The Pharma Innovation Journal 2023; 12(3): 5540-5550

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(3): 5540-5550
© 2023 TPI
www.thepharmajournal.com
Received: 02-12-2022
Accepted: 07-01-2023

Muneer Ahmad
Division of Entomology
Dryland agriculture research station Rangreth, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar Srinagar, Kashmir, Jammu and Kashmir, India

Shafeeq Hakeem
Division of Entomology
Dryland agriculture research station Rangreth, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar Srinagar, Kashmir, Jammu and Kashmir, India

Sushil Kumar
Division of Entomology
Dryland agriculture research station Rangreth, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar Srinagar, Kashmir, Jammu and Kashmir, India

Zewar Husain
Division of Entomology
Dryland agriculture research station Rangreth, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar Srinagar, Kashmir, Jammu and Kashmir, India

Nadeem Dar
Division of Entomology
Dryland agriculture research station Rangreth, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar Srinagar, Kashmir, Jammu and Kashmir, India

Corresponding Author:
Muneer Ahmad
Division of Entomology
Dryland agriculture research station Rangreth, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar Srinagar, Kashmir, Jammu and Kashmir, India

The social insect honey bees and their heritable trait for the hygienic behavior for combating biotic threats: A review

Muneer Ahmad, Shafeeq Hakeem, Sushil Kumar, Zewar Husain and Nadeem Dar

Abstract
It has often been said that bees are responsible for one out of every three bites of food we eat. Most crops grown for their fruits (including vegetables such as squash, cucumber, tomato and eggplant), nuts, seeds, fiber (such as cotton), and hay (alfalfa grown to feed livestock), require pollination by insects. Pollinating insects also play a critical role in maintaining natural plant communities and ensuring production of seeds in most flowering plants. Pollination is the transfer of pollen from the male parts of a flower to the female parts of a flower of the same species, which results in fertilization of plant ovaries and the production of seeds. The main insect pollinators, by far, are bees, and while European honey bees are the best known and widely managed pollinators, there are also hundreds of other species of bees, mostly solitary ground nesting species, that contribute some level of pollination services to crops and are very important in natural plant communities. Hygienic behavior of honeybees involves inspection, uncapping and removal of diseased and dead brood from the colony. The objective of this review is to study the activities involved in hygienic behavior of individually tagged bees from selected hygienic (H) and non-hygienic (NH) colonies in the presence of chalk brood infected brood (*Ascosphaera apis*) or pin-killed brood or varroa infected brood. The hygienic behavior of honey bee workers contributes to the social immunity of colonies. The ability of workers to detect and remove unhealthy or dead brood prevents the transmission of brood diseases inside the colony. Over the last five decades, this trait has been extensively studied and improved in several research and breeding programs. Given the strong interest for hygienic behavior, we here review the costs and benefits associated with this trait, extending preceding reviews on this subject from the late 1990s. Since the 1990s, there have been no major new insights on the efficiency of this behavior against American foulbrood and chalk brood. However, the number of publications on hygienic behavior against the mite *Varroa destructor* has considerably increased, fueling the debate regarding the efficiency of hygienic behavior against this parasite. Breeding programs have shown that selection for a specific trait might also impact other traits. Thus, we also review the cost of trade-offs between hygienic behavior and other economically important traits for bee breeders. Overall, the benefits of hygienic behavior seem to largely outweigh its costs for both colonies and bee breeders. Hygienic behavior is a heritable genetic trait, which is commonly taken into account in A. Mellifera breeding programs in order to improve the vitality of the stocks. Such programs have been running for several years and the hygienic abilities and disease resistance of breeding A. mellifera colonies have largely been strengthened. The assay consisting in monitoring the removal of freeze-killed brood from a comb section has been acknowledged as the most conservative and reliable screening procedure to quantify the hygienic behavior of a colony.

Keywords: Traits, Hygenic Behaviour, Honey Bees, Social Insect

Introduction

Hygienic behavior in honey bees is a heritable trait of individual workers that confers colony-level resistance against various brood diseases. Hygienic workers detect and remove dead or diseased brood from sealed cells

Hygienic behavior is an important form of social immunity (Cremer *et al*. 2007) [12] for a number of social insect species. The term hygienic behavior was coined by Rothenbuhler (1964) [76] to describe the process of detection and elimination of diseased brood by adult honey bees (*Apis mellifera*). The term "*Varroa*-sensitive hygiene" (VSH) was coined more recently (Harris 2007) [32] to describe the detection and removal of brood infested with the parasitic mite *Varroa destructor* by honey bees (Harbo and Harris 2005) [29]. The behavioral sequence of uncapping and removing the brood, as first described (Rothenbuhler 1964) [76], is the same whether the brood is diseased, mite-infested, or dead,

But this motor pattern may be triggered by the detection of different odorants associated with the health status of the brood. In honey bee colonies, elimination of brood consists of adult bees removing and/or cannibalizing the abnormal brood from individual cells, either intact or in pieces, and discarding remains outside the hive; in *Reticulitermes* termites, it consists of cannibalization (Davis *et al*. 2018) [17] and in *Lasius* ants of destructive disinfection by dismembering the infected pupa and then disinfecting with venom (Pull *et al*. 2018) [70]. Hygienic behavior helps maintain the health of densely populated insect societies by limiting horizontal transmission of pathogens and population growth of parasites. Workers that destructively eliminate already infected or infested individuals protect the colony, or super organism, in a similar way to immune cells that protect an organism from pathogen spread throughout the body (Cremer and Sixt 2009) [13]. In recent years, research on hygienic behavior in honey bees has increased with the aim of understanding and restoring colony health. The early research on this behavior was in relation to honey bee resistance to American foulbrood (caused by *Paenibacillus larvae*) and to chalk brood (caused by *Ascosphaera apis*) diseases. Focus shifted to the relationship between hygienic behavior and resistance to the parasitic mite, *Varroa* in the 1990s (Leclercq *et al*. 2018a [46]; Mondet *et al*. 2020) [57]. This review emphasizes the underlying behavioral mechanisms of hygienic behavior in honey bees and when known, in other social insects. The goals of this review are to (1) explore the relationship between honey bee hygienic behavior toward diseased brood and *Varroa*-parasitized brood and (2) provide avenues for future research that would benefit honey bee health and survivorship.

Three recommended methods to test for hygienic behavior

1. The freeze killed brood assay

In this assay, a comb section of sealed brood containing approximately 100 cells on each side (2 inches by 2.5 inches, or 5 centimeters by 6 centimeters) is cut from a frame and frozen for 24 hours at -10°F (-20°C). The frozen comb section is inserted into a frame of sealed brood in the colony being tested (Figure 2). Tests have shown that it does not matter if the frozen section comes from the same colony from which it was removed or from a different colony. The frame with the freeze-killed brood insert is placed in the center of the brood nest. One day (24 hours) later the frame is removed and the number of sealed cells remaining is recorded. A hygienic colony will have uncapped and removed over 95% of the frozen brood within 24 hours. A non-hygienic colony will take over six days to completely remove the frozen brood.

2. Liquid nitrogen

Freezing the brood with liquid nitrogen is more efficient and less destructive to the combs than cutting, freezing and replacing comb inserts. Liquid nitrogen is relatively inexpensive and easy to obtain; check with your local gas and welding suppliers, veterinary practice or livestock artificial insemination firm. There are no laws in any state restricting the use of industrial grade liquid nitrogen by individuals. It must be kept in an appropriate tank (e.g., a Dewar tank, which can be purchased through gas and welding supply houses), and the tank should be securely fastened to the truck during travel to avoid spillage.

Common sense and several precautions must be used when handling liquid nitrogen. It has a boiling temperature of -195°C (-320°F), which means that it is extremely cold and will kill skin (causing severe frostbite) on contact. We recommend that users read the material safety data sheet on liquid nitrogen from the supplier.

