*Journal of Insect Science*, (2021) 21(6): 4; 1–13
https://doi.org/10.1093/jisesa/ieab064
Research

ENTOMOLOGICAL SOCIETY OF AMERICA
SHARING INSECT SCIENCE GLOBALLY

OXFORD

# Hygiene-Eliciting Brood Semiochemicals as a Tool for Assaying Honey Bee (Hymenoptera: Apidae) Colony Resistance to *Varroa* (Mesostigmata: Varroidae)

**K. Wagoner,[1,7] J. G. Millar,[2] J. Keller,[3] J. Bello,[2,4] P. Waiker,[1] C. Schal,[3] M. Spivak,[5] and O. Rueppell[6]**

[1]Department of Biology, University of North Carolina Greensboro, P.O. Box 26170, Greensboro, NC 27402, USA, [2]Department of Entomology, University of California Riverside, 165 Entomology Building, Citrus Drive, Riverside, CA 92521, USA, [3]Department of Entomology, North Carolina State University, Campus Box 7613, Raleigh, NC 27695, USA, [4]Current address: Provivi Inc., 1701 Colorado Avenue, Santa Monica, CA 90404, USA, [5]Department of Entomology, University of Minnesota, 1980 Folwell Avenue, St. Paul, MN 55108, USA, [6]Department of Biological Sciences, University of Alberta, CW-405 Biological Sciences Building, Edmonton, Alberta T6G 2E9, Canada, and [7]Corresponding author, e-mail: kaira.wagoner@gmail.com

Subject Editor: Michael Simone-Finstrom

Received 13 May 2021; Editorial decision 9 August 2021

## Abstract

Despite numerous interventions, the ectoparasitic mite *Varroa* (*Varroa destructor* Anderson and Trueman [Mesostigmata: Varroidae]) and the pathogens it vectors remain a primary threat to honey bee (*Apis mellifera* Linnaeus [Hymenoptera: Apidae]) health. Hygienic behavior, the ability to detect, uncap, and remove unhealthy brood from the colony, has been bred for selectively for over two decades and continues to be a promising avenue for improved *Varroa* management. Although hygienic behavior is expressed more in *Varroa*-resistant colonies, hygiene does not always confer resistance to *Varroa*. Additionally, existing *Varroa* resistance selection methods trade efficacy for efficiency, because those achieving the highest levels of *Varroa* resistance can be time-consuming, and thus expensive and impractical for apicultural use. Here, we tested the hypothesis that hygienic response to a mixture of semiochemicals associated with *Varroa*-infested honey bee brood can serve as an improved tool for predicting colony-level *Varroa* resistance. In support of our hypothesis, we demonstrated that a mixture of the compounds (*Z*)-10-tritriacontene, (*Z*)-8-hentriacontene, (*Z*)-8-heptadecene, and (*Z*)-6-pentadecene triggers hygienic behavior in a two-hour assay, and that high-performing colonies (hygienic response to ≥60% of treated cells) have significantly lower *Varroa* infestations, remove significantly more introduced *Varroa*, and are significantly more likely to survive the winter compared to low-performing colonies (hygienic response to <60% of treated cells). We discuss the relative efficacy and efficiency of this assay for facilitating apiary management decisions and selection of *Varroa*-resistant honey bees, as well as the relevance of these findings to honey bee health, pollination services, and social insect communication.

**Key words:** hygienic behavior, social immunity, chemical communication, alkene, unhealthy brood odor

The honey bee (*Apis mellifera*) is the most important crop pollinator worldwide, contributing an estimated $235–$577 billion to annual global crop value (Quigley et al. 2019). Recent agricultural demand for honey bee pollination services is growing at a faster rate than the managed honey bee population (Aizen and Harder 2009), threatening global food security (Marshman et al. 2019). Despite their importance as pollinators, the health of honey bees is declining, with annual colony loss rates as high as 45% in the United States (Kulhanek et al. 2017). The primary biological threat to honey bee health is the parasitic mite *Varroa destructor* (Traynor et al. 2020), which completes reproduction inside honey bee brood cells (Martin 1994) and is both a physiological burden and disease vector to its honey bee host (Bowen-Walker et al. 1999, Ramsey et al. 2019).

Numerous interventions exist to control *Varroa*, including chemical treatments such as synthetic miticides, organic acids, and essential oils, and mechanical techniques such as drone brood removal, brood interruption, and use of *Varroa*-resistant honey bee stocks. Drawbacks, efficacy, and adoption vary greatly among these methods. The majority of beekeepers in the United States currently use synthetic miticides to control *Varroa* (Haber et al. 2019) despite evidence of negative sublethal effects such as reduced queen and drone reproductive health (Rangel and Tarpy 2015, Rangel and Fisher 2019), and reduced worker memory, foraging, and hive maintenance (Gashout et al. 2020a,b). The use of previously effective miticides such as coumaphos and fluvalinate is now associated with high overwintering losses (Haber et al. 2019) due to the evolution of

© The Author(s) 2021. Published by Oxford University Press on behalf of Entomological Society of America.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (https://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com



Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

*Varroa* resistance (Sammataro et al. 2005, González-Cabrera et al. 2016). While the miticide amitraz is currently associated with the lowest overwintering colony losses (Haber et al. 2019), *Varroa* resistance to amitraz has been reported (Elzen et al. 1999, Elzen et al. 2000, Maggi et al. 2010, Rinkevich 2020), which is particularly concerning given the extent to which the beekeeping industry currently relies on the compound for *Varroa* control. Though *Varroa* resistance to organic acids such as formic acid and essential oil compounds such as thymol has not been reported, these treatments can contaminate hive products (Bogdanov 2006), are temperature dependent (Imdorf et al. 1995, Imdorf et al. 1999, Tihelka 2018), and are less effective than other interventions (Haber et al. 2019). Mechanical techniques such as drone comb removal and brood interruption can be effective at slowing *Varroa* population growth. However, these methods tend to be prohibitively labor intensive (Wilkinson et al. 2002, Jack et al. 2020), and additional interventions are typically required to achieve adequate *Varroa* control (Delaplane et al. 2005, Wantuch and Tarpy 2009, Jack et al. 2020). Integrated Pest Management (IPM) strategies that combine regular *Varroa* monitoring with carefully timed treatments can be implemented for more sustainable *Varroa* control. IPM labor is intensive, however, and requires region-specific analysis of critical *Varroa* infestations (Noël et al. 2020).

While numerous chemical and mechanical strategies have been implemented to reduce the threat of *Varroa*, the drawbacks of existing interventions combined with consistently high annual colony losses (Kulhanek et al. 2017) suggest that more suitable interventions may be needed. One promising avenue for achieving sustainable, effective *Varroa* control is the selective breeding of *Varroa*-resistant honey bees. Although *Varroa*-resistant honey bees can be selected based on overall low mite population growth, the mechanisms underlying mite population control may differ within and among apiaries, making it difficult to distinguish brood, adult, and environmental effects. A more common breeding strategy involves the selection of hygienic honey bees that demonstrate an enhanced ability to detect and remove unhealthy brood from the colony. Selective breeding of hygienic behavior has the potential to be a sustainable, long-term solution for *Varroa* control because it places the burden of mite control on the bees rather than the beekeeper, it is not susceptible to the development of resistance by *Varroa*, and it does not harm bees or contaminate hive products. However, while the hygienic trait is elevated in many *Varroa*-resistant colonies, hygienic performance does not always confer *Varroa* resistance (Spivak and Reuter 2001b).

Several techniques for selective breeding of bees with augmented hygienic traits have been developed, such as 1) measuring removal of freeze- or pin-killed brood, 2) quantifying uncapping and/or removal of *Varroa*-infested brood, and 3) evaluating mite reproductive success in brood cells. However, existing selection methods tend to trade efficacy for efficiency, because those achieving the highest levels of *Varroa*-specific hygiene are time-consuming and require greater technical skill (Leclercq et al. 2018b, Mondet et al. 2020), and are thus expensive and impractical for commercial use. For example, although hygienic behavior can be selected for relatively easily through quantification of freeze-killed or pin-killed brood removal, hygienic response to killed brood does not always confer *Varroa* resistance (Leclercq et al. 2018a, Spivak and Danka 2021), as some colonies deemed 'hygienic' using these methods still require miticides to limit *Varroa* infestations (Spivak and Reuter 2001b). In contrast, selecting for traits such as *Varroa*-sensitive hygiene (VSH) or low mite reproductive success can reliably achieve *Varroa* resistance (Locke 2016), but these techniques are skilled-labor intensive, making them impractical for most mid- to large-scale commercial operations (Noël et al. 2020).

In honey bees, hygienic behavior is regulated by chemical communication between developing brood and adult nurse bees, where quantitative (Salvy et al. 2001, Nazzi et al. 2004, Schöning et al. 2012, McAfee et al. 2017, Wagoner et al. 2019, Liendo et al. 2021) and qualitative (Kathe et al. 2021, Mondet et al. 2021) changes of brood odor profiles can signal health status and trigger hygienic uncapping and removal of brood. Of particular interest are cuticular hydrocarbons (CHCs), which form a waxy layer on insect cuticles to prevent desiccation (Jackson and Baker 1970, Blomquist et al. 1987) and facilitate communication, including nestmate recognition (Howard and Blomquist 2005, van Zweden and d'Ettorre 2010) and task performance (Greene and Gordon 2003). Compared to alkanes, alkenes elicit stronger behavioral responses (Dani et al. 2005) and are more easily discriminated by honey bee workers (Châline et al. 2005), suggesting their relative importance in nestmate communication. Interestingly, numerous monoalkenes with similar structures but wide-ranging volatilities have been associated with honey bee health status and hygienic behavior (Nazzi et al. 2002, Nazzi et al. 2004, Wagoner et al. 2019). Substantial differences in the volatility of monoalkenes associated with hygienic behavior support a two-step model of hygiene communication (McAfee et al. 2018), where smaller, more volatile compounds attract workers, and larger less-volatile compounds trigger hygienic behavior. However, given that CHCs with a wide range of volatility can trigger hygiene (Wagoner et al. 2020), less volatile CHCs may also help workers more accurately pinpoint the location of compromised cells. Such a system could both recruit specialized workers (Barrs et al. 2021) to a problem area, and prevent costly mistakes associated with accidental uncapping and/or removal of healthy brood. Efficient hygiene communication combined with our ability to understand, quantify, and manipulate this natural social immune mechanism provides substantial potential for the development of improved control of honey bee pests and pathogens.

Recent appeals for improved hygiene selection tools emphasize that ideal stimuli used to induce brood removal would closely resemble stimuli found in unhealthy brood cells in the colony environment (Leclercq et al. 2018b). The discovery of enhanced olfactory sensitivity in hygiene-performing adults (Spivak et al. 2003) and the recent identification and synthesis of the hygiene-inducing compounds (*Z*)-10-tritriacontene (Z10-$C_{33}$), (*Z*)-8-hentriacontene (Z8-$C_{31}$), (*Z*)-8-heptadecene (Z8-$C_{17}$) and (*Z*)-6-pentadecene (Z6-$C_{15}$) (Wagoner et al. 2020), which are naturally elevated in *Varroa*-parasitized brood (Nazzi et al. 2002, Nazzi et al. 2004, Wagoner et al. 2019, Wagoner et al. 2020, Mondet et al. 2021) and adults (Nation et al. 1992), offer a potential path for the development of improved tools to facilitate apiary management and the selective breeding of *Varroa*-resistant honey bees. Here we tested the hypothesis that hygienic response to a mixture of semiochemicals associated with *Varroa*-infested honey bee brood could serve as an improved tool for predicting colony-level *Varroa* resistance. Specifically, we tested the predictions that two-hour colony response to a mixture of Z10-$C_{33}$, Z8-$C_{31}$, Z8-$C_{17}$, and Z6-$C_{15}$ applied to honey bee brood cell caps would be 1) negatively correlated with colony *Varroa* infestation, 2) positively correlated with colony *Varroa* removal, and 3) predictive of overwintering success in colonies not treated chemically for *Varroa*. We compared the performance of this assay to that of the established Freeze-Killed Brood (FKB) assay, and a control mixture containing the structurally similar compounds (*Z*)-16-dotriacontene (Z16-$C_{32}$), (*Z*)-15-triacontene (Z15-$C_{30}$), (*Z*)-7-heptadecene (Z7-$C_{17}$), and (*Z*)-7-pentadecene (Z7-$C_{15}$). Control

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

compounds were chosen based on their similarity in size and structure to UBO compounds, and because they have not been detected on honey bee cuticles.

