BEHAVIORAL MECHANISMS UNDERLYING PARASITE SPREAD AND HOST SURVIVAL IN THE VARROA DESTRUCTOR / APIS MELLIFERA PARASITE/HOST SYSTEM

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BEHAVIORAL MECHANISMS UNDERLYING PARASITE SPREAD AND HOST SURVIVAL IN THE VARROA DESTRUCTOR / APIS MELLIFERA PARASITE/HOST SYSTEM

David Thomas Peck, Ph.D.

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This work has focused on understanding the remarkable survival of the honey bees (*Apis mellifera*) living without beekeeper intervention in Cornell University's Arnot Forest, despite the bees' infestation with the devastating parasitic mite *Varroa destructor*. The various mechanisms of intercolony transmission of the Varroa mite were investigated by examining the behavior of both mites and their honey bee hosts. Behavioral observations of mites and bees interacting on flowers demonstrated the plausibility of indirect mite transmission between colonies, using flowers as an intermediate substrate and staging ground for the infestation of new hosts. Experimentally placed boxes containing honey in combs were robbed by free-living bees in a forest setting, demonstrating the likelihood of mite transmission between colonies via honey robbing behavior. Detailed observations of worker and drone bees from light-colored colonies infested with a large number of Varroa mites and from nearby dark-colored colonies infested with few mites allowed us to correlate bee behavior with changes in mite populations. These correlations reveal that mites from dying colonies are carried into healthy colonies when the healthy colonies rob honey from the sick ones, and to a lesser extent when infested bees drift from the sick colonies into the nearby healthy ones. These investigations, taken together, show that the mechanisms of horizontal intercolony transmission have been underestimated in forests like the Arnot Forest. After demonstrating these multiple mechanisms of intercolony mite transmission, even when colonies are widely spaced as in forests, we reconsidered the earlier hypothesis that Varroa mites in the Arnot Forest had evolved avirulence. Instead, we investigated the behavioral mechanisms the Arnot Forest bees may be using to resist infestation by Varroa mites. We have demonstrated that the Arnot Forest bees possess multiple behavioral traits (brood hygienic behavior and mite grooming behavior) at high levels, which appear to confer partial resistance to Varroa mites. These traits are expressed at levels higher than is found in unselected populations, but lower than is found in populations that have been consistently selected by queen breeders for each trait, though whether this intermediate expression is adaptive or the result of incomplete evolution is still unclear.

BIOGRAPHICAL SKETCH

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David Thomas Peck, a native of Towson, Maryland, spent his early life developing a love of, and fascination with, the natural world. This was driven in part by his science teachers at the Park School of Baltimore, and in part by his participation in outdoor activities in the Boy Scouts of America. At Tufts University he studied biology, biopsychology, and environmental science. He participated in field and laboratory research on the behavior and stress physiology of Bobolinks and European starlings, learned about parasitic manipulation of host behavior for the first time, and opened his first bee hive with his academic mentor Phil Starks. David continued his scientific development at Cornell University's Department of Neurobiology and Behavior, where he worked closely (at various times and in various capacities) with each of his committee members and many other scientific mentors. His Ph.D. began with an investigation of the behavior of black-legged ticks carrying the bacterium that causes Lyme disease, continued with research into the capacity of *Chlamydia muridarum* to manipulate the mating behavior of infected laboratory mice, and finally settled into the studies presented in this dissertation on the behavioral components of the parasite-host relationship between Varroa mites and honey bees. David is, as ever, utterly fascinated by the complex behaviors that parasites and hosts engage in to try to gain advantage over each other in parasite-host conflicts.

DEDICATION

None of this work would have been possible without the assistance of many organisms. I cannot possibly thank them all in this space, but I would like to particularly thank each of my committee members for their unique styles of mentorship, my wife Samantha for her unending patience with my eccentricities, and most of all the countless honey bees, Varroa mites, mice, ticks, and bacteria that have given their lives over the course of my graduate research career.

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TABLE OF CONTENTS

Chapter 1: *Varroa destructor* mites can nimbly climb from flowers onto foraging honey bees

David T. Peck, Michael L. Smith, Thomas D. Seeley

Abstract
Varroa destructor, the introduced parasite of European honey bees associated with massive colony deaths, spreads readily through populations of honey bee colonies, both managed colonies living crowded together in apiaries and wild colonies living widely dispersed in natural settings. Mites are hypothesized to spread between most managed colonies via phoretically riding forager bees when they engage in robbing colonies or they drift between hives. However, widely spaced wild colonies show *Varroa* infestation despite limited opportunities for robbing and little or no drifting of bees between colonies. Both wild and managed colonies may also exchange mites via another mechanism that has received remarkably little attention or study: floral transmission. The present study tested the ability of mites to infest foragers at feeders or flowers. We show that *Varroa destructor* mites are highly capable of phoretically infesting foraging honey bees, detail the mechanisms and maneuvers by which they do so, and describe mite behaviors post-infestation.

Introduction

The parasitic mesostigmatid mite *Varroa destructor* (Anderson & Trueman 2000) is a highly damaging pest of both managed and wild colonies of European honey bees (*Apis mellifera*). The parasitism of the mites, and the spread of the viruses that they vector during their feeding (Kevan et al. 2006, Martin et al. 2012), causes devastation in honey bee colonies. The exponential reproduction of the mites builds their population in a bee colony to extraordinary heights, causing the demise of most untreated host colonies within a few years (Korpela et al. 1992, Fries et al. 2006). *Varroa* infestation has been identified as the primary factor contributing to high overwinter colony mortality in some analyses (Guzman-Novoa et al. 2009, Genersch et al. 2010). Mites can spread through the bee population both vertically and horizontally. Vertical transmission occurs when honey bee colonies cast reproductive swarms, and the phoretic mites travel upon the swarming bees to the new nest site. Horizontal transmission of mites between colonies is thought to take place primarily through drift of worker bees into colonies other than their own, robbing of honey stores from weak colonies by stronger ones, and the movement of

infested brood or bees by beekeepers. However, these avenues of horizontal transmission may be absent or reduced in isolated wild colonies, such as the feral honey bee population of the Arnot Forest in New York State (Seeley 2007). We investigated the plausibility of an additional horizontal transmission mechanism: mites passing between colonies by infesting foragers from a mite-free colony after having been groomed from infested bees and onto flowers.

There is already evidence that *Varroa* mites occasionally wind up on flowers. In 2000, a USDA inspector found a live mite in a refrigerated shipment of flowers from the Netherlands (Pettis et al. 2003). An exhaustive search of the flower shipment did not reveal any honey bees, suggesting that the mite had survived for an extended time on a flower. The authors speculated that such an infested flower placed in an open-air flower market might allow the spread of this mite across international borders. One author conducted "limited floral surveys" around honey bee colonies for *Varroa destructor*, but found none (Pettis et al. 2003). Another report recounts the discovery of a live mite on a dead honey bee contained in a shipment of cut flowers from South America (Kevan et al. 1990). The repeated detection of these international mite biocontainment breaches prompted our investigation into whether mites on flowers are capable of infesting a honey bee during natural foraging.

Research on the relationship between *Varroa destructor* and flowers has been modest. Hartwig and Jedruszuk (1987) as well as Smirnov (1975) showed that mites can survive on flowers for as long as six days, depending on flower species. Gromyko (1982) maintained live *Varroa* on flowers for as long as six days under controlled conditions, and reported that some mites were able to climb onto dead bees presented to them afterwards on a watch glass. Hartwig and Jedruszuk (1987) reported that some mites climbed onto live workers held against infested flowers for "about 30 seconds," but the presentation of the bee to the floral mite was not

naturalistic. Also, no investigation was made of anti-mite grooming by the foragers, and no information was reported about the mites' behavior during and after infestation. Thus, there exists suggestive evidence that *Varroa destructor* can move from flowers onto foraging bees, but there are few observations and no quantification of this phenomenon in a naturalistic context. As the mites lack eyes and likely rely on their chemosensory forelegs to detect potential hosts (Le Conte et al. 1989, Rickli et al. 1992, Pernal et al. 2005) we began this study with a genuine doubt that a mite on a flower would be capable of the sensory discrimination and rapid acrobatics required to detect and mount a foraging honey bee before it flew away.

Eickwort (1994) described phoresy (one organism moving by attaching itself to another) as "the principal adaptation required of a mite in order to become an important associate of bees." Schwarz & Huck (1997) clearly demonstrated that floral waystations are used during bee-to-bee phoretic jumps by parasitic mites of bumble bees, and the phoretic interaction between mites, flowers, and hummingbirds has been elegantly described by Colwell (1985). It is unknown whether *Varroa jacobsoni*, the mites from which *Varroa destructor* recently evolved, displays behaviors that would enable mite transfer on flowers, but it is likely that these parasites have evolved a diverse range of phoretic dispersal mechanisms to move between widely spaced colonies of their host bee *Apis cerana*. The question which drove the present study was whether *Varroa destructor* mites can infest Western honey bees at flowers and so be carried to a new host colony, and whether this is a mechanism for mite dispersal between widely spaced colonies. We investigated mite behavior during flower-to-bee infestations, as mite success in these behaviors is critical to the floral transmission hypothesis. This study also helps us understand the nature of mite infestation of honey bees generally, as our methods allowed us to observe infestations in exquisite detail. We found that the behavior of mites on flowers results in efficient infestation of

foragers, and that the behavior of mites on foragers results in avoidance of the honey bee's behavioral resistance to mite infestation (i.e., grooming.)

Methods

We conducted our studies at the Cranberry Lake Biological Station (44°09'N 74°48'W) in July of 2014. The station is owned by the State University of New York College of Environmental Science and Forestry, who generously allowed our work to proceed alongside their summer field training program. This field station is nestled in an extraordinarily nectar-poor area of the Adirondack State Park in northern New York State, where forager bees are easily trained to visit experimental food sources. To determine whether mites can infest foraging bees, we placed mites on focal flowers (Fig. 1.1) or a sugar-water feeder (see Fig. 4.5 in Seeley 1995) and observed the mites' interactions with workers from a nearby honey bee colony that were visiting these food sources. The bees' arrivals and departures were recorded as were all mite orientations, movements, and interactions with the bees. We used both video recordings and direct observations to determine the behavior of both focal mites and honey bee foragers during each trial. Mites were placed on the grooved base of the feeder, on the yellow inflorescence or white petal of a daisy flower (*Bellis* cultivar), on the spikes in the center of an Echinacea flower (*Echinacea* cultivar), or on the petal of a speedwell flower (*Veronica* cultivar). White sheets of paper (22cmx28cm) and a white cloth (1mx1.9m) were spread underneath our equipment so we could detect any mites that fell off of bees within a half-meter radius of the focal flower or feeder.

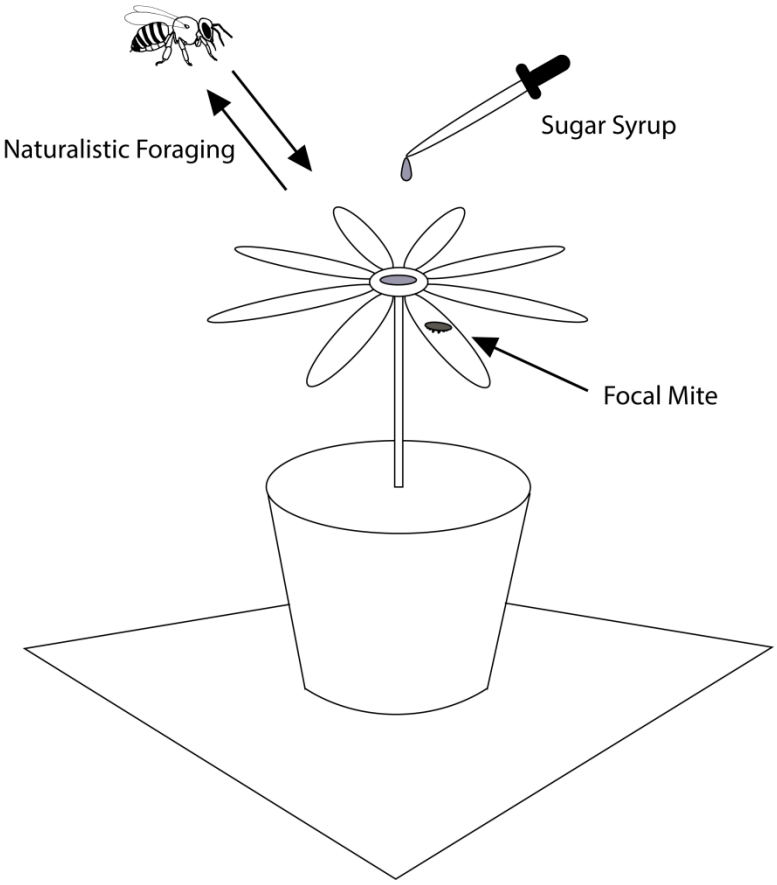

Figure 1.1: Experimental setup to monitor mite behavior towards foraging honey bees. To see whether mites fell off bees after climbing onto them, the top of the cup holding the focal flower was covered in white paper, as was the stool on which the cup sat. The entire apparatus was underlain by a 1m by 2m sheet of white cloth.

Because our flowers provided an artificially rich food source in an otherwise forage-poor environment, each flower received much more forager attention than would be expected in nature. We cannot, therefore, consider the time that each mite spent on a flower before infesting a bee as a realistic indication of how long a mite waits to infest a forager in nature. Therefore, we report instead the total number of "bee-seconds" of forager activity experienced by a flower bearing a mite before the mite climbed onto a bee. This indicates the average amount of time a forager would spend on a mite-bearing flower before she becomes infested.

To maintain forager interest in the focal feeders and flowers, bees were offered sucrose solutions scented with anise extract. The molarity of the sugar solution was adjusted (0.25M to 2M) so that only one to four foragers visited the focal feeder or flower at any one time. Sucrose droplets were pipetted onto the center of all focal flowers, roughly at the position of the flowers' own natural nectaries.

All bees were from a colony of European honey bees with a naturally mated queen. The colony occupied a ten-frame Langstroth hive body, with six frames that contained brood and were covered in adult bees, and four frames that held empty comb to motivate foraging. We selected this colony due to its easily portable size and its ability to provide us with plentiful mites to use in our tests (average mite density: 9.3 phoretic mites per 100 bees) as measured by sugar shake (Dietemann et al. 2013).

Varroa were obtained from the honey bee colony that also provided our foragers. We used the sugar shake method (Dietemann et al. 2013) to obtain live phoretic-stage mites. After removal from their bee hosts, each mite was removed from the powdered sugar with a toothpick, cleaned with chlorine-free water, examined for damage, and then placed in a shaded plastic container until being used in the experiment. Humidity in the container was maintained by a damp paper towel. No mite spent more than two hours in the container before being used in a trial.

Results

Infestation Success Rates:

Of 31 mites placed on the glass feeder, 29 infested a bee; the two that did not were blown off the feeder by wind. In 12 of the 29 infestations (40%), the bee immediately groomed herself, but in only 3 instances was the mite successfully dislodged. Consequently, 26 of the 31 mites

(84%) left the feeder attached to a bee. Of 43 mites placed on flowers, all 43 infested a bee, and almost every one (41 of 43) left the flower on the bee it had infested. One mite fell off its forager, and one was groomed off. The average time taken by a mite placed on a flower to infest a foraging bee was 119 bee-seconds (Fig. 1.2). The most rapid infestation from a flower took only 2 bee-seconds, while the longest took 840 bee-seconds.

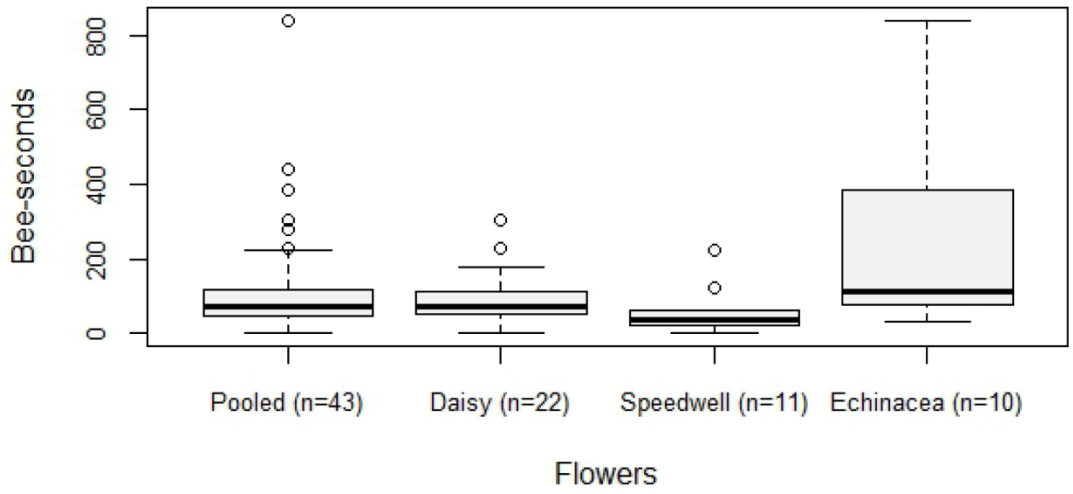

Figure 1.2: The number of bee-seconds before a honey bee forager was infested by the focal mite. Boxplots shown for each of the three species used, and pooled data from all three species. Boxes span the first through third quartiles, and whiskers span the range of non-outlier values.

When parsing the pooled flower data by flower species, we found that mites on Echinacea flowers experienced significantly more bee-seconds of foraging before infestation (243.1 +/- 253.9) compared to mites on daisies (92.6 +/- 72.6) ($t_{30} = 2.60$; $P = 0.014$) but not compared to mites on speedwell (59.1 +/- 63.8) ($t_{19} = 2.33$; $P = 0.031$) using a Bonferroni-corrected alpha of 0.016. Mites on daisies and speedwell were not significantly different from one another ($t_{31} = 1.30$; $P = 0.204$). Mites tested on daisy flowers were placed alternately on either the yellow center of the flower head or the white "petals" (ray florets). The bee-seconds

before infestation did not differ significantly between these two groups (103.5 +/- 25.4 vs. 83.5 +/- 19.5) ($t_{20} = 0.12$; $P = 0.908$). The behaviors of the mites during infestations on each flower type were not discernably different and so were pooled with the feeder observations and videos for further analysis.

