus titers (a pathogen commonly transmitted and spread by *Varroa* mites) than those that did not swarm (Loftus et al. 2016). Additionally, absconding is a social immunity behavior commonly induced by pathogen stress (Simone-Finstrom 2017). We suggest that future studies measure the mite infestation levels and rates of absconding and swarming in feral versus managed colonies, particularly with respect to Africanized bees. In our study, 38.46% of feral colonies and 26.32% of managed colonies turned over. We could not tell if these colonies died, absconded, or swarmed, something that detailed video monitoring could resolve. In addition, further research should be conducted to explore this foreleg biting and its efficacy in reducing mite infestations.

Figures

Figure 1. Approximating honey bee colony size with visual estimates. Using GIMP 2.10 software, a grid was overlaid on pictures of frames to assist with the estimation of areas the bees occupied, before multiplying that area with the number of bees per cell within the grid.

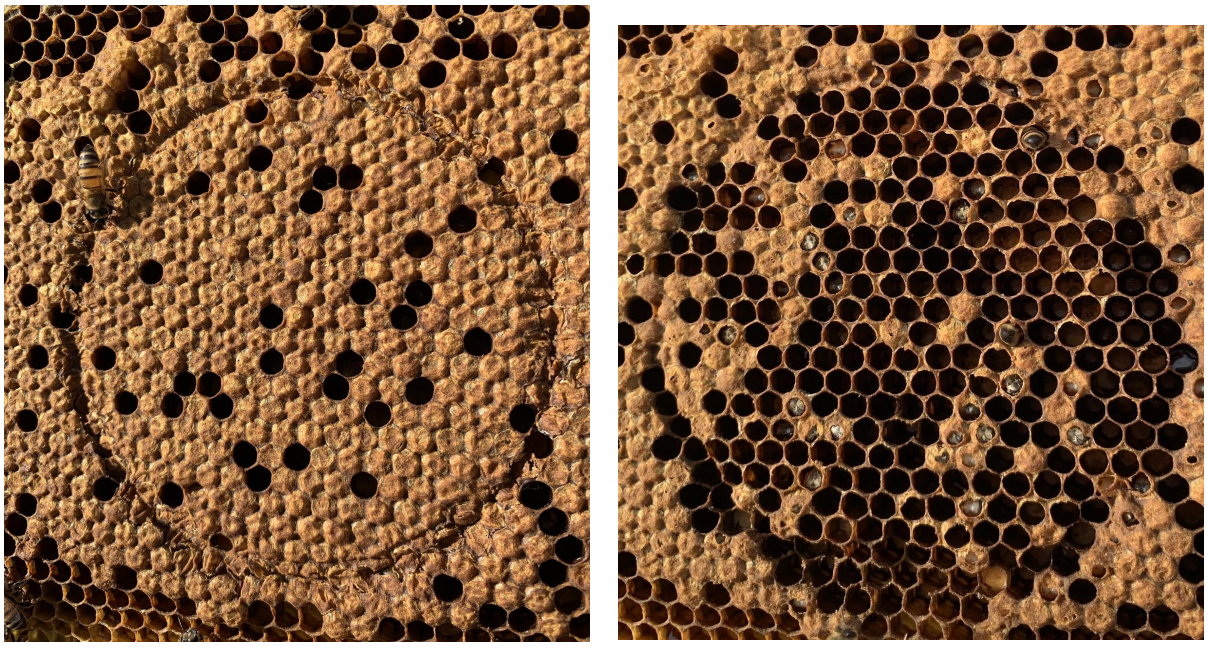

Figure 2. Pin-kill assay treatment for hygienic behavior. A side-by-side comparison of the brood frame when the pin-kill assay was conducted (left) and the same section of brood 24 hours later (right) after the colony had removed pin-killed brood.

Figure 3. Self-grooming behavioral assay setup. Two Petri dishes with press-in beeswax comb foundation are mounted vertically so they were level with the camcorder. An LED lamp was placed above to illuminate the video further.