You will need to construct (or find) a hollow cylinder into which you will pour the liquid nitrogen to freeze a circular section of sealed brood. We have been using a 3-inch (75 millimeter) diameter PVC pipe. The cylinder must be at least 4 inches (100 millimeters) long because the nitrogen will boil on contact with the brood.

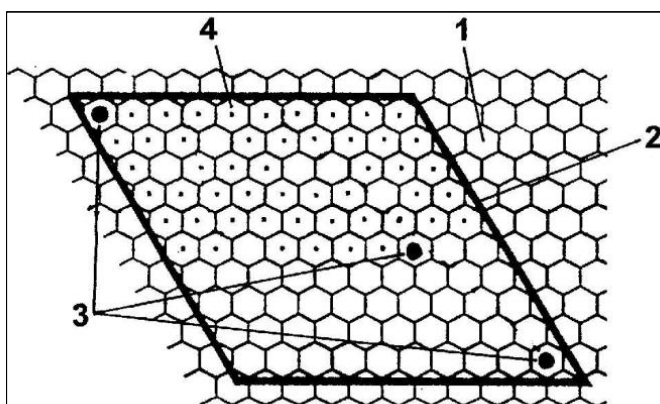
A minimum of 10 ounces (300 milliliters) of liquid nitrogen is needed to freeze-kill all the brood (approximately 160 cells) within a 3-inch diameter cylinder. A smaller amount will not kill all of the brood, leading to erroneous results. Use a 10-ounce or larger polystyrene foam coffee cup for measuring and pouring. Other materials may shatter on contact with the liquid nitrogen.

Select a frame with at least a 3-inch diameter circle of sealed brood containing fewer than 30 unsealed cells within the circle. Lay the frame horizontally across a support (e.g., an empty super). Twist the cylinder into the sealed brood until it reaches the midrib. Record the number of unsealed cells inside the cylinder. Pour 50–60 milliliters of the liquid nitrogen into the cylinder and wait for it to freeze the edges or evaporate. Then pour the remainder of the liquid nitrogen into the cylinder. Wait to remove the cylinder until it thaws, which may take three to 10 minutes (Figure 3). If you have additional cylinders, you can start the next test while you are waiting for previous ones to thaw. We put a drawing pin (thumbtack) in the top of the frame to mark the frame and the location of the test on the frame. Some hygienic colonies clean and repair the comb so quickly that it is hard to locate the test when you return. Place the frame in the center of the brood nest (Figure 4). Remove the frame containing the frozen brood 24 hours later and record the number of sealed cells remaining within the circle. When testing a colony that has been required, six to eight weeks must elapse after requiring for the bees in the colony to be daughters of the new queen.

Fig 1: Nitrogen Killing and Freez killing Techniques of Bee Brood

3 Pin Killing Brood technique

This technique is done in which section of Brood on frame is killed by Pinning on sealed brood and the frame is given back to the colony and the removal of dead brood timing is measured.



Hygienic Behaviour

Hygienic behavior in honey bees is a heritable trait of individual workers that confers colony-level resistance against various brood diseases. Hygienic workers detect and remove dead or diseased brood from sealed cells.

Varroa sensitive hygiene (VSH) is a behavioral trait of honey bees *(Apis mellifera)* in which bees detect and remove bee pupae that are infested by the parasitic mite *Varroa destructor*. *V. destructor* is considered to be the most dangerous pest problem for honey bees worldwide. VSH activity results in significant resistance to the mites.

Timing of hygienic removal of diseased brood

The most serious disease of honey bees was American foulbrood. Beekeepers and researchers (Park 1937) noted that some colonies did not succumb to this disease and they considered these colonies to be resistant. They observed that "the bees sometimes remove and dispose of larvae very soon after they die, thus eliminating the evidence." Following these observations, it was determined that first instar larvae derived either from resistant or from susceptible colonies were equally susceptible to American foulbrood, but larvae inoculated more than 2 days and 5 h after hatching from the egg did not become infected (Woodrow 1942). It was later observed that the adult bees from resistant colonies removed the majority of the diseased brood from the cells whereas bees from susceptible colonies did not, and concluded that colony resistance depended on behavioral removal of diseased brood by adult bees, rather than physiological resistance of the brood (Woodrow and Holst 1942) [99]. These findings were later confirmed by Rothenbuler and others (Spivak and Gilliam 1998a) [80].

The experiments by Woodrow and Holst also revealed that the timing of adult bees' removal of the infected brood was key to understanding the apparent resistance (Woodrow and Holst 1942) [99]. After suspending known quantities of *P. larvae* spores in the food surrounding individual first instar larvae, they noted that the resistant colonies started eliminating the infected larvae on the sixth day after inoculation (the day the cell containing a 5th instar is capped with wax) and had removed all of infected brood by day 11. They collected intact brood that was removed from the hive by the bees and found the brood had only the non-infectious rod form of *P. larvae*, indicating the bees were removing the brood from the nest while the non-infectious rods were multiplying within them. In contrast, bees in a susceptible colony did not begin removing infected brood until day 9 after inoculation, and not all of the diseased brood was removed from the cells; some was uncapped but later recapped with wax. The bacterium reached the highly infectious spore stage in the remaining brood of the susceptible colonies, and bees from susceptible colonies were sometimes removing the brood while bacteria were infectious, potentially spreading the disease. Woodrow and Holst concluded that "…resistance to American foulbrood in the honey bee colony consists in its ability to detect and remove diseased brood before the causative organism… reaches the infectious spore stage in the diseased larvae." Observations of hygienic activity against brood infected with *Mellisococcus plutonius* prompted J. I. Hambleton to report that "American foulbrood resistant strains are highly susceptible to European foulbrood" (Root 1966) [74]. The apparent susceptibility may have been because the bees were actively handling younger honey bee larvae which have infectious *M. plutonius*but non-infectious *P. larvae*; this possibility requires further study.

The timely detection and removal of brood was demonstrated after bees were challenged with a different pathogen, the chalkbrood fungus (Invernizzi *et al*. 2011) [37]. The most hygienic colonies, those that uncapped pin-killed brood (see section on "Assays" below), also tended to uncap cells and cannibalize the chalk brood-infected brood before the brood was consumed by fungal mycelia and became infectious "mummies." Colonies with numerous intact chalk brood mummies on the bottom board of the colony indicated that bees were not hygienic because the infected brood was removed after it reached the spore stage, increasing the risk of horizontal transmission.

The timely elimination of infected brood is important for other social insects, such as colonies of the invasive garden ant, *Lasius neglectus* (Tragust *et al*. 2013 [88]; Pull *et al*. 2018) [70], and the subterranean termite, *Reticulitermes flavipes* (Davis *et al*. 2018) [17]. These social insect colonies nest in the soil where they may become exposed to the soil-borne fungal entomopathogens such as *Metarhizium*. Adult ants in the genus *Lasius* groom infectious conidiospores of *Metarhizium*

from the brood into infrabuccal pouches and disinfect the fungal pellets in their pouches with their antimicrobial venom (Tragust *et al*. 2013) [88]. If the fungus is undetected on the cuticle of some ants and germinates into the pupal body, upon detection of the infected pupa, the adult ants unpack it from its cocoon, dismember it, and disinfect the pupal remains with venom (Pull *et al*. 2018) [70]. The detection and destructive disinfection of the infected pupa occurs when the pathogen is in the non-infectious incubation period, similar to how honey bees detect and remove infected, but not infectious pupae from the nest. The destructive disinfection prevented the pathogen from completing its life cycle, thus preventing intra-colony disease transmission (Pull *et al*. 2018) [70]. In *R. flavipes* termite colonies, *Metarhizium* conidiospores are groomed from infected individuals, but once the fungus enters the body, termites cannibalize the infected nest mate (Davis *et al*. 2018) [17]. It was not determined if cannibalism occurred during the non-infectious incubation period; however, the switch from sanitary prevention (allo-grooming) to elimination (cannibalism) was clear, suggesting that termites also are able to detect the stage of infection (Davis *et al*. 2018) [17].