Approximately half of the mites, both on feeder (59%) and on flowers (49%), required only one contact with a bee before infesting it. The mean number of mite-bee contacts prior to and including infestation on the feeder and flowers was 2.1. The most mite-bee contacts any mite experienced was 7, most of which were contacts with the bee's tarsal claw. Of the 22 out of 43 focal mites on flowers which did not infest a bee on first contact, 15 (68%) made contact with the tarsal claw of the bee on the first contact, and then infested the bee upon the next contact with a different body part.

Mites on both substrates (feeder or flowers) rarely walked more than one centimeter. Their activity level ranged from orientation and walking <2mm towards nearby foragers to standing still with no movement other than the occasional extension of the forelegs. The mites frequently engaged in the same repeated foreleg extension behavior: the chemosensory forelegs were extended forward and upwards. This behavior was infrequent when there were no bees on the forage, and increased in frequency when foragers moved to within a few centimeters of the mite. Some, but not all, mites oriented towards nearby foragers, and walked towards bees prior to infestation. Mites on flowers were frequently observed to move to the edges of petals and other floral structures and then remain facing outward. For example, mites placed on the center of Echinacea flowers universally moved to the tips of the spine-like disc florets and oriented their forelegs upwards.

Mite Behavior During and After Infestation:

Combining data obtained from observations on the feeder and the flowers, we saw 74 infestations. In 71, we observed the initial point of contact between bee and mite, and in 58 we observed the mite long enough to see the location on the bee upon which the mite stopped moving before the forager departed. The mounting of the foraging bees by the mites was rapid, and was followed immediately by movement from the site of first contact to one of a number of apparent refugia on the bee's body (Fig. 1.3). Analysis of 12 particularly detailed infestation videos showed that the mean time between the beginning of mite-bee contact and the mite coming to rest on the bee was extremely short, just 3.48 ± 1.85 s (range 1.58-6.98 s). Most initial contacts with the bee's thorax and abdomen were with the ventral surface, meaning perhaps some of these infestations first observed on the thorax and abdomen may in fact have begun on the forager's legs, which were the most common sites of first mite-bee contact. In every infestation, the first parts of the mite's body to make contact with the bee were the extended forelegs, and in many cases mites were observed to latch onto a host with their forelegs and then rapidly (in < 0. 03 s) flip their body upside down to bring the rest of their legs in contact with the host. The locations where the mites settled (n=58) most often were the dorsal intertagmal regions of the "neck" and "waist" (17% and 17%), the anterior dorsolateral portion of the first visible segment of the abdomen (16%), and the dorsal surfaces of the trochanter or femur (28%). Less common settling locations were the central dorsal thorax (10%), and the ventral surfaces of the thorax or abdomen (5% and 7%).

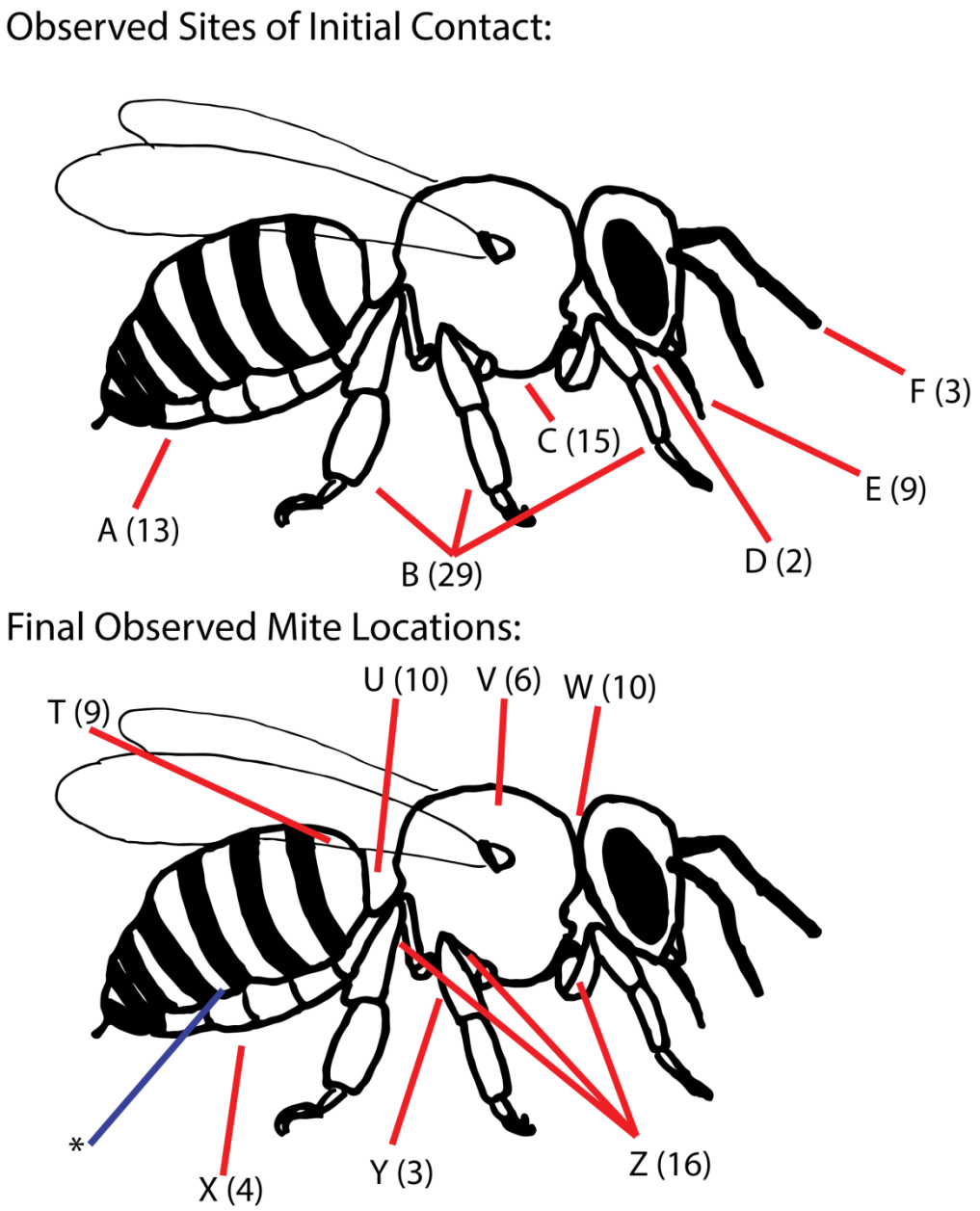

Figure 1.3: Sites of initial contact between foragers and mites (n=71), and the sites at which mites came to rest (n=58). Numbers in parentheses denote the number of mites observed interacting with each host body part. Data are pooled from feeder and flower observations. A: Any part of the ventral abdomen; B: Legs, above the tarsal claw; C: Ventral or lateral thorax or proximal surface of legs; D: Ventral surface of head; E: Proboscis; F: Antenna. T: Anterior dorsolateral abdomen, 1st segment; U: Intertagmal region between thorax and abdomen ("waist"); V: Central dorsal thorax; W: Intertagmal region between head and thorax ("neck"); X: Ventral abdomen; Y: Ventral thorax; Z: Dorsal trochanter or femur. The asterisk refers to the space between the 3rd and 4th tergites: the most common location for *Varroa* found on hive bees in past studies.

We did not observe the bees perform conspicuous avoidance behavior toward the mites at the feeder or the flowers. The bees did, however, respond to infestation; in many cases, foragers took off from the flower within one or two seconds of being infested. Of the 64 infestations in which we could evaluate grooming responses in real time or in the video recording, 20 bees (31%) began grooming themselves at the feeder or flower, though only 4 of these grooming events led to the mites detaching from the bee, and in 3 of those 4 cases the mite immediately mounted the same or a nearby forager. One instance of allogrooming was observed (on a flower), but the grooming bee failed to dislodge the mite from the groomed bee.

Discussion

Varroa destructor mites are able to rapidly infest honey bees foraging at a feeder or at flowers of several species. Our observations reveal that mites can quickly mount honey bees engaged in foraging, and that despite efforts by the bees to groom off the mites, they almost always succeed in leaving the forage site still attached to a bee. *Varroa* transfer from flower to bee can occur in just 2 seconds of foraging activity on a flower. It is not yet clear how significant this mode of transmission may be for mite spread between colonies because little is known about how frequently mites wind up on flowers. Our study examined only the transfer of mites from flowers to bees but not from bees to flowers. Therefore our data are most relevant to situations such as those reports in the literature where live mites have been transported on flowers through biocontainment barriers (Kevan et al. 1990, Pettis et al. 2003) but they also support the plausibility of mite transfers between bee colonies via flowers. At this point, however, we do not claim that this is a common phenomenon.

We have also found that the behavior of *Varroa* upon infestation enables them to evade the bees' grooming defense, giving them a chance to travel home with the hapless bee to her

colony's nest, where they may be able to begin reproducing. Thorp (2000) discusses similar "safe spaces" on various bee species which allow passively acquired pollen grains to evade bee grooming, and refers to Kimsey's (1984) work on mite exploitation of these same refugia from grooming in euglossine bees. *Varroa* success during the forager's flight back to the nest is unknown, though we monitored mite falls in a 1m radius around the forage source and across all of our observations fewer than 14% of our focal mites were recovered within this area. Mite success upon return to the hive is also unknown. Bees are also known to engage in allogrooming solicitation dances (Land & Seeley 2004), which may or may not significantly reduce the survival of mites brought home by foragers.

Our use of mites from a single bee colony does not invalidate the conclusion that *Varroa* mites have the sensory and behavioral capacity to infest freely behaving forager bees at flowers. The colony from which we obtained our mites was selected not for any remarkable feature of its mites, but simply because it was a mite-laden colony in our bee yard which was small enough to be transported via boat to the study site. However, the fact that our focal mites and foraging bees were both from the same colony does mean that this study neglects possible effects of chemical adaptation between the mites and bees. *Varroa* mites have been shown to passively absorb the cuticular hydrocarbon profile of their host hive (Kather et al. 2015a, Kather et al. 2015b). Therefore, a forager might have had an easier time detecting and removing a mite if the mite had the hydrocarbon profile of a foreign colony, (though mite grooming-avoidance behavior suggests increased detectability may not be sufficient to stop infestation.) Also notably, the mites in our study infested bees from their original hive instead of ignoring familiar-smelling hosts for the chance to disperse on a foreign bee. Further studies will be needed to tease apart whether or not colony-specific chemical cues play any part in *Varroa* infestation behaviors on flowers.

The behavior of the hostless mites that we observed suggests that they employ a "sit and wait" strategy, wherein little movement takes place except when cues from a nearby honey bee are detected. The foreleg extension behavior of the mites is comparable to the "questing" behavior seen in host-seeking ticks (Lees 1948), but is difficult to assign definite function to it as the extension of the forelegs may serve a sensory or a mechanical purpose, or both. Mites perhaps use the chemosensory setae on their forelegs to "sniff" for potential hosts (Le Conte et al. 1989, Rickli et al. 1992, Pernal et al. 2005) or this foreleg extension may simply enable the mites to easily grasp a passing bee, or both. Once a bee approached, mites commonly but not universally oriented towards and approached the bee. More than half (53%) of the successful mites infested a forager upon first contact, while the other 47% made repeated contacts before successfully infesting a bee (usually the same one.) Most of the initial, unsuccessful contacts were between the mite and a tarsal claw of the bee, suggesting that tarsal claws lack the cues necessary to trigger mite behavior, that mites detect but do not attempt to climb the tarsal claws of honey bees, or that mites are unable to grab onto a bee's tarsal claw when they are stepped on.

The mite behaviors that we observed during infestation show how foragers can become infested at flowers, but may also relate to infestations which take place in the nest. The initial refugia on forager bees that we observed (Fig. 1.3) are not the sites that other investigators have reported for mites found on bees collected from colonies, but they may be the sites that provide combined safety from grooming and rapid accessibility. Perhaps we have identified the mites' preliminary refugia. The preferred feeding attachment site of *Varroa destructor* on bees in hives is between the third and fourth ventro-lateral tergites (Delfinado-Baker et al. 1992, Bowen-Walker et al. 1997). Whether mites move to our "preliminary refugia" during in-hive transfers between bees is unknown. It is currently unknown if the behaviors we observed are specially

adapted for infesting bees engaged in foraging or are simply the typical first sites where a mite settles on any newly infested bee before moving to a preferred long-term location. Since mites prefer to use nurse bees as hosts over foragers (Kraus et al. 1986, Le Conte & Arnold 1987, Kuenen & Calderone 1997) perhaps movement to these non-feeding refugia provides a safe place for a mite to wait until the forager it has infested brushes past a nurse bee in the hive. Kather and colleagues have shown that mites entering a new colony require a period of at least three hours to chemically adapt to the odors of their new hosts, (2015b) which offers another explanation for why mites on flowers would initially move to non-feeding sites which offer refuge from host grooming.

The actual likelihood of a mite transferring between two colonies via this floral transfer pathway is unknown, and further study is required to determine whether this is a major, minor, or completely negligible transmission phenomenon in nature. Optimal virulence theory predicts that parasites and pathogens should evolve lower virulence in systems wherein horizontal transmission is low relative to vertical transmission (Ewald 1995, Schmid-Hempel 2011). In a eusocial insect like the honey bee, the colony can be thought of as a superorganism, so colony-to-colony mite transmission can be considered horizontal transmission, and infestation persistence in mother swarms and daughter colonies can be considered vertical transmission. A complete understanding of both vertical transfer mechanisms (i.e. swarming) and horizontal mechanisms (e.g. drift of infested bees, robbing between colonies, floral transfers between foragers, etc.) is required before accurate predictions can be made about the direction of virulence evolution in the mites and mite-vectored viruses of honey bees living under natural conditions, (but see Fries & Camazine (2001) for an excellent review of what is known). Floral transfer may represent an important avenue for horizontal mite transmission between widely

spaced wild colonies. Whether or not floral mite transfer occurs often in nature, mites infesting managed colonies may be experiencing selection for higher virulence due to high rates of horizontal mite transmission from crowding bees into apiaries (leading to high rates of drift and robbing) and from beekeeping practices that may facilitate mite transmission (such as the moving of brood from one colony to another). Research has shown that closely spaced colonies appear to share *Varroa* via drift or robbing from heavily infested colonies, but that such pronounced *Varroa* spread does not occur between colonies spaced more widely apart (Seeley & Smith 2015), as is typical in the wild. Thus, it is unclear whether mites in unmanaged (wild) colonies may evolve avirulence or virulence in the absence of human interference, and virulence theory can only offer accurate predictions if we first understand all mechanisms of mite transmission between colonies and their relative importance in the spread of mites through susceptible host populations.

Some risks demonstrated by these data have immediate relevance. For instance, the Isle of Man was declared a *Varroa*-free region by the European Union's Department of the Environment, Food, and Agriculture in early 2015 (Isle of Man Government, 2015), but by early 2016 reports of illegally imported and likely *Varroa*-contaminated bees called the safety of the Manx bees into question. Likewise, Newfoundland, Iceland, and a number of other island regions are currently considered free from *Varroa*. Great efforts have been taken to protect Australian beekeeping operations from invasion by *Varroa destructor*, with apparent success thus far (Department of Agriculture and Water Services Australia 2016). Any region free from the parasite should carefully consider both the movement of bees and bee products, but also potentially flowers and other substrates on which *Varroa* could survive transport and which may subsequently attract honey bee foragers. Even regions already containing *Varroa* should

consider any avenue of horizontal transmission that might allow locally absent bee viruses or acaricide resistance genes to be spread by a foreign mite to local bees. Though our data do not demonstrate that *Varroa* transmission on forage is a common occurrence, we have demonstrated the ease with which a floral mite may breach biocontainment and infest a new region. Since every phoretic mite is female and may be pregnant, and since *Varroa* are highly resistant to the costs of inbreeding because mating normally takes place between siblings, only a single biocontainment breakdown is required for *Varroa* to invade a new population of previously mite-free bees.

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Supporting Information

Video available with original publication in PLOS One – https://doi.org/10.1371/journal.pone.0167798.s001
Higher quality video available: https://www.youtube.com/watch?v=Oij1HOxD3iU
1.S1 Video: A recording of a floral mite infestation. In this video, a mite on a daisy can be seen responding to the arrival of a foraging bee, approaching and then mounting the bee, and then crawling along the bee's abdomen to the space between the bee's abdomen and thorax.

Dataset available with original publication in PLOS One - https://doi.org/10.1371/journal.pone.0167798.s002
1.S2 Behavior Dataset: All behavioral observations analyzed in this manuscript are included as a digital spreadsheet document.

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Chapter 2: Robbing by honey bees in forest and apiary settings: implications for horizontal transmission of the mite *Varroa destructor*

David T. Peck and Thomas D. Seeley,

Abstract

Honey bees are well known to rob honey from neighboring colonies in apiaries, but this behavior has not been studied in natural contexts. We found that bees living in widely dispersed nests in a forest discovered unguarded honeycombs and did so rapidly enough to acquire parasites and pathogens. However, the rate of discovery by robbers in the forest was markedly lower than in apiaries. Evidently, the capacity for robbing-mediated disease transmission is lower among wild colonies than among managed colonies. Observations of the behavior of robbers collecting honey from a dead colony revealed that it is ideal for transmitting parasites, particularly *Varroa destructor*. The implications of these findings for the evolution of avirulence in the disease agents of the honey bee are discussed. / (123 words, 673 characters)

Introduction

The life of a honey bee colony revolves around the production, storage, and consumption of honey. Honey enables a colony to survive long nectarless periods, including winters in temperate regions of the world. Honey bees are lauded as diligent laborers, but they will sometimes take a work shortcut when possible. Park (1949) reported that the average load of nectar collected during a nectar flow weighs 40mg (confirmed by von Frisch, 1967 and Wells and Giacchino, 1968). Park also reported that the typical load of honey collected by a robber nearly matches the forager's body weight of 82mg. Besides being twice the weight of the average load of nectar, the average load of honey is twice as valuable per unit weight: about 80% sugar (White 1975) for honey vs. about 40% sugar for nectar (Park 1949, Southwick & Pimentel 1981). Therefore, milligram-for-milligram, stolen honey is about twice as valuable as foraged nectar. In addition, a bee collecting nectar must visit and manipulate a multitude of flowers to acquire a full load, while a bee robbing honey needs only to visit the robbing site, chew open a honey cell, and imbibe the honey. Therefore, unless she is attacked by defenders , a

honey robber can make many, highly profitable trips each day, while a nectar forager will make fewer trips, each only about one quarter as profitable as the robber's. The energetic advantages of honey robbing over nectar foraging clearly favor theft, so long as the risks to the robbers are low.