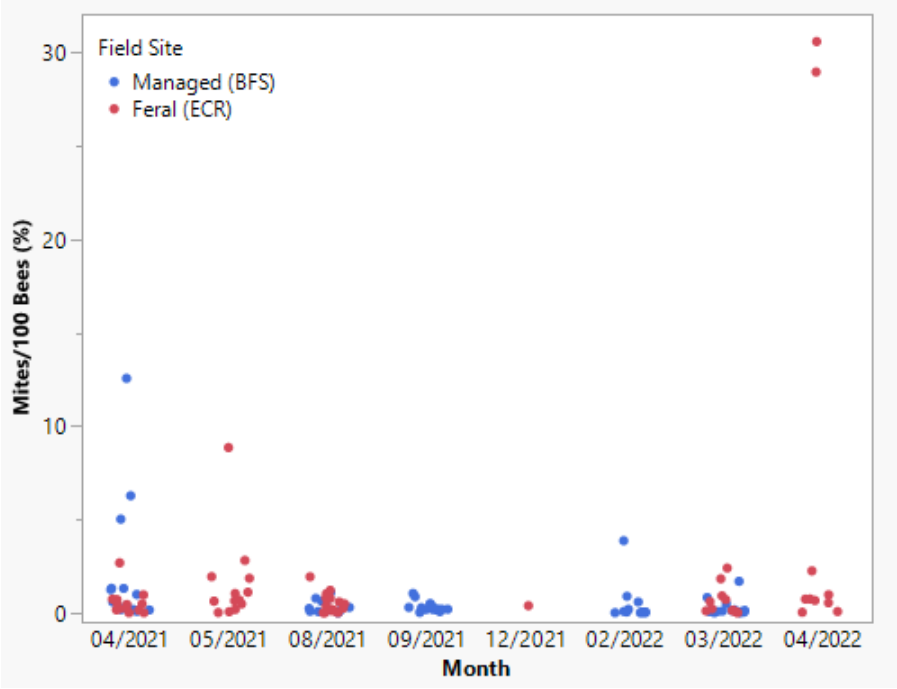

Figure 4. Mite infestation rates throughout the year. There was no significant change in infestation over time ($p = 0.06$), but it was somewhat higher in April and May and may have slightly declined in Fall and Winter.

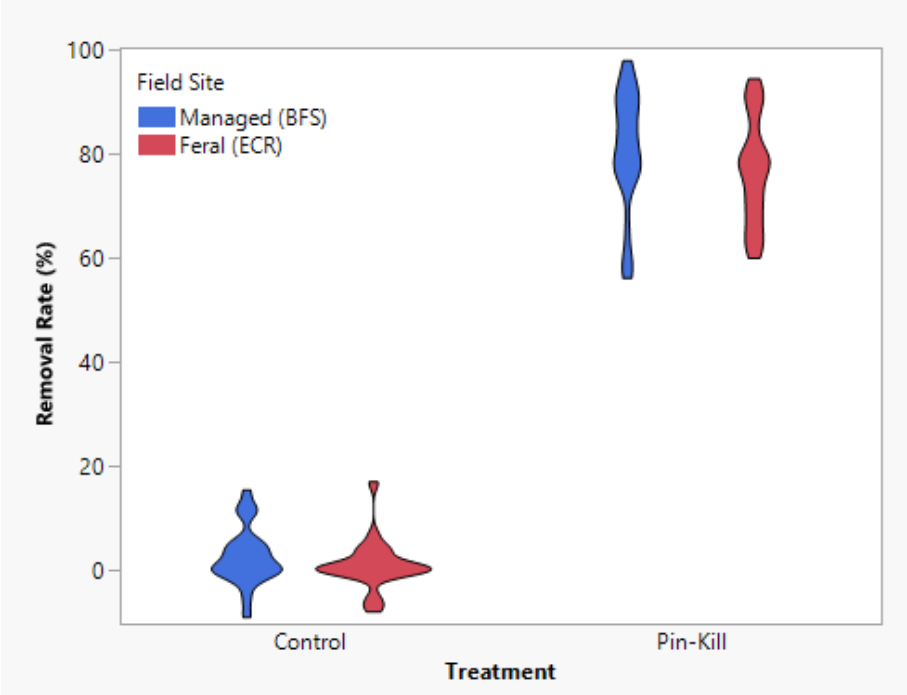

Figure 5. No difference in hygienic behavior of feral and managed bees. Pin-kill assays assessed colonies' removal of dead brood over 24 hours. Pin-kill assays significantly increased larval removal rate ($F_{1,80} = 1801.78,\ p \prec 0.0001$), but there were no differences between feral and managed bees ($F_{1,11} = 0.23,\ p = 0.64$).