## Materials and Methods

### Overview

Experiments at five sites across three study years (Table 1) were performed to test our hypothesis that honey bee hygienic response to the unhealthy brood odors Z10-$C_{33}$, Z8-$C_{31}$, Z8-$C_{17}$, and Z6-$C_{15}$ can predict colony-level resistance to *Varroa*. Sites included a private beekeeper yard in Vass, North Carolina (Vass), a temporary apiary in Lamberton, Minnesota (Lamb), and apiaries at the University of Minnesota (UMN), the University of North Carolina Greensboro (UNCG), and North Carolina State University (NCSU). Study years varied at different sites as follows: Vass (2019), Lamb (2019), UMN (2019-2020), UNCG (2018-2020), and NCSU (2020). Colonies from Vass, Lamb, and UMN (2019 only) were chemically treated for *Varroa* in the spring (before data collection) and fall (after data collection), and colonies at NCSU and UMN (2020) were treated for *Varroa* in late summer (after data collection). Chemical treatments used included amitraz (Lamb and NCSU), and formic acid (UMN), and were not disclosed by the beekeeper for Vass. UNCG colonies were never treated for *Varroa*. Only colonies that maintained the same queen from the beginning of experiments through data collection were included in the analyses. For comparison with results from other sites, the study at NCSU was conducted as an independent investigation in which colony evaluations were performed blindly with respect to *Varroa* infestation levels. Freeze-killed brood (FKB) assays and assays with experimental compounds Z10-$C_{33}$, Z8-$C_{31}$, Z8-$C_{17}$, and Z6-$C_{15}$ were tested in all colonies, at all sites, in all years. Assays with control compounds Z16-$C_{32}$, Z15-$C_{30}$, Z7-$C_{17}$, and Z7-$C_{15}$ were tested in Vass, Lamb, UNCG (2018-2019), and UMN (2019). *Varroa* infestation was measured at all sites, *Varroa* removal assays were performed only at UNCG and UMN (all years) due to their technical and labor-intensive nature, and overwintering analysis was performed only at UNCG, where colonies were never treated with miticides.

### Chemical Synthesis

Syntheses of Z6-$C_{15}$, Z7-$C_{15}$, Z16-$C_{32}$, and Z10-$C_{33}$ have been described previously (Wagoner et al. 2020). Syntheses of the other experimental and control compounds used in the study are described below.

### Synthesis of (*Z*)-8-Heptadecene (Z8-$C_{17}$)

A solution of octyltriphenylphosphonium bromide (11.4 g, 25 mmol) in 125 ml dry THF under argon atmosphere was cooled to 0°C, and sodium hexamethyldisilazide (1 M solution in THF, 26 ml, 26 mmol) was added over 30 min. The solution was warmed to room temp and stirred 1 hr, then cooled to ~−15°C in an ice-salt bath, and a solution of freshly distilled nonanal (3.12 g, 22 mmol) in 10 ml THF was added by syringe pump over 1 hr. The resulting mixture was allowed to slowly warm to room temp overnight, then the resulting slurry was quenched with saturated $NH_4Cl$, and extracted with 200 ml hexane. The hexane layer was washed sequentially with 1 M aq. HCl, saturated aq. $NaHCO_3$, and brine, then dried over anhydrous $Na_2SO_4$ and concentrated. The crude product was taken up in 100 ml hexane, cooled to 0°C, and filtered with suction, rinsing the precipitated triphenylphosphine oxide with ice-cold hexane. The hexane solution was flushed through a pad of silica gel, rinsing with hexane, then concentrated. The resulting liquid was Kugelrohr distilled (bp ~80°C, 0.5 mm Hg), yielding 4.75 g (91%, 94% pure by GC) of (*Z*)-8-heptadecene. MS (*m/z*, abundance): 238 (14), 210 (1), 182 (1), 168 (1), 154 (1), 140 (3), 139 (3), 125 (15), 111 (40), 97 (82), 83 (93), 69 (94), 55 (100), 43 (67), 41 (65).

**Table 1.** Sample size and miticide use across study site and year

| Site | Year(s) | Colonies (n) | Spring Miticide? |
|---|---|---|---|
| Vass | 2019 | 18 | Yes |
| Lamb | 2019 | 24 | Yes |
| UMN | 2019 | 12 | Yes |
| | 2020 | 13 | No |
| UNCG | 2018 | 12 | No |
| | 2019 | 11 | No |
| | 2020 | 13 | No |
| NCSU | 2020 | 26 | No |

### Synthesis (*Z*)-7-Heptadecene (Z7-$C_{17}$)

(*Z*)-7-Heptadecene was synthesized in analogous fashion and yield from heptyltriphenyl-phosphonium bromide and decanal, bp ~80°C, 0.05 mm Hg. MS (*m/z*, abundance): 238 (18), 210 (1), 182 (1), 168 (1), 154 (2), 140 (4), 139 (4), 125 (15), 111 (42), 97 (87), 83 (98), 69 (100), 55 (98), 43 (62), 41 (65).

### Synthesis of (*Z*)-15-Triacontene (Z15-$C_{30}$)

(*Z*)-15-Triacontene was prepared in an analogous fashion from pentadecanal and pentadecyltriphenylphosphonium bromide. After the initial workup, the crude product was concentrated, then taken up in hexane, and filtered with suction through a bed of silica gel, rinsing well with hexane. The resulting solution was concentrated, giving a clear oil which partially crystallized on standing. The material was taken up in hot acetone, and the solution was chilled overnight at 4°C. The solution was filtered cold, producing pure (*Z*)-15-triacontene as a fluffy white, low-melting solid in 38% yield. MS (*m/z*, abundance): 420 (6), 292 (1), 262 (1), 250 (1), 238 (1), 224 (1), 208 (2), 196 (2), 182 (2), 167(4), 153 (6), 139 (11), 125 (27), 111 (54), 97 (100), 83 (89), 69 (65), 57 (84), 55 (62), 43 (60, 41 (30).

### Synthesis of (*Z*)-8-Hentriacontene (Z8-$C_{31}$)

A solution of 1-nonyne (4.96 g, 40 mmol) and ~100 mg triphenylmethane indicator in 150 ml dry THF was cooled in an icebath under Ar, and butyllithium in hexanes (2.5 M, 17 ml) was added dropwise until the solution turned pink, indicating an excess of butyllithium. NaI (0.6 g, 4 mmol) was added in one portion, followed by dropwise addition of docosanyl bromide (7.79 g, 20 mmol) in THF. The resulting mixture was heated to 60°C for 3 d. The mixture was then cooled to room temp, quenched with saturated aq. $NH_4Cl$, and extracted with hexane. The hexane extract was washed with brine, dried over anhydrous $Na_2SO_4$, and concentrated. The residue was taken up in hexane and filtered through a 4 cm plug of silica gel, rinsing well with hexane. After concentration, the resulting white solid was taken up in 150 ml hot acetone, and recrystallized overnight at 4°C. The mixture was filtered cold with suction, rinsing the solids with ice-cold acetone, providing 8-hentriacontyne as fluffy white crystals (8.02 g, 93%).

A slurry of Lindlar catalyst (1 g) and pyridine (1 g) in 50 ml hexane was flushed with $H_2$ without stirring, then the flask was sealed under $H_2$ atmosphere and stirred for 15 min. A solution of the alkyne (4.34 g, 10 mmol) in 50 ml hexane was added in one portion by syringe, and the mixture was stirred until all the starting material

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

had been consumed (~5 hr), monitoring the uptake of $H_2$ with a gas burette. The mixture was then filtered through a bed of Celite filtering aid with suction, rinsing well with hexane. The resulting hexane solution was washed twice with 1M HCl to remove pyridine, once with brine, then concentrated. The residue was dissolved in 50 ml hot acetone, and recrystallized overnight at 4°C, yielding (*Z*)-8-hentriacontene (4.23 g, 97%, 99.6% pure by GC) as white crystals. MS (*m/z*, abundance): 434 (7), 406 (1), 334 (1), 320 (1), 306 (1), 292 (1), 278 (1), 264 (1), 252 (1), 238 (1), 224 (1), 210 (1), 195 (2), 181 (3), 167 (4), 153 (6), 139 (11), 125 (28), 111 (55), 97 (100), 83 (89), 69 (72), 57 (84), 55 (69), 43 (62), 41 (30).

## UBO, FKB, and CON Assays

Unhealthy brood odor (UBO) and Freeze-killed brood (FKB) assays were conducted at all five sites. Initial UBO assays were performed in June to test the predictive ability of the assay, and to allow a minimum of six weeks for population turnover in colonies with newly-introduced queens. Assays were repeated in August to allow a comparison of the correlation between assay response and mite infestation across seasons. As illustrated in Fig. 1, unhealthy brood odor (UBO) assays were performed by applying 0.5 ml of a hexane solution containing 2.5 mg each Z10-$C_{33}$, Z8-$C_{31}$, Z8-$C_{17}$, and Z6-$C_{15}$ to a small circular region of capped, non-emerging honey bee brood cells, and quantifying hygienic response after 2 hr (Wagoner and Rueppell 2019; 2020a,b). Assay areas contained up to approximately 50 cells and were isolated using a short section of PVC pipe with a 3.8 cm inner diameter. To reduce distortion of the wax cells, a lathe was used to narrow the base of the PVC pipe, forming a cutaway approximately 2.4 mm long, and 0.8 mm thick. To mark the outside perimeter of the assay area, the cylinder was lightly pressed and twisted into the wax, cutaway side down, with care not to injure the brood underneath. For each assay, the number of capped cells in the test area at time zero ($T_0$) was recorded, including all cells for which >50% of the cell cap was located inside the test area. Solutions were applied using 5 ml glass spray bottles, and the frame was left undisturbed until the solvent appeared to have evaporated (~15 s). Treated frames were then returned to their respective colonies. After 2 hr ($T_2$), frames were recollected, and capped cells in the assay regions were recounted. Assay scores were calculated as the percentage of the capped cells at $T_0$ that were manipulated (any uncapping including piercing) at $T_2$. Control alkene (CON) assays were performed and evaluated in the same manner as UBO assays, using 0.5 ml of a hexane solution containing 2.5 mg each Z16-$C_{32}$, Z15-$C_{30}$, Z7-$C_{17}$, and Z7-$C_{15}$.

Freeze-killed brood (FKB) assays were performed on UBO-tested colonies after completion of the June UBO assays, as previously described (Büchler et al. 2013). Briefly, a section of PVC pipe approximately 7.5 cm in diameter was pressed firmly into a region of capped, non-emerging honey bee brood cells. Liquid nitrogen was then poured into the PVC pipe, freeze-killing the brood within. After allowing the assay region to thaw, the PVC pipe was removed, and the frames were returned to their colony of origin. After 24 hr ($T_{24}$), frames were recollected, and the total number of cells containing any pupae were counted. Assay scores were calculated as the percentage of the capped brood at $T_0$ that was completely removed at $T_{24}$.