Previous work on robbing has focused on either the behavior of individual robbers at the nest entrance (v. Buttel-Reepen 1900, Cale 1946, Free 1954) or the mechanisms by which guard bees detect and exclude robbers (Butler and Free 1952, Kalmus and Ribbands 1952, Ribbands 1953, Collins 1985). Little is known about how robbing targets are discovered, except that robbing is more common when nectar is sparse (Butler and Free 1952, Ribbands 1954). Moreover, no attention has been paid to robbing when colonies live at natural densities, or to how quickly honey is discovered and robbed in apiaries. Our goal was to better understand the opportunities for robbing in both natural and apiary settings.

Besides being a form of intraspecific kleptoparasitism, robbing may also give rise to interspecific parasitism. The risk of acquiring parasites from robbing is strongly influenced by the timing of the robbed colony's illness and death. If a colony succumbs to a *Varroa destructor* (Anderson and Trueman 2000) infestation in autumn but is not robbed until spring, then all the mites will be long dead. However, if a colony is robbed before or shortly after its death, it will contain live mites which can infest robbers and travel home with them (Gromyko 1982, De Guzman et al. 1993). If a colony dies in winter, as most do (Seeley 2017) some pathogens can still be transmitted during springtime robbing, (e.g. American Foulbrood, European Foulbrood, Chalkbrood disease, *Nosema cerana*, *Nosema apis*, etc.) but others are rendered harmless, (e.g. *Varroa* mites, tracheal mites, mite-vectored bee viruses, etc.) Therefore, to understand the risks

of parasite and pathogen transmission via robbing, we must know how long a disease agent can survive in dying/dead colonies, and how quickly such colonies are found by robbers.

To understand how robbing might contribute to pathogen spread, we investigated how quickly unguarded honey was discovered by both wild colonies living in a forest, and managed colonies living in apiaries. Studies of the forest populations used in this study (Seeley 2007; Seeley et al. 2015) and other natural habitats (Moritz et al. 2007; Hinson et al., 2015) have found colony densities of 1-3 colonies per km^2. Therefore, colonies living in the wild are spaced much more widely than colonies packed in an apiary. A second motivation for this study was to determine whether the *V. destructor* infesting the survivor population of honey bees living in the Arnot Forest have evolved avirulence due to few opportunities for horizontal transmission (Ewald 1987; Ewald 2004). Mite avirulence has been hypothesized, based on the expectation that robbing and drift between colonies living in this forest are unlikely (Seeley 2007).

Material and Methods

Animals and Setting:

Two groups of European-derived honey bee colonies were used. One group comprised the unmanaged colonies living in trees in the Arnot Forest (42°17'N, 76°39'W, altitude 585 m), a mostly forested 17 km^2 research preserve owned by Cornell University, outside the town of Cayuta, in Schuyler and Tompkins Counties, New York State, USA. The other group comprised the managed colonies living in five apiaries owned by Cornell University in Ithaca, New York (*42*°26′N, 76°30′W). The Arnot Forest bees have been studied previously to assess their density and genetics (Seeley 2007; Seeley et al. 2015) and to understand their survival despite being infested with *V. destructor* (Mikheyev et al. 2015; Locke 2015). Repeated surveys of the Arnot Forest have found a population of ca. 18 colonies in the forest, and that each colony's nest is

within the foraging range (6+km, Visscher and Seeley 1982) of approximately three other colonies. The apiary colonies were housed in Langstroth hives and were closely spaced, with at least 15 colonies crowded within a 10 x10 m area.

Small-scale tests in 2013 and 2014:

Preliminary studies were conducted in October in 2013 and 2014. Unoccupied hives containing honey were placed 7-8 m off the ground on platforms in trees and were monitored weekly to see if/when they would be discovered by robbers. In 2013, three hives were placed in sites A, B, and C, and in 2014, four were placed in sites A, B, C, and D (Fig. 2.1). All were 5-frame Langstroth hives with either one frame at least two-thirds full of honey (2013), or 2-3 frames at least two-thirds full of honey (2014). Hives were spaced 750+ m apart. We diagnosed robbing of a hive by noticing bees at the entrance, and then opening the hive to check that honey had been removed. We estimated the fraction of honey cells that had been emptied to nearest 5%. Also, to assess whether the wild colonies in the Arnot Forest were seeking unguarded honey during October in 2013, we set out two unoccupied hives with frames of honey at the bases of two occupied bee trees.

Large-scale tests in 2015:

Ten unoccupied 5-frame robbing target boxes (RTBs) containing frames of honey were set out in the Arnot Forest (Fig. 2.1) and one was set out in each of 5 managed apiaries ca. 25 km from the Arnot Forest. The RTBs in the Arnot Forest were hung from tree limbs, to minimize risk of destruction by black bears (*Ursus americanus*). We made the RTBs from used, 10-frame Langstroth hive bodies cut in half. Each had a 5-cm^2 entrance opening in one end wall. To prevent interference by ants, we coated the rope used to hang each hive with Tanglefoot Insect Barrier. To prevent hives from spinning, we secured each one with a secondary rope tied to a

nearby tree. Each RTB contained three frames of empty comb and one frame of capped honey. All frames had been used by bees for at least one year. To test whether our hanging boxes would be readily robbed by bees, we hung two in trees within 100m of an apiary in June 2015; both were being robbed when checked 48 hours later.

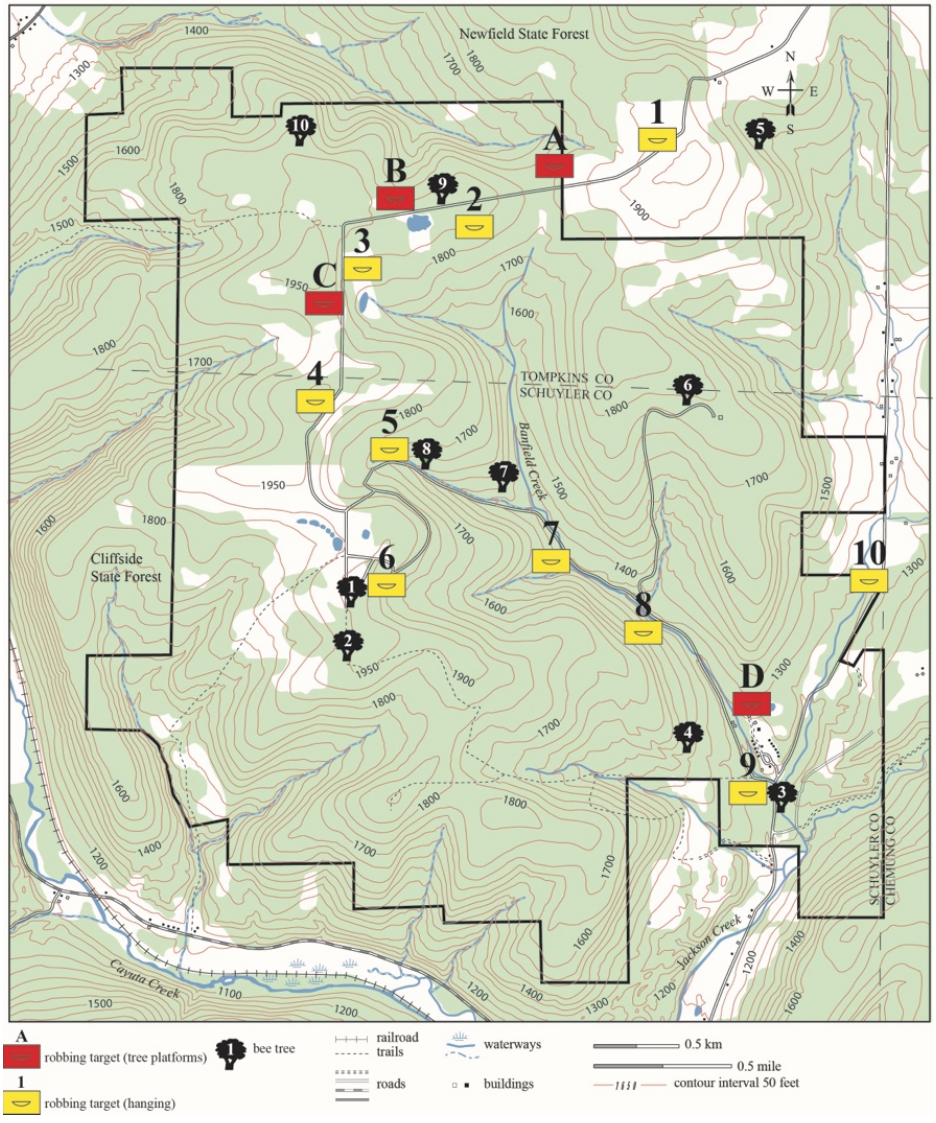

Figure 2.1: Map of Arnot Forest showing locations of robbing test boxes. Letters A-D denote locations used in 2013 and 2014. Numbers 1-10 denote locations used in 2015, spaced roughly 1km from one another along the central road, as terrain and tree growth allowed. Also shown are the locations of bee trees found during survey in 2011.

As shown in Fig. 2.1, we spaced the RTBs as evenly as possible along the central roadway in the Arnot Forest. We lacked an up-to-date map of the colonies occupying trees in the forest, so we could not equalize the distance of our RTBs to the nearest bee tree. Previous surveys of wild colonies in the forest (Seeley 2007; Seeley et al. 2015) indicate that the density of our RTBs was probably higher than usually exists in nature, but was not utterly unrealistic.

On 28 July 2015, we began our first large-scale robbing test. We hung 10 RTBs in the Arnot Forest and we set 5 RTBs on cement blocks in 5 apiaries (one per apiary). We checked all 15 RTBs at least once per week for robbers. On 1 September, the test was ended and all honey frames were removed. By 13 September, all RTBs were removed from the forest and the apiaries, so they could not be discovered and remembered by foragers when a second round of testing began in October.

On 8 October 2015, we again hung 10 RTBs in the forest. That evening, after all bee flight had ceased, we placed two RTBs in each of 5 apiaries. Five were identical to the boxes hung in the forest and were hung from a tree branch 20-40 m from the hives. The other 5 RTBs were placed on cement blocks within the apiary, as in the July trial. We checked all the RTBs between 1600 and 1900 h on every day (n=17) that had at least a 30-min period of rainless weather above 15° C. To prevent the wild colonies in the Arnot Forest from filling their nests with stolen honey, we removed each RTB as soon as we found it being robbed.

On a few unseasonably warm days starting 4 November 2015, we conducted a final test of robbing. We hung a RTB in a tree within 20 m of the center of each of the 5 test apiaries. The boxes were set out before the temperature warmed above 15°C, and we observed them late in the afternoon to see how many were discovered and what percentage of the cells in each frame still contained any honey.

Focal observations in 2017:

During a nectar dearth in July 2017, we noticed bees robbing honey from an unoccupied hive at the home of one of the authors (TDS). We reoriented the robbers to a 2-frame observation hive (see Seeley 1995, pp. 72-73) in which we had installed two frames of honey. On 2-3 July, we observed the robbers at work inside the observation hive, noting especially the time each bee spent in the hive on each robbing foray.

Results

Small-scale robbing tests in October 2013 and 2014:

In 2013, we found that between 14 Oct and 17 Nov, 0 of 3 hives mounted in trees was robbed. We also found, however, that both hives placed on the ground beside two occupied bee trees on the morning of 21 October were being robbed at day's end. In 2014, we found that between 12 Oct and 28 Oct, 1 of 4 hives was robbed. We also discovered, on 28 Oct, that a bear had found and destroyed one the hives while removing its honey.

Large-scale robbing tests in 2015:

Fig. 2.2 shows that in our first experiment, conducted during a nectar dearth from late July through late August, the unguarded honey combs in boxes placed in apiaries were robbed much more quickly than the unguarded honey combs in boxes placed throughout the forest. Three of the 5 boxes in apiaries were robbed within the first 3 days. Examination of the remaining two RTBs revealed large infestations of ants (*Camponotus* spp. and *Formica*. spp.) which probably prevented the boxes from being robbed. Figure 2.2 also shows that many of the RTBs in the Arnot Forest were gradually discovered and robbed between 28 July and 1 September. A honey flow from goldenrod (*Solidago spp.*) began in late August, so we ended our first test then.

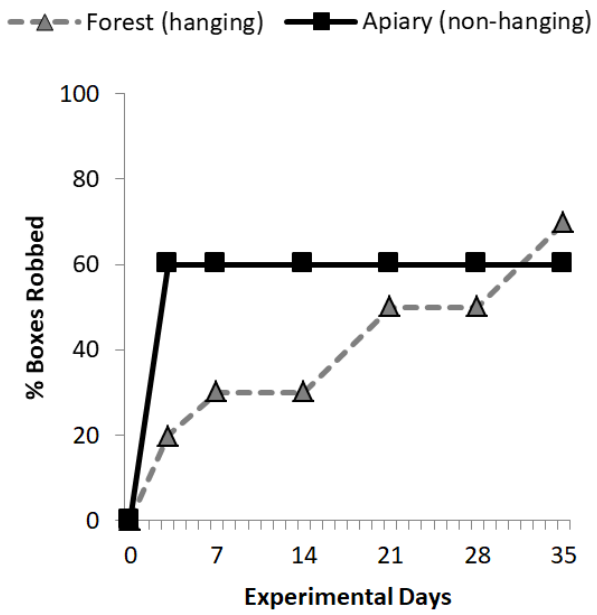

Figure 2.2: Records of discovery of robbing target boxes in Arnot Forest (n=10) and in apiaries (n=10), 28 July to 1 Sept 2015.

Figure 2.3 shows that in our second experiment, conducted in October when virtually no nectar was available, the unguarded honey combs in RTBs placed in apiaries were discovered within the first day of the experiment. No difference in discovery rate was found between non-hanging RTBs set among hives and hanging RTBs set 20-40m from the center of each apiary. Figure 2.3 also shows that RTBs in the Arnot Forest were discovered at a rate of about one box per day of weather suitable for honey bee flight (i.e., temperature above ca. 15° C and not raining).

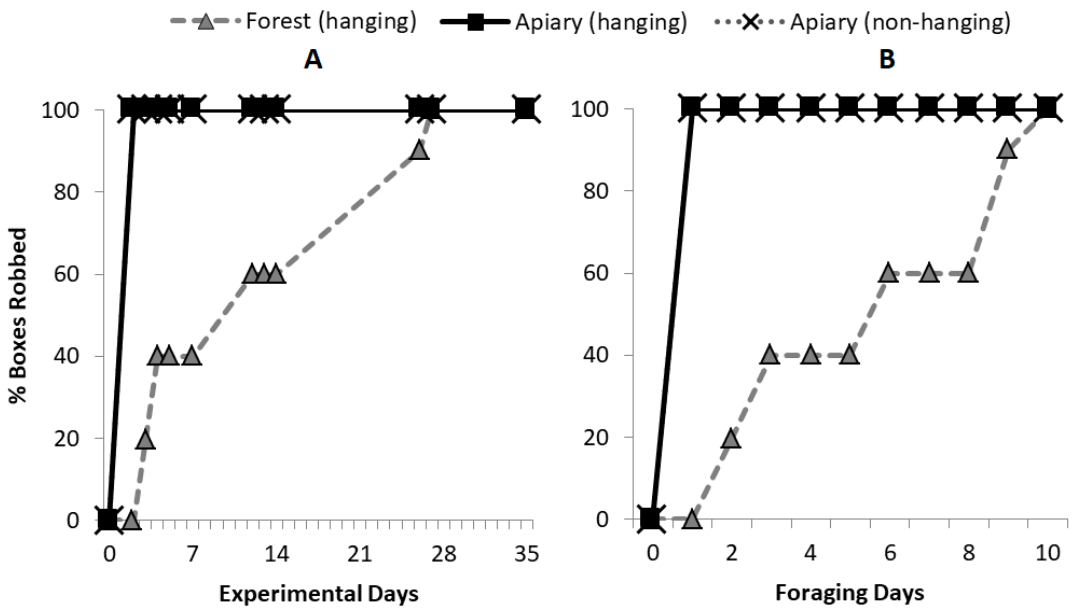

Figure 2.3: Records of discovery of robbing target boxes in Arnot Forest (n=10) and in apiaries (n=5 hanging; 5 non-hanging), 8 Oct to 12 Nov 2015. Time scale in A is days from the start of the experiment. Time scale in B is days in which weather conditions allowed for forager flight.

The final robbing test, in which one RTB was hung within 20 m of the center of each of 5 apiaries on 4 November, showed again that unguarded honey in an apiary was prone to rapid discovery and removal. Boxes were positioned before the weather was warm enough for bee flight, at 9am, and were examined at 4:30pm. One box contained less than 5% of a frame of honey, and the other 4 contained no honey, despite every box starting with a frame 90-100% full of capped honey.

Of the 20 RTBs placed near apiaries in 2015 (10 hanging and 10 on the ground), the majority were being robbed within 24 hours and 90% were being robbed within 3 days. Of the 27 RTBs placed throughout the Arnot Forest, 18 (67%) were robbed: 0 of 3 from 14 Oct-17 Nov 2013, 1 of 4 from 12 Oct-12 Nov 2014, 7 of 10 from 28 Jul-1 Sep 2015, and 10 of 10 from 8 Oct-4 Nov 2015. The observations with the best temporal resolution were made 8 Oct–4 Nov

2015. During this test, 100% of the RTBs in the forest were robbed within 27 days, which included only 10 days with weather suitable for bee flight. A Wilcoxon-Mann-Whitney test on these data from 8 Oct-4 Nov 2015 confirmed that interval between box placement and discovery by bees was greater for boxes in the forest (median= 6 days; n=10) compared to boxes in or near apiaries (median= 1 day; n=10) $W=0.0$, $p<0.0001$.