In sum, the *timing* of detection and elimination of the diseased brood by adult social insects seems to be a critical component in preventing pathogen transmission within these social insect colonies, and thus in colony-level resistance. It would be to the pathogen's advantage for individuals within the colony to handle diseased brood when infectious because it would increase the risk of pathogen transmission, whereas it would be to the colony's advantage if individuals eliminate the brood before it is infectious because it would limit pathogen spread. Whether the timing of the elimination of brood when mite-infested is similarly important is discussed below.

Assays for honey bee hygienic behavior

Bioassays for hygienic behavior were recently reviewed in depth (Leclercq *et al*. 2018a) [46] and thus, only some points are highlighted here. The best way to determine if a colony of honey bees (or other social insects) can detect and remove diseased brood is to challenge individual bees or larvae, or an entire colony, with a known dose of a pathogen and observe the response of adult nest mates to infected individuals. Due to the risks involved in challenging honey bee colonies with potentially lethal and highly infectious pathogens such as *P. larvae*, researchers began exploring assays that would not involve inoculating larvae with a pathogen. As a proxy for diseased brood, cyanide-killed brood was presented in colonies to facilitate experiments using lines of bees already selected for resistance and susceptibility to American foulbrood (Jones and Rothenbuhler 1964) [76]. Later, researchers began screening unselected colonies for hygienic behavior using freeze-killed brood (Spivak and Gilliam 1998b) [81], or pin-killed brood (Newton and Ostasiewski 1986) [61].

How quickly a colony could detect and remove the experimentally killed brood did not always correspond with the colony's ability to remove diseased brood (Gilliam *et al*. 1983) [23]. Thus, after screening colonies using a freeze-killed (or pin-killed) brood assay, it is important to subsequently challenge colonies with a pathogen to determine if they are behaviorally resistant (Spivak and Reuter 2001a) [84]. As a recent example, an imperfect correspondence was found between the removal of freeze-killed brood and physiological resistance to chalk brood in Australian honey bee colonies (Gerdts *et al*. 2018) [22]. Of 649 colonies tested for hygienic behavior using the freeze-killed brood assay, 16% were considered highly hygienic (removed 95% of the freeze-killed brood within 24 h), suggesting they should not have signs of disease within the colony, but in fact, 23% of these highly hygienic colonies presented signs of chalk brood disease. These results provide an example of how the freeze-killed brood assay does not fully predict behavioral resistance in the test population.

Of note is that colonies that remove less than 95% of the freeze-killed brood within 24 or 48 h tend to remove little, if any, pathogen infected brood after challenge; they tend not to be resistant to American foulbrood or chalk brood (M Spivak, unpublished data). This observation begs the question of why highly hygienic colonies are rare in nature and whether there are associated fitness costs with the trait (Mondragon *et al*. 2005 [59]; Bigio et al. 2014 [5]; Leclercq *et al*. 2017 [47]). We speculate that resistance does not depend solely on hygienic behavior but likely involves a combination of other physiological factors in honey bees, including the immune response (Evans and Spivak 2010) [21], trans generational immune priming (Hernandez Lopez *et al*. 2014) [35], micro biome community (Raymann and Moran 2018) [72], antimicrobial activity of larval food (Rose and Briggs 1969 [75]), presence of propolis in the nest (Borba *et al*. 2015) [8], and other factors yet to be discovered.

In sum, and as pointed out previously (Leclercq *et al*. 2018b) [46], assays for hygienic behavior, like the freeze-killed or pin-killed brood assays, are not necessarily useful predictors of pathogen resistance in a colony or population of colonies. They are useful to screen colonies for the ability of the adult bees to quickly remove dead brood (e.g., > 95% removal within 24 h for the freeze-killed brood test) and these colonies can be subsequently challenged to quantify pathogen resistance. In other words, the assays are used to narrow down the number of colonies to be challenged, to increase the chances of finding resistant colonies.

Hygienic behavior in relation to *Varroa*

Although some ant and termite colonies have brood parasites (Korb and Fuchs 2006; Lachaud *et al*. 2016), studies of their hygienic response are limited; e.g., the ant *Ecatomma tuberculatum* detects and removes parasitic wasps (Perez-Lachaud *et al*. 2015) [68] and other nest intruders (Perez-Lachaud *et al*. 2019) [69]. Thus, this section will concentrate on honey bees' response to *Varroa destructor*. When *V. destructor* spread through *A. mellifera* colonies in Europe and North America, researchers looked to this mite's original host species, *A. cerana*, to determine how it survived without succumbing to the parasite. A number of potential resistance mechanisms were described, hygienic behavior being one of them (Peng *et al*. 1987a; Peng *et al*. 1987b) [66, 67]. In *Apis cerana*, *Varroa* reproduces only on seasonally produced drone brood and does not reproduce on worker brood. If the mite infests worker brood (or are experimentally introduced onto worker pupae), the pupa dies, due to a toxic salivary gland secretion injected by mite (Zhang and Han 2018) [100] and the bees hygienically remove the dead brood from the nest (Page *et al*. 2016) Page. The signal or cue from the dying pupa was termed "altruistic suicide" and the removal "social apoptosis"; the combination was hypothesized to increase inclusive fitness benefits to the colony (Page *et al*. 2016) [64]. In *A.*

mellifera, *Varroa* reproduces successfully on both drone and worker brood, and worker pupae do not die if infested with the mites, although they could if also infected with high enough virus levels.

After *Varroa* spread through Europe, *A. m. carnica* colonies in Germany were tested for their ability to detect and remove *Varroa*-infested brood (Boecking and Drescher 1992) [6]. The removal of infested brood would be a form of mite resistance because it would increase mite mortality or disrupt mite reproductive success (Leclercq *et al*. 2018a) [46]. In the Boecking and Drescher study (Boecking and Drescher 1992) [6], the colonies were not previously selected for hygienic behavior or mite resistance. After experimentally introducing mites into recently capped brood cells, 29% of the infested brood were removed after 10 days when one mite per cell was introduced and 55% were removed when two mites per cell were introduced, indicating that in fact, some *A. mellifera* colonies could detect and remove some mite-infested pupae, even though they were naïve hosts to this parasite.

A significant negative relationship between the results of the freeze-killed brood assay and mite population growth over one season was found in the UK (Toufailia *et al*. 2014) [87]. The statistical significance was driven by eight of the 42 colonies that removed > 95% of the freeze-killed brood within 48 h and were thus highly hygienic, again confirming that screening for these highly hygienic colonies based on the freeze-killed brood assay will help locate colonies with a relatively higher potential of removing mite-infested brood. Colonies that removed less than 95% of the freeze-killed brood showed no significant relationship between hygienic behavior and mite growth (Toufailia *et al*. 2014) [87], which was also observed in Mexico (Mondragon *et al*. 2005) [59].

A large population derived from diverse sources of colonies in western Canada was selected over three generations for hygienic behavior using either the freeze-killed brood test or peptide biomarkers from bees' antennae, with the goal of testing the utility of marker-assisted selection for hygienic behavior (Guarna *et al*. 2017) [27]. Eleven of the 13 protein markers were linked to hygienic behavior (including two linked to VSH, see section below), and two were linked to grooming behavior. This remarkable study showed two things: that protein biomarkers can be used successfully in breeding bees (and possibly other livestock) and that compared to unselected stocks, colonies selected using either the freeze-killed brood assay or peptide biomarkers had increased hygienic behavior, showed no loss of honey production, and had increased survival when challenged with either *P. larvae* or *Varroa.*

Researchers in Germany have used the pin-killed brood assay in breeding programs to successfully reduce mite loads. Other researchers reported no correlation between the removal of freeze-killed or pin-killed brood and the mite infestation of colonies, reviewed in Locke (2016) [49]. However, the latter studies used these assays to try to determine the mechanism of resistance of a population, not to screen and narrow down the number of colonies for subsequent challenge to quantify potential resistance, or to use in breeding programs.