## Varroa Infestation

*Varroa* infestation was measured in June and August for colonies at all five sites using standard methods (Dietemann et al. 2013). Briefly, approximately 300 adult bees were collected from brood frames and rinsed thoroughly with 75% ethanol. Bee and *Varroa* numbers were counted and recorded, and the percent infestation (number of *Varroa* per 100 bees) was calculated for each sample. Hereafter, '*Varroa* infestation' refers to the infestation of adult honey bees.

## Varroa Removal

*Varroa* removal experiments were performed in August at UNCG and UMN. To mimic natural brood cell infestation, transparent plastic sheets were used to mark the locations of uncapped brood cells containing 5th larval instars. Experimental frames were

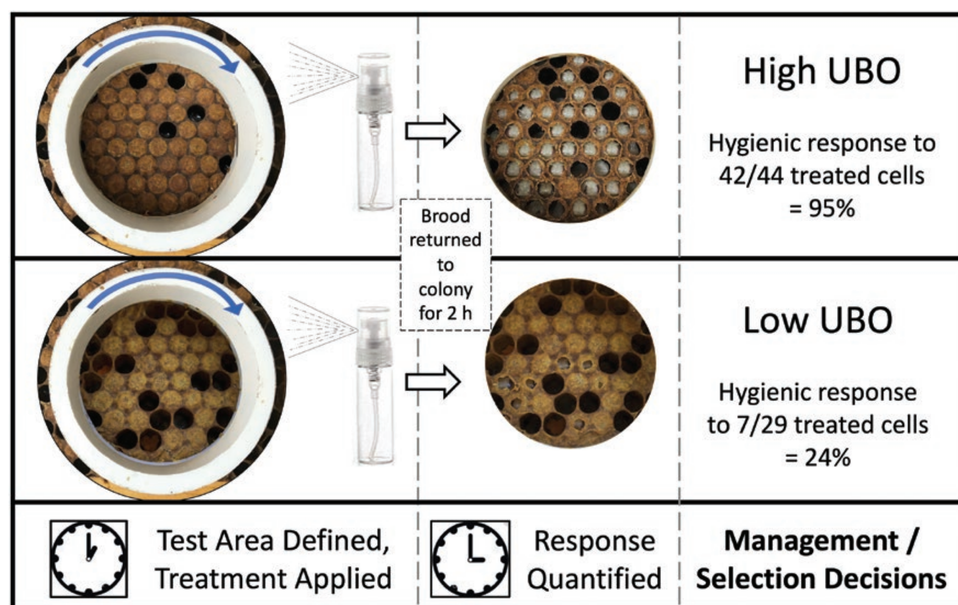


**Fig. 1.** Example of unhealthy brood odor (UBO) assays for colonies demonstrating high and low UBO responses. For each colony, a section of PVC pipe was placed on an area of non-emerging capped brood and twisted gently into the wax to define the test area. The number of capped cells for which >50% of the cell cap was located inside the test area were counted, the UBO mixture was applied to test areas by spraying, and the frames were returned to their respective colonies. After two hours the frames were recollected, and UBO scores were calculated based on the percentage of capped cells remaining in the test area.

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

returned to their colony of origin, and recollected within 16 hr to ensure that experimental cells were appropriately aged for *Varroa* introduction (Frey et al. 2013). *Varroa* were introduced into approximately one-half of recently capped cells by cutting and lifting one side of the cell cap with a razor blade, and gently introducing a mite on the tip of a small paintbrush. The number of cells that received the *Varroa* treatment was dependent on the availability of *Varroa* and appropriately aged brood, and ranged from 13–50 cells per colony. The number of cells that received *Varroa* and control treatments was consistent within each colony. *Varroa* used for removal assays were collected by sugar shake as previously described (Dietemann et al. 2013), and only active *Varroa* able to cling to the paintbrush bristles were considered viable, and introduced to brood cells. The other half of recently capped cells were opened and resealed without *Varroa* introduction, serving as controls. Brood frames were then returned to their colony, and hygienic uncapping and removal of control and infested brood were recorded 2- and 8-d post-capping. Because hygienic behavior is not typically performed at this stage in development (Harris 2007), removal of brood on day 2 post capping was considered to be an experimental artifact of cell cap manipulation during *Varroa* introduction, and these cells were excluded from data analysis.

### Overwintering

Only colonies from UNCG were used for overwintering analysis because they were the only population without any (chemical) *Varroa* treatment. Such treatments against *Varroa* significantly affect colony survival (Amdam et al. 2004, Locke et al. 2014), and thus potentially obscure natural survival differences among colonies. Thus, overwintering success after chemical control of *Varroa* in Vass, Lamb, UMN, and NCSU was not considered a useful measure of the predictive value of the UBO assay. Overwintering was defined as the survival of a queen and workers from August of one year until March of the following year.

### Statistical Analysis

Spearman's correlations were used to compare assay scores and indicators of *Varroa* resistance. Welch's *t*-tests were used to compare assay thresholds that best distinguish high and low *Varroa* infestation. Welch's *t*-tests were also used to compare indicators of *Varroa* resistance for high and low UBO, CON, and FKB colonies, where 'high' colonies were defined as those scoring ≥60% on UBO and CON assays, and ≥95% on FKB assays. The thresholds distinguishing 'low' and 'high' UBO assay scores were selected based on results from a series of t-tests comparing 'low' and 'high' thresholds from 1 to 99% (see Results). These categorical evaluations were intended to describe effects quantitatively, rather than for hypothesis testing. Welch's *t*-test was used because sample sizes were uneven and Levene's test indicated that the assumption of homogeneity of variance was violated for *Varroa* infestation data. When all colonies for which *Varroa* infestation data were available were included in the analysis, Levene's test indicated that variances in June colony responses were not equal for low and high scoring colonies based on the UBO assay ($F_{1,99}$ = 7.6, $P$ = 0.01), CON assay ($F_{1,39}$ = 4.1, $P$ = 0.05), or FKB assay ($F_{1,99}$ = 3.9, $P$ = 0.05). Similarly, variances in August colony responses were not equal for low and high scoring colonies based on the UBO assay ($F_{1,124}$ = 11.4, $P$ < 0.01), CON assay ($F_{1,50}$ = 7.5, $P$ = 0.01), or FKB assay ($F_{1,124}$ = 5.9, $P$ = 0.02). *Varroa* infestation data also failed to meet assumptions of normality. However, use of the parametric Welch's test was deemed appropriate because sample sizes were sufficiently large (Kallenberg and Kallenberg 1997), it enabled consistent analyses across experiments, and a secondary analysis of the data using the nonparametric Mann–Whitney *U* test gave similar results (data not shown). All analyses, except threshold analyses, were performed using IBM SPSS Statistics version 26.0.0.0. Threshold analyses were performed using RStudio version 1.2.5033, R base package version 3.6.2.

## Results

### Varroa Infestation

Colonies that exhibited strong responses in UBO and FKB assays had lower *Varroa* infestations than colonies that exhibited weak responses, especially when colonies chemically treated for *Varroa* in spring (and thus less informative about their intrinsic ability to resist *Varroa*) were excluded from the analysis. Compared to untreated colonies, colonies treated in spring with miticides had significantly reduced *Varroa* infestations in June ($F_{1, 99}$ = 13.3, $P$ < 0.001) and August ($F_{1, 124}$=20.8, $P$ < 0.001). In miticide-treated colonies, no correlations were found between June UBO assay responses and *Varroa* infestations in June (rs = −0.01, d.f. = 52, $P$ = 0.46) or August (rs = −0.09, d.f. = 52, $P$ = 0.26; Fig. 2a). Similarly, in miticide-treated colonies no correlations were found between FKB assay responses and *Varroa* infestations in June (rs = −0.02, d.f. = 52, $P$ = 0.44) or August (rs = 0.06, d.f. = 52, $P$ = 0.33; Fig. 2b). For colonies that were not chemically treated for *Varroa* in spring, June UBO assay scores were negatively correlated with *Varroa* infestation levels in both June (rs = −0.60, d.f. = 45, $P$ < 0.001) and August (rs = −0.54, d.f. = 70, $P$ < 0.001; Fig 2c). FKB assay scores in untreated colonies were negatively correlated with *Varroa* infestations in August (rs = −0.30, d.f. = 70, $P$ = 0.005; Fig 2d), but not June (rs = −0.18, d.f. = 45, $P$ = 0.12). There was no evidence of a correlation between CON assay scores and *Varroa* infestations in either June (rs = 0.13, d.f. = 9, $P$ = 0.35) or August (rs = −0.19, d.f. = 20, $P$ = 0.20). There was a significant negative correlation between August UBO assay scores and *Varroa* infestation levels (rs = −0.35, d.f. = 68, $P$ = 0.001), but no significant correlation between *Varroa* removal and *Varroa* infestations (rs = −0.17, d.f. = 43, $P$ = 0.13).

To understand the predictive ability of the UBO assay among colonies with low mite populations, the relationship between UBO response and *Varroa* infestation was reevaluated for colonies that were not treated in spring and that had infestations below the economic treatment threshold of 3%. In this subset of colonies, there was a significant negative correlation between June UBO assay scores and August *Varroa* infestations (rs = −0.41, d.f. = 34, $P$ = 0.006; Fig. 3a) and suggestive evidence of negative correlations between August UBO assay scores and August *Varroa* infestations (rs = −0.28, d.f. = 32, $P$ = 0.052), and between FKB assay scores and August *Varroa* infestations (rs = −0.24, d.f. = 34, $P$ = 0.084; Fig. 3b).

### Categorical Analysis of *Varroa* Infestation

For a comparison of the UBO assay and the FKB assay, in which the use of a 95% threshold and categorical analysis are standard practice, we sought to determine a similarly informative threshold for the UBO assay. Our data further justified such an analysis because they suggested not a linear relationship between UBO assay scores and Varroa infestation levels, but a threshold above which infestation fell significantly (Fig. 2c). To empirically establish the most biologically meaningful threshold for distinguishing 'low' and 'high' UBO assay scores we varied the threshold for classifying 'low' and 'high' from 1 to 99% and ran a series of t-tests comparing mean August *Varroa* infestation between the resulting groups (Fig. 4a).