Observations of robbing behavior in 2017:

At first, the robbers moved hesitantly at the hive entrance, as if they were nervous. After two hours of robbing, however, they entered and exited the hive entrance without hesitation. Once inside, they displayed remarkably consistent behavior. Sometimes, when a robber was approached or touched by another bee—always another robber—the newly arrived bee would either rear up on her hind legs and charge at the other bee, or she would drop from the comb onto the hive floor and then run out the entrance. Usually, however, a robber would calmly join a cluster of other robbers imbibing honey from an opened cell. Each robber walked over hundreds of cells of capped honey to get to and join such a cluster. The opened cells had not been wholly uncapped by the first robber to arrive – instead, the first robber at each cell cut/chewed a hole no larger than was needed for her to insert her tongue into the cell. These pin-prick openings were gradually enlarged when additional bees tried to drink from the same cell. Doing so often led to two bees standing head-to-head, pushing each other back and forth. As the robbers jostled over the honey, shards of cappings wax would fall to the hive's floor, creating a carpet of wax particles like what we found in all of our RTBs that had been robbed. We never saw a bee insert her body or even her entire head into a cell. The average time a robber spent inside the hive was 689 ± 248 s, (range = 280-1338 s, n=16). Upon arrival, a robber generally

took 30-120 s to find a spot to start sucking up honey, after which she worked steadily until her abdomen was bulging, at which point she scurried out of the hive.

Discussion

Our data show that bees living in a forest setting are both willing and able to find and steal unguarded honey. Our data also show that during a nectar dearth unguarded honey combs in an apiary are discovered and robbed rapidly, within a matter of hours, but that more naturally dispersed honey combs in a forest are discovered and robbed over the course of days or weeks (Fig. 2.2; Fig. 2.3A). Some authors have hypothesized that robbing is so rare or so slow in the wild that the death of a mite-infested wild colony "probably means the demise of both the bees and the mites" (DeGrandi-Hoffman et al. 2017). The speed at which our boxes of unguarded honey were exploited indicates that there are opportunities in both apiaries and forests for parasites and pathogens to be transmitted during robbing.

Our observations of the behavior of robbers plundering the honey stores inside the nest of a dead colony revealed their within-nest behavior as they steal honey from an unguarded nest. When the bees first approached the hive, they behaved as has been reported: with "an air of roguery and a nervous and guilty agitation" (Cale 1946). Since this behavior eventually abated during our two days of observations, it seems that the jerky, horizontal flight of robbers arriving at a fresh source of honey is either maintained by the presence of resident guard bees in the robbed hive, or is simply tentative orientation by robbers arriving at the entrance of another colony's nest (Free 1954, Winston 1987). Once robbers entered the dead colony's nest, they never inserted their heads or bodies into cells while they imbibed honey, perhaps because robbing often occurs in defended nests, so it is adaptive for robbers to remain vigilant while loading. This, as well as the apparently fearful responses by robbers to other bees when inside

the foreign nest, suggests that robbers risk being killed by guards of the colony they are robbing. Curiously, they are vigilant even when they are robbing from a nest whose colony is long-dead and therefore lacks defenders. When robbers are feeding, however, they behave calmly. The fact that robbers often spend several minutes standing completely motionless drinking honey from a single cell shows that they present ample opportunity for lingering *V. destructor* to climb onto robbers and then be airlifted back to a healthy colony.

An open question in understanding the impact of *V. destructor* on honey bee health is how these mites have spread so effectively from infested colonies to uninfested colonies when they have arrived in each new region around the globe. This mystery remains of great concern in regions that are still free of the parasite, such as Australia and Newfoundland. It is easy to see how these mites have been able to spread within an apiary and between apiaries, through the movement (by beekeepers) of mite-infested bees and brood between colonies, drift of foragers between colonies, transmission of mites on shared floral resources, and robbing. What is harder to explain, however, is how these mites have been able to spread throughout wild populations of honey bees living in natural landscapes over the course of only a few decades. Our studies show that mites have opportunities for intercolony transmission through robbing, even in remote settings like the Arnot Forest.

Many of our robbing target boxes were robbed quickly, in just a day or two. This shows that there will be times when the mites lingering on the combs of a colony that has collapsed will survive until the dead colony's nest is discovered and robbed. De Guzman et al. (1993) examined the survival of female mites that were taken from capped drone brood cells and then placed on dead bees and non-bee substrates that differed in temperature and humidity. They found that most mites survived for 1-3 days (5 days max) at temperatures seen in Ithaca in early

autumn. One of us (DTP) has observed mites survive for more than six days on a dead bee pinned to a flower in a humid, 23ºC laboratory setting (unpubl. data). Salchenko (1972) reported mite survival without food for up to 9 days at 28ºC and 85% relative humidity. Grobov (1977) summarized various investigations and concluded that mites can survive an average of 3-5 days without food (honey bee hemolymph). When mites have access to live bees or brood on which to feed, they can survive much longer. Indeed, caged bees in a laboratory can sustain mites for a week or more (Dietemann et al. 2012). Since mite-induced colony death does not kill every bee in the colony simultaneously, mites can persist on a dying colony's remaining bees and brood, which stretches the time period in which viable mites can be transmitted to robbers. Our data show that even a mite in a dead colony's nest in a forest still has some chance of dispersing to another colony on a robber, bringing with her whatever viruses or miticide-resistance genes that she carries. Moreover, our data show that a mite in a dead colony's hive in an apiary is likely to make it into a new colony.

Since the robbing target boxes we used were identical and conspicuous, and since worker honey bees are excellent learners of the visual properties of food sources (reviewed by Menzel and Eckoldt, 2016), it is possible that discovering honey in one box sensitized bees to search for similar boxes. This could explain why a higher fraction of our RTBs were discovered in 2015, when these boxes were numerous, identical, and conspicuous, than in 2013 and 2014, when the boxes were rare, not identical, and less conspicuous (placed against tree trunks). Natural nest sites, as described by Seeley and Morse (1976), may be more difficult to find than our RTBs. Our data may, therefore, over-estimate the speed with which honey in natural nests is discovered and robbed.

Honey bees are not the only animals interested in unguarded honey. Other flying insects, including yellow jacket wasps (*Vespula* spp.) and bumble bees (*Bombus* spp.), entered about half of our RTBs, though only a few individuals were observed at any one time. Bears are a threat to colonies in beekeepers' hives, but appear to be less dangerous to colonies living in tree nests. One of us (TDS) has monitored 9 bee tree colonies for 15 years and 10 additional colonies for 7 years. In that time, only one colony's nest was discovered by a bear (as indicated by claw marks) and that occurred after the tree blew down in a storm (Seeley 2007, Seeley et al. 2015). Ants are commonly seen in hives at ground level, as we experienced in our RTBs in apiaries in early 2015. However, no ants were observed in the RTBs set out in 2013 and 2014 on wooden platforms in contact with the trees. Bait hives placed in five similar locations in 2003 were also not invaded by ants, and indeed avoiding ants is one of the reasons beekeepers are told to place bait hives above ground level (Seeley 2012). These observations suggest to us that the discovery and robbing of unguarded honey in natural nests is done mainly by honey bees, and that isolating our RTBs from bears and ants made them more accurate facsimiles of natural tree nests. Nonetheless, it is possible that some natural nests may be destroyed or degraded by other species before bees are able to rob them of their honey.

Colonies of Arnot Forest survivor bees are able to survive in trees without beekeeper interventions for years at a time, and repeated surveys of the forest in 1978, 2001, and 2011 show that the density of wild colonies in the forest is the same before and after the arrival of *V. destructor* (in mid 1990s) (Visscher and Seeley 1982; Seeley 2001; 2011). Genetic analyses of modern (2011) and historical (1977) wild colonies living in the forests around Ithaca, NY suggest, however, that the population of wild honey bee colonies in the Arnot Forest experienced a dramatic selection event around the time *V. destructor* arrived, and that it experienced strong

selection on genes relevant to mite resistance (Mikheyev et al. 2015). If the arrival of mites precipitated the death of a large percentage of the colonies, our data suggest that those mites may have then spread via robbing from dying colonies into healthier colonies, including some that may have had genes conferring mite resistance. This would mean that even in a forested landscape, a resistant colony may run the risk of absorbing mites from less resistant colonies, killing or weakening the resistant colony before it can spread its own genes into the population. Such an effect would be more pronounced in an apiary setting where robbing is likely to follow colony death more rapidly, while more mites are apt to be alive on the combs. Great care should be taken to prevent robbing in any program attempting to breed mite-resistant bees.

Until we understand all of the mechanisms by which the agents of honey bee disease can be transmitted horizontally (between unrelated colonies) and vertically (between mother and daughter colonies during swarming), we cannot accurately predict the optimal virulence level of the parasites and pathogens in the system (Ewald 1987; Fries and Camazine 2001, Ewald 2004; DeGrandi-Hoffman et al. 2017). Robbing is one of a number of mechanisms for horizontal transmission of mites between colonies, and our data show that its effect cannot be ignored when attempting to predict whether mites and viruses in wild populations of honey bee colonies will experience selection for increased or decreased virulence. It has been hypothesized that the survival of some bee populations with *V. destructor* may be the result of low horizontal transmission of mites between these colonies, which would favor avirulence in the mites or the viruses they carry (Seeley, 2007). The apparent ease of robbing in forested settings shows that this hypothesis is probably incorrect, and that instead these bees have probably evolved resistance and tolerance mechanisms to allow them to persist with the mites.

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Chapter 3: Mite Bombs or Robber Lures? The roles of drifting and robbing in *Varroa destructor* transmission from collapsing colonies to their neighbors

David T. Peck, Thomas D. Seeley

Abstract

When honey bee colonies collapse from a high infestation of Varroa mites, neighboring colonies often experience surges in their mite population. The collapsing colonies, or "mite-bombs", seem to pass their mites to neighboring colonies, either through infested workers drifting from the sick hive, honey robbers entering the sick hive, or both. To study inter-colony mite transmission, we positioned colonies of black-colored bees around a cluster of mite-laden colonies of yellow-colored bees; we monitored the movement of bees before, during, and after mite-induced colony collapse; and we tracked each colony's change in mite level. Our findings suggest that mite-infested colonies slowly pass mites to nearby colonies through worker and drone drift, and that late in the season mites are spread to both nearby and distant colonies through the robbing of sick hives. We suggest that "mite leakers" or "robber lures" are better terms than "mite-bombs" for describing collapsing colonies.

Introduction

The parasitic mite *Varroa destructor* is a recently speciated parasite of the western honey bee *Apis mellifera*, (Anderson and Trueman, 2000). The parasite feeds upon both juvenile and adult honey bees, and is known to transmit harmful viruses between colonies which cause additional direct harm (Kevan et al. 2006, Martin et al. 2012). The mites are wingless, eyeless, and unable to crawl between widely spaced honey bee nests. Despite these limitations, honey bee colonies are almost universally infested with these mites, including colonies that have been recently purged of mites by use of chemical treatments (Greatti et al. 1992; Sakofski et al. 1990; Frey & Rosenkranz, 2014, Frey et al. 2015), and wild colonies spaced widely in isolated environments (Seeley 2007).

Bees can ferry mites between colonies either indirectly or directly. Indirectly, a mite can move from one bee to a neutral location like a flower, and from there to a bee from another colony. Mites are certainly agile enough to achieve this (Peck et al. 2016) but this indirect

mechanism is unlikely to move large numbers of mites between colonies. Instead, it is likely that most mite transmission occurs directly, when a bee flies between its nest and another colony's nest while carrying a mite. Drifting, when a bee leaves its natal nest and takes up residence in another colony's nest, presents such an opportunity. Robbing, when bees enter another colony's nest to remove honey and then bring it back to their own nest, offers another route of bee-mediated mite transmission. Worker bees can drift or rob, while drone bees can only drift. Drifting supports unidirectional mite transmission from the bee's original colony to its new colony, unless the bee subsequently drifts back (and 15% of drifted bees in one study drifted 3 or more times in their lives (Pfeiffer & Crailsheim 1998).) Robbing supports bidirectional transmission, since mites can ride the robbers and infest the robbed colony, or they can infest the robbers and ride them home.

Our study focused on understanding the mechanisms underlying the widely reported phenomenon that when one colony dies with a large population of mites, the mite populations in neighboring colonies often skyrocket at roughly the same time (Loftus et al. 2016, Oliver 2018). This has been framed as a "mite bomb" phenomenon whereby mites are propelled as "shrapnel" into neighboring colonies via the drift of infested workers and drones out of the dying colony. It has been suggested that such drift may even be a manipulation of bee behavior by the mites themselves (Kralj & Fuchs 2006).) We tested the hypothesis that this mite bomb phenomenon exists, and we measured the relative roles of drifting and robbing in the spread of these mites.

In addition, we tested whether it protects a colony to be farther from a collapsing colony. Large intercolony distances may reduce drifting, robbing, or both. This mite-spreading phenomenon is known from apiaries, but perhaps there is less mite spread in natural (forest) settings, where colonies are widely spaced (Galton 1971, Seeley 2007, Seeley et al. 2015). A

protective effect of distance may explain why some populations of free-living colonies are surviving with Varroa (Seeley 2007, reviewed in Locke 2016), and may suggest optimal spacing of hives by beekeepers.

Drifting between wild colonies is considered uncommon given the wide spacing of their nests (Fries & Camazine 2001; Seeley et al. 2015). Meanwhile, drifting between apiary colonies is certainly common. Jay (1965; 1966a) found that when hives painted white were placed in rows spaced 1 m apart, between 4% and 96% of marked workers drifted into other colonies, depending upon wind direction, distance from landmarks, and the number of colonies in the row. Pfeiffer and Crailsheim (1998) found that up to 90% of marked bees drifted out of their parental hive, and estimated that up to 40% of all bees in some hives in apiaries may have drifted in from elsewhere. However, Goodwin et al. (2006) found only 0-3% drift between nearby colonies, suggesting that while some drift in apiaries is common, the amount of drift which takes place is highly variable. Drone drift of at least 50% has been measured at intercolony spacings typical of apiaries (<1m), but was barely detectable (0-2%) when colonies were separated by 40-100m (Seeley & Smith 2015). Thus drift of both workers and drones is common within apiaries, but is greatly reduced when colonies are spaced more widely.

Robbing between colonies has also been implicated as a mechanism of mite transmission. Sakofski et al. (1990) reported highest mite introgression into treated colonies during the late summer, when robbing was most common in their study region. Greatti et al. (1992) reported high rates of mite introgression during periods of nectar dearth, and hypothesized that robbing of feral colonies was the likely cause. Frey et al. (2015) monitored mite invasion of colonies at various distances (1m to 1.5km) from infested colonies and found no protective effect of distance, proposing that all colonies had robbed the mite source colonies during a nectar dearth.

All of these studies measured the fall of dead mites below colonies that were continuously treated with miticides. One of us (Seeley 2007) presumed robbing to be unlikely among bees living in forests due to their wide spacing, but recent work has shown that bees from widely spaced forest colonies will quickly find and rob from unguarded honey combs in their environment (Peck & Seeley 2018). Thus, robbing is likely common even across large distances.

Our goal was to determine whether robbing, drifting, or a combination of the two best explained any observed mite transmission from dying mite-infested colonies to healthy ones. We predicted that if robbing is the primary mechanism of inter-colony mite transmission, then we would see sudden increases in the phoretic mite levels of healthy colonies coinciding with the onset of robbing, followed by decreases in phoretic mites soon afterwards as the imported mites entered cells to reproduce. We predicted that if normal intercolony drifting is the primary mechanism, then we would see constant worker and drone drift rates as mite levels correspondingly climb in the healthy colonies (Fig. 3.1). We also considered the alternative possibility that sudden increases in worker and drone drift rates during collapse of the infested colonies could corresponded to sudden and simultaneous skyrocketing of the mite levels in the healthy colonies. In addition to investigating these mechanisms, we also tested the prediction that larger intercolony distance between dying and healthy colonies confers protection to the healthy colonies by reducing the opportunities for mites to spread from the dying colonies, primarily by reducing drift.

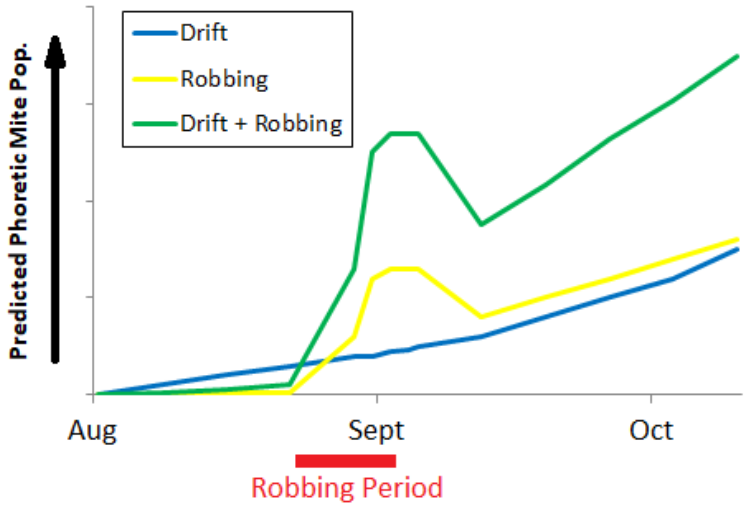

Figure 3.1: Predicted phoretic mite population levels in healthy colonies near mite-infested colonies in three hypothesized scenarios: (1) Mites primarily spread via consistent drift, (2) Mites primarily spread by robbing during the autumn period of intense robbing, and (3) Mites spread by both drift and robbing. At greater distance from the collapsing colonies, mite increases due to drift were hypothesized to be lower, while increases caused by robbing were not predicted to be significantly impacted.

Methods

We conducted this experiment in a field in Ithaca NY (42°29'43.5"N 76°25'53.7"W). This location provided isolation from beekeepers' colonies and from wild colonies (the surrounding land is mostly wetland and young forest). This site was used in a previous study of colony spacing and drift (Seeley & Smith 2015). As shown in Fig. 3.2, we established an apiary containing three mite-donor colonies (MDCs) arranged in a line and spaced 0.5 m apart, and two mite-receiver colonies (MRCs) spaced 1m from the MDCs. Two more MRCs were placed 50m from the MDCs, in opposite directions, and another two MRCs were placed 300m from the MDCs, also in opposite directions. We used this symmetrical array to replicate our test of distance effects.