In sum, the freeze-killed and pin-killed brood assays for hygienic behavior are useful screening tools to find colonies that may remove diseased and mite-infested brood upon subsequent challenge. For *Varroa* in particular, selecting bees based on these assays will yield colonies with lower mite loads relative to unselected colonies (Spivak and Reuter 1998; Spivak and Reuter 2001b; Büchler *et al*. 2010 [11]; Guarna *et al*. 2016; Guarna *et al*. 2017 [26, 27]) but to date, selection using these assays has not resulted in populations resistant to mites; that is, populations that do not require treatment to survive. Thus, these field assays should not be used as sole tests or indicators of *Varroa* resistance, as other traits contribute to various degrees to this resistance, reviewed in Mondet *et al*. (2020) [57].

Varroa -sensitive hygiene

Varroa-sensitive hygiene is a specialized term for the hygienic trait in which honey bees detect and remove brood specifically infested with *Varroa.* VSH activity is largely the same as that of the hygienic trait; the bees perform the hygienic behavioral sequence of uncapping and removing brood, but the removal in this case is triggered by the detection of mite-infested brood, rather than diseased or dead brood. Note that the term VSH also is often used for lines of bees bred for enhanced expression of the trait. Bees that express high levels of VSH show clear resistance to *Varroa* in that they do not require treatments to survive mite infestations, as has been demonstrated by USDA researchers in Baton Rouge, LA, USA. Of note, a critical experiment has not been conducted which could clarify the relationship between colonies selected for VSH and those selected for hygienic behavior based on the freeze-killed or pin-killed brood assay. It would be informative to challenge colonies that express VSH with *P. larvae* or *A. apis* pathogen to determine if bees that express VSH only respond to mite-infested brood, or if they also detect and remove diseased brood and thus, are hygienic in general.

This history of bees with VSH-based mite resistance, and how it has been selected over the years is somewhat convoluted. Harbo and Hoopinger began by searching for colonies that displayed resistance to *Varroa* with no a priori assumptions about which traits would be involved (Harbo and Hoopingarner 1997) [31]. They inoculated 43 colonies with known quantities of *Varroa* at the beginning of the season and quantified mite loads after ~ 10 weeks. They found three colonies with fewer mites at the end of the test than were originally inoculated. After running a number of tests to determine the mechanism for active resistance against the mites, they concluded that the factor that best explained the apparent resistance was the low reproductive success of the mites on worker brood. They selectively bred a line from several of the highest-performing colonies and gave it the name suppression of mite reproduction or SMR. The mechanism for how bees or brood from the SMR colonies could reduce mite reproductive success was unknown. The mites entered worker brood cells to feed and reproduce; however, the authors reported that the mites died in the cell without reproducing, produced no progeny, produced males only, or produced progeny too late to mature.

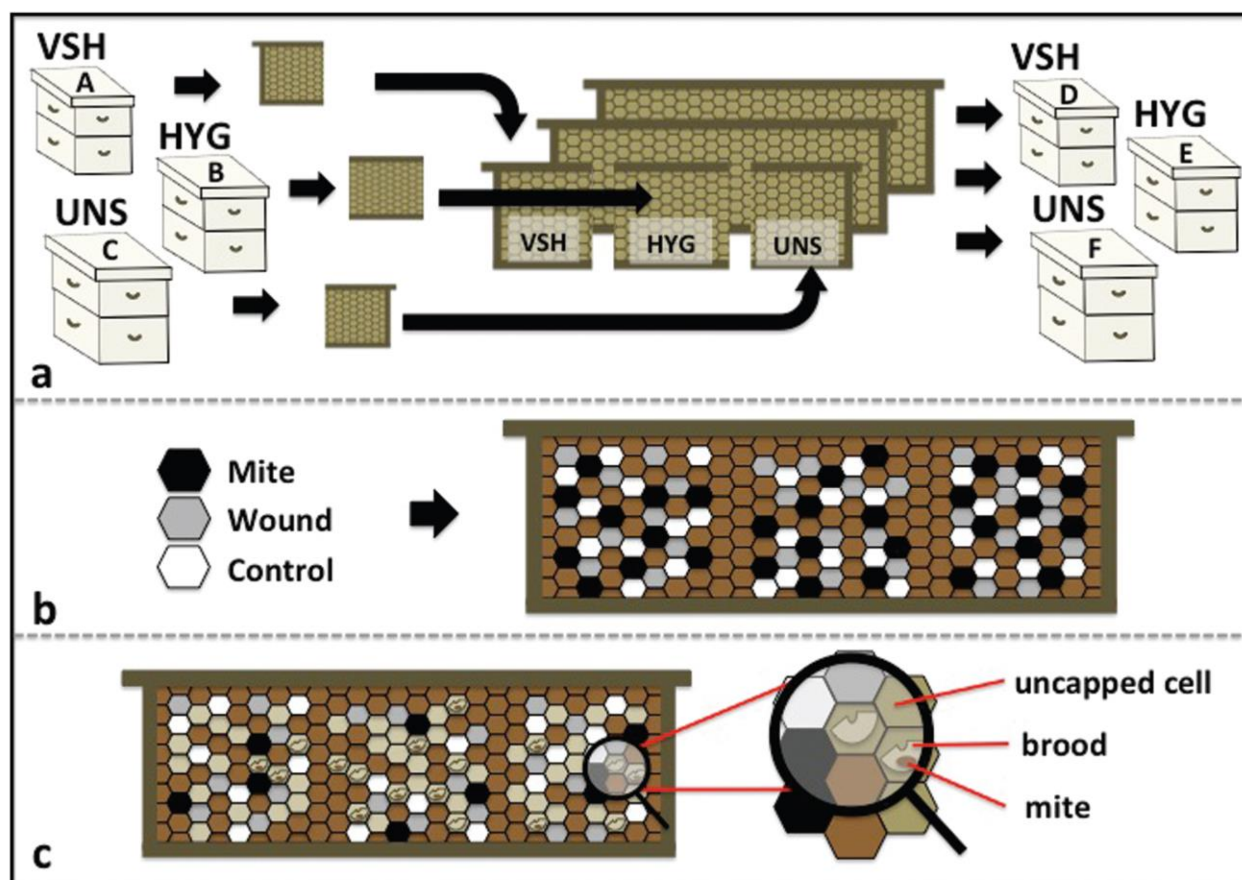
SMR colonies removed > 95% of the freeze-killed brood within 48 h, which indicated that the bees were expressing a high level of hygienic behavior (Ibrahim and Spivak 2006) [36]. These results were surprising because the SMR line was selectively bred for reduced mite reproduction, not for hygienic behavior (Harbo and Harris 1999) [28]. It was hypothesized that the SMR bees could be detecting and removing pupae on which the mites were reproducing, leaving pupae with mites that did not reproduce successfully.

This hypothesis was tested in two ways. In one test, recently capped brood combs with known percentages of mite infestation were introduced into colonies with and without the SMR trait (Harbo and Harris 2005). After 8 days, the SMR colonies had significantly lower mite infestation (2%) compared to the controls (9%). Of the mites that remained, the SMR colonies had a lower proportion of reproductive mites, 20% vs. 71%, suggesting the SMR bees were targeting pupae with reproductive mites. In a second test, mites were experimentally introduced onto individual pupae of two types of colonies: SMR bees and Minnesota Hygienic bees that had been selected using the freeze-killed brood assay (Ibrahim and Spivak 2006) [36]. The SMR colonies removed significantly more mite-infested pupae than colonies from the hygienic line. Together, these findings indicated that bees bred for SMR express hygienic behavior and that adult bees may selectively remove pupae infested with reproductive mites. In addition, hygienic activity may disrupt the reproduction of mites on targeted pupae and some of these mites may re-invade other open brood cells and later be counted as non-reproductive. In 2007, Harris renamed the line from SMR to VSH to reflect that the main mechanism that leads to non-reproductive mites (and thus mite resistance) apparently is hygienic behavior rather than the ability of the brood to somehow reduce mite reproductive success.