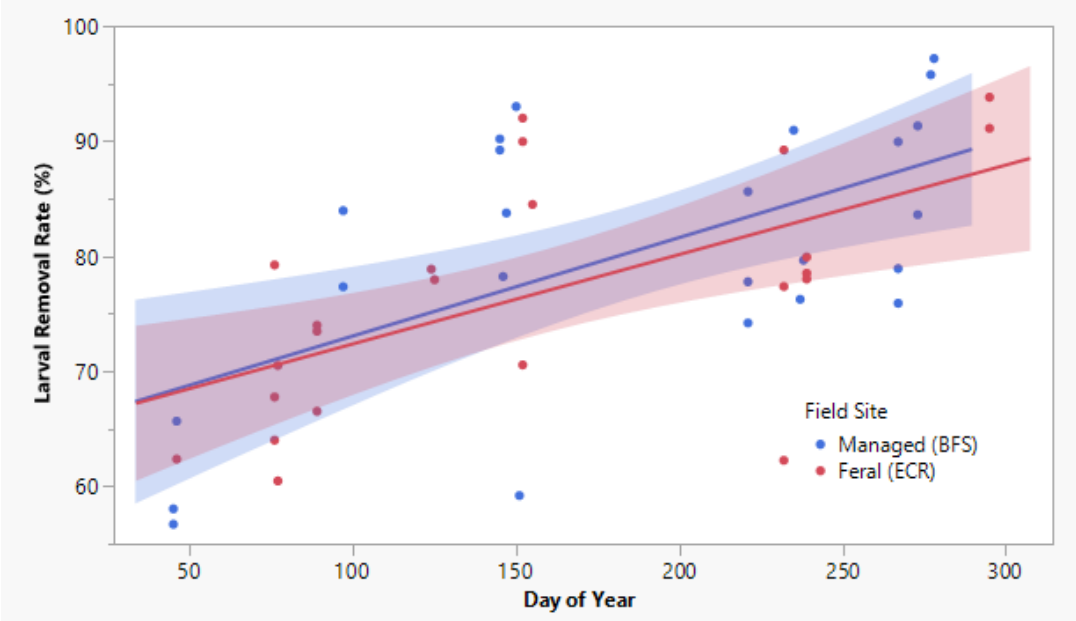

Figure 6. Hygienic behavior increases across the year. Larval removal rate as measured by pin-kill assays increased from the beginning to end of the year ($F_{1,33} = 27.17,\ p \prec 0.0001$). Lines of best fit are plotted with 95% confidence intervals shaded.