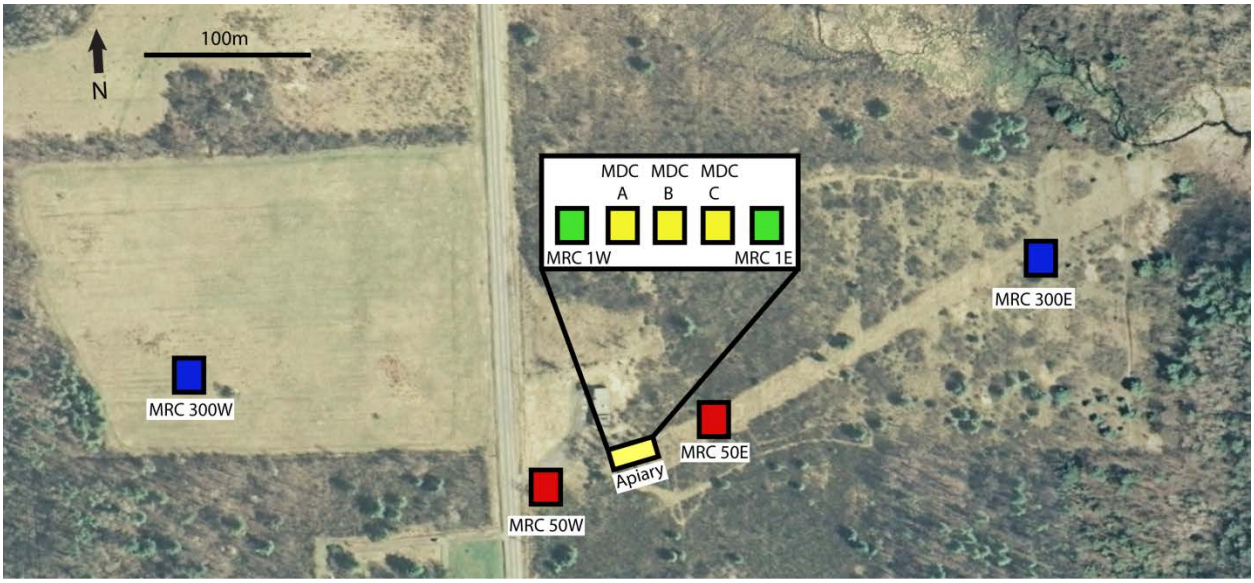

Figure 3.2: Birds-eye view of study site, showing the array of three MDCs and six MRCs. All hive entrances faced south.

To distinguish between resident and foreign bees at each hive, we used bees of two distinct colors. In mid-May 2017 we obtained 10 Cordovan Italian and 17 New World Carniolan queens from C.F. Koehnen and Sons, Inc. in California. The Cordovan queens (producing bright yellow offspring) were installed in nucleus colonies made from colonies with high mite infestations (1-3 mites per 300 bees) as determined by sugar shake (Dietemann et al. 2013). The New World Carniolan queens (producing dark offspring) were installed in nucleus colonies made from colonies with low mite counts (0-1 mites per 300 bees). In early August, the three Cordovan colonies with the highest mite populations and only yellow workers, and the six New World Carniolan colonies with the darkest workers were selected for use in the experiment.

MDCs were housed in two deep 10-frame hive bodies below a queen excluder, with a deep honey super on top to encourage use of the brood combs for brood rearing (to foster mite reproduction). MRCs were housed similarly, but with a screened bottom board beneath the bottom brood box for sampling of falling mites. Hives were painted various colors and assigned

among the colonies to make them distinctive. To boost the mite populations in the MDCs, we provided each one with 4 frames of drone comb. To prevent mite movement between MDCs and MRCs before the experiment began, we kept them in two isolated apiaries until we moved them to the experimental site. Once they were moved to this site, we placed an inverted hive cover in front of each hive and regularly inventoried its contents, to monitor the numbers of dead adult bees, immature bees, and mites leaving each colony.

We considered continuously treating the MRCs with miticides so that any observed mites could be counted as immigrants from the MDCs (as per Sakofski et al. 1990, Greatti et al. 1992, Frey et al. 2011). However, we did not, because continuous treatment could have left chemical residues repellent to mites, altered the behavior of the bees in the treated colonies, or tagged the MRC bees with an odor identifiable by guard bees in the MDCs. Withholding miticides also let us monitor the MRCs for mite-induced colony mortality.

We moved the MDCs to the experimental apiary on 16 August 2017 and the MRCs the next morning. From this point on, we took data at regular intervals through December 2017. Specific dates of data collection were adjusted for weather, but our target schedule was the following. (1) Every 10 days, get a sugar-shake count of the phoretic mites in each colony. (2) Every 5 days, count the mites that had fallen onto oiled boards beneath the MRCs, visually distinguishing (by color) between adult (dark) and juvenile (light) mites. (3) Every 4 days (as weather permitted), count 100 workers and drones entering or leaving each hive, noting how many were of the "wrong" color for the hive. If fewer than 100 workers or drones were observed in 5 minutes, we noted the percent of off-color bees seen (e.g. yellow bees entering or leaving a dark-bee hive.) After late November, we halted most data collection, but knocked on each MRC hive on 20 Dec, 20 Feb, and 20 April and listened for buzzing, to determine winter mortality.

When examining each colony we also assessed aggression at each hive entrance, to see if robbing was occurring. Specifically, we noted for each colony whether there was (1) no aggression, (2) worker-worker aggression but no other obvious signs of robbing, or (3) worker-worker aggression and other clear signs of robbing (e.g. hairless bees that had been stripped by combat with guards, and distinctive, side-to-side flight at hive entrance (v. Buttel-Reepen 1900, Cale 1946, Free 1955).) We also noted for each colony whether many (>20) dead, off-color bees had accumulated in front of its hive since the last observation, indicating fighting and killing at the hive. If we saw all these indicators at once, we describe the colony as experiencing intense robbing. When we saw robbing of the MDCs by dark bees, we tracked robbers to their hives using a modified version of Nickel Jacob's 16th century method (Fraser 1947): After dusting robbers and guards at the entrances of all three MDCs with powdered sugar, we examined each MRC and counted how many of 100 returning bees had powdered sugar on them, and then repeated the process a second time.

Results

Mite Population Dynamics:

We achieved rapid mite growth in three of our MDCs while at the same time we kept mite populations low in our MRCs before the start of the experiment (Fig. 3.3). The phoretic mite populations of the MDCs climbed to high levels in early August, peaked between mid-August and early September, then dropped precipitously as the colonies died. The phoretic mite populations in the MRCs began increasing soon after they were placed in the experimental array, and dramatic increases occurred between mid and late September, after the mite populations in the MDCs had peaked. The phoretic mite populations in the MRCs declined soon after these

spikes, and then remained at intermediate levels (between 5 and 20 mites/300 bees) for a few weeks until increasing dramatically (MRC 1E) or modestly (all others) by the last measurement on 28 Nov. There were no statistically significant differences, as determined by one-way ANOVA tests, between the different distance treatments for the MRCs in peak phoretic mite population ($F(2,1)= 0.845$, $p= 0.51$), final phoretic mite population ($F(2,1)= 1.91$, $p= 0.29$), or peak adult mite drop ($F(2,1)= 0.758$, $p= 0.54$).

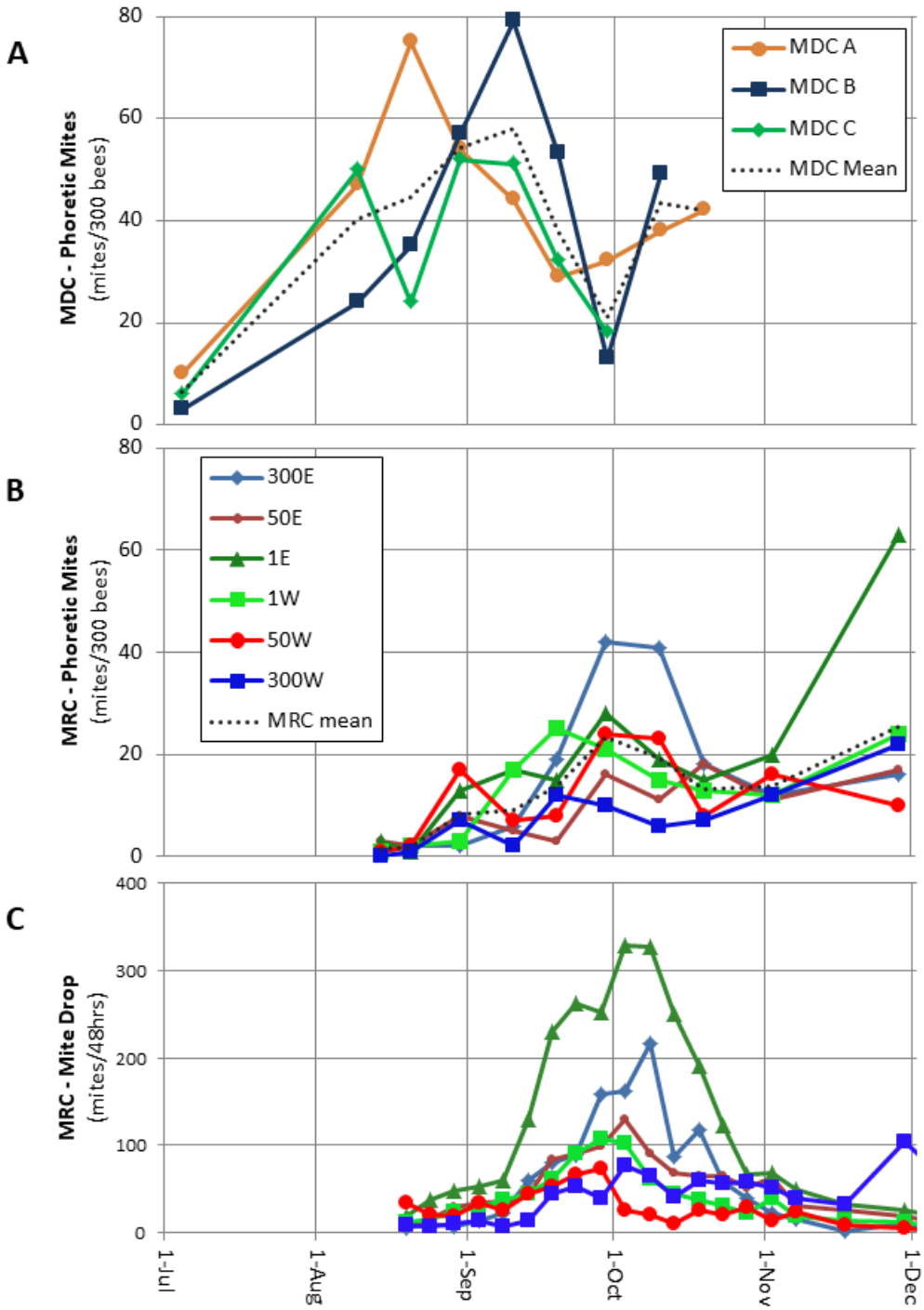

Figure 3.3: Phoretic mite infestation levels, as determined by powdered sugar shake method for MDCs (A) and MRCs (B), and mature (dark colored) mite drops onto oiled boards underneath MRCs (C). Phoretic mite measurements of MDCs ended when each colony died. Lines in bottom graph continue towards counts from last measurement date (12 Dec).

Robbing Dynamics:

We first detected worker-worker aggression at the entrances to some MDCs and MRCs on 21 Sept. We observed intense robbing of the MDCs between late September and mid October, the time period when the phoretic mite population in each MRC rose and then fell (Fig. 3.4). Between 10 Sep and 29 Sept all MRCs showed an increase in phoretic mite population, and four of the six showed a dramatic increase between 19 Sep and 29 Sep, which encompassed the first days of intense robbing of the MDCs.

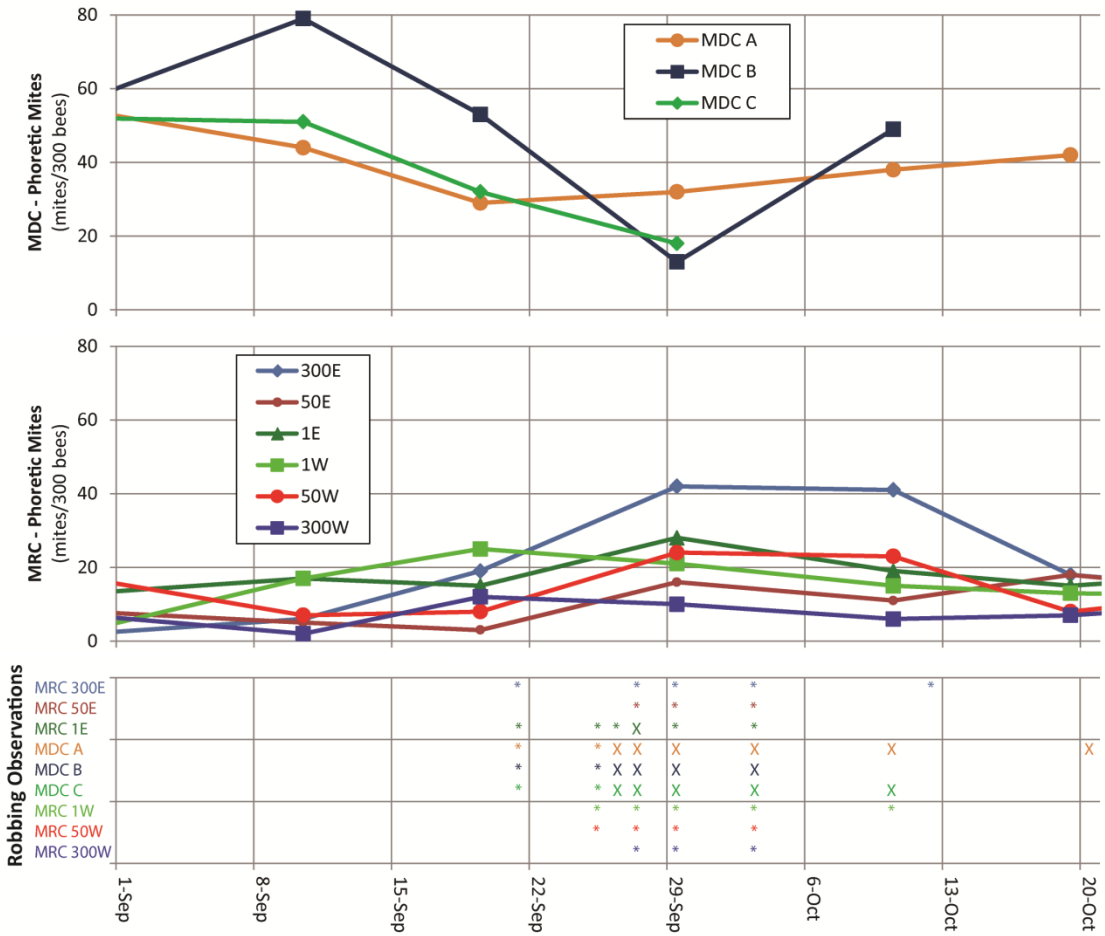

Figure 3.4: Robbing observations at MRCs and MDCs from 1 Sept to 20 Oct, with phoretic mite populations in MDCs and MRCs during that period. For robbing observations, the * signifies instances of worker-worker aggression, while X indicates more significant robbing, as indicated by characteristic robber bee behavior and large numbers of dead bees in front of the robbed hive.

Worker Drift Dynamics:

Figure 3.5 shows counts of yellow bees (from the MDCs) at the entrances of MRCs from mid August to late October. Before the robbing of the MDCs in late September, yellow bees were seen mostly in MRCs 1W and 1E. During the intense robbing of the MDCs on 25 and 27 Sept, yellow bees were seen entering most MRCs, but particularly MRCs 50W and 1W.

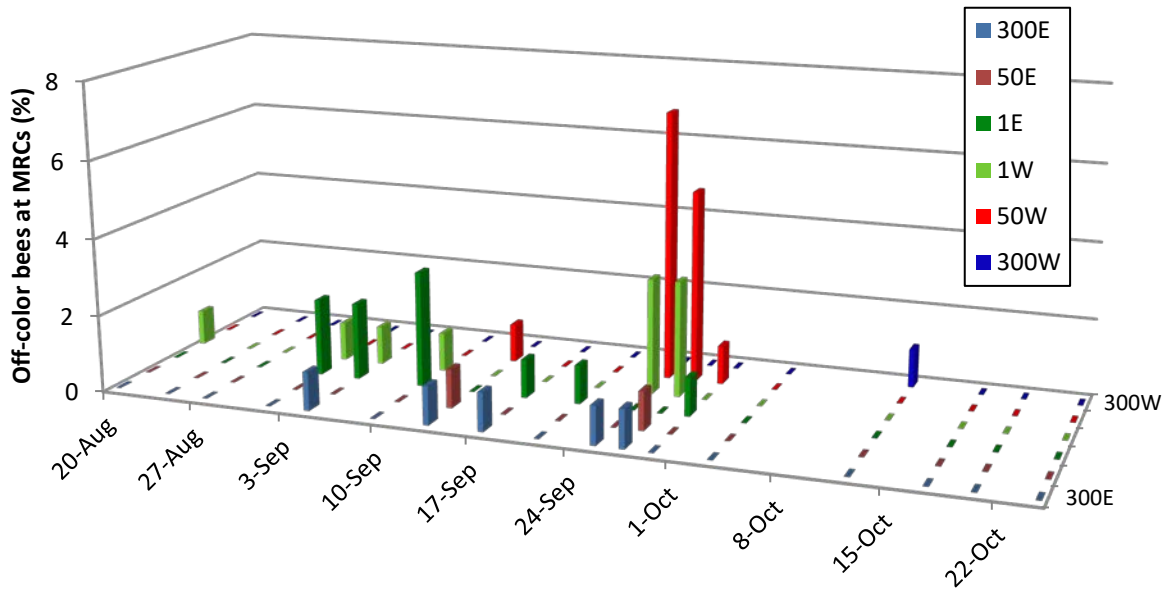

Figure 3.5: Percent of yellow workers at the hive entrance of each MRC, which each produced only dark workers. Off-color bees may have drifted, or were bees trying to rob from the MRCs.

Drone Drift Dynamics:

Soon after setting out the MDCs and MRCs in the array, we saw high levels of drone drift between the MDCs and the MRCs spaced at 1m (Fig. 3.6). Yellow drones from the MDCs were seen in MRCs at all three distances tested, but the vast majority (92.3% of all off-color drones in the MRCs) were seen in the two MRCs at 1m from the MDCs. The MDCs also readily accepted drifting black drones from the MRCs such that from 20 Aug to 15 Sept, on average 11.7%

(s.d.=8.1) of drones counted in all three MDCs were black. Before placing the colonies in the experimental array, we had confirmed that the MDCs contained only yellow Cordovan drones, and the MRCs contained only black drones.