A further finding was that the reproductive success (fertility and number of viable female offspring) of *Varroa* on pupae not hygienically removed by bees was significantly lower in VSH colonies than in Minnesota Hygienic colonies (Ibrahim and Spivak 2006) [36]. This suggests an additional effect of VSH pupae that reduced mite reproductive success, indicating that hygienic behavior alone was not completely responsible for the mite resistance in this line. Recent studies also suggest a brood effect that suppresses mite reproduction (Wagoner *et al*. 2019) [95]. Such an effect originating from brood could be a valuable trait to support mite resistance. However, a brood-based effect was not increased reliably in an attempt to select and breed for it.

The methods used for selecting *Varroa*-resistant bees by the USDA researchers in Baton Rouge has varied through time. Progress originally came by quantifying the relative population growth of the mites over a short period, typically ~ 10 weeks (Harbo and Hoopingarner 1997) [31]. Colonies later were selected based on the frequency of non-reproductive mites in them, after this factor was determined to be the principal determinant of resistance. The frequency of non-reproductive mites has been the most extensively used criterion for selection and continues to be used today. After the role of hygiene was discovered, some selection involved introducing combs containing known percentages of mite-infested brood and quantifying the decrease in infestation after 1 week. This method requires more replication to be accurate when colonies being tested have low mite resistance. Experience with these three methods suggests that highly mite-resistant colonies (i.e., those that require no treatment against *Varroa*) generally have mite population growth of ≤ 1.0 per reproductive cycle and ≥ 60% of mites that are non-reproductive, and remove ≥ 80% of mite-infested brood after 1 week (Danka *et al*. 2016) [92].

Measuring mite population growth, the frequency of non-reproductive mites or the removal of mite-infested brood is technically difficult and tedious, and these issues have limited bee breeders' selection for the VSH trait. To date, there is no simple field assay that will yield the high *Varroa* resistance of the bees selected with these technical methods. Selection based on the freeze-killed brood assay will not be sufficient (and discussed earlier). Some resistant populations, particularly the "survivor" stocks that thrive without treatment indicate that hygienic behavior, however assayed, may not be the main mechanism for all populations, e.g., African populations in Africa and the neotropics, plus populations in Sweden, France, and the Arnot Forest in New York (Locke 2016; Mondet *et al*. 2020 [49, 57]). Populations of highly resistant bees, including survivor populations (Locke 2016) [49] and Russian bees (Rinderer *et al*. 2001), display non-reproduction of mites or low mite population growth, but the lack of, or slow, mite increase may be due to a combination of inter-related factors that range from life-history traits (e.g., high swarming frequency) to distinct behavioral traits (VSH or grooming).



Timing of removal of *Varroa*-infested and virus-infected brood

It is not known if the timing of detection and removal of *Varroa*-infested brood is as critical of a component in preventing parasite transmission as it is for pathogen transmission during removal of diseased brood. This issue has not been studied. The timing of hygiene may not depend on the presence of the mite per se but on the virus levels in the pupae, such as deformed wing virus (DWV), which are induced to replicate and vectored by the mites as the mite feeds. The bees' removal of mite-infested brood tends to increase 72 h after the larvae is capped with wax (Spivak 1996 [36]; Harris 2007), which is when the larva initiates metamorphosis into a pupa and when the mite feeds and begins reproducing in the cell (Donzé and Guerin 1994 [19]; Martin 1995 [50]; Donzé and Guerin 1997 [20]). The removal process can continue for the duration of pupal development (Vandame *et al*. 2002) [90]. Hygienic handling of the virus-infested brood could either increase or decrease transmission of the pathogen. The risk of increasing transmission would depend on the type and level of the virus infection, which could depend on the stage of bee pupal development, and the relative infectivity and virulence of the virus to the bees. This area requires testing because these factors are only beginning to be understood in honey bees (Brutscher *et al*. 2016 [10]; Grozinger and Flenniken 2019[25]).

A few studies have shown a link between hygienic behavior and reduction in virus-infested brood. Hygienic colonies, determined based on the pin-killed brood assay, tended to remove worker pupae infected with DWV (Schöning *et al*. 2012) [77]. Highly hygienic colonies, determined based on the freeze-killed brood assay, also had significantly lower levels of DWV in addition to lower mite population growth over the season (Toufailia *et al*. 2014) [87]. Brood infected with DWV produced chemical compounds that when experimentally applied to brood elicited hygienic behavior (Wagoner *et al*. 2019) [95]. The correspondence between mite infestation, virus load, and stimulus intensity has not been explored relative to the timing of hygienic detection and removal by honey bees. Understanding the relationship among these factors will not be easy, nor necessarily robust from one population of bees to the next, but is worthy of study.

Mechanisms of detection of diseased and *Varroa*-infested brood by adult bees

To study the mechanisms underlying how adult honey bees detect diseased brood before the pathogen reaches the infectious spore stage, it was hypothesized that hygiene was mediated by olfactory stimuli emitted from diseased brood (Spivak *et al*. 2003) [24]. It was not known if the odorant was passively or actively emitted, i.e., whether it was a cue or signal (Maynard Smith and Harper 2003 [55]; Leonhardt *et al*. 2016) [48]. A number of neuroethological methods were employed to test the olfactory hypothesis, using chalk brood as the test pathogen, and the line of honey bees selectively bred for hygienic behavior based on the colony response to a freeze-killed brood assay (Arathi *et al*. 2000 [1]; Masterman *et al*. 2000 [54]; Gramacho and Spivak 2003 [24]; Spivak *et al*. 2003 [24]). Based on the results of these experiments, it was concluded that bees from hygienic colonies were able to detect and discriminate between odors of diseased and healthy brood at a lower stimulus level compared to bees from non-hygienic colonies. Non-hygienic bees would, and do, detect and remove diseased brood, but only when the pathogen is infectious and the stimulus level is very high (Figure 2), increasing the risk of pathogen transmission.

Testing Honey Bee Colonies for Hygienic Behavior It is relatively easy to determine if a colony of bees displays hygienic behavior. If you are curious whether your bees express the behavior, you can test them using one of these methods. (Also see Spivak and Reuter, 1998b) [83]. They involve presenting bees with freeze killed or pin-killed brood and determining the colony's rate of removal of the dead brood. The ability of a colony to quickly remove freeze-killed or pin-killed brood corresponds generally with how quickly the colony detects and removes diseased or mite-infested brood. These methods are used as an initial screen to find colonies with hygienic tendencies. This initial assay should be followed by more detailed tests of a colony's ability to detect and remove actual diseased or mite-infested brood. Two Recommended Methods to Test for Hygienic Behavior 1. The Freeze Killed Brood Assay In this assay, a comb section of sealed brood containing approximately 100 cells on each side (2 inches by 2.5 inches. which you will pour the liquid nitrogen to freeze a circular section of sealed brood. We have been using a 3-inch (75 millimeter) diameter PVC pipe. The cylinder must be at least 4 inches (100 millimeters) long because the nitrogen will boil on contact with the brood. A minimum of 10 ounces (300 milliliters) of liquid nitrogen is needed to freeze-kill all the brood (approximately 160 cells) within a 3-inch diameter cylinder. A smaller amount will not kill all of the brood, leading to erroneous results. Use a 10-ounce or larger polystyrene foam coffee cup for measuring and pouring. Other materials may shatter on contact with the liquid nitrogen. Select a frame with at least a 3-inch diameter circle of sealed brood containing fewer than 30 unsealed cells within the circle. Lay the frame horizontally across a support (e.g., an empty super). Twist the cylinder into the sealed brood until it reaches the midrib. Record the number of unsealed cells inside the cylinder. Pour 50–60 milliliters of the liquid nitrogen into the cylinder and wait for it to freeze the edges or evaporate. Then pour the remainder of the liquid nitrogen into the cylinder. Wait to remove the cylinder until it thaws, which may take three to 10 minutes (Figure 3). If you have additional cylinders, you can start the next test while you are waiting for previous ones to thaw. We put a drawing pin (thumbtack) in the top of the frame to mark the frame and the location of the test on the frame. Some hygienic colonies clean and repair the comb so quickly that it is hard to locate the test when you return. Place the frame in the center of the brood nest. Remove the frame containing the frozen brood 24 hours later and record the number of sealed cells remaining within the circle. When testing a colony that has been required.