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

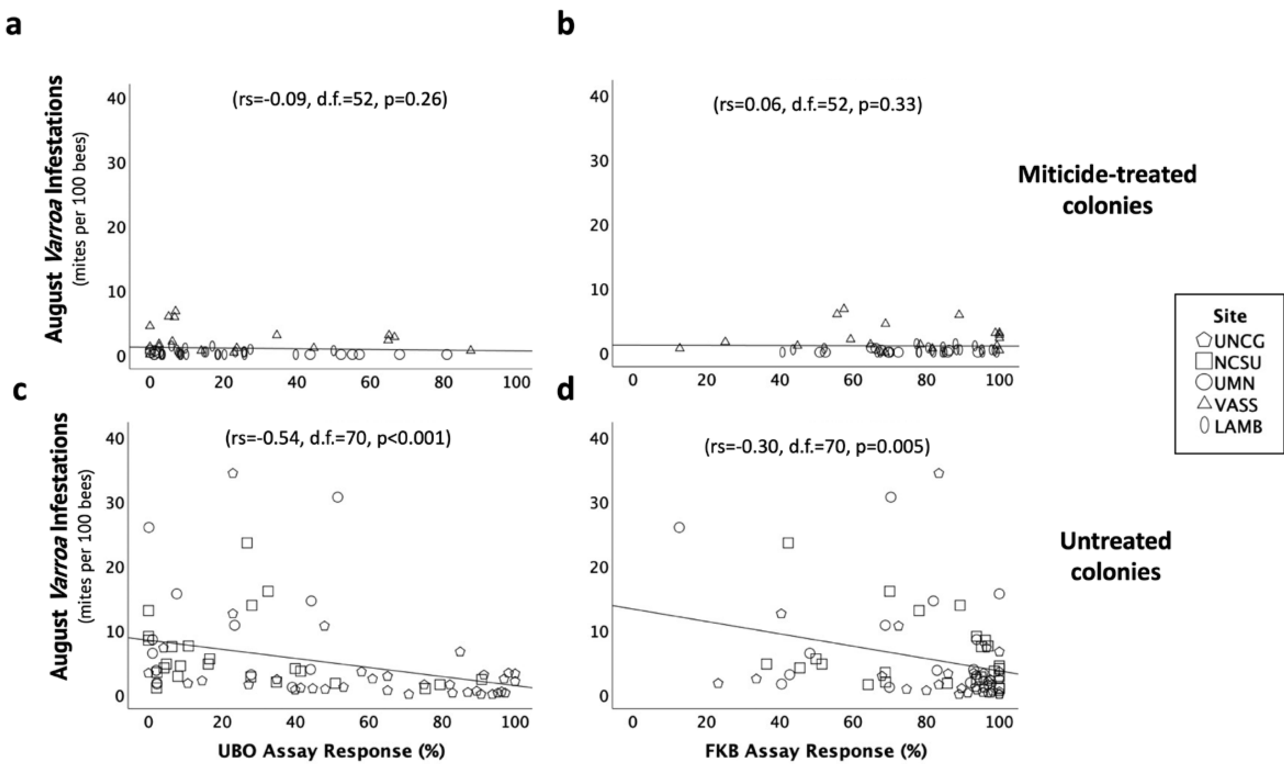


**Fig. 2.** Spearman's correlations between assay responses and August *Varroa* infestations in untreated and miticide-treated colonies. Untreated colonies that exhibited strong responses in unhealthy brood odor (UBO) and freeze-killed brood (FKB) assays had lower *Varroa* infestations than colonies that exhibited weak assay responses. Data from miticide-treated colonies was collected at three sites including Vass, North Carolina (Vass), Lamberton, Minnesota (Lamb), and the University of Minnesota (UMN) ($n$ = 18, 24, and 12, respectively). Data from untreated colonies was also collected at three sites including the University of North Carolina Greensboro (UNCG), the University of Minnesota (UMN), and North Carolina State University (NCSU) ($n$ = 35, 13, and 24, respectively). Each data point represents a single colony. Symbol shape corresponds to study site. The UMN site included both spring-treated and non-spring-treated colonies, depending on year. In miticide-treated colonies, no correlations were observed between August *Varroa* infestations (percent of adult bees infested) and responses in the UBO assays (a) and in the FKB assays (b). In untreated colonies there was significant evidence of negative correlations between August *Varroa* infestation and responses in the UBO (c) and FKB (d) assays.

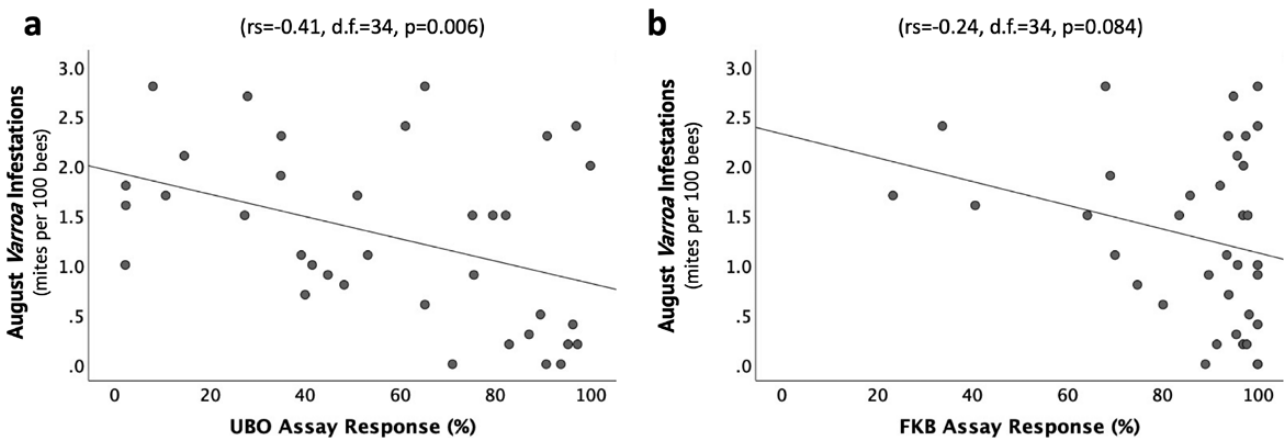


**Fig. 3.** Spearman's correlation of August *Varroa* infestations with June unhealthy brood odor (UBO) assay responses and freeze-killed brood (FKB) assay responses for colonies that were not chemically treated for *Varroa* in spring but had August *Varroa* infestations below 3% ($n$ = 36 for each assay type). Each data point represents a single colony. A significant negative correlation was observed between June UBO assay responses and August *Varroa* infestations (a). There was suggestive evidence of a negative correlation between freeze-killed brood (FKB) assay responses and August *Varroa* infestations (b).

Comparison of mean August *Varroa* infestation between colonies with UBO assay scores <62% and ≥62% resulted in the smallest $P$-value ($8.61 * 10^{-6}$). However, the largest drop in $P$-value ($-6.48 * 10^{-3}$) occurred between UBO assay scores 51% and 52%. Based on these combined results, we chose to use a threshold of 60% ($P = 9.37 * 10^{-6}$) to distinguish the most biologically relevant low

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

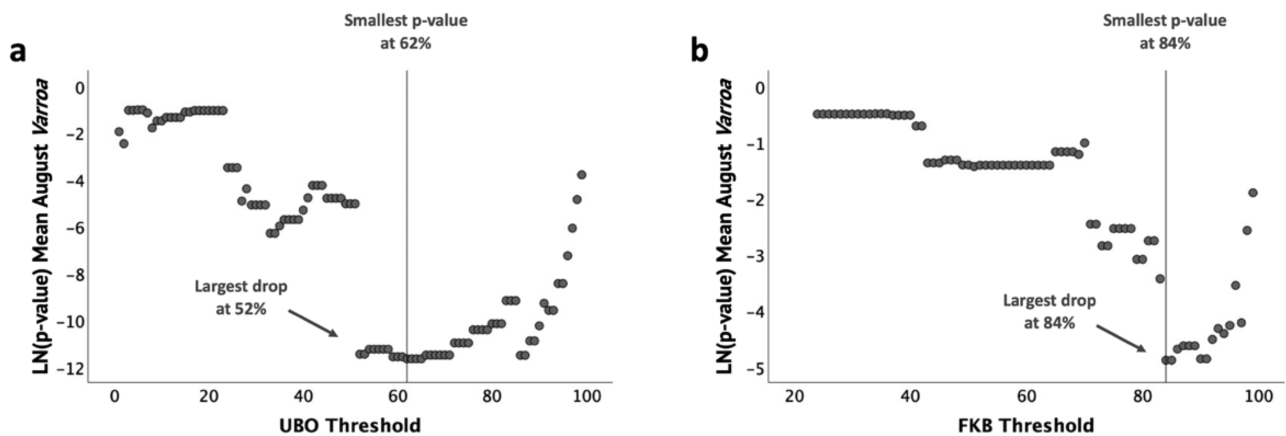


**Fig. 4.** Relative strength of unhealthy brood odor (UBO) and freeze-killed brood (FKB) thresholds for classifying 'low' and 'high' assay scores from 75 honey bee colonies. Each data point represents a statistical test result (log-normal *P*-value) as a function of the threshold (1–99%). For the UBO assay (a), the smallest *P*-value and largest change in *P*-value were found at 62% and 52%, respectively. For the FKB assay (b), the smallest *P*-value and largest change in *P*-value both occurred at 84%.

and high scores in the UBO assay. The 60% threshold resulted in a *P*-value similar to the 62% threshold, was sufficiently distant from the large change in *P*-value observed at the 52% infestation level to minimize misclassification, and was the nearest round number to the 62% threshold. Thus, hereafter 'low' and 'high' UBO colonies refer to colonies that scored <60% and ≥60% in the UBO assay, respectively. A similar analysis performed for the FKB assay (Fig. 4b) indicated that the comparison of mean August *Varroa* infestation at FKB assay scores <84% and ≥84% resulted in the smallest *P*-value ($7.6 * 10^{-3}$). The largest drop in *P*-value ($6.49 * 10^{-5}$) occurred between FKB assay scores 83% and 84%. For reasons listed above and because it is the established practice, we chose to use the conventional threshold of 95% (resulting in $P = 0.014$) to distinguish 'low' and 'high' FKB colonies. Hereafter 'low' and 'high' FKB colonies refer to colonies that scored <95% and ≥95% in the FKB assay, respectively.

Colonies that were classified as highly hygienic based on June UBO assays had significantly fewer *Varroa* in June and August than low UBO colonies ($F_{1,35} = 16.4$, $P < 0.001$ and $F_{1,56} = 23.8$, $P < 0.001$, respectively). Mean *Varroa* infestations for colonies with low and high June UBO scores were 2.6% and 0.4% respectively for June (Fig. 5a), and 7.2% and 1.6% respectively for August (Fig. 5c). For August UBO assays, August *Varroa* infestation was also significantly lower in high UBO colonies than low UBO colonies ($F_{1,68} = 8.5$, $P = 0.005$). Mean *Varroa* infestations for colonies with low and high August UBO scores were 6.7% and 2.8%, respectively. To evaluate the predictive value of the UBO assays conducted in June and August for late-season mite control applications, UBO scores for colonies above the economic threshold of *Varroa* infestation (3%) were compared. In June UBO assays, 3 of 29 colonies (10%) that scored ≥ 60% had August *Varroa* infestations over the economic treatment threshold of 3%, with maximum and average *Varroa* infestations of 6.6% and 4.4%, respectively. In contrast, 6 of 25 colonies (24%) that scored ≥60% in the August UBO assay had August *Varroa* infestations over 3%, with maximum and average *Varroa* infestations of 30.6% and 11%, respectively.

For FKB assays, August but not June *Varroa* infestations differed significantly between low and high FKB colonies ($F_{1,63} = 6.4$, $P = 0.014$ and $F_{1,39} = 3.3$, $P = 0.078$, respectively). Mean June *Varroa* infestations for low and high FKB colonies were 2.4% and 1.2%, respectively (Fig. 5b). Mean August *Varroa* infestations for low and high FKB colonies were 6.8% and 3.2%, respectively (Fig. 5d). For CON assays, neither June nor August *Varroa* infestation differed significantly between low and high CON colonies ($F_{1,1} = 0.02$, $P = 0.91$ and $F_{1,15} = 0.25$, $P = 0.63$, respectively). Mean June *Varroa* infestation was 0.4% for colonies with both low and high CON scores. Mean August *Varroa* infestations for colonies with low and high CON scores were 3.1% and 4.6%, respectively.

## Relationship Between UBO, FKB, and CON Assays

In untreated colonies, June UBO and FKB assay scores were positively correlated with each other (rs = 0.28, d.f. = 70, $P = 0.009$; Fig. 6a). With respect to UBO and FKB assay thresholds, colonies fell into one of four possible categories: low UBO/low FKB (46%, $n = 33$), low UBO/high FKB (22%, $n = 16$), high UBO/low FKB (15%, $n = 11$), or high UBO/high FKB (17%, $n = 12$). Only high UBO colonies (regardless of FKB classification) had *Varroa* infestation levels significantly lower than colonies in the low UBO/low FKB category (Fig. 6b). Colonies that scored low in both the UBO and FKB assays had significantly higher *Varroa* loads than colonies with both high UBO/low FKB scores ($P = 0.029$) and high UBO/high FKB scores (0.009) but did not differ in *Varroa* load from colonies with low UBO/high FKB scores (0.38). *Varroa* infestations of colonies that scored low in the UBO assay but high in the FKB assay did not differ from those of colonies in the high UBO/low FKB ($P > 0.99$) or high UBO/high FKB ($P = 0.98$) categories. *Varroa* infestations of colonies that scored high in the UBO assay did not differ significantly by FKB assay response ($P > 0.99$). Though UBO and CON assay scores were significantly correlated (rs = 0.71, d.f. = 73, $P < 0.001$), colony responses to CON assays did not have the same predictive ability with respect to *Varroa* infestation as colony response to UBO assays.