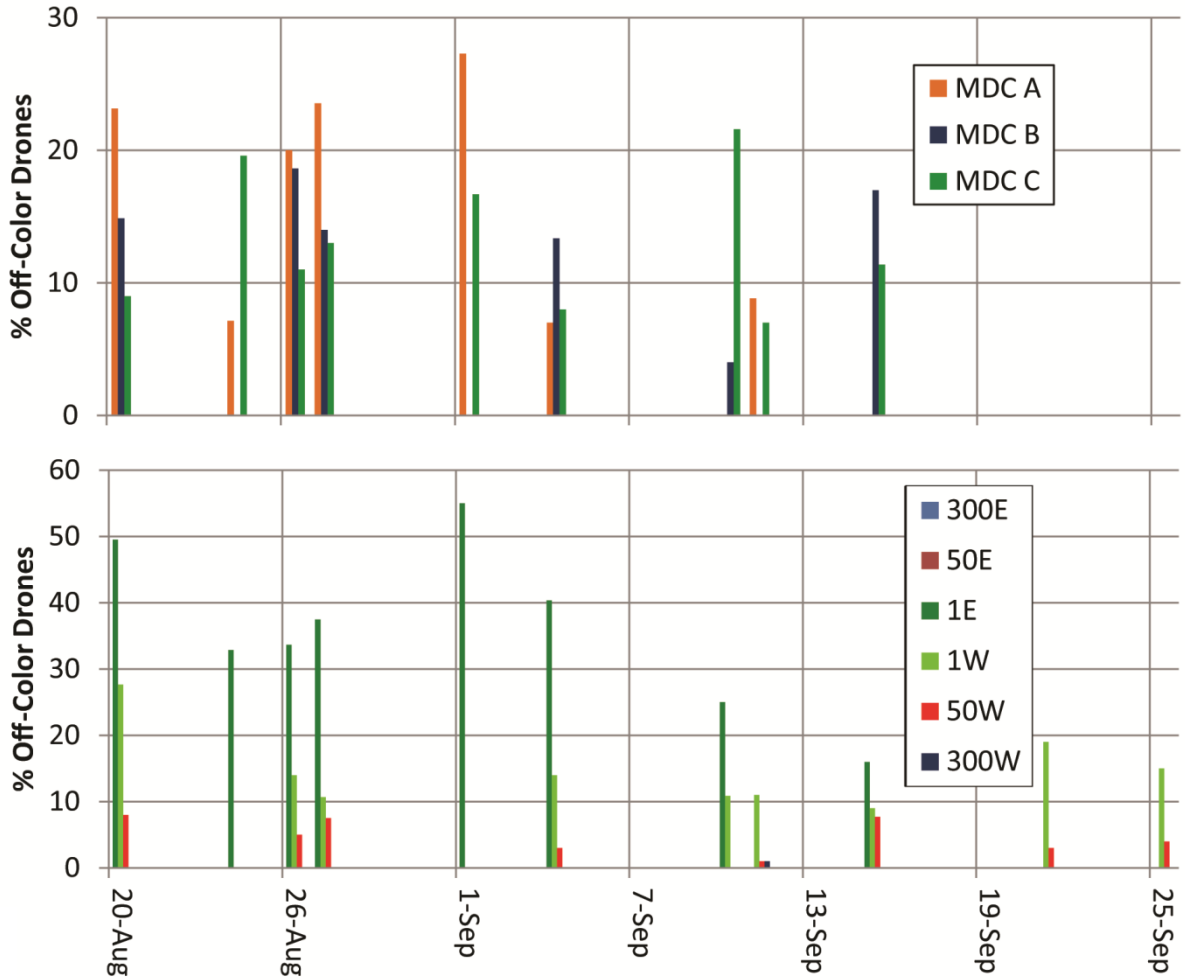

Figure 3.6: Drone drift into MDCs and MRCs over course of experiment, until all drones were evicted from colonies

Dead Mites and Bees Outside of Hives:

To document colony collapse in action, we placed a clean, inverted hive cover under the entrance to MDC A on 23 August, 6 days after the colonies were assembled in the array. We analyzed the contents of the cover each evening for 3 days, and we found that MDC A had

disgorged 111 cordovan drones, 66 cordovan workers, 121 drone pupae, 11 worker pupae, and 1241 mites, of which 799 showed a mature coloration. Exhaustion of the researcher counting the mites, not the mite supply, halted further data collection at the same detail, but comparable numbers of dead bees and mites were found in the hive cover on each day over the next week. As MDC B and C collapsed, we saw patterns of mite and bee outflow that matched those of MDC A. Some dead yellow bees in front of each MDC had shriveled or malformed wings, consistent with infection with Deformed Wing Virus. The only other striking change in number or identity of dead bees at the entrances of the MDC's hives occurred during the period of intense robbing in late September, when dead MRC (black) workers were suddenly found in large numbers outside all of the MDCs. Between 25-27 September, MDC A accumulated 500 dead workers, 90% of them black; MDC B accumulated 1340 dead workers, 88% black; and MDC C accumulated 215 workers, 72% of them black. For comparison, during this same time period, MRCs 1E and 1W accumulated 175 and 185 dead workers, 7% and 6% of them yellow, respectively. When we checked the MRCs for survival on 18 Dec, many (>100) dead black workers were found at the entrances of MRCs 1E, 1W, 300E, and 300W.

Sugar Dusting of Robbers:

On 27 September, a day with strong robbing (Fig. 3.4) of all three MDCs, we used powdered sugar to dust all bees at the hive entrances of the MDCs, and then we looked for the dusted robbers entering their home nests. We counted four dusted bees entering both MRCs at 300m, two entering the MRCs 50W, 1W, and 1E, and five entering MRC 50E. At the same time, a few (1-3) yellow workers without powdered sugar were seen entering MRCs 300E, 1E, 50W, and 300W, indicating that during this period of robbing of the MDCs there was wide-ranging intercolony traffic of workers.

Colony Mortality:

All three MDCs were dead (had a dead queen and no live bees) by 10 Oct, 19 Oct, and 2 Nov, respectively. After the deaths of the MDCs, we checked the MRCs bimonthly to monitor their winter mortality. MRC 1E was dead by 20 Dec. MRCs 1W, 300W, and 300E were dead by 20 Feb 2018. Both MDCs 50W and 50E survived to the spring of 2018.

Discussion

The patterns reported here with respect to the Varroa population dynamics in the MRCs, the robbing of the MDCs by workers from the MRCs, and the drifting of workers and drones from the MDCs, all help us understand the processes that cause the "mite bomb" phenomenon: when some colonies in a location collapse from high mite loads, the mite loads in neighboring colonies often surge to high levels. In our experiment, three MDCs and six MRCs were brought together in the middle of August, when the phoretic mite counts in the MDCs were high and rising rapidly, but these counts were low in the six MRCs. In late September, however, the phoretic-mite counts and the mite-drop counts in the MRCs suddenly started to rise strongly (Fig. 3.3). What process explains this pattern of surging mite loads in the MRCs? Our data suggest that robbing of the MDCs by workers from the MRCs played the largest role in producing the pattern (Fig. 3.4), but that drifting of mite-infested workers and drones from the MDCs to the MRCs was evidently also important, especially for MRCs closest to the MDCs.

As the MDCs sickened and died over the course of the experiment, the previously healthy MRCs developed large mite populations. The average maximum phoretic mite count in the MRCs was 32.3 mites per 300 bees. One of our goals was to determine ideal apiary layouts and management practices to minimize mite transmission from collapsing colonies. Though the first MRC to die in winter was 1m from the MDCs, the only colonies to survive until the next spring

were those spaced 50m from the MDCs, counter to our hypothesized protective effect of intercolony distance. Past studies of mite population growth where "mite resistant" and "mite susceptible" colonies were housed in the same apiaries may have to be revisited. The ease with which these parasites can spread reveals the danger of assuming that all mites found in a colony were born there.

On 10 Sep, before the first observation of worker-worker aggression or overt robbing of the MDCs, the phoretic mite populations in the MRCs ranged from 2 to 17 mites per 300 bees (Fig. 3.4). By 29 Sep, after all three MDCs had been intensely robbed, MRC phoretic mite counts ranged from 10 to 42, with an average increase of 14.5 mites per 300 bees. During this same 19-day period, the phoretic mite measurements of the MDCs fell from 51 to 18, 79 to 13, and 44 to 32 mites, thus with an average decrease of 37 mites per 300 bees. Thus, it appears that robbing of collapsing colonies is responsible for the movement of large numbers of mites to relatively healthy colonies. The sugar dusting of robbers confirmed that every MRC engaged in robbing of the MDCs, exposing the MRCs to mites regardless of distance from the MDCs; The most dramatic post-robbing spike in phoretic mites was seen in MRC 300E. We have previously shown (Peck & Seeley 2018) that honey combs spaced 1km apart in a forest inhabited by naturally distributed wild colonies (roughly 1-3 colonies per km^2 (Seeley 2007, Seeley et al. 2015, Moritz et al. 2007, Hinson et al. 2015)) are rapidly and efficiently discovered and robbed, meaning intercolony distances greater than 300m may still offer little mite protection. The calm behavior of robbers inside robbed hives (Peck & Seeley 2018) likely makes it easy for mites to infest them, especially once the robbed hive's guards have been overcome.

Drifting appears to have also played a role in mite transmission from collapsing colonies to healthy ones, particularly those within the central apiary. Prior to the onset of robbing most

worker and drone drift from the MDCs was into MRCs 1E and 1W (Fig. 3.5, Fig. 3.6) and on 10 Sep these MRCs at 1m had more phoretic mites than those at 50m or 300m. However, after one week of robbing, this was no longer the case. The highest drone drift into the MRCs was seen the day after these colonies were moved to the array, and this was almost exclusively into the MRCs 1m from the MDCs (Fig. 3.6). This short range suggests that drones probably drift due to orientation errors, and not as part of an adaptive invasion of other colonies. Drift may have been higher had our hives been painted the same color; Jay (1966b) found that installing colored boards above hive entrances could reduce the number of marked workers drifting within an apiary from an average of 39% to an average of 9%. Despite the low levels of drift, the steady increase in MRC mite populations from the experiment's start until robbing began (Fig. 3.3, Fig. 3.4) suggests that mites do migrate into new colonies via worker and drone drift well before the onset of robbing. Close proximity to colonies with high mite levels, therefore, imposes a steady disease risk to healthy colonies. We regularly observed a few drifted workers from the MDCs in the MRCs 300m away, suggesting that completely protecting a colony from drift would require intercolony distances even greater than 300m.

If drift into the MRCs increased as the population of mites in the MDCs increased, for either workers (Fig. 3.5) or drones (Fig. 3.6) we did not detect it. Though phoretic mites typically infest younger bees, some can be found infesting older bees like foragers (Kuenen & Calderone 1997) especially when phoretic mite infestations climb above 20% (Cervo et al. 2014). Some have proposed that these mites may be adapted to dispersal by selectively infesting foragers, perhaps even increasing the rate at which infested bees drift by impeding their orientation (Kralj & Fuchs 2006). This conforms to the concept of the "mite bomb" blasting mite-infested bees into neighboring colonies in a sudden, shrapnel-like explosion. Our data (Fig.

3.5, Fig. 3.6) concur with those of Goodwin et al. (2006) who found no dramatic increase in drift during the mite-induced death of a colony, but instead relatively constant rates of drift out of the dying colonies. We observed only one brief but dramatic increase in the number of off-color workers entering the MRCs (Fig. 3.5), and it occurred precisely as we observed intense robbing in the MDCs (Fig. 3.4). However, the total number of off-color bees in the MRCs was considerably lower than the 40% drift that has been estimated for colonies in apiaries (Pfeiffer & Crailsheim 1998). The increase in MDC workers in the MRCs correlated with the onset of robbing, not the peak mite levels in the MDCs. In addition, worker drift should have led to a sustained increases in the number of off-color bees in the MRCs, not the ephemeral increase we observed, suggesting that this increase in off-color bees entering the MRCs may have been bold robbers from the MDCs stealing honey for their failing colonies, or disoriented MDC bees who quickly died or made their way home after the robbing had ended. This suggests that this elevated "drift" may still ultimately have been caused by robbing, and not by a manipulation of the bees from the MDCs.

We did not treat our MRCs with miticides, so some of the mite growth in these colonies was the result of natural reproduction and not introgression from other colonies. Not treating allowed us to detect mite-related mortality, and left us confident that the natural behavior of the mites and bees wasn't altered by the presence of chemical miticides. We decided to perform this experiment with a cluster of three MDCs, instead of a single collapsing colony for two reasons. First, three collapsing colonies provided three times the mites a single colony would have provided, increasing the magnitude of the mite influxes we could observe in the six MRCs. Second, using three colonies increased the likelihood that at least one colony would collapse during our observations. Consequently, some of the bees and mites leaving each MDC might

have entered the other two MDCs, which we could not detect. Therefore, we cannot tie specific mite level increases in the MRCs to specific changes in any one MDC. Future studies must balance the need to observe mite-induced colony death and the desire to assign individual drifting or robbing bees to their natal hives. No feral colonies were known in the landscape around our study site, and the area does not contain mature forest likely to provide many nesting cavities, but robbing from feral colonies is a possible confound of our distance test. However, the fact that robbers from every MRC were found to be robbing the MDCs suggests that the collapsing colonies in the center of our experimental array are at least among the sources of late-season mites found in even the most distant MRCs.

Our data carry a troubling implication – that the largest, strongest, and healthiest hives in a landscape may be the ones most likely to rob, and thereby acquire a large number of mites from sickly colonies. This suggests that even in naturally living bee populations such as those reviewed by Locke (2016), natural selection for mite resistance among untreated colonies may be undercut by the robbing-mediated mite-induced death of otherwise healthy colonies. "Treatment-free" beekeepers attempting to breed mite-resistant bees by withholding chemical treatment may improve their breeding outcomes if they decline mite treatments throughout the year, but treat after local robbing to eliminate mites imported from nearby mite-susceptible colonies. Oliver (2018) describes how he may have mistakenly rejected some colonies for queen breeding due to high autumn mite levels caused by mite importation via robbing.

Our findings confirm that mite-infested colonies pose a risk to nearby colonies, but the colloquial terminology for these colonies - "mite bombs" - does not accurately reflect the mechanisms of mite transmission, as there is no sudden "explosion" of mite-infested bees from the sick colonies. Instead, infested colonies slowly pass mites through worker drift (largely to

nearby colonies, Fig. 3.5) and drone drift (almost exclusively to nearby colonies, Fig. 3.6) until their weakness and a local nectar dearth make the infested colonies an irresistible robbing target to stronger colonies in the area. We suggest that depending on circumstances such sickly colonies be framed as "mite leakers" for the risk they pose to hives within their apiary, or "robber lures" for the temptation they offer colonies in a broad area to rob them. This distinction is important: If the sickness of the host doesn't reduce (or increases) the opportunities for the parasite to spread, the parasite should evolve higher virulence (Ewald 1987; 2004). If mites spread primarily through robbing, selection should favor mites (and mite-borne viruses) that severely weaken their host colonies, to make them more attractive targets to robbers. If instead drift of manipulated bees was the primary mechanism of mite dispersal, selection might favor mites and viruses that left colonies healthy enough to produce large numbers of drones and workers (see: DeGrandi-Hoffman et al. 2017). The moderate, local mite transmission risk from drifting and the high, long-distance risk from robbing indicate that managed bee colonies must either be completely isolated from one another, or must be managed with the expectation that a significant importation of mites will occur.

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Chapter 4: Multiple mechanisms of behavioral resistance to an introduced parasite, *Varroa destructor* in a survivor population of European honey bees

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Abstract

Mesostigmatid mites of the genus *Varroa* are specialist parasites of honey bees (genus *Apis*.) A recent (c. 100 years) host shift event enabled mites specialized to exploit Asiatic honey bees (*Apis cerana*) to infest colonies of European honey bees (*Apis mellifera*) and speciate into *Varroa destructor*, causing economic and ecological harm. We investigated how a population of western honey bees living without human management or mite treatment in the Arnot Forest near Ithaca, New York is able to survive with these parasites. We describe multiple mite-resistance phenotypes, including high levels of hygienic behavior and grooming behavior, which may synergize to significantly limit the reproduction of *Varroa destructor* in these colonies. Comparing observed levels of mite-resistance phenotypes to historic measurements of similar unselected populations of honey bees, we infer baseline levels of these mite-resistance phenotypes. Our data are consistent with the hypothesis that the Arnot Forest survivor bees have rapidly evolved behavioral resistance to these parasites through increased expression of multiple, apparently independent, mite-resistance mechanisms.

Introduction

The ectoparasitic mesostigmatid mite *Varroa destructor* is currently a major health threat to European honey bees (*Apis mellifera*) (Dahle 2010, Genersch et al. 2010, Neumann & Carreck 2010, Rosenkranz et al. 2010, VanEngelsdorp et al. 2011). As mites feed on both adult and juvenile bees, they vector pathogenic bee viruses with disastrous consequences for bee health (Kevan et al. 2006, Schroeder & Martin 2012, Martin et al. 2012). The mite *Varroa jacobsoni* experienced a recent (<100 yrs) host shift from the Asian honey bee (*Apis cerana*) onto the European honey bee (*Apis mellifera*) and in so doing speciated into *Varroa destructor* (Anderson & Trueman 2000). Biocontainment efforts to limit the spread of the mite were largely unsuccessful, and the mite is now present in bee populations globally, with the exception of Australia and a few island populations (Isle of Man Government 2015, D.A.W.S. Australia 2016). *V. destructor* was first documented in the United States in the mid-1980s meaning free-

living populations of bees have had only a few decades in which to adapt to the mite, or die. Globally, bee colonies of European stock that do not receive beekeeper-applied mite treatments normally succumb to the mites and viruses (Sammataro et al. 2000, Dainat et al. 2012) typically within 2-3 years (Rosenkranz et al. 2010).

With the great economic and ecological threat posed by this emerging parasite, the need to understand Varroa-resistance in honey bees is of paramount importance. Various Varroa-resistance phenotypes have been identified in honey bees (Rinderer et al. 2001, Harris et al. 2010, Locke et al. 2014; reviewed in Boecking & Spivak 1999). Bee breeders have sought to produce queens whose colonies suppress mite populations so that beekeeping can return to its pre-Varroa state, often focusing on single resistance mechanisms. In addition to these focused breeding programs, a small number of so-called "survivor bee" populations have been identified across the globe which survive without chemical Varroa treatment or beekeeper intervention (Fries et al. 2006, Le Conte et al. 2007, Seeley 2007, Locke & Fries 2011, Locke et al. 2012, Oddie et al. 2017; reviewed in Locke 2016). Whether the Varroa-resistance of these naturally selected populations is greater or less than that of artificially selected bees remains an open question.