Reference

1. Arathi HS, Burns I, Spivak M. Ethology of hygienic behaviour in the honey bee Apis mellifera L. (Hymenoptera: Apidae): Behavioural repertoire of hygienic bees. Ethology. 2000;106:365-379. https://doi.org/10.1046/j.1439-0310.2000.00556.xArticleGoogle Scholar
2. Arathi HS, Ho G, Spivak M. Inefficient task partitioning among nonhygienic honeybees, Apis mellifera L., and implications for disease transmission. Anim Behav. 2006;72:431-38. https://doi.org/10.1016/j.anbehav.2006.01.018
3. Aumeier P, Rosenkranz P, Goncalves L. A comparison of the hygienic response of Africanizied and European (Apis mellifera carnica) honey bees to Varroa-infested brood in tropical Brazil. Genet Molec Biol. 2000;23:787-791.
4. Baracchi D, Mazza G, Turillazzi S. From individual to collective immunity: the role of the venom as antimicrobial agent in the Stenogastrinae wasp societies. J Insect Physiol. 2012;58:188-193. https://doi.org/10.1016/j.jinsphys.2011.11.007
5. Bigio G, Al Toufailia H, Ratnieks FL. Honey bee hygienic behaviour does not incur a cost via removal of healthy brood. J Evol Biol. 2014;27:226-230. https://doi.org/10.1111/jeb.12288
6. Boecking O, Drescher W. The removal response of Apis mellifera L. colonies to brood in wax and plastic cells after artificial and natural infestation withVarroa jacobsoni Oud and to freeze-killed brood. Exp & Appl Acarol. 1992;16:321-329. https://doi.org/10.1007/bf01218574
7. Boecking O, Spivak M. Behavioral defenses of honey bees against Varroa jacobsoni Oud. Apidologie. 1999;30:141-158. https://doi.org/10.1051/apido:19990205
8. Borba RS, Klyczek KK, Mogen KL, Spivak M. Seasonal benefits of a natural propolis envelope to honey bee immunity and colony health. J Exp Biol. 2015;218:3689-3699. https://doi.org/10.1242/jeb.127324
9. Borba RS, Spivak M. Propolis envelope in Apis mellifera colonies supports honey bees against the pathogen, Paenibacillus larvae. Sci Rep. 2017;7:11429. https://doi.org/10.1038/s41598-017-11689-w
10. Brutscher LM, McMenamin AJ, Flenniken ML. The buzz about honey bee viruses. PLoS Pathog. 2016;12:e1005757. https://doi.org/10.1371/journal.ppat.1005757
11. Büchler R, Berg S, Le Conte Y. Breeding for resistance to Varroa destructor in Europe. Apidologie. 2010;41:393-408. https://doi.org/10.1051/apido/2010011
12. Cremer S, Armitage SA, Schmid-Hempel P. Social immunity. Curr Biol. 2007;17:R693-702. https://doi.org/10.1016/j.cub.2007.06.008
13. Cremer S, Sixt M. Analogies in the evolution of individual and social immunity. Philos Trans R Soc Lond B Biol Sci. 2009;364:129-142. https://doi.org/10.1098/rstb.2008.0166
14. Dainat B, Evans JD, Chen YP, Gauthier L, Neumann P. Dead or alive: deformed wing virus and Varroa destructor reduce the life span of winter honeybees. Appl Environ Microbiol. 2012;78:981-987. https://doi.org/10.1128/AEM.06537-11
15. Ahad MA. Evolution without Wilson's Sociobiology: Is Sociobiology a special branch of Entomology that deals with social insect. International Journal of Entomology Research. 2022;7(12):161-73.
16. Danka RG, Rinderer TE, Spivak M, Kefuss J. Comments on: "Varroa destructor: research avenues towards sustainable control". J Apicul Res. 2013;52:69-71. https://doi.org/10.3896/Ibra.1.52.2.14
17. Davis HE, Meconcelli S, Radek R, McMahon DP. Termites shape their collective behavioural response based on stage of infection. Sci Rep. 2018;8:14433. https://doi.org/10.1038/s41598-018-32721-7
18. De Miranda JR, Genersch E. Deformed wing virus. J Invertebr Pathol. 2010;103 Suppl 1:S48-61.

