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

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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)-8-heptadecene, (E)-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 $\geq$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 (Trueman et al. 2020), which completes reproduction inside healthy 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 reproducative 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...
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 monoalkanes 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 monoalkanes 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-C33), (Z)-8-hentriacontene ((Z8-C32), (Z)-8-heptadecene ((Z8-C17), and (Z)-6-pentadecene ((Z6-C15)) (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-C15, Z8-C17, Z8-C15, and Z6-C15 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-C32), (Z)-15-triacontene ((Z15-C30), (Z)-7-heptadecene ((Z7-C17), and (Z)-7-pentadecene ((Z7-C15). Control
compounds were chosen based on their similarity in size and structure to UBQ 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-C15, Z8-C17, Z8-C19, and Z6-C13 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-C15, Z8-C17, Z8-C19, and Z6-C13 were tested in all colonies, at all sites, in all years. Assays with control compounds Z16-C32, Z15-C30, Z7-C17, and Z7-C15 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-C13, Z7-C17, Z16-C32, and Z10-C15 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-C17)

A solution of octyltriphenylphosphonium bromide (11.4 g, 26 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 NH4Cl, and extracted with 200 ml hexane. The hexane layer was washed sequentially with 1 M aq. HCl, saturated aq. NaHCO3, and brine, then dried over anhydrous Na2SO4 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 iced 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 (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)-7-Heptadecadene (Z7-C17)

(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-C30)

(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), 210 (1), 182 (1), 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-C36)

A solution of 1-nonyne (4.96 g, 40 mmol) and ~100 mg triphenylmethane indicator in 150 ml dry THF was cooled in an ice-bath 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, 82 mmol) in 1 M aq. HCl, saturated aq. NaHCO3, and brine, then dried over anhydrous Na2SO4 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 H2 without stirring, then the flask was sealed under H2 atmosphere and stirred for 15 min. A solution of the alkyn (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
had been consumed (~5 hr), monitoring the uptake of H2 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-C33, Z8-C31, Z8-C17, and Z6-C15 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 (T0) 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 (T2), frames were recollected, and capped cells in the assay regions were recounted. Assay scores were calculated as the percentage of the capped cells at T2 that were manipulated (any uncapping including piercing) at T2. 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-C32, Z15-C30, Z7-C17, and Z7-C15.

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 (T24), 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 T0 that was completely removed at T24.

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...
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 (F1,99 = 7.6, P = 0.01), CON assay (F1,99 = 4.1, P = 0.05), or FKB assay (F1,99 = 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 (F1,124 = 11.4, P < 0.01), CON assay (F1,124 = 7.5, P = 0.01), or FKB assay (F1,124 = 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 (Kallenbgerg and Kallenbgerg 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 (F1,99 = 13.3, P < 0.001) and August (F1,124 = 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).
Comparison of mean August *Varroa* infestation between colonies with UBO assay scores <62% and ≥62% resulted in the smallest $P$-value ($8.61 \times 10^{-6}$). However, the largest drop in $P$-value ($-6.48 \times 10^{-3}$) occurred between UBO assay scores 51% and 52%. Based on these combined results, we chose to use a threshold of 60% ($P = 9.37 \times 10^{-6}$) to distinguish the most biologically relevant low
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 large change in P-value observed at 64%.

For FKB assays, August but not June Varroa infestations differed significantly between low and high FKB colonies (Fig. 5a). 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 = 41), 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 = 0.37, 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
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 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).
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 ($r_s = 0.36$, 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 ($F_{34,1} = 1.0$, $P = 0.33$) 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
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 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).
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 removal and FKB assay responses. 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-C14 and Z8-C14 are elevated in response to DWV infection regardless of Varroa infestation status (Wagoner et al. 2019), combined with evidence that Z10-C14 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.

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Author Contribution

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