## *Varroa* Removal

Colonies that exhibited strong responses in June UBO, August UBO, and FKB assays demonstrated higher *Varroa* removal than colonies that exhibited weak assay responses. Removal of experimentally introduced *Varroa* was significantly positively correlated with June UBO (rs = 0.51, d.f. = 55, $P < 0.001$; Fig. 7a), August UBO (rs = 0.57, d.f. = 43, $P < 0.001$), and FKB (rs = 0.36, d.f. = 55, $P = 0.003$; Fig. 7b) assay scores. There was no evidence of a relationship between

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

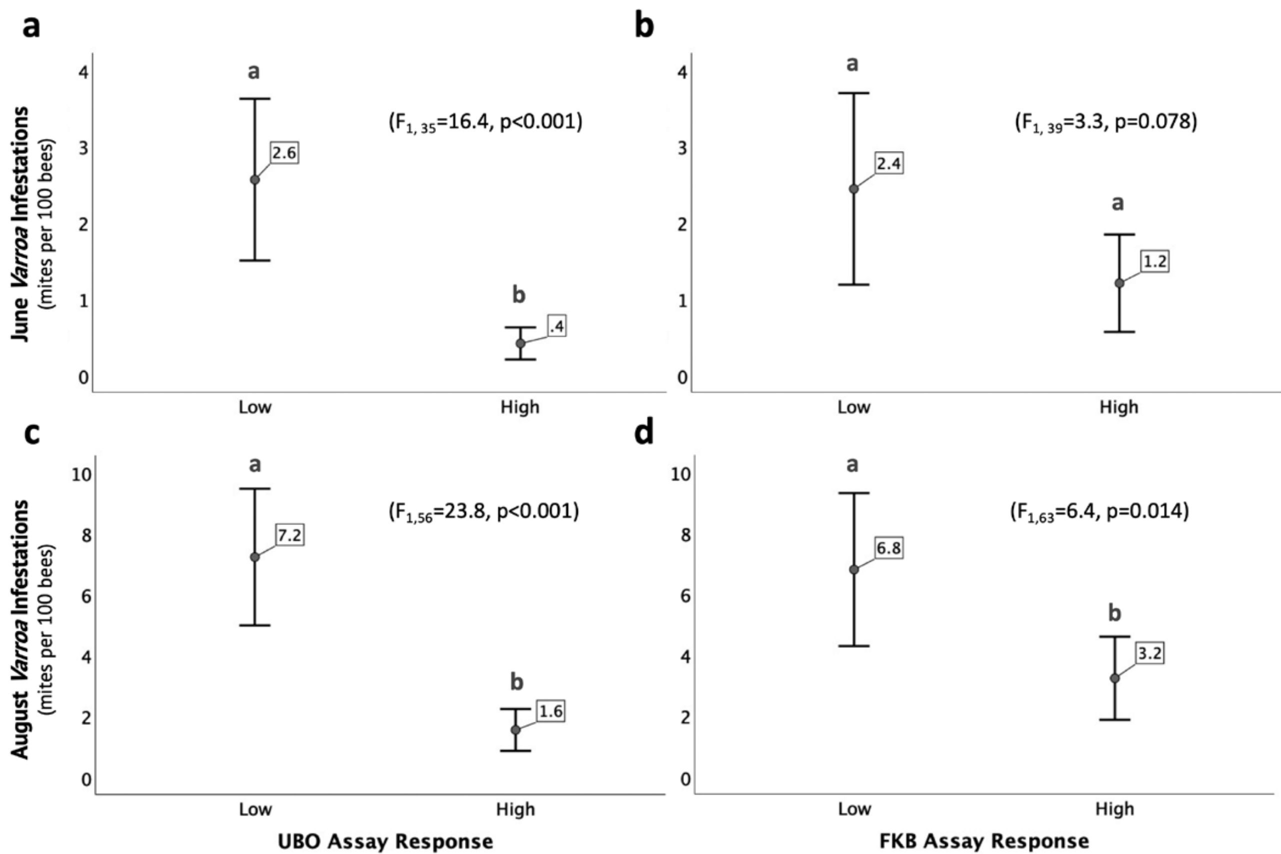


**Fig. 5.** Welch's tests comparing colony *Varroa* infestations for high and low UBO and FKB assay scores. In colonies not treated chemically for *Varroa* in spring, colonies that exhibited strong responses in unhealthy brood odor (UBO) and freeze-killed brood (FKB) assays had lower *Varroa* infestations than colonies that exhibited weak UBO and FKB assay responses. For each mean, 95% confidence intervals are provided. Different letters indicate significant differences in *Varroa* infestations for colonies with low and high assay responses. June *Varroa* infestations were significantly higher for colonies that scored low in the UBO assay ($n$ = 47) (a). There was suggestive evidence that June *Varroa* infestations were higher for colonies that scored low in the FKB assay ($n$ = 47) (b). August *Varroa* infestations were significantly higher for colonies that scored low in both UBO ($n$ = 72) (c) and FKB ($n$ = 72) (d) assays.

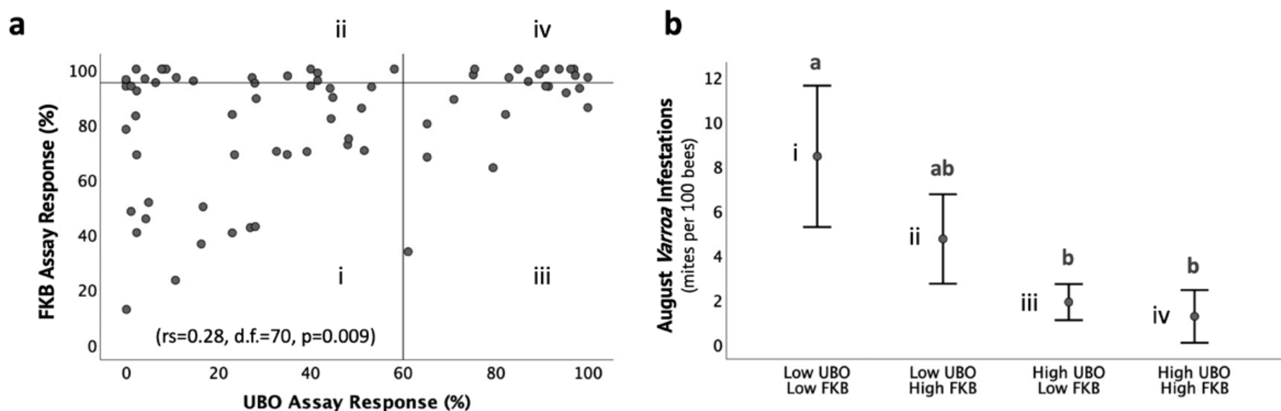


**Fig. 6.** Evidence that despite a significant Spearman's correlation between unhealthy brood odor (UBO) and freeze-killed brood (FKB) assay responses, the UBO assay is a more accurate predictor of adult honey bee *Varroa* infestation ($n$ = 72). Each data point in (a) represents a single colony. For each mean in (b), 95% confidence intervals are provided. The numbers i–iv represent the four possible UBO by FKB categorical outcomes, and different letters indicate significant differences in *Varroa* infestations of colonies in these four groups. UBO and FKB assay responses were significantly positively correlated (a). The vertical line at 60% and horizontal line at 95% indicate UBO and FKB thresholds, respectively. *Varroa* infestations only differed significantly between colonies in the low UBO/low FKB category, and colonies in the high UBO/low FKB ($P$ = 0.029), and high UBO/high FKB ($P$ = 0.009) categories (b).

removal of experimentally introduced *Varroa* and CON assay scores (rs = 0.10, d.f. = 31, $P$ = 0.29). Mean percent removal of experimentally introduced *Varroa* was significantly higher for colonies that scored high in June UBO, August UBO, and FKB assays than for low-scoring colonies ($F_{48,1}$ = 12.9, $P$ = 0.001, Fig. 7c; $F_{39,1}$ = 16.4, $P$ < 0.001; and $F_{34,1}$ = 6.6, $P$ = 0.015, Fig. 7d, respectively). There was

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

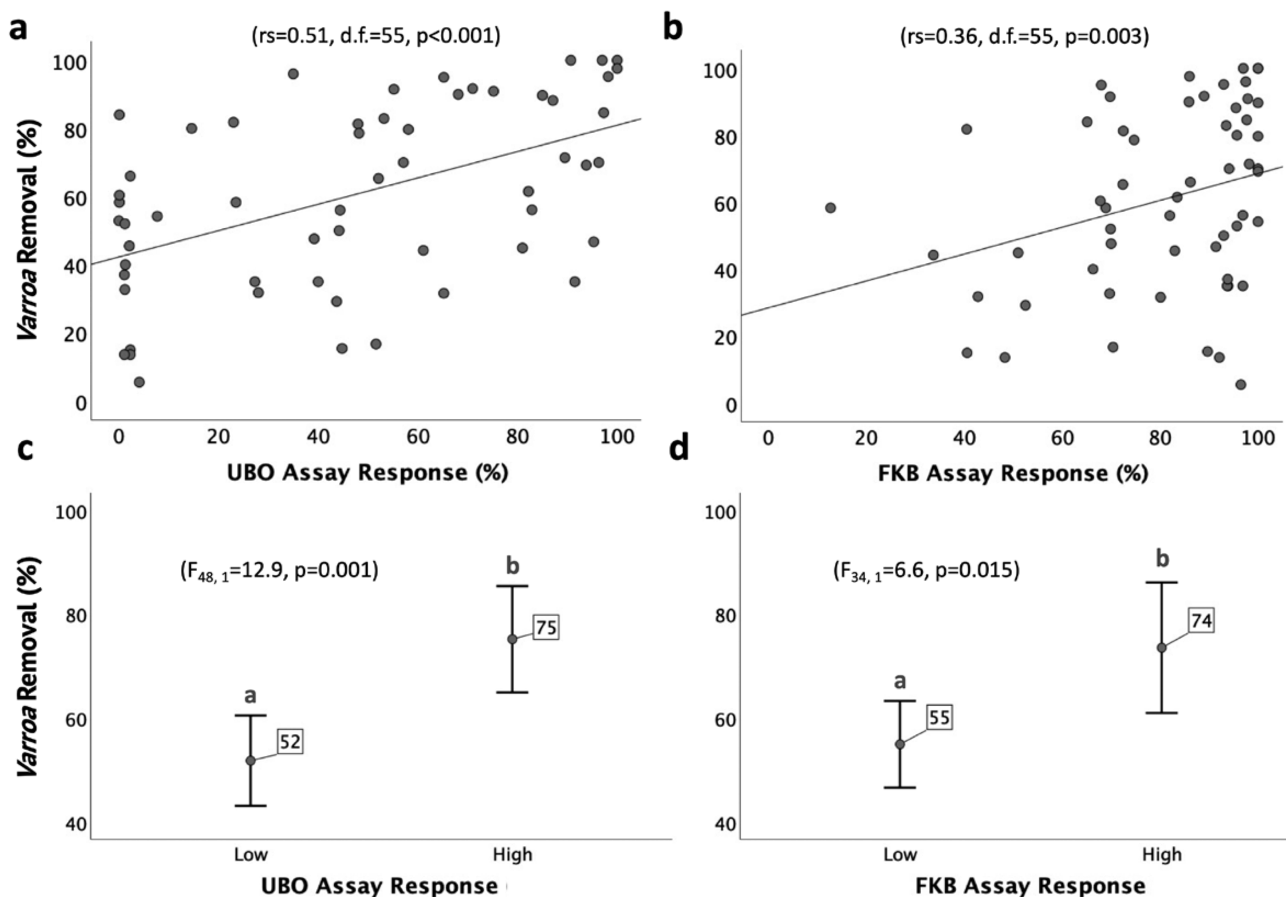


**Fig. 7.** Spearman's correlations and Welch tests comparing *Varroa* removal and UBO/FKB assay scores. Colonies that exhibited strong responses in unhealthy brood odor (UBO) and freeze-killed brood (FKB) assays were more likely to remove *Varroa* in brood cells than colonies that exhibited weak UBO and FKB assay responses. Each data point in (a) and (b) represents a single colony. For each mean in (c) and (d), 95% confidence intervals are provided. Different letters indicate significant differences in *Varroa* removal for colonies with low and high assay responses. *Varroa* removal was significantly positively correlated with (a) June UBO assay response and (b) FKB assay response ($n$ = 57 colonies each). Mean percent removal of experimentally introduced *Varroa* was significantly higher for colonies that scored high in (c) June UBO and (d) FKB assays than for low-scoring colonies ($n$ = 45 colonies each).

no significant difference in the removal of experimentally introduced *Varroa* for colonies that scored low and high in the CON assay ($F_{31,1}$ = 5.7, $P$ = 0.46). Removal of *Varroa*-infested and control brood was significantly positively correlated (rs = 0.36, d.f. = 55, $P$ = 0.003). Mean percent removal of *Varroa*-infested brood was significantly higher than removal of control brood (61% and 8% respectively, $t$ = 14.9, d.f. = 56, $P$ < 0.001).