https://doi.org/10.1016/j.jip.2009.06.012
19. Donzé G, Guerin PM. Behavioral attributes and parental care of Varroa mites parasitizing honeybee brood. Behav Ecol Sociobiol. 1994;34:305-319.
20. Donzé G, Guerin PM. Time-activity budgets and space structuring by the different life stages of Varroa jacobsoni in capped brood of the honey bee, Apis mellifera. J Ins Behav. 1997;10:371-393.
21. Evans JD, Spivak M. Socialized medicine: individual and communal disease barriers in honey bees. J Invertebr Pathol. 2010;103 Suppl 1:S62-72. https://doi.org/10.1016/j.jip.2009.06.019
22. Gerdts J, Dewar RL, Simone Finstrom M, Edwards T, Angove M. Hygienic behaviour selection via freeze-killed honey bee brood not associated with chalkbrood resistance in eastern Australia PLoS One. 2018;13:e0203969. https://doi.org/10.1371/journal.pone.0203969
23. Gilliam M, Taber S, Richardson GV. Hygienic behavior of honey bees in relation to chalk brood disease. Apidologie. 1983;14:29-39. https://doi.org/10.1051/apido:19830103
24. Gramacho KP, Spivak M. Differences in olfactory sensitivity and behavioral responses among honey bees bred for hygienic behavior. Behav Ecol Sociobiol. 2003;54:472-479. https://doi.org/10.1007/s00265-003-0643-y
25. Grozinger C, Flenniken ML. Bee viruses: Ecology, pathogenicity, and impacts. Annu Rev Entomol. 2019;64:205-226. https://doi.org/10.1146/annurev-ento-
26. Guarna M, *et al*. Expression biomarkers used for the selective breeding of complex polygenic traits. bioRxiv; c2016. https://doi.org/10.1101/076174
27. Guarna MM *et al*. Peptide biomarkers used for the selective breeding of a complex polygenic trait in honey bees. Sci Rep. 2017;7:8381. https://doi.org/10.1038/s41598-017-08464-2
28. Harbo JR, Harris JW. Selecting honey bees for resistance to Varroa jacobsoni. Apidologie. 1999;30:183-196. https://doi.org/10.1051/apido:19990208
29. Harbo JR, Harris JW. Suppressed mite reproduction explained by the behaviour of adult bees. J Apicul Res. 2005;44:21-23. https://doi.org/10.1080/00218839.2005.11101141
30. Harbo JR, Harris JW. Responses to Varroa by honey bees with different levels of Varroa Sensitive Hygiene. J Apicul Res. 2009;48:156-161. https://doi.org/10.3896/Ibra.1.48.3.02
31. Harbo JR, Hoopingarner RA. Honey Bees (Hymenoptera: Apidae) in the United States that express resistance to Varroa jacobsoni (Mesostigmata: Varroidae). J Econ Entomol. 1997;90:893-898.
32. Harris JW. Bees with Varroa Sensitive Hygiene preferentially remove mite infested pupae aged five days post capping. J Apicul Res. 2007;46:134-139. https://doi.org/10.1080/00218839.2007.11101383
33. Harris JW, Danka RG, Villa JD. Changes in infestation, cell cap condition, and reproductive status of Varroa destructor (Mesostigmata: Varroidae) in brood exposed to honey bees with Varroa sensitive hygiene. Ann Entomol Soc Amer. 2012;105:512-518. https://doi.org/10.1603/An11188
34. Harris JW, Harbo JR. Changes in reproduction of Varroa destructor after honey bee queens were exchanged between resistant and susceptible colonies. Apidologie. 2000;31:689-699.
35. Hernandez Lopez J, Schuehly W, Crailsheim K, Riessberger-Galle U. Trans-generational immune priming in honeybees. Proc Biol Sci. 2014;281:20140454. https://doi.org/10.1098/rspb.2014.0454
36. Ibrahim A, Spivak M. The relationship between hygienic behavior and suppression of mite reproduction as honey bee (Apis mellifera) mechanisms of resistance to Varroa destructor. Apidologie. 2006;37:31-40. https://doi.org/10.1051/apido:2005052
37. Invernizzi C, Rivas F, Bettucci L. Resistance to chalkbrood disease in Apis mellifera L. (Hymenoptera: Apidae) colonies with different hygienic behavior. Neotropical Entomol. 2011;40:28-34
38. Jones RL, Rothenbuhler WC. Behaviour genetics of nest cleaning in honey bees. II. Responses of two inbred lines to various amounts of cyanide-killed brood. Anim Behav. 1964;12:584-588. https://doi.org/10.1016/0003-3472(64)90083-1
39. Kawasaki T, Kawai T. Discrimination between self and non-self-nucleic acids by the innate immune system. Int Rev Cell Mol Biol. 2019;344:1-30. https://doi.org/10.1016/bs.ircmb.2018.08.004
40. Kirrane MJ, De Guzman LI, Rinderer TE, Frake AM, Wagnitz J, Whelan PM. Asynchronous development of honey bee host and Varroa destructor (Mesostigmata: Varroidae) influences reproductive potential of mites. J Econ Entomol. 2011;104:1146-1152. https://doi.org/10.1603/ec11035
41. Korb J, Fuchs A. Termites and mites – adaptive behavioral responses to infestation. Behaviour. 2006;143:891-907
42. Lachaud JP, Klompen H, Perez-Lachaud G. Macrodinychus mites as parasitoids of invasive ants: An overlooked parasitic association. Sci Rep. 2016;6:29995. https://doi.org/10.1038/srep29995
43. Le Conte Y, Arnold G, Trouiller J, Masson C, Chappe B. Identification of a brood pheromone in honeybees. Naturwissenschaften. 1990;77:334-336. https://doi.org/10.1007/bf01138390
44. Le Conte Y, Hefetz A. Primer pheromones in social Hymenoptera. Annu Rev Entomol. 2008;53:523-542. https://doi.org/10.1146/annurev.ento.52.110405.091434
45. Leclercq G, Francis F, Gengler N, Blacquière T. Bioassays to quantify hygienic behavior in honey bee (Apis mellifera L.) colonies: A review. J Apicul Res. 2018a;57:663-673. https://doi.org/10.1080/00218839.2018.1494916
46. Leclercq G, Blacquière T, Gengler N, Francis F. Hygienic removal of freeze-killed brood does not predict Varroa-resistance traits in unselected stocks. J Apicul Res. 2018b;57:292-299. https://doi.org/10.1080/00218839.2018.1426350
47. Leclercq G, Pannebakker B, Gengler N, Nguyen BK, Francis F. Drawbacks and benefits of hygienic behavior in honey bees (Apis mellifera L.): A review. J Apicul Res. 2017;56:366-375. https://doi.org/10.1080/00218839.2017.1327938
48. Leonhardt SD, Menzel F, Nehring V, Schmitt T. Ecology and evolution of communication in social insects. Cell. 2016;164:1277-1287.

https://doi.org/10.1016/j.cell.2016.01.035
49. Locke B. Natural Varroa mite-surviving Apis mellifera honeybee populations. Apidologie. 2016;47:467-482. https://doi.org/10.1007/s13592-015-0412-8
50. Martin S. Ontogenesis of the mite Varroa jacobsoni Oud in drone brood of the honeybee Apis mellifera L. under natural conditions. Exp & Appl Acarology. 1995;19:199-210. https://doi.org/10.1007/BF00130823
51. Martin SJ. The role of Varroa and viral pathogens in the collapse of honeybee colonies. J Appl Ecol. 2001;38:1082-1093.
52. Martin SJ, Hawkins GP, Brettell LE, Reece N, Correia-Oliveira ME, Allsopp MH. Varroa destructor reproduction and cell re-capping in mite-resistant Apis mellifera populations. Apidologie; c2019. https://doi.org/10.1007/s13592-019-00721-9
53. Masterman R, Ross R, Mesce K, Spivak M. Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (Apis mellifera L.). J Comp Phys A. 2001;187:441-452. https://doi.org/10.1007/s003590100216
54. Masterman R, Smith BH, Spivak M. Brood odor discrimination abilities in hygienic honey bees (Apis mellifera L.) using proboscis extension reflex conditioning. J Ins Behav. 2000;13:87-101. https://doi.org/10.1023/A:1007767626594
55. Maynard Smith J, Harper D. Animal signals. Oxford series in ecology and evolution, 1st edn. Oxford University Press, New York; c2003.
56. Medzhitov R, Janeway CAJ. Decoding the patterns of self and nonself by the innate immune system. Science. 2002;296:298-300.
57. Mondet F, *et al*. Honey bee survival mechanisms against the parasite Varroa destructor: A systematic review of phenotypic and genomic research efforts. International J Parasitol. 2020;50:433-447. https://doi.org/10.1016/j.ijpara.2020.03.005
58. Mondet F, Kim SH, de Miranda JR, Beslay D, Le Conte Y, Mercer AR. Specific cues associated with honey bee social defence against Varroa destructor infested brood. Sci Rep. 2016;6:25444. https://doi.org/10.1038/srep25444
59. Mondragon L, Spivak M, Vandame R. A multifactorial study of the resistance of honeybees Apis mellifera to the mite Varroa destructor over one year in Mexico. Apidologie. 2005;36:345-358. https://doi.org/10.1051/apido:2005022
60. Nazzi F, Della Vedova G, D'Agaro M. A semi chemical from brood cells infested by Varroa destructor triggers hygienic behaviour in Apis mellifera. Apidologie. 2004;35:65-70. https://doi.org/10.1051/apido:2003065
61. Newton DC, Ostasiewski NJJ. A simplified bioassay for behavioral resistance to American foulbrood in honey bees. Amer Bee J. 1986;126:278-281.
62. Oddie M *et al*. Rapid parallel evolution overcomes global honey bee parasite. Sci Rep. 2018;8:7704. https://doi.org/10.1038/s41598-018-26001-7
63. Oddie M *et al*. Response to: A small shift in VSH-gene frequency instead of rapid parallel evolution in bees. A comment on Oddie; c2018. https://doi.org/10.13140/RG.2.2.14662.22085
64. Page P, *et al*. Social apoptosis in honey bee super organisms. Sci. Rep. 2016;6:27210. https://doi.org/10.1038/srep27210
65. Park OW. Testing for resistance to American foulbrood in honeybees. J Econ Entomol. 1937;30:504-512. https://doi.org/10.1093/jee/30.3.504
66. Peng CS, Fang Y, Xu S, Ge L, Nasr ME. Response of foster Asian honeybee (Apis cerana Fabr.) colonies to the brood of European honeybee (Apis mellifera L.) infested with parasitic mite, Varroa jacobsoni Oudemans. J Invertebr Pathol. 1987a;49:259-264.
67. Peng YS, Fang YZ, Xu SY, Ge LS. The resistance mechanism of the Asian honey bee, Apis cerana Fabr, to an ectoparasitic mite, Varroa jacobsoni Oudemans. J Invertebr Pathol. 1987b;49:54-60. https://doi.org/10.1016/0022-2011(87)90125-X
68. Perez-Lachaud G, Bartolo-Reyes JC, Quiroa-Montalvan CM, Cruz-Lopez L, Lenoir A, Lachaud JP. How to escape from the host nest: imperfect chemical mimicry in eucharitid parasitoids and exploitation of the ants' hygienic behavior. J Insect Physiol. 2015;75:63-72. https://doi.org/10.1016/j.jinsphys.2015.03.003
69. Perez-Lachaud G, Rocha FH, Valle-Mora J, Henaut Y, Lachaud JP. Fine-tuned intruder discrimination favors ant parasitoids. PLoS One. 2019;14:e0210739. https://doi.org/10.1371/journal.pone.0210739
70. Pull CD, *et al*. Destructive disinfection of infected brood prevents systemic disease spread in ant colonies. Elife; c2018. p. 7. https://doi.org/10.7554/eLife.32073
71. Rath W. Co-adaptation of Apis cerana Fabr and Varroa jacobsoni Oud Apidologie. 1999;30:97-110.
72. Raymann K, Moran NA. The role of the gut microbiome in health and disease of adult honey bee workers. Curr Opin Insect Sci. 2018;26:97-104. https://doi.org/10.1016/j.cois.2018.02.012
73. Rinderer TE, *et al*. Resistance to the parasitic mite Varroa destructor in honey bees from far-eastern Russia. Apidologie. 2001;32:381-394
74. Root AI. The ABC and XYZ of Bee Culture. 33rd Edition. 33th edition edn. The A. I. Root Co., Medina, Ohio; c1966.
75. Rose R, Briggs JD. Resistance to American foulbrood in honey bees IX. Effects of honey-bee larval food on the growth and viability of Bacillus larvae. J Invertebr Pathol. 1969;13:74-80.
76. Rothenbuhler WC. Behaviour genetics of nest cleaning in honey bees. I. Responses of four inbred lines to disease-killed brood. Anim Behav. 1964;12:578-583. https://doi.org/10.1016/0003-3472(64)90082-x
77. Schöning C, Gisder S, Geiselhardt S, Kretschmann I, Bienefeld K, Hilker M, *et al*. Evidence for damage-dependent hygienic behaviour towards Varroa destructor-parasitised brood in the western honey bee, Apis mellifera J Exp Biol. 2012;215:264-271. https://doi.org/10.1242/jeb.062562
78. Spivak M. Honey bee hygienic behavior and defense against Varroa jacobsoni. Apidologie. 1996;27:245-260. https://doi.org/10.1051/apido:19960407
79. Spivak M, Gilliam M. Facultative expression of hygienic behavior of honey bees in relation to disease resistance. J Apicul Res. 1993;32:147-157. https://doi.org/10.1080/00218839.1993.11101300
80. Spivak M, Gilliam M. Hygienic behaviour of honey bees and its application for control of brood diseases and varroa Part I. Hygienic behaviour and resistance to