Of bees possessing naturally selected resistance for Varroa mites, the honey bees of the Arnot Forest near Ithaca, New York are particularly intriguing. The capture of mite-infested swarms from this forest in summer 2003 (Seeley 2007) reveal that the bees of this forest have been infested with Varroa mites for at least 15 years. Remarkably, when the density of colonies living in this forest was measured after the arrival of Varroa (in 2002 and 2011) it was found to be the same as in 1978, well before the arrival of Varroa (Visscher and Seeley 1982, Seeley 2007, Seeley et al. 2015). Furthermore, genetic comparisons between bees collected in the

Arnot Forest (and surrounding forests) in 1977 and in 2011 showed that the Arnot Forest bees are not recently immigrated swarms from local managed bees, but instead are the descendants of the bees that were living in the forest forty years ago (Mikheyev et al. 2015, Seeley et al. 2015). These genetic analyses also yielded two potentially important insights into the history of the Arnot Forest bees, namely (1) that some of the genetic changes between 1977 and the present are in genes implicated in learning, development, and behavior, suggesting that behavioral evolution might have taken place, and (2) that the bees experienced a dramatic matrilineal bottleneck between 1977 and 2011, which is presumed to have been caused by the arrival of Varroa (Mikheyev et al. 2015). Therefore, a pressing need exists to identify and characterize precisely how natural selection has solved the problem of the Varroa mite in this population of free-living honey bees.

Varroa-sensitive hygienic behavior, the selective removal of pupae infested with reproducing mites, is a direct mechanism by which bees might suppress mite reproduction (Boecking & Drescher 1992, Harbo & Harris 2005, Harbo & Harris 2015). However, due to the difficulty of precisely monitoring whether brood hygiene is sensitive to Varroa-infestation, many breeding programs have instead focused on promoting hygienic behavior that is not specifically sensitive to Varroa mites, i.e. the detection and rapid removal of any dead or unhealthy pupae (Spivak 1996, Spivak & Downey 1998, Dietemann et al. 2013). When mites phoretically infest adult worker bees, high levels of mite-focused grooming behavior would allow a colony to directly kill and remove mites, either through self-grooming or allogrooming, and some breeding programs have therefore focused on breeding bees that groom and damage large numbers of mites from adult bees (Hunt et al. 2016). Recent work on a number of resistant bee populations has proposed a different mechanism of mite control: high levels of uncapping and recapping the

cells of pupating bees might disrupt mite reproduction enough to confer significant mite-control to the colony (Oddie & Büchler et al. 2018). Various mechanisms have been hypothesized to increase the percentage of mites in brood that fail to reproduce, leaving a large number of non-reproductive mites detectable in the brood (Harbo & Harris 2005, Ibrahim & Spivak 2006).

In this investigation, we obtained a number of genetically pure Arnot Forest swarms and housed them in moveable frame hives. We examined mite-resistance traits in this population under conventional beekeeping conditions, using assays that have previously been used on both mite-resistant and mite-susceptible bee populations, thereby allowing comparison of the Arnot Forest bees to those populations. By assaying brood hygienic behavior, colony-level mite grooming behavior, brood uncapping and recapping behavior, mite reproductive success, and by analyzing worker comb cell dimensions, we can offer a glimpse into the nests of these wild-living survivor bees and suggest how they may be persisting with Varroa.

Methods

Source of Bees:

The resistant bees we investigated came from the bee population of the Arnot Forest (42°17'N, 76°39'W, altitude 585 m), a 17 km^2 teaching and research forest preserve owned by Cornell University. The Arnot Forest is a high-elevation (310-620 m) rugged forest which spans southern Tompkins County, and northern Chemung and Schuyler Counties, New York. The region is mostly (96%) forested with both hardwood and softwood forest in a wide range of successional stages (Odell et al. 1980) and the Arnot Forest is surrounded by similarly forested land with few dwellings (Seeley et al. 2015). The honey bee population living in the Arnot Forest has been repeatedly surveyed and studied, (Seeley 2007, Tarpy et al. 2015, Seeley et al.

2015) and represents a unique population that has been genetically confirmed to have persisted in the wild through the arrival and establishment of the Varroa mite (Mikheyev et al. 2015). The unique advantage of studying this population is that we can be reasonably assured that the bees in the forest truly represent an established population of surviving bees, and not colonies resulting from repeated spillover from a beekeeper's managed colonies.

We obtained bees from the Arnot Forest population by hanging bait hives from tree limbs and by placing bait hives on hivestands or buildings throughout the forest. Bait hives were placed along the forest's central roadway at 1km intervals. Each bait hive was comparable to a Langstroth nucleus hive, and contained four frames of drawn wax comb. The risk of bear attacks necessitated hanging most of the bait hives, and also necessitated removing caught swarms from the forest and conducting experiments at Cornell University's bee yards in Ithaca, NY. When swarms of bees occupied the boxes, they were screened and removed from the forest after sunset. In addition to colonies obtained via swarm capture, additional Arnot colonies were made by splitting existing Arnot colonies, letting them rear queens, mating those daughter queens in the Arnot Forest on "bear-proof" mating stands, and then returning these colonies to Ithaca for testing. If swarming or queen mortality caused an Arnot queen to be replaced while in Ithaca, the colony was removed from the study population despite its Arnot Forest matriline, to ensure that only pure Arnot population genetics were evaluated.

All the Arnot-derived colonies brought to Ithaca were widely spaced within an apiary to minimize drift or robbing which might lead to an exchange of bees or mites. All colonies were placed at least 50 meters from the nearest colony, except one pair that were placed 8m apart as space became limited. To further protect bees from navigation errors, all colonies were housed in differently colored hives whose entrances faced in various directions. All colonies were

housed in 10-frame ‘deep’ Langstroth beekeeping equipment, and were managed to prevent swarming and the filling of the broodnest with honey.

We also obtained queens from three sources – a commercial queen breeder operating out of California, a commercial queen breeder operating out of Vermont, and a population of “yard” bees from a local apiary that had been regularly and consistently treated for Varroa mites prior to the start of the experiment, hence had no strong selective pressure to evolve resistance to Varroa infestation. Colonies headed by these queens were used as contemporaneous controls in our various tests, as described below.

Behavioral Resistance Tests:

Hygienic Assay:

Brood hygienic behavior was studied through use of the freeze killed brood assay. Ongoing work (Spivak & Downey 1998, Ibrahim & Spivak 2006) to breed Varroa-resistance by selecting bees for hygienic behavior has provided a standardized methodology to evaluate this trait (Dietemann et al. 2013). Circles of capped purple-eyed worker brood were frozen using liquid nitrogen, and then placed back into the colonies and checked after 24 hours to determine how many cells had been uncapped, and of those how many had been partially or fully emptied. After recording these data, each frame was returned to its colony’s hive. If any pupae remained capped, a second measurement was taken 48 hours after freezing, except for measurements in August of 2015, which were conducted only after 24 hours. Measurements were taken over the course of three summers (2014-2016) but only if a colony met certain conditions: it was rearing at least 7 ‘deep’ frames of brood, and it had enough bees to cover at least 15 ‘deep’ Langstroth frames. These requirements ensured that colony size and demographics did not cause variation in each colony’s performance on the assay (Spivak & Gilliam 1993). When possible, we

conducted repeated measurements on the same colonies, though swarming, slow growth, and overwinter colony losses sometimes prevented this. For colonies that could be tested more than once, all measurements on a colony were averaged together to produce a phenotype level for each colony. In addition to the Arnot Forest bees, we also measured this trait in our local miticide-treated bees (2015), and our colonies headed by commercial queens from California (2015) and Vermont (2016).

Grooming Assay:

We assessed the mite-grooming behavior of the Arnot Forest bees by finding the percentage of adult mites collected from the bottom of each hive with damage to their legs and/or dorsal plates, as described by Hunt et al. (2016). Eight Arnot Forest colonies were evaluated from July 22nd to July 24th 2016, and simultaneous control assays were conducted on four colonies headed by queens from the commercial breeder in Vermont who is not known to systematically select for grooming behavior. Each colony contained between 15 and 20 'deep' frames of bees, were assessed by placing a corrugated plastic board covered in canola oil on the solid bottom board under each colony, beneath a screened bottom board (Dadant and Sons, Illinois). The layer of oil was sufficient to kill and hold in place any mites that fell from the colony above, as well as to prevent invasion by any ants that may have otherwise interfered with the fallen mites. When the sticky boards were removed, the minimally adhesive oil let us easily examine each fallen mite under a dissecting microscope to assess damage. First, each mite was categorized as either a juvenile or an adult, based on the color of the exoskeleton. Then, the number of legs which were damaged or missing on the mite was recorded, as was any apparent damage to the mite's dorsal plate. "Dimples" in the mites dorsum were excluded from consideration. If a colony had more than 120 mites on the oil board, only 120 mites were

sampled, and these were taken from vertical and horizontal transects across the board to ensure a random sample of mites were scored for grooming damage.

Uncapping/Recapping Behavior and Mite Reproduction Rate:

Recently, Oddie & Büchler et al. (2018) have suggested that some colonies protect themselves from Varroa mites by uncapping cells containing reproducing mites, disrupting the mite's reproductive process, and then recapping the cell to allow the bee inside to continue pupating. This uncapping/recapping behavior can be detected by cutting the caps off of cells over pupating bees nearing emergence, and examining them for a characteristic light spot where the pupal cocoon was broken during uncapping, and subsequently filled back in with wax during recapping. On 4 Sept, 2016 we froze a frame from each of 8 Arnot forest colonies, containing a cohort of capped workers within 48 hours of expected emergence. We examined 150 cells from each frame (1200 total), and determined how many of the cells had been uncapped and recapped (Oddie & Büchler et al. 2018). For these cells and others (2103 total) we determined the percent of cells infested by mites, and the percent of those infested cells likely to have produced at least one mature and mated daughter mite (i.e. containing at least one mother mite, at least one male mite, and at least one daughter mite whose developmental state made it likely to be mature and mated by the time the bee emerged from the cell (Martin 1994, Martin 1995, Dietemann et al. 2013).

Cell Size Measurements:

It has also been suggested that honey bees might control Varroa reproduction by building smaller cells in which to rear young bees, therefore reducing the amount of space available for mites to maneuver and mate on a developing bee pupa (Erickson et al. 1995). Three of the hanging bait hives set out in the Arnot Forest for this study attracted swarms which built some

quantity of comb before they were recovered and moved into standard Langstroth beekeeping equipment. All were combs of worker cells. To analyze average cell size, we used calipers to measure the internal distance between parallel faces of 10 cells on each side of each lobe of comb.

Historic Data on Resistance Phenotypes:

The great strength of the Arnot Forest population is that we have historic measurements from the forest in the form of preserved bees and ecological surveys from the 1970s, well before the arrival of Varroa. However, despite these advantages compared to other purported survivor populations, we have no direct measurements of pre-Varroa behavioral resistance traits among the Arnot Forest bees. Therefore, we designed our studies to allow justifiable comparisons between our measurements of the Arnot Forest bees and other populations of bees that were measured before they had experienced either natural or artificial selection for Varroa resistance.

Varroa was first detected at Cornell University bee yards in 1994 and likely arrived in the Arnot Forest around the same time, so we use data from two published studies of hygienic behavior measured in 1995 in unselected bees. The first study, Spivak and Downey (1998), used the same freeze-killed brood assay to study hygienic behavior both in bees that had been actively selected for hygienic behavior, as well as a number of colonies that had not been actively selected for hygienicity. The second study, Oldroyd (1996), reported the hygienic behavior of bees from 10 different breeders in Australia which had not (and still have not) been exposed to Varroa mites.

The work by Hunt et al. (2016) at Purdue University has showed that with attentive selection, the mite grooming tendency of honey bees can be artificially increased. We include two data points from their published report: The baseline mean mite grooming performance of

the unselected population before their selection work began in 2007, and the mean mite grooming performance of the population in 2015 after 8 years of steady selection for grooming behavior.

To infer change in the cell size of the Arnot Forest bees we compared data from combs built by these bees to data from combs taken from three local honey bee colonies in tree cavities, located, dissected, and photographed in 1975. (Seeley 1976) These photographs contained rulers on the same plane as the combs, and therefore allowed us to measure the dimensions of cells obtained decades before the arrival of Varroa.

Results

Hygienic Behavior:

Bees from the Arnot Forest population showed high freeze-killed brood assay responses. The average hygienic behavior of all Arnot Forest colonies assayed was 72.9% (s.d.= 21.3, n=12) in 24 hours, and 96.5% (s.d.= 6.4, n=11) in 48 hours. (Fig. 4.1) Control bees from two commercial queen breeders in Vermont and California also demonstrated high levels of the hygienic trait, while queens from our research apiaries that had been treated for mites regularly and so were not subjected to selection for hygienic behavior demonstrated much lower levels. Figure 4.1 also includes reproduced data from past studies of hygienic and unselected bees in Minnesota, and from unselected queen lines from ten breeders in Australia. Figure 4.1 shows data for cells emptied in 24 and 48hr, while Table 4.S1 also includes data on the rate of cell uncapping.

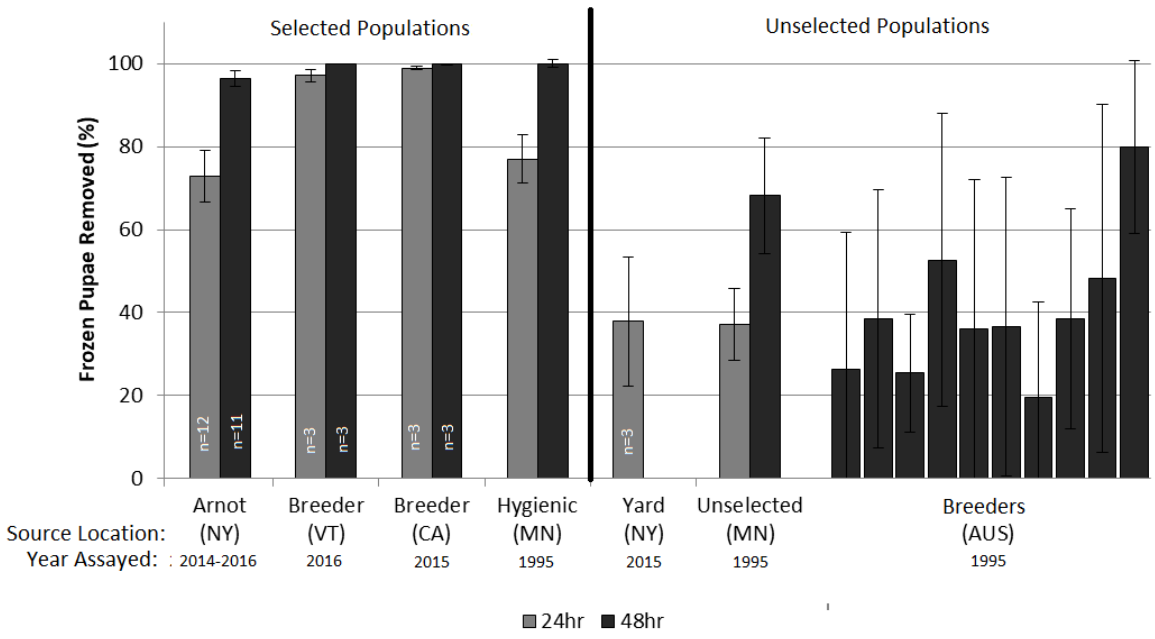

Figure 4.1: Mean percentage (± SEM) of freeze-killed brood removed from colonies with various backgrounds, assessed at 24 and 48 hour marks. Data on both Minnesota and Australian bees were obtained from published reports: Hygienic and Unselected Minnesota bees were tested in 1995, and reported in Spivak and Downey 1998. Australian bees from 10 different queen breeders were tested in 1995, and reported in Oldroyd 1996.

Grooming:

On average, 34% (s.d.=7.4) of the mites collected under the Arnot Forest colonies were damaged, which is a significantly higher percentage than was found the same year (2016) under control colonies from a queen breeder in Vermont, where only 20% (s.d.=6.5) of mites were damaged, t two-tailed (10)= 3.071, p= 0.012 (Fig. 4.2). Data from a population of bees at Purdue University are reproduced in Fig. 4.2, showing that the percent of damaged mites recovered beneath colonies climbed from less than 5% before selection began to nearly 50% after eight years of queen breeding and selection targeting grooming behavior.

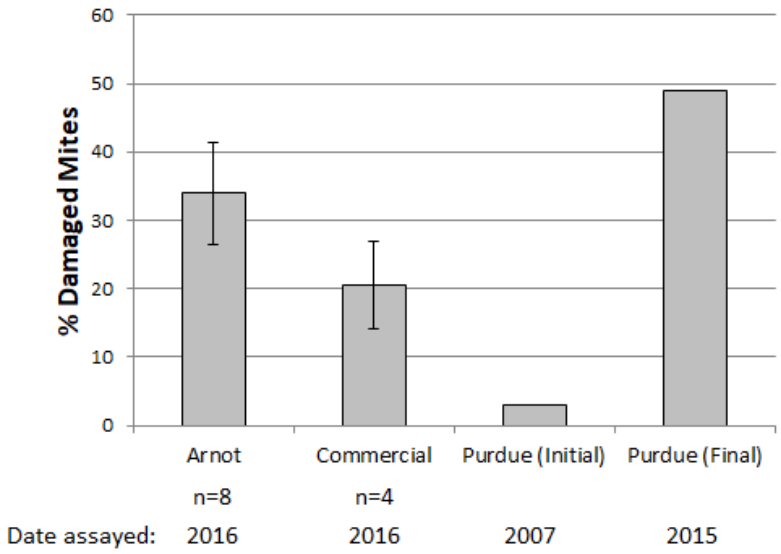

Figure 4.2: Percent damaged mites (±s.d.) recovered from oil-coated boards placed beneath screened boards under colonies with various backgrounds. Data from Purdue bees come from initial baseline and most recent published results of program breeding bees for grooming performance, (Hunt et al. 2016).

Uncapping/Recapping Behavior and Mite Reproduction:

By opening a cohort of capped cells containing worker pupae approximately one day before bee emergence, we determined the incidence of cell uncapping and recapping by the bees and mite reproductive success rate. Table 4.1 shows the percent of 150 cells in each of eight Arnot Forest colonies that had been uncapped and recapped. Of the 2103 cells examined, 410 were infested with mites, with an average infestation of 20.1% (s.d.= 12.07). Of the infested cells, 74.2% (s.d.= 13.35) contained one or more mature female mites, at least one male mite, and at least one daughter mite old enough relative to the age of the bee to mate prior to the bee's emergence from the cell. We also checked 1200 of the cells we opened for recapping, finding 28.4% (s.d.= 19.76) of all cells had been recapped. Colony 1 was the only one of these to live through the following winter.