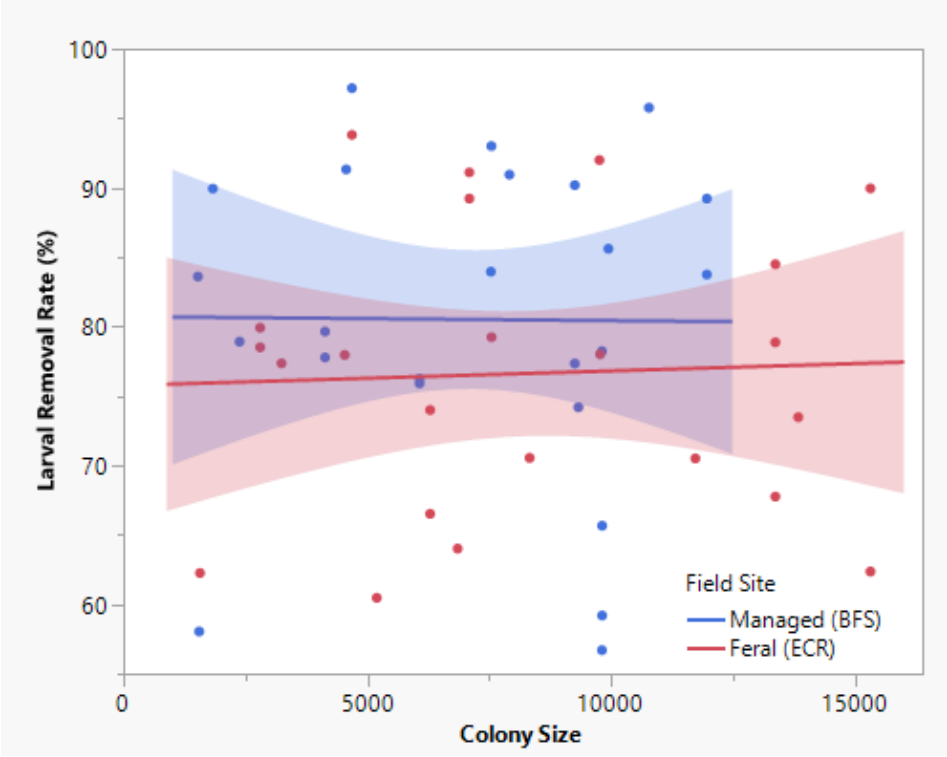

Figure 7. Hygienic behavior slightly increased with colony size. Hygienic behavior measured through pin-kill assays are plotted with corresponding colony sizes. There was a very slight increase in larval removal rate with colony size (1% per increase of 1000 bees, $t_{28} = 2.77$, p=0.0098). Lines of best fit are plotted with 95% confidence intervals shaded.

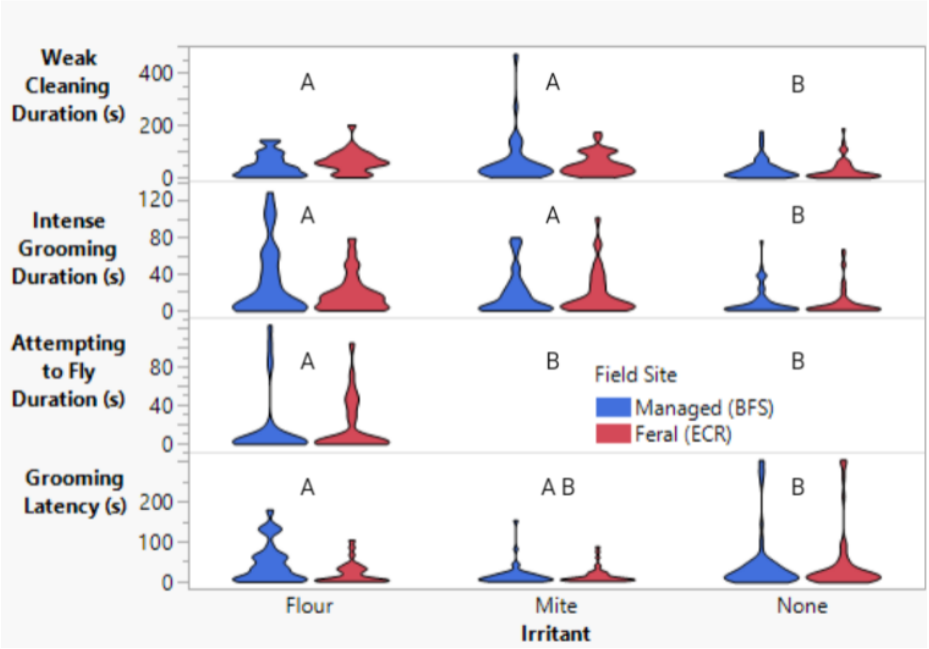

Figure 8. Self-grooming duration and response time does not differ between feral and managed bees. Differences between irritant groups were assessed by a Tukey-HSD test. Groups sharing letters are not significantly different. Overall, bees with irritants tended to weakly and intensely groom for longer durations than those without irritants ($F_{1,240} \geq 26.66,\ p \leq 0.0001$). There are no differences in self-grooming durations or latency between feral and managed bees ($F_{1,10} \leq 1.02,\ p \geq 0.34$).