American foulbrood. Bee World. 1998a;79:124-134. https://doi.org/10.1080/0005772x.1998.11099394
81. Spivak M, Gilliam M. Hygienic behaviour of honey bees and its application for control of brood diseases and varroa - Part II. Studies on hygienic behaviour since the Rothenbuhler era. Bee World. 1998b;79:169-186. https://doi.org/10.1080/0005772x.1998.11099408
82. Spivak M, Masterman R, Ross R, Mesce KA. Hygienic behavior in the honey bee (Apis mellifera L.) and the modulatory role of octopamine. J Neurobiol. 2003;55:341-354. https://doi.org/10.1002/neu.10219
83. Spivak M, Reuter G. Performance of hygienic honey bee colonies in a commercial apiary. Apidologie. 1998;29:291-302.
84. Spivak M, Reuter GS. Resistance to American foulbrood disease by honey bee colonies Apis mellifera bred for hygienic behavior. Apidologie. 2001a;32:555-565.
85. Spivak M, Reuter GS. Varroa destructor infestation in untreated honey bee (Hymenoptera Apidae) colonies selected for hygienic behavior. J Econ Entomol. 2001b;94:326-331.
86. Swanson JA, Torto B, Kells SA, Mesce KA, Tumlinson JH, Spivak M. Odorants that induce hygienic behavior in honeybees: identification of volatile compounds in chalkbrood-infected honeybee larvae. J Chem Ecol. 2009;35:1108-1116. https://doi.org/10.1007/s10886-009-9683-8
87. Toufailia HMA, Amiri E, Scandian L, Kryger P, Ratnieks FL. Towards integrated control of varroa: effect of variation in hygienic behaviour among honey bee colonies on mite population increase and deformed wing virus incidence. J Apicul Res. 2014;53:555-562.
88. Tragust S, Mitteregger B, Barone V, Konrad M, Ugelvig LV, Cremer S. Ants disinfect fungus-exposed brood by oral uptake and spread of their poison. Curr Biol. 2013;23:76-82. https://doi.org/10.1016/j.cub.2012.11.034
89. Van Alphen JJM, Fernhout. A small shift in VSH-gene frequency instead of rapid parallel evolution in bees. A comment on Oddie, *et al*. 2018 Peer J Preprints; c2019. p. 7. https://doi.org/10.7287/peerj.preprints.27938v2
90. Vandame R, Morand S, Colin M-E, Belzunces L. Parasitism in the social bee Apis mellifera: Quantifying costs and benefits of behavioral resistance to Varroa destructor mites. Apidologie. 2002;33:443-445. https://doi.org/10.1051/apido:2002025
91. Villa JD, Danka RG, Harris JW. Simplified methods of evaluating colonies for levels of Varroa Sensitive Hygiene (VSH). J Apicul Res. 2009;48:162-167. https://doi.org/10.3896/Ibra.1.48.3.03
92. Villa JD, Danka RG, Harris JW. Selecting honeybees for worker brood that reduces the reproduction of Varroa destructor. Apidologie. 2016;47:771-778. https://doi.org/10.1007/s13592-016-0433-y
93. Villa JD, Danka RG, Harris JW. Repeatability of measurements of removal of mite-infested brood to assess Varroa Sensitive Hygiene. J Apicul Res. 2017;56:631-634. https://doi.org/10.1080/00218839.2017.1369707
94. Villegas AJ, Villa JD. Uncapping of pupal cells by European bees in the United States as responses to Varroa destructor and Galleria mellonella. J Apicul Res. 2006;45:203-206. https://doi.org/10.1080/00218839.2006.11101348
95. Wagoner K, Spivak M, Hefetz A, Reams T, Rueppell O. Stock-specific chemical brood signals are induced by Varroa and Deformed Wing Virus, and elicit hygienic response in the honey bee. Sci Rep. 2019;9:8753. https://doi.org/10.1038/s41598-019-45008-2
96. Wagoner KM, Spivak M, Rueppell O. Brood affects hygienic behavior in the honey bee (Hymenoptera: Apidae). J Econ Entomol. 2018;111:2520-2530. https://doi.org/10.1093/jee/toy266
97. Wilson EO, Durlach NI, Roth LM. Chemical releasers of necrophoric behavior in ants. Psyche. 1958;65:108-114.
98. Woodrow AW. Susceptibility of honeybee larvae to individual inoculations with spores of Bacillus larvae. J Econ Entomol. 1942;35:892-895. https://doi.org/10.1093/jee/35.6.892
99. Woodrow A, Holst E. Removal of diseased brood in colonies infected with American foulbrood. Am Bee J. 1942;83:22-23.
100. Zhang Y, Han R. A saliva protein of Varroa mites contributes to the toxicity toward Apis cerana and the DWV elevation in A. mellifera. Sci Rep. 2018;8:3387. https://doi.org/10.1038/s41598-018-21736-9
101. Danka RG, Harris JW, Dodds GE. Selection of VSH-derived “Pol-line” honey bees and evaluation of their Varroa-resistance characteristics. Apidologie. 2016;47:483-490. https://doi.org/10.1007/s13592-015-0413-7