Table 4.1: For eight Arnot Forest colonies, the percent of assayed cells that had been uncapped and recapped, the percent of assayed cells that were infested with mites, and the percent of infested cells containing at least one daughter mite likely to be mated, for both singly infested cells and cells with any number of foundress mites.

Colony ID	% Recapped **(cells assayed)**	% Infested **(cells assayed)**	% Infested successfully reproducing (one foundress)	% Infested successfully reproducing (all infested)
1	9.3 **(150)**	8 **(350)**	46.4	46.4
2	14 **(150)**	15.9 **(251)**	75.0	85.0
3	4 **(150)**	4.8 **(250)**	91.7	91.7
4	34 **(150)**	20.4 **(250)**	49.0	72.6
5	54 **(150)**	40.4 **(250)**	48.5	71.3
6	16.7 **(150)**	20.4 **(250)**	49.0	72.6
7	48 **(150)**	16.4 **(250)**	75.6	80.5
8	47.3 **(150)**	34.1 **(252)**	57.0	73.3

Cell Size:

The internal wall-to-wall dimension of the worker cells obtained from the three Arnot Forest swarms was 5.14mm for the first swarm, 5.27mm for the second, and 5.24 for the third, for an average dimension of 5.22mm (s.d.= 0.056) (n=3). For historic comparison, three bee trees in the forests around Ithaca, NY that were cut open and dissected in 1975 had worker combs with wall-to-wall dimensions of 5.19mm, 5.12mm, and 5.25mm for an average historic dimension of 5.19mm.

Discussion

The honey bees of the Arnot Forest express both hygienic behavior and Varroa-grooming behavior at levels seen in populations of bees artificially selected for these traits over periods of years. They also show varied levels of cell uncapping/recapping behavior, and have relatively small cell sizes compared with commercially available combs. The number of colonies we checked for these various phenotypes (n=8-12 colonies for all except cell size) are likely

representative of the entire population of bees within the Arnot Forest, which has been repeatedly estimated at ca. 20 colonies. (Seeley 2007, Seeley et al. 2015) While we sampled bees only from the Arnot Forest population, these represent only a portion of the population of hundreds of colonies living in the forests of the region. Because there are not beekeepers in or near the forest (Seeley et al. 2015), and because the extant bee population can be genetically traced to the bees living in the forest in 1977 (Mikheyev et al. 2015), we feel confident that modern Arnot Forest bee resistance is the product of natural selection, and not introgression of artificially selected resistance genes. Our data show that the Arnot Forest bees are not using just one resistance mechanism to survive with Varroa, but instead a combination of at least two mechanisms. Similarly, mite-resistant bees from Primorsky, Russia have experienced strong selection for mite-resistance, and express multiple mite resistance traits (hygienic and grooming behaviors, see Table 4.2).

Hygienic Behavior:

The Arnot Forest bees express hygienic behavior at higher levels than populations in the US and Australia that have not experienced selection for this trait (Fig. 4.1). Our colonies headed by queens purchased from breeders from California and Vermont expressed equivalently high levels of the trait, while the Arnot Forest bees performed almost identically to the early "hygienic bees" tested in 1995 (Spivak & Downey, 1998). Meanwhile, the three local mite-susceptible colonies we tested showed substantially lower levels of hygienic behavior (and few data were available from them as their lineages soon died out.) Comparing the Arnot Forest bees to the historic controls, it is clear that they outperform the unselected bees tested in Spivak & Downey (1998), and on average outperform all ten of the Australian queen sources tested in Oldroyd (1996). Though our assay did not test Varroa sensitivity per se, performance on the

freeze-killed brood assay is strongly correlated with sensitivity to brood Varroa infestation (Ibrahim & Spivak 2006).

Grooming Behavior:

Mites collected from the floors of the Arnot Forest colonies showed higher grooming damage levels (34%) than those collected from colonies from a commercial queen breeder (20%) whose queen breeding does not include selection for mite grooming. The comparison of the Arnot Forest bees to the unselected bees used as the source of the Purdue "Ankle-Biter" population suggests the Arnot Forest bees are outperforming unselected populations of bees. The level of grooming seen in the Arnot Forest colonies matches the performance of the Purdue bees after ca. 6 years of active selection for the grooming trait (Hunt et al. 2016). However, considerably higher levels of grooming damage have been found in the Purdue colonies that have experienced continued selection, though that breeding program experienced difficulty exceeding 50% in the years of selection after 2015 (Greg Hunt, personal communication).

Many of the historic data on levels of mite grooming are flawed by the misattribution of dimples on the dorsal surfaces of mites to grooming, when they are instead merely developmental defects (Davis 2009). Other historic data cannot be meaningfully compared to the Arnot Forest data since often the colonies assayed have experienced selection for Varroa resistance, or are fully or partially derived from non-European lineages (mostly African.) However, a few studies offer suitable comparisons: Arechavaleta-Velasco and Guzman-Novoa (2001) tested colonies from multiple independent queen breeders in Mexico and found that only 7-13% of mites recovered from beneath colonies showed damage from grooming. Ruttner and Hanel (1992) studied five of a beekeeper's most mite-resistant *A. m. carnica* colonies, and reported that between 7.1% and 28.1% of dead mites falling beneath the colonies showed

damage. Invernizzi et al. (2015), working in Uruguay, reported 18% of mites collected beneath *A. mellifera ligustica* hives and 29% beneath Africanized (*A. m scutellata* hybrid) hives were damaged. Rinderer et al. (2001) found that 42% of mites collected from beneath colonies of mite-resistant Russian bees were damaged, compared to only 28% of mites collected from mite-susceptible colonies.

Uncapping/Recapping Behavior and Mite Reproductive Success:

Table 4.1 shows that the Arnot Forest bees are capable of uncapping and recapping the cells of developing brood. Unlike grooming and hygienic behavior, where all Arnot Forest colonies demonstrated intermediate or high levels of the trait on all assessments, some Arnot Forest colonies demonstrated extremely low levels of recapping while others demonstrated high levels, with the eight colonies sampled ranging from 4% to 54% (mean=28.4%) recapping. Oddie & Büchler et al. (2018) have recently reported recapping rates which also ranged widely, but which tended to be higher in four untreated mite-resistant populations than in local mite-susceptible populations. That team also experimentally demonstrated that uncapping and recapping of cells disrupts the reproduction of mites inside them. However, our data suggest that the level of recapping tightly correlated with colony level infestation – the percent of cells that were infested and the percent of cells that were recapped in each colony are very similar to one another in all colonies except #7 (Fig. 4.S1). Infested cells were not all recapped (on average 49.1% were, s.d.= 21.2) and recapped cells were not all infested (on average 36.3% were, s.d.= 14.1) (Table 4.S2). This suggests that measured cell recapping may be an artifact of a facultatively expressed behavior, with more recapping occurring the higher the colony's level of infestation, at least in the Arnot Forest population. The mite reproductive rates that we measured (Table 4.1) suggest that mite reproduction was taking place normally in these colonies (a review

by Martin (1998) found that typical reproductive success for mites is about 70% in worker cells). The only colony to survive the following winter, colony #1, was the only one that demonstrated a low rate of mite reproduction.

Cell Size:

Our data on cell size were collected opportunistically from only three Arnot Forest swarms, but the cells from these combs were similar to one another, ranging from 5.14-5.27mm in wall-to-wall cell diameter. They were not appreciably different from the historic comb samples from 1973, which ranged from 5.12-5.25mm. Thus, the Arnot Forest bees have not changed the size of their cells in response to Varroa. It is possible that this was a preexisting trait that has been preserved in the population due to some conferred Varroa resistance, but a number of studies have suggested that small worker cell size does not protect colonies from ballooning mite populations (Liebing & Aumeier 2007, Taylor et al. 2008, Ellis et al. 2009, Berry et al. 2010, Seeley & Griffin 2011). The historic and modern Arnot Forest cell sizes are considerably larger than commercially available small-cell comb foundation (Dadant and Sons, Illinois) which measures approximately 4.9mm from wall to wall. The cells made in the Arnot Forest are indeed smaller than those made using commercial honey bee worker foundation, (compare to Walter T. Kelley company's standard worker comb foundation, which yields cells of 5.38mm when built out) but the significance of this for Varroa resistance is unclear.

Table 4.2: Summary of resistance traits demonstrated by Arnot Forest bee population compared with other known populations of European honey bees surviving without Varroa treatment

Trait	**Arnot Forest Bees**	**Other Survivor Populations with Trait**
Hygienic Behavior	Present	Primorsky, Russia (de Guzman et al. 2002)
Grooming Behavior	Present	Primorsky, Russia (Rinderer et al. 2001)
Small Colony Size	Present	Gotland, Sweden (Locke & Fries 2011)
Uncapping/Recapping	Equivocal	Oslo, Norway (Oddie & Büchler et al. 2018) Gotland, Sweden (Oddie & Büchler et al. 2018) Avignon, France (Oddie & Büchler et al. 2018) Sarthe, France (Oddie & Büchler et al. 2018)
Small Cell Size	Absent	n.d.

Our contemporaneous controls serve to validate our methodologies, but very few data were available from modern mite-susceptible colonies in our region. Since many local beekeepers buy queens from out-of-state queen breeders annually to recover from winter losses, we did not have access to a pool of locally adapted bees that had not experienced selection for mite resistance traits. While we kept our study population of bees genetically pure by only allowing them to mate in the Arnot Forest, other studies have faced confounds when their mite-resistant and mite-susceptible populations have been housed within drone flight range of one another, and have therefore had opportunities to interbreed (Oddie & Büchler et al. 2018). Comparison of our bees to historic populations is likely more informative than comparison to modern bees which have been subjected to rigorous selection for mite-resistance behavior, often making selection decisions using the same assay we used to characterize hygienic behavior in the Arnot Forest bees.

Though we cannot directly demonstrate changes in resistance phenotypes from the ancestral Arnot Forest bees to the modern resistant bees, we have compared our bees to bees

measured using identical tests prior to or soon after the arrival of Varroa, hence before much natural or artificial selection had occurred for any of these traits. Genetic comparisons of modern Arnot Forest bees to their pre-Varroa ancestors suggest that these bees have experienced strong selective pressures since the 1970s, and that the genes most affected by this selection are involved in learning, and development (Mikheyev et al. 2015). This raises the possibility of naturally selected behavioral change in these bees. Though we think it most parsimonious that the Arnot Forest bees have evolved high levels of these mite-resistance traits, it is possible that these traits are expressed now at the same levels they were prior to Varroa. Hypothesized evolutionary history aside, these traits are currently helping these bees persist in the face of a novel parasite that has devastated both wild and managed bee populations worldwide.

We found no evidence to support the hypothesis that the Arnot Forest's mites have evolved avirulence (Seeley 2007). Some Arnot Forest test colonies showed dramatic increases in mite population during our studies (see, for example, late season brood infestation rates in Table 4.1). However, since we conducted our assays in Ithaca, and not the Arnot Forest, it is possible that drift or robbing may have introduced mites to the colonies we were studying which may have outcompeted avirulent mites from the forest. The fact that the Arnot Forest colonies were not able to completely suppress mite growth in our equipment is not surprising, since we kept them in large hives and prevented them from swarming. Research has shown that both frequent swarming and small nest sizes are correlated with lower Varroa mite populations (Loftus et al. 2016, Seeley & Smith 2015). Colony monitoring around Ithaca, New York shows that the Arnot Forest bees and other bees living in small, unmanaged nests swarm regularly (annually, 87% of queens are replaced) (Seeley 2017). Also, the natural cavities these bees normally occupy are often isolated from each other by hundreds or thousands of meters, instead of the mere

centimeters separating many commercial honey bee colonies from one another, which likely reduces the risk of mite transmission and accumulation by drift (Peck and Seeley in prep) though evidently not from robbing (Peck & Seeley 2018). It is likely that the protective effects of widely spaced nests, the mite-suppression benefits of living in small nests and swarming frequently, and the multiple behavioral mechanisms of mite resistance that we have demonstrated here synergize to protect these bees from Varroa.

Selection optimizes phenotypes based on costs incurred and benefits accrued, suggesting pre-Varroa levels of hygienic and grooming phenotypes were likely optimal for the fitness of pre-Varroa bees, and that a new balance between costs and benefits must be struck in the presence of Varroa mites. The data presented here led to the construction of a theoretical framework to predict how selection will act on multiple host resistance phenotypes in a coevolutionary arms race between a host and a parasite (Peck & Reeve, in prep). The model allows hosts to evolve any number of different resistance traits to oppose parasite growth and reproduction, and also allows parasites to evolve counters to these resistance traits. In the Apis-Varroa system, the bees may evolve to smell and remove reproducing mites, while the mites may evolve to produce fewer detectable odors during reproduction. Our model assumes that increased expression of a resistance trait will allow the host to better resist the parasite, but nonlinearly (sigmoidally), with diminishing returns as host resistance increases. Returns diminish because the host survival probability for a given host fitness component must have an upper asymptote of 1.0 no matter how high its resistance level. At the same time, increasing investment in resistance will apply a stronger selection pressure on the parasite to counter this resistance. This game-theoretic "tug-of-war" model also assumes that higher levels of a resistance trait are more costly to other components of fitness (including other kinds of resistance

traits) and that this tradeoff affects the optimal level of the resistance trait. For example moderately hygienic bees might open and empty 50% of pupal cells containing mites, but also accidentally open and empty 5% of cells containing only healthy pupae. Meanwhile a highly hygienic colony might open and empty 75% of infested cells, but also open and empty 30% of healthy cells, imposing a much higher cost to the colony in the form of lost pupae. The model predicts that optimal host resistance will resemble the data we have presented here: a mix of different resistance phenotypes corresponding to different host fitness components, each expressed at a higher level than in the absence of the parasite, but expressed less than the theoretical maximum level of expression of which the host is capable. Co-evolution of host resistance and parasite virulence leads to a host's more even allocation of investment across different host resistance phenotypes. Others have considered and attempted to quantify such costs for brood hygienic behavior in bees (Vandame et al. 2002), and reached a similar conclusion – that intermediate levels of multiple resistance phenotypes may provide the maximal colony net benefit.

Conclusion:

The honey bees of the Arnot Forest display intermediate levels of multiple mite-resistance traits (Table 4.2). By studying the resistance traits that emerge when a novel parasite invades a naïve host lets us understand how parasite resistance emerges in a natural selective landscape. This natural experiment offers a counterpoint to the strategy employed by some researchers and queen breeders to produce mite-resistant bees by selecting for a single "silver bullet" mite-resistance phenotype. Such a breeding strategy may impose unnecessary costs on the bees, by over-elaborating traits that should instead be expressed at intermediate levels in conjunction with other resistance traits. We cannot say whether breeding programs should

emulate the solution of the Arnot Forest bees, or if other survivor populations will reach the same balance of phenotypes. We simply present one solution to the Varroa problem which has been provided entirely by natural selection: a diverse arsenal of behavioral resistance traits and protective life history traits, instead of a single silver bullet strategy.

Supplementary Materials

Table 4.S1: Performance on the freeze-killed brood (FKB) hygienic behavior assay from all populations studied, including % cells emptied and % cells uncapped but not necessarily emptied, at 24 hour and 48 hour time points. "Arnot (repeated)" includes mean performance of all colonies which were measured more than once, while "Arnot (all)" averages mean performance of repeatedly measured colonies with measurements of colonies measured only once.

2014 + 2015 + 2016:	24hr FKB Emptied	s.d.	24hr FKB Uncapped	s.d.	*n*		48hr FKB Emptied	s.d.	48hr FKB Uncapped	s.d.	*n*
Arnot (repeated)	**81.66**	13.9	**91.03**	7.48	7		**97.72**	2.54	**98.59**	1.15	5
Arnot (all)	**72.85**	21.29	**86.17**	13.16	12		**96.45**	6.39	**97.71**	4.00	11
Control (California)	**98.93**	0.74	**99.35**	0.64	3		**99.78**	0.34	**100.00**	0.00	3
Control (VT)	**97.16**	2.62	**100.00**	0.00	3		**100.00**	0.00	**100.00**	0.00	3
Control (yard)	**37.87**	26.77	**45.45**	31.42	3		n.d.		n.d.		
Control (all)	**77.99**	32.97	**81.60**	31.34	9		**99.89**	0.27	**100.00**	0.00	6

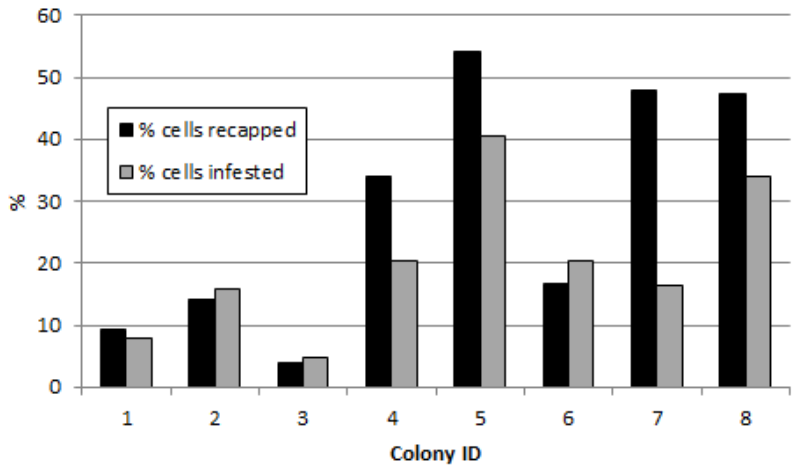

Figure 4.S1: Relationship between the percent of cells uncapped and the percent of cells infested for each of the 8 Arnot Forest colonies assayed. 150 cells were opened for each colony. The uncapping/recapping behaviors of the Arnot Forest bees may be sensitive to the level of Varroa infestation in the colony.

Table 4.S2: Correlation between whether an assayed cell was recapped and whether it was infested in the recapping behavior assay. 150 cells were opened for each colony.

Colony	% of infested cells that were recapped	% of recapped cells that were infested
1	21.4	21.4
2	50.0	42.9
3	20.0	33.3
4	52.9	35.3
5	68.4	48.1
6	37.5	24.0
7	68.0	23.6
8	74.6	62.0
mean	49.1 ± 21.2	36.3 ± 14.1

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