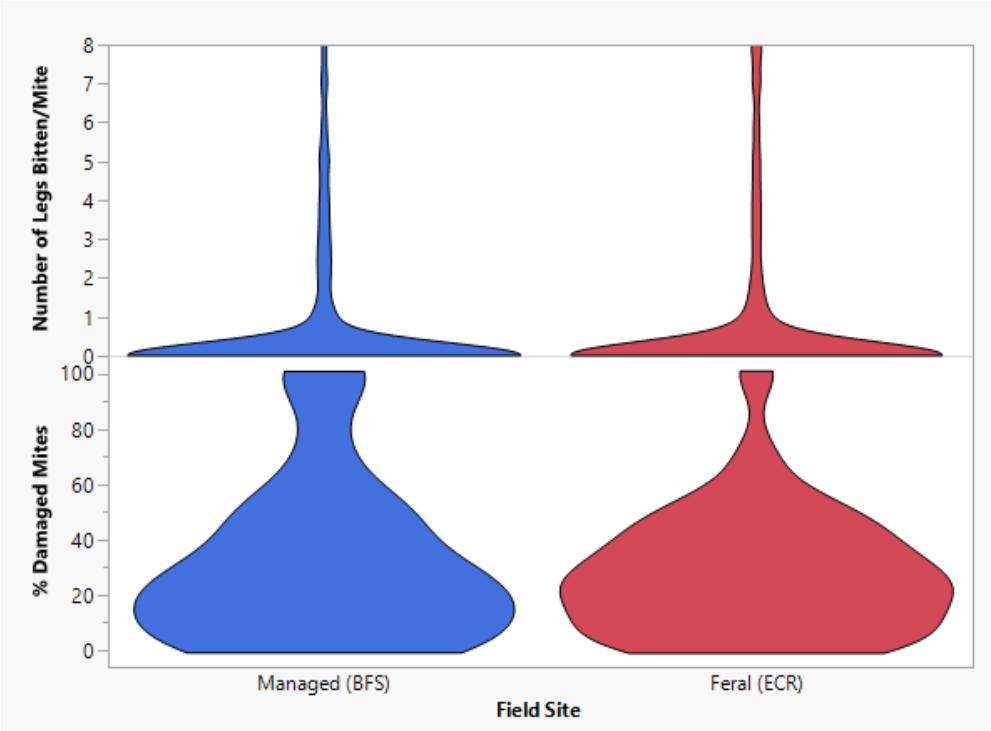

Figure 9. Mite biting intensity does not vary between feral and managed bees. After collecting *Varroa destructor* in sticky traps under honey bee colonies, their damage was categorized underneath a dissecting microscope. Ultimately, feral and managed bees bite similar amounts of of mite legs and bite similar percentages of the mite population ($F_{1,24} \leq 0.02,\ p \geq 0.90$).