### Overwintering

Colonies that exhibited strong responses in UBO assays were more likely to overwinter successfully than colonies that exhibited weak UBO assay responses. Overwintering survival was higher in colonies that scored high in June ($F_{31,1}$ = 5.1, $P$ = 0.032) and August ($F_{32,1}$ = 6.6, $P$ = 0.015, Fig. 8a) UBO assays. Overwintering survival did not differ significantly for colonies with low versus high scores in FKB ($F_{33,1}$ = 2.5, $P$ = 0.12, Fig. 8b) or CON ($F_{19,1}$ = 1.0, $P$ = 0.33) assays. Average survival of colonies that scored high in June and August UBO assays were 57% and 65%, respectively. Average survival of colonies that scored low in June and August UBO assays were 21% and 24%, respectively. Removal of experimentally introduced *Varroa* was significantly higher in colonies that survived overwinter than those that died ($F_{30,1}$ = 4.7, $P$ = 0.038). Mean *Varroa* removal of surviving colonies was 80%, compared to 61% for colonies that did not survive overwinter.

## Discussion

Based on previous findings linking specific cuticular hydrocarbons (CHCs) to honey bee hygienic behavior and *Varroa* infestation (Nazzi et al. 2002, Nazzi et al. 2004, Wagoner et al. 2019, Wagoner et al. 2020), we tested the hypothesis that hygienic response to unhealthy brood odors (UBOs) could serve as an improved tool for predicting colony-level *Varroa* resistance. Our results support our hypothesis, showing that colony responses in the two-hour UBO assay predicted honey bee colony *Varroa* infestation level, *Varroa* removal, and overwintering success. Predictive ability of the UBO assay was more powerful for colonies that were not treated for *Varroa* in spring, as the *Varroa* infestation levels and overwintering outcomes of these colonies more accurately reflected their innate *Varroa*-resistance traits. Colony response in the UBO assay served as a faster and more accurate predictor of *Varroa* resistance than colony response to a mixture of control alkenes (CON) which have not been associated with *Varroa* infestation, the freeze-killed brood (FKB) assay, or removal of experimentally introduced *Varroa*. The hygienic response observed in CON assays was not wholly unexpected given that the control alkenes used have not been identified on honey bee cuticles, and are thus likely perceived as foreign substances. Response to the CON assay indicates that hygiene can be triggered by different stimuli. However, the lack of evidence of a

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

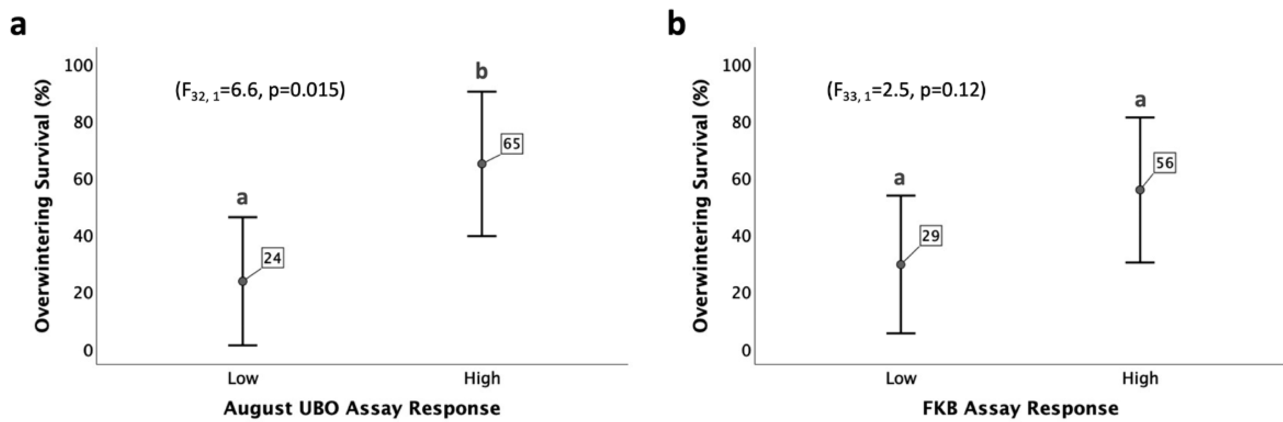


**Fig. 8.** Welch's tests comparing overwintering success with UBO and FKB assay scores. Mean overwintering survival with 95% confidence intervals are provided for colonies categorized as high and low in unhealthy brood odor (UBO) and freeze-killed brood (FKB) assays. Colonies that exhibited strong responses in UBO assays were more likely to overwinter successfully than colonies that exhibited weak UBO assay responses (a). There was no evidence that overwintering survival differed for colonies that scored high and low in the FKB assay (b). Different letters indicate significant differences in overwintering survival for colonies with low and high assay responses (*n* = 35 colonies each).

relationship between responses in the CON assay and any of the three measures of *Varroa* resistance, combined with clear evidence for such a relationship between the UBO assay and all three measures of *Varroa* resistance, supports the notion that compounds in the UBO assay are distinct chemical markers of honey bee stress. Furthermore, these findings suggest that hygienic uncapping and removal of *Varroa*-infested brood are triggered by these specific unhealthy brood odors, rather than a more general sensitivity to abnormal olfactory stimuli. As an improved tool for the identification of *Varroa*-resistant honey bee colonies, the UBO assay has the potential to improve honey bee health by facilitating the selective breeding of more *Varroa*-resistant honey bees. Furthermore, the ability of the UBO assay to predict *Varroa* resistance could inform apiary management decisions such as queen sourcing, colony placement (e.g., isolation of resistant colonies), and need for *Varroa* monitoring and timing of miticide use.

As expected, *Varroa* infestations in the experimental colonies increased from early to late summer (Martin 1998, Messan et al. 2021). Our first prediction was that colony responses to the UBO mixture would be negatively correlated with colony *Varroa* infestations. While this trend was apparent when all colonies were included in the analysis, use of chemical miticides in spring had the expected effect of reducing colony *Varroa* infestation levels regardless of their natural defenses, weakening the predicted relationship. Therefore, colonies not chemically treated in spring provided a more accurate representation of the relationship between colony assay responses and *Varroa* infestation levels. Because *Varroa* removal assays are sometimes used as a measure of colony *Varroa*-specific hygiene, we analyzed *Varroa* removal as both a dependent variable, indicative of the abilities of UBO, FKB, and CON assays to predict colony *Varroa* resistance, and as an independent variable, similar to the UBO, FKB, and CON assays. In colonies not treated for *Varroa* in spring, responses in the June and August UBO assays served as better predictors of *Varroa* infestation than responses in the CON assay, the FKB assay, or colony *Varroa* removal. The lack of a correlation between natural August *Varroa* infestation and *Varroa* removal after the experimental introduction was unexpected, and suggests that the removal of experimentally introduced *Varroa* collected by sugar shake may not be a reliable predictor of colony *Varroa* resistance. This could be due to artifacts of experimental *Varroa* introduction, or to variation in virus loads and/or reproductive status of *Varroa* collected from adult honey bees. The strong negative correlation between responses in the June UBO assay and August *Varroa* infestation among untreated colonies with infestation rates below the 3% economic treatment threshold for *Varroa* suggests that the UBO assay may provide high resolution information on colony *Varroa* resistance, distinguishing the most *Varroa*-resistant colonies even within groups of colonies capable of maintaining relatively low *Varroa* populations.

The correlation analyses were conducted to test our hypotheses but the relationships between assay responses and *Varroa* infestations were also quantified categorically, given that the use of a response threshold is required for practical application and the relationship between UBO assay scores and *Varroa* infestation appear to follow a non-linear, threshold function. Our systematic search for the most meaningful UBO threshold value indicated a similar separation of colony *Varroa* resistance between 52% and 62%, resulting in our adoption of a 60% threshold UBO score for practical purposes. The same analysis of the FKB assay indicated that the most powerful distinction could be found around 84%. However, to avoid misclassification (since the largest drop in *P*-value was also associated with the 84% threshold) and to ensure compatibility with other studies and common practice, the conventional 95% threshold was selected for the FKB assay. In addition to providing statistical support for selection of thresholds in the present study, this analysis supports the established use of a 95% threshold for FKB assays.

Results from categorical analyses were useful for quantifying potential selection effects and comparing UBO assay performance with that of the FKB assay for practical purposes, because FKB categorization is currently used for the identification of hygienic breeder queens (Spivak et al. 2009, Büchler et al. 2013). High scores in June UBO and FKB assays were associated with mean August *Varroa* infestations of 1.6% and 3.2% respectively, indicating that high UBO colonies are twice as effective at controlling *Varroa* as high FKB colonies. The proximity of the mean *Varroa* infestation of high FKB colonies (3.2%) to the 3% infestation level commonly used as an economic threshold for *Varroa* treatment may explain variability in reports of the FKB assay's ability to predict *Varroa* resistance (Leclercq et al. 2017). Though UBO and FKB assay scores were positively correlated with each other, only colonies with high UBO scores had significantly lower *Varroa* infestations, supporting previous claims that FKB response is not necessarily a reliable predictor of *Varroa*-specific hygiene (Leclercq et al. 2018a).

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

We also found support for our second prediction, that colony response in the UBO assay would be positively correlated with colony removal of experimentally introduced mites. Colony response in the FKB assay was also positively correlated with *Varroa* removal. In both the continuous and categorical data analyses, the statistical relationship between *Varroa* removal and UBO assay responses was stronger than the relationship between *Varroa* removal and FKB assay responses. This supports the notion that, while a colony's ability to remove dead brood in the FKB assay may serve as some indication of its capacity for *Varroa* detection and removal, *Varroa* infestation induces the production of specific unhealthy brood odors that are different from odors released by dead brood. This is consistent with previous evidence that the odors emanating from dead brood are different in composition and strength from the odors produced by parasitized or otherwise unhealthy brood (Spivak and Downey 1998, Nazzi et al. 2004, McAfee et al. 2018, Wagoner et al. 2019, Wen 2020). Thus, colony propensity for olfactory recognition of and response to unhealthy brood odors is a better indicator of *Varroa* resistance than colony recognition of and response to dead brood signals. This result also calls into question whether there is indeed a link between hygienic specialists and necrophoric behavior (Perez and Johnson 2019) in all contexts.