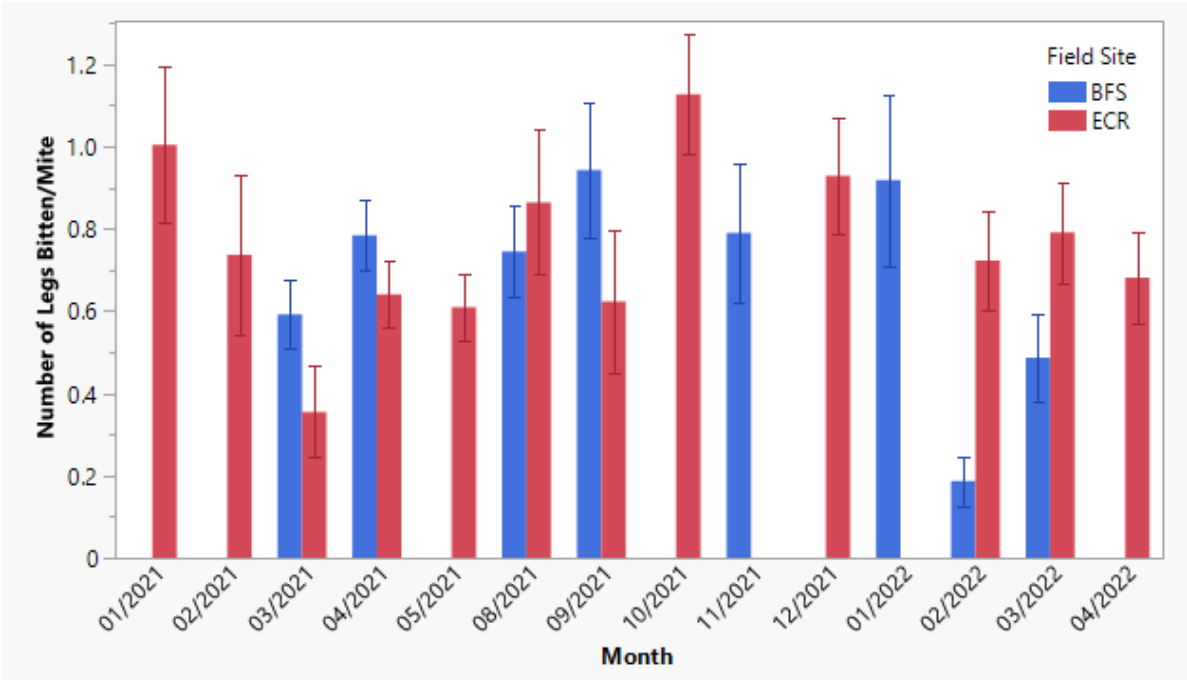

Figure 10. Number of legs bitten per mite slightly increases throughout the year. About 0.00025 more legs are bitten with each day ($F_{1,3870} = 5.84,\ p < 0.02$). Means are plotted for each month with one standard error bar.

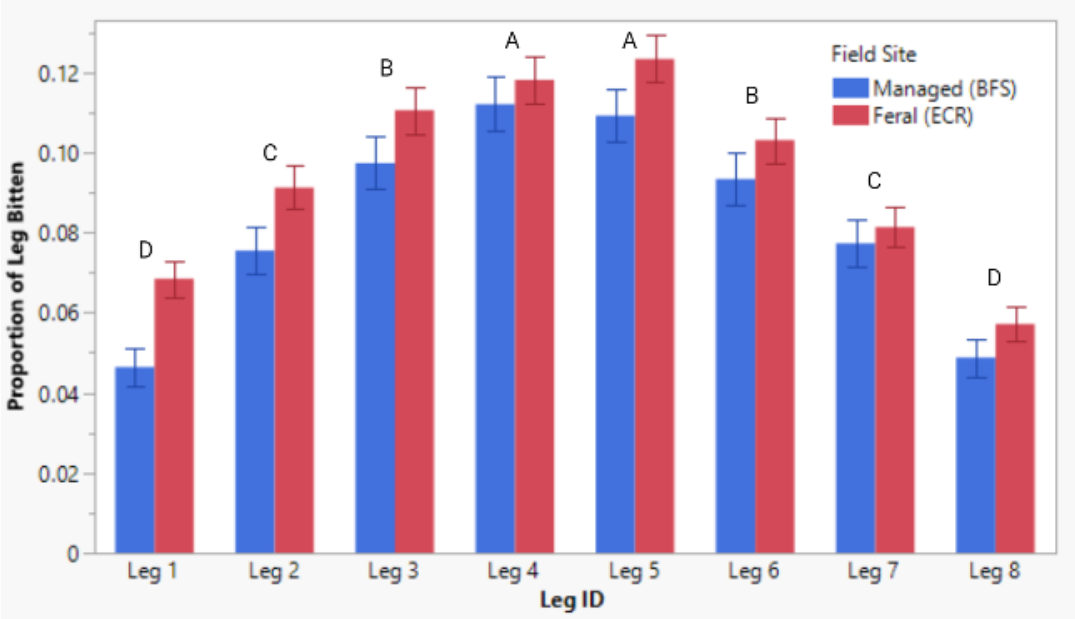

Figure 11. No difference in mite leg biting preferences between feral and managed bees, but preferential biting of mite forelegs.
Proportion of each mite leg was categorized underneath a dissecting microscope. Feral and managed bees bit mite legs in a similar pattern ($p<0.83$). Mite forelegs were bitten off at higher proportions by both feral and managed bees when compared to other mite legs ($F_{1,28889} = 103.69,\ p < 0.0001$).

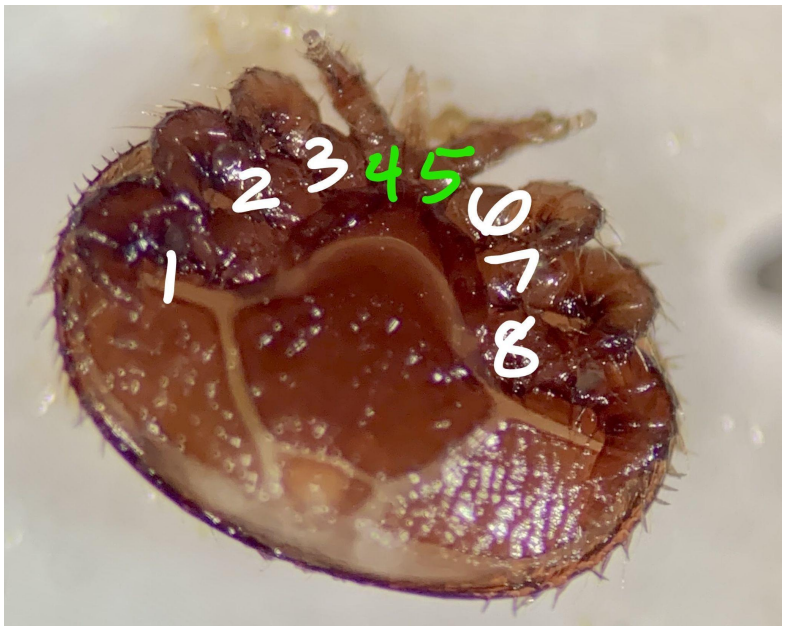

Figure 12. Mite leg identification. Mite legs were numbered 1-8 to detail whether particular legs were being bitten off at higher rates compared to others. Legs 4 and 5 (in green) are the mite's forelegs, which house the chemosensing tarsal pit organ.

Tables

Table 1. Hygienic behavior measured by pin-kill assays

	Managed (BFS)				Feral (ECR)			
	Control Treatment		Pin-Kill Treatment		Control Treatment		Pin-Kill Treatment	
	Mean	Std Error	Mean	Std Error	Mean	Std Error	Mean	Std Error
Larval Removal Rate (%)	2.5332 ± 1.0912		80.5153 ± 2.3550		0.9516 ± 0.9924		76.6255 ± 2.1193	

Table 2. Self-grooming trial treatment groups

	Control Treatment	Experimental Treatment	
	No Irritant	Mite Irritant (*V. destructor*)	Wheat Flour Irritant
Managed (BFS)	n = 67	n = 38	n = 31
Feral (ECR)	n = 60	n = 38	n = 27

Table 3. Self-grooming trials: Durations and latency

	Managed (BFS)						Feral (ECR)					
	No Irritant (Control)		Mite Irritant		Flour Irritant		No Irritant (Control)		Mite Irritant		Flour Irritant	
	Mean ± Std Error		Mean ± Std Error		Mean ± Std Error		Mean ± Std Error		Mean ± Std Error		Mean ± Std Error	
Weak Cleaning Duration (s)	33.407	4.5455	61.0020	14.0099	47.7360	7.8117	29.9920	4.8863	52.5310	6.7454	61.1350	8.0591
Intense Cleaning Duration (s)	8.597	1.7964	17.2300	3.7701	29.8750	6.8211	7.4020	1.9378	18.1580	3.8904	18.3660	4.0004
Attempting to Fly Duration (s)	0	0	0.0000	0.0000	14.3090	6.0512	0.0000	0.0000	0.0000	0.0000	17.2670	5.3476
Total Time Grooming (s)	42.004	6.0289	76.6670	16.5939	91.9200	13.6477	36.2840	6.5840	72.7400	9.7101	96.7680	12.7009
Grooming Latency (s)	41.25400	8.89420	15.4210	4.4419	45.0000	8.8584	40.3170	9.7167	13.8680	3.2681	20.8520	5.2267

Table 4. Autogrooming trials: Mite removal

	Managed (BFS)		Feral (ECR)	
	Mean ± Std Error		Mean ± Std Error	
Mite Removals	4.0000	0.0505	6.0000*	0.0708
Time to 1st Removal (s)	202.5	35.4877	75.6000	37.1400

*One feral bee removed its mite twice during the same trial as the mite remounted on to its host

Table 5. Mite biting intensity

	Managed (BFS)		Feral (ECR)	
	Mean ± Std Error		Mean ± Std Error	
Proportion of Leg Bitten	0.0825	0.0021	0.0942	0.0019
Number of Legs Bitten/Mite	0.6586	0.0394	0.7472	0.0364
% Damaged Mites	31.1807	2.9773	27.3348	2.2154

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