Our third prediction, that colony response to the UBO mixture would be indicative of overwintering success without *Varroa* treatment, was also supported by our results. While the removal of experimentally introduced *Varroa* was also predictive of overwintering success, colony response in the FKB assay did not predict overwintering outcomes. It is interesting to note that the best predictors of overwintering success were assays that measured hygienic behavior in August. This also may have been the case for FKB assays, but August FKB assays were not performed. Compared to early season assays, late season assays may better indicate winter bee physiology, colony health going into winter, and/or colony ability to control *Varroa* during the time of year when *Varroa*-vectored virus loads are highest (Tentcheva et al. 2004, Traver et al. 2018).

In these experiments, the UBO assay outperformed the FKB assay, as a faster and more accurate predictor of colony *Varroa* resistance. This finding was robust across the three measures of *Varroa* resistance tested, as well as across experimental sites and years. In the FKB assay, the intensity of the hygiene-inducing stimulus increased with time, resulting in a high threshold (95%) that decreased measurement resolution at the most important part of the parameter space. In contrast, stimulus intensity in the UBO assay decreased with time, enabling rapid, high-resolution hygienic evaluation that can be fine-tuned through manipulation of the doses applied. This improved resolution of colony hygienicity enables differentiation of resistance levels at the upper end, facilitating the identification of the very best colonies capable of surviving without *Varroa* treatments. These findings highlight the significant potential of the UBO assay to contribute to the control of *Varroa* through improved breeding, and by informing management decisions such as if and when to implement *Varroa* control treatments, and when and where to move colonies for purposes of pollination or isolation. As an efficient and effective tool to predict *Varroa* resistance, the UBO assay has the potential to significantly improve honey bee health, and thus strengthen global pollination services and food security. However, while hygienic colonies identified based on the FKB assay have demonstrated resistance to other important honey bee diseases (Spivak and Gilliam 1998, Spivak and Reuter 2001a), the relationship between colony response in the UBO assay and general colony disease resistance has not yet been tested. Evidence that Z10-$C_{33}$ and Z8-$C_{31}$ are elevated in response to DWV infection regardless of *Varroa* infestation status (Wagoner et al. 2019), combined with evidence that Z10-$C_{33}$ is elevated in pathogen-infected ant pupae targeted for removal (Pull et al. 2018) suggests that these UBOs are produced as part of a general disease response, rather than a *Varroa*-specific response. Thus, honey bee colony response in the UBO assay may indicate disease resistance in addition to *Varroa* resistance. Future studies should aim to test the relationship between hygienic response to the UBO chemical mixture and colony resistance to relevant pathogens such as chalkbrood, European foulbrood, and deformed wing virus.

Other compounds associated with *Varroa* infestation and hygienic removal have been identified (Salvy et al. 2001, Mondet et al. 2021). It is unclear, however, whether some of these compounds originate from the brood, or from the *Varroa* family, eliciting a truly *Varroa*-specific response by honey bee adults. Regardless, it is likely that the UBO compounds used in the present study represent only a portion of the chemical blend produced by unhealthy honey bee brood. Thus, honey bee hygienic responses to mixtures of these UBOs with other relevant compounds should be evaluated. Another direction for future research is analysis of smoker use on colony hygienic response in the UBO assay. Smoke has been shown to temporarily interfere with honey bee olfactory perception (Visscher et al. 1995). Although antennal responsiveness has been shown to return to normal 10–20 min after smoke exposure, this timeframe represents 8-17% of a two-hour assay, and thus smoke use could have a substantial adverse effect on colony UBO assay response. The heritability of traits associated with UBO assay performance, and the relationships between colony UBO assay response and other important traits such as honey production and brood pattern also remain to be tested.

Finally, the alkene Z10-C33 was recently associated with pathogen-infected ant pupae
targeted for unpacking (Pull et al. 2018), a behavior comparable to the hygienic removal of honey bee brood. This suggests the possible conservation of certain monoalkenes as triggers for hygiene-like behavior across social insect species, warranting further analysis of the biochemistry of hygiene-like behavior in other social insects, and opening the potential for the development of semiochemically based products to control social pests, such as certain species of ants, wasps, and termites. Future research should address these knowledge gaps in order to inform best practices for implementation of the UBO assay as a tool to improve honey bee health, and to expand our understanding of the role of chemical communication in social insect immunity.

## Acknowledgments

We thank Katie Lee, Bob Danka, Jeff Hull (Triad Bee Supply), and Calvin Terry (Midnight Bee Supply) for their respective contributions to the content and completion of this work. This work was funded and made possible by the United States Department of Agriculture (National Institute for Food and Agriculture, grant number 2017-68004-26321) and the North Carolina Biotechnology Center (2016-TEG-1503).

## Author Contribution

K.W.: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing—original draft. J.G.M.: Conceptualization; Funding acquisition; Methodology; Project Administration; Resources; Writing—review and editing. J.K.: Investigation; Validation. J.B.: Resources. P.W.: Formal analysis. C.S.: Conceptualization; Funding acquisition; Methodology; Project Administration; Writing—re-

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

view and editing. M.S.: Conceptualization; Funding acquisition; Investigation; Methodology; Project Administration; Supervision; Validation; Writing—review, and editing. O.R.: Conceptualization; Funding acquisition; Methodology; Project Administration; Supervision; Writing—review and editing.

## References Cited

**Aizen, M. A., and L. D. Harder. 2009**. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Curr. Biol. 19: 915–918.

**Amdam, G. V., K. Hartfelder, K. Norberg, A. Hagen, and S. W. Omholt. 2004**. Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the mite *Varroa destructor* (Acari: Varroidae): a factor in colony loss during overwintering? J. Econ. Entomol. 97: 741–747.

**Barrs, K. R., M. O. Ani, K. K. Eversman, J. T. Rowell, K. M. Wagoner, and O. Rueppell. 2021**. Time-accuracy trade-off and task partitioning of hygienic behavior among honey bee (*Apis mellifera*) workers. Behav. Ecol. Sociobiol. 75: 1–13.

**Blomquist, G. J., D. R. Nelson, and M. De Renobales. 1987**. Chemistry, biochemistry, and physiology of insect cuticular lipids. Arch. Insect Biochem. Physiol. 6: 227–265.

**Bogdanov, S. 2006**. Contaminants of bee products. Apidologie. 37: 1–18.

**Bowen-Walker, P. L., S. J. Martin, and A. Gunn. 1999**. The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite varroa jacobsoni Oud. J. Invertebr. Pathol. 73: 101–106.

**Büchler, R., S. Andonov, K. Bienefeld, C. Costa, F. Hatjina, N. Kezic, P. Kryger, M. Spivak, A. Uzunov, and J. Wilde. 2013**. Standard methods for rearing and selection of *Apis mellifera* queens. J. Apicult. Res. 52: 1–30.

**Châline, N., J. C. Sandoz, S. J. Martin, F. L. Ratnieks, and G. R. Jones. 2005**. Learning and discrimination of individual cuticular hydrocarbons by honeybees (*Apis mellifera*). Chem. Senses. 30: 327–335.

**Dani, F. R., G. R. Jones, S. Corsi, R. Beard, D. Pradella, and S. Turillazzi. 2005**. Nestmate recognition cues in the honey bee: differential importance of cuticular alkanes and alkenes. Chem. Senses. 30: 477–489.

**Delaplane, K. S., J. A. Berry, J. A. Skinner, J. P. Parkman, and W. M. Hood. 2005**. Integrated pest management against *Varroa* destructor reduces colony mite levels and delays treatment threshold. J. Apicult. Res. 44: 157–162.

**Dietemann, V., F. Nazzi, S. J. Martin, D. L. Anderson, B. Locke, K. S. Delaplane, Q. Wauquiez, C. Tannahill, E. Frey, and B. Ziegelmann. 2013**. Standard methods for *Varroa* research. J. Apicult. Res. 52: 1–54.

**Elzen, P., J. Baxter, M. Spivak, and W. Wilson. 1999**. Amitraz resistance in *Varroa*: new discovery in North America. Am. Bee J. 139: 362-362.

**Elzen, P. J., J. R. Baxter, M. Spivak, and W. T. Wilson. 2000**. Control of *Varroa jacobsoni* Oud. resistant to fluvalinate and amitraz using coumaphos. Apidologie. 31: 437–441.

**Frey, E., R. Odemer, T. Blum, and P. Rosenkranz. 2013**. Activation and interruption of the reproduction of *Varroa* destructor is triggered by host signals (*Apis mellifera*). J. Invertebr. Pathol. 113: 56–62.

**Gashout, H. A., E. Guzman-Novoa, and P. H. Goodwin. 2020a**. Synthetic and natural acaricides impair hygienic and foraging behaviors of honey bees. Apidologie. 51: 1155–1165.

**Gashout, H. A., E. Guzman-Novoa, P. H. Goodwin, and A. Correa-Benítez. 2020b**. Impact of sublethal exposure to synthetic and natural acaricides on honey bee (*Apis mellifera*) memory and expression of genes related to memory. J. Insect Physiol. 121: 104014.

**González-Cabrera, J., S. Rodríguez-Vargas, T. G. Davies, L. M. Field, D. Schmehl, J. D. Ellis, K. Krieger, and M. S. Williamson. 2016**. Novel mutations in the voltage-gated sodium channel of pyrethroid-resistant *Varroa* destructor populations from the southeastern USA. PLoS One. 11: e0155332.

**Greene, M. J., and D. M. Gordon. 2003**. Social insects: cuticular hydrocarbons inform task decisions. Nature. 423: 32.

**Haber, A. I., N. A. Steinhauer, and D. vanEngelsdorp. 2019**. Use of chemical and nonchemical methods for the control of *Varroa* destructor (Acari: Varroidae) and associated winter colony losses in U.S. beekeeping operations. J. Econ. Entomol. 112: 1509–1525.

**Harris, J. W. 2007**. Bees with Varroa sensitive hygiene preferentially remove mite infested pupae aged ≤ five days post capping. J. Apicult. Res. 46: 134–139.

**Howard, R. W., and G. J. Blomquist. 2005**. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. Annu. Rev. Entomol. 50: 371–393.

**Imdorf, A., S. Bogdanov, V. Kilchenmann, and C. Maquelin. 1995**. Apilife VAR: a new varroacide with thymol as the main ingredient. Bee World. 76: 77–83.

**Imdorf, A., S. Bogdanov, R. I. Ochoa, and N. W. Calderone. 1999**. Use of essential oils for the control of *Varroa jacobsoni* Oud. in honey bee colonies. Apidologie. 30: 209–228.

**Jack, C. J., E. van Santen, and J. D. Ellis. 2020**. Evaluating the efficacy of oxalic acid vaporization and brood interruption in controlling the honey bee pest *Varroa* destructor (Acari: Varroidae). J. Econ. Entomol. 113: 582–588.

**Jackson, L. L., and G. Baker. 1970**. Cuticular lipids of insects. Lipids. 5: 239–246.

**Kallenberg, O., and O. Kallenberg. 1997**. Foundations of modern probability, vol. 2, Springer.

**Kathe, E., K. Seidelmann, O. Lewkowski, Y. Le Conte, and S. Erler. 2021**. Changes in chemical cues of *Melissococcus plutonius* infected honey bee larvae. Chemoecology. 31: 189–200.

**Kulhanek, K., N. Steinhauer, K. Rennich, D. M. Caron, R. R. Sagili, J. S. Pettis, J. D. Ellis, M. E. Wilson, J. T. Wilkes, and D. R. Tarpy. 2017**. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. J. Apicult.. Res. 56: 328–340.

**Leclercq, G., B. Pannebakker, N. Gengler, B. K. Nguyen, and F. Francis. 2017**. Drawbacks and benefits of hygienic behavior in honey bees (*Apis mellifera* L.): a review. J. Apicult. Res. 56: 366–375.

**Leclercq, G., T. Blacquière, N. Gengler, and F. Francis. 2018a**. Hygienic removal of freeze-killed brood does not predict *Varroa*-resistance traits in unselected stocks. J. Apicult. Res. 57: 292–299.

**Leclercq, G., F. Francis, N. Gengler, and T. Blacquière. 2018b**. Bioassays to quantify hygienic behavior in honey bee (*Apis mellifera* L.) colonies: a review. J. Apicult. Res. 57: 663–673.

**Liendo, M. C., I. Muntaabski, R. M. Russo, S. B. Lanzavecchia, D. F. Segura, M. A. Palacio, J. L. Cladera, P. C. Fernández, and A. C. Scannapieco. 2021**. Temporal changes in volatile profiles of *Varroa* destructor-infested brood may trigger hygienic behavior in *Apis mellifera*. Entomol. Exp. Appl. 169: 563–574.

**Locke, B. 2016**. Inheritance of reduced Varroa mite reproductive success in reciprocal crosses of mite-resistant and mite-susceptible honey bees (*Apis mellifera*). Apidologie. 47: 583–588.

**Locke, B., E. Forsgren, and J. R. de Miranda. 2014**. Increased tolerance and resistance to virus infections: a possible factor in the survival of *Varroa* destructor-resistant honey bees (*Apis mellifera*). PLoS One. 9: e99998.

**Maggi, M. D., S. R. Ruffinengo, P. Negri, and M. J. Eguaras. 2010**. Resistance phenomena to amitraz from populations of the ectoparasitic mite *Varroa* destructor of Argentina. Parasitol. Res. 107: 1189–1192.

**Marshman, J., A. Blay-Palmer, and K. Landman. 2019**. Anthropocene crisis: climate change, pollinators, and food security. Environments. 6: 22.

**Martin, S. J. 1994**. Ontogenesis of the mite *Varroa jacobsoni* Oud. in worker brood of the honeybee *Apis mellifera* L. under natural conditions. Exp. Appl. Acarol. 18: 87–100.

**Martin, S. 1998**. A population model for the ectoparasitic mite *Varroa jacobsoni* in honey bee (*Apis mellifera*) colonies. Ecol. Model. 109: 267–281.

**McAfee, A., T. F. Collins, L. L. Madilao, and L. J. Foster. 2017**. Odorant cues linked to social immunity induce lateralized antenna stimulation in honey bees (*Apis mellifera* L.). Sci. Rep. 7: 46171.

**McAfee, A., A. Chapman, I. Iovinella, Y. Gallagher-Kurtzke, T. F. Collins, H. Higo, L. L. Madilao, P. Pelosi, and L. J. Foster. 2018**. A death pheromone, oleic acid, triggers hygienic behavior in honey bees (*Apis mellifera* L.). Sci. Rep. 8: 5719.

**Messan, K., M. M. Rodriguez, J. Chen, G. DeGrandi-Hoffman, and Y. Kang. 2021**. Population dynamics of *Varroa* mite and honeybee: effects of parasitism with age structure and seasonality. Ecol. Model. 440: 109359.

**Mondet, F., M. Parejo, M. D. Meixner, C. Costa, P. Kryger, S. Andonov, B. Servin, B. Basso, M. Bieńkowska, and G. Bigio. 2020**. Evaluation of

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021

suppressed mite reproduction (SMR) reveals potential for *Varroa* resistance in European honey bees (*Apis mellifera* L.). Insects. 11: 595.

**Mondet, F., S. Blanchard, N. Barthes, D. Beslay, C. Bordier, G. Costagliola, M. R. Hervé, B. Lapeyre, S. H. Kim, B. Basso, et al. 2021**. Chemical detection triggers honey bee defense against a destructive parasitic threat. Nat. Chem. Biol. 17: 524–530.

**Nation, J., M. Sanford, and K. Milne. 1992**. Cuticular hydrocarbons from *Varroa jacobsoni*. Exp. Appl. Acarol. 16: 331–344.

**Nazzi, F., N. Milani, and G. D. Vedova. 2002**. (Z)-8-heptadecene from infested cells reduces the reproduction of *Varroa* destructor under laboratory conditions. J. Chem. Ecol. 28: 2181–2190.

**Nazzi, F., G. Della Vedova, and M. D'Agaro. 2004**. A semiochemical from brood cells infested by *Varroa* destructor triggers hygienic behaviour in *Apis mellifera*. Apidologie. 35: 65–70.

**Noël, A., Y. Le Conte, and F. Mondet. 2020**. Varroa destructor: how does it harm *Apis mellifera* honey bees and what can be done about it? Emerg. Top. Life Sci. 4: 45–57.

**Perez, A. A., and B. R. Johnson. 2019**. Task repertoires of hygienic workers reveal a link between specialized necrophoric behaviors in honey bees. Behav. Ecol. Sociobiol. 73: 1–12.

**Pull, C. D., L. V. Ugelvig, F. Wiesenhofer, A. V. Grasse, S. Tragust, T. Schmitt, M. J. Brown, and S. Cremer. 2018**. Destructive disinfection of infected brood prevents systemic disease spread in ant colonies. Elife. 7: e32073.

**Quigley, T. P., G. V. Amdam, and G. H. Harwood. 2019**. Honey bees as bioindicators of changing global agricultural landscapes. Curr. Opin. Insect Sci. 35: 132–137.

**Ramsey, S. D., R. Ochoa, G. Bauchan, C. Gulbronson, J. D. Mowery, A. Cohen, D. Lim, J. Joklik, J. M. Cicero, J. D. Ellis, et al. 2019**. Varroa destructor feeds primarily on honey bee fat body tissue and not hemolymph. Proc. Natl. Acad. Sci. U. S. A. 116: 1792–1801.

**Rangel, J., and A. Fisher. 2019**. Factors affecting the reproductive health of honey bee (Apis mellifera) drones—a review. Apidologie. 50: 759–778.

**Rangel, J., and D. R. Tarpy. 2015**. The combined effects of miticides on the mating health of honey bee (Apis mellifera L.) queens. J. Apicult. Res. 54: 275–283.

**Rinkevich, F. D. 2020**. Detection of amitraz resistance and reduced treatment efficacy in the Varroa Mite, Varroa destructor, within commercial beekeeping operations. PLoS One. 15: e0227264.

**Salvy, M., C. Martin, A. G. Bagnères, E. Provost, M. Roux, Y. Le Conte, and J. L. Clément. 2001**. Modifications of the cuticular hydrocarbon profile of Apis mellifera worker bees in the presence of the ectoparasitic mite Varroa jacobsoni in brood cells. Parasitology. 122: 145–159.

**Sammataro, D., P. Untalan, F. Guerrero, and J. Finley. 2005**. The resistance of Varroa mites (Acari: Varroidae) to acaricides and the presence of esterase. Int. J. Acarol. 31: 67–74.

**Schöning, C., S. Gisder, S. Geiselhardt, I. Kretschmann, K. Bienefeld, M. Hilker, and E. Genersch. 2012**. Evidence for damage-dependent hygienic behaviour towards Varroa destructor-parasitised brood in the western honey bee, Apis mellifera. J. Exp. Biol. 215: 264–271.

**Spivak, M., and R. G. Danka. 2021**. Perspectives on hygienic behavior in Apis mellifera and other social insects. Apidologie. 52: 1–16.

**Spivak, M., and D. L. Downey. 1998**. Field assays for hygienic behavior in honey bees (Hymenoptera: Apidae). J. Econ. Entomol. 91: 64–70.

**Spivak, M., and M. Gilliam. 1998**. 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. 79: 169–186.

**Spivak, M., and G. S. Reuter. 2001a**. Resistance to American foulbrood disease by honey bee colonies Apis mellifera bred for hygienic behavior. Apidologie. 32: 555–565.

**Spivak, M., and G. S. Reuter. 2001b**. Varroa destructor infestation in untreated honey bee (Hymenoptera: Apidae) colonies selected for hygienic behavior. J. Econ. Entomol. 94: 326–331.

**Spivak, M., R. Masterman, R. Ross, and K. A. Mesce. 2003**. Hygienic behavior in the honey bee (Apis mellifera L.) and the modulatory role of octopamine. J. Neurobiol. 55: 341–354.

**Spivak, M., G. S. Reuter, K. Lee, and B. Ranum. 2009**. The future of the MN hygienic stock of bees is in good hands. Am. Bee J. 149: 965–967.

**Tentcheva, D., L. Gauthier, N. Zappulla, B. Dainat, F. Cousserans, M. E. Colin, and M. Bergoin. 2004**. Prevalence and seasonal variations of six bee viruses in Apis mellifera L. and Varroa destructor mite populations in France. Appl. Environ. Microbiol. 70: 7185–7191.

**Tihelka, E. 2018**. Effects of synthetic and organic acaricides on honey bee health: a review. Slov. Vet. Res. 55: 119–140.

**Traver, B. E., H. K. Feazel-Orr, K. M. Catalfamo, C. C. Brewster, and R. D. Fell. 2018**. Seasonal effects and the impact of in-hive pesticide treatments on parasite, pathogens, and health of honey bees. J. Econ. Entomol. 111: 517–527.

**Traynor, K. S., F. Mondet, J. R. de Miranda, M. Techer, V. Kowallik, M. A. Y. Oddie, P. Chantawannakul, and A. McAfee. 2020**. Varroa destructor: a complex parasite, Crippling honey bees worldwide. Trends Parasitol. 36: 592–606.

**van Zweden, J. S., and P. d'Ettorre. 2010**. Nestmate recognition in social insects and the role of hydrocarbons, insect hydrocarbons: biology, biochemistry and chemical ecology. *In* G. J. Blomquist, and A. G. Bagnères, (eds.), vol. 11. Cambridge University Press.

**Visscher, P. K., R. S. Vetter, and G. E. Robinson. 1995**. Alarm pheromone perception in honey bees is decreased by smoke (Hymenoptera: Apidae). J. Insect Behav. 8: 11–18.

**Wagoner, K., and O. Rueppell,** inventors; University of North Carolina Greensboro, assignee. **2019**. Methods and compositions for inducing hygienic behavior in honey bees. U.S. patent 10,512,251 B2.

**Wagoner, K., and O. Rueppell,** inventors; University of North Carolina Greensboro, assignee. **2020a**. Methods and compositions for inducing hygienic behavior in honey bees. U.S. patent 10,524,455 B2.

**Wagoner, K., and O. Rueppell,** inventors; University of North Carolina Greensboro, assignee. **2020b**. Synergistic mixture for inducing hygienic behavior in honey bees, and related compositions and methods. U.S. patent application 62/831,277.

**Wagoner, K., M. Spivak, A. Hefetz, T. Reams, and O. Rueppell. 2019**. Stock-specific chemical brood signals are induced by Varroa and Deformed Wing Virus, and elicit hygienic response in the honey bee. Sci. Rep. 9: 8753.

**Wagoner, K. M., J. G. Millar, C. Schal, and O. Rueppell. 2020**. Cuticular pheromones stimulate hygienic behavior in the honey bee (Apis mellifera). Sci. Rep. 10: 7132.

**Wantuch, H. A., and D. R. Tarpy. 2009**. Removal of drone brood from Apis mellifera (Hymenoptera: Apidae) colonies to control Varroa destructor (Acari: Varroidae) and retain adult drones. J. Econ. Entomol. 102: 2033–2040.

**Wen, P. 2020**. Death recognition by undertaker bees. bioRxiv. https://www.biorxiv.org/content/10.1101/2020.03.05.978262v1.abstract

**Wilkinson, D., G. C. Smith, S. Hutton, and Y. York. 2002**. Modeling the efficiency of sampling and trapping Varroa destructor in the drone brood of honey bees (Apis mellifera). Am. Bee J. 142: 209–212.

Downloaded from https://academic.oup.com/jinsectscience/article/21/6/4/6414651 by guest on 04 November 2021