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Chemical detection triggers honey bee defense against a destructive parasitic threat

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Invasive species events related to globalization are increasing, resulting in parasitic outbreaks. Understanding of host defense mechanisms is needed to predict and mitigate against the consequences of parasite invasion. Using the honey bee *Apis mellifera* and the mite *Varroa destructor*, as a host–parasite model, we provide a comprehensive study of a mechanism of parasite detection that triggers a behavioral defense associated with social immunity. Six *Varroa*-parasitization-specific (VPS) compounds are identified that (1) trigger *Varroa*-sensitive hygiene (VSH, bees' key defense against *Varroa* sp.), (2) enable the selective recognition of a parasitized brood and (3) induce responses that mimic intrinsic VSH activity in bee colonies. We also show that individuals engaged in VSH exhibit a unique ability to discriminate VPS compounds from healthy brood signals. These findings enhance our understanding of a critical mechanism of host defense against parasites, and have the potential to apply the integration of pest management in the beekeeping sector.

Infectious diseases involving vector-borne pathogens such as parasites are a global problem that has been exacerbated by invasive species events associated with globalization[1]. Invasive species that reveal themselves as new parasites disrupt established host–pathogen dynamics and can, if commercially valuable plants or animals are targeted, cause immense economic damage[2–4]. In the face of such threats, an in-depth understanding of host defense mechanisms can provide a valuable tool not only to predict, but also to mitigate against, the consequences of parasite invasion.

Organisms that live in groups are particularly susceptible to invasions by new parasites, because the risks of disease spread and transmission of parasites between individuals are high[5]. However, many social animals have evolved mechanisms that allow effective defense at the group level[6,7]. Collective behavioral defenses—also known as social immunity—serve two key functions: to reduce exposure of individuals to sources of infections and/or to increase the level of resistance to pathogens and parasites[6,8]. Thus, all these collective strategies involve one crucial step: detection of either the pathogen or the parasite itself, or of infected/infested individuals.

Surprisingly little is known about the cues that trigger collective behavioral defenses, or how such signals are detected. This is problematic, in particular, because detection sensitivity and recognition accuracy have profound effects on the efficacy of the defense. What is known, however, is that chemical communication plays a key role in host–parasite interactions[9] and, moreover, diseased individuals harbor specific odorant profiles that are distinct from healthy conspecifics[10]. In social insects, cuticular hydrocarbons (CHCs), which play a central role in nestmate recognition[11], are affected by disease states. Changes in CHC profiles on parasite infestation have been identified in ants, honey bees and termites, and associated with the specific targeting of infested nestmates[12–18]. CHCs, ketones, alcohols and fatty acids have all been identified as candidates for social immunity triggering, and behavioral responses and/or an infested/infected state shows high degrees of correlation with some of these semiochemicals. However, causal studies confirming the role of these compounds are extremely rare: a single study in termites identified a blend of ketones, alcohol and fatty acids that can trigger corpse burial[19]; a study in ants suggests that linoleic and oleic acids may be responsible for necrophoric response toward fungus-infected corpses[20]; a study in honey bees showed that phenethyl acetate is associated with hygienic behavior toward a fungal pathogen killing the brood[17]; and two further studies in honey bees revealed that (*Z*)-pentadec-6-ene and (*Z*)-10-tritriacontene are associated with hygienic behavior toward the brood parasite, *Varroa destructor*[16,21]. However, final confirmation of a candidate compound's role in social defense requires that the level of behavior triggered by the compound at the colony or group level in bioassays mirror the intrinsic ability of colonies/groups to display this behavioral defense. In the present study, we provide a comprehensive description of all steps of a mechanism of parasite detection that triggers a behavioral defense known to confer social immunity and associated resistance in the host.

The honey bee, *Apis mellifera*, is an iconic example of an economically relevant social insect facing detrimental invasive species events associated with globalization. In recent times, the parasitic mite, *V. destructor*, moved from the Asian honey bee, *A. cerana*, to the European honey bee, *A. mellifera*, a host switch accompanied by a loss of balance in the host–parasite relationship[22]. *A. mellifera* is capable of displaying an array of behavioral defenses against this parasite[23], one of the most effective of which is known as VSH, which involves the selective removal of parasitized individuals from the brood nest[24], a behavior that interrupts the reproductive cycle of the parasite, providing the honey bee with a critically important means of resistance to *Varroa* sp. The chemical

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compounds bees use to selectively target *Varroa*-infested brood cells have remained elusive.

Chemical communication has been strongly implicated in host detection of mite-infested brood cells, and chemical signaling is also considered to be a strong determinant of VSH behavior[25–27]. Stimuli originating from mites, as well as from a parasitized brood, have been examined closely. CHCs[13,16,18,28], cuticular polar compounds[29], mite pheromones[30] and brood esters[31,32] are all strong candidates. To date, however, only two compounds, (*Z*)-pentadec-6-ene and (*Z*)-10-tritriacontene, have been directly implicated in VSH behavior using behavioral tests[16,21]. One study focused on VSH behavior responses during the first phase of post-capping development, and more specifically on the pre-pupal stage[16]. However, VSH behavior has been shown to occur throughout post-capping development, and to be particularly intensive during pupal development[27,33,34]. In cells containing pupal bees, female mites, if present in the cells, will be actively reproducing[35]. In the present study, we test whether honey bee colonies can react to odorants coming from reproducing *Varroa* females, their offspring and/or the parasitized pupae.

The present study achieves three important goals: (1) six naturally occurring chemical cues that trigger VSH behavior are identified, (2) we demonstrate that synthetic combinations of the compounds identified can be used to evaluate the level of VSH behavior in field colonies and (3) insight is provided into why some, but not all, worker bees perform VSH behavior.

Results

Identification of chemical cues that trigger VSH. To begin, a bioassay was performed under field conditions to identify *Varroa*-related extracts that trigger VSH behavior. After the injection of *Varroa* mite family extracts, 48.3% of the cells were uncapped (Fig. 1; '*Varroa*'). This level of response is significantly higher than that observed after treatment with solvent alone (negative control 'Iso', $z=5.00$, $P<0.001$), but lower than the level of response obtained with dead bee extracts (positive control, $z=-4.82$, $P<0.001$).

Individuals forming a mite family were also separated into two groups so that founder females could be analyzed separately from their offspring. Injection of mite offspring extracts resulted in 74.6% of injected cells being uncapped (Fig. 1; 'Offspring'), a level of response significantly higher than that triggered by injection of solvent alone ($z=5.38$, $P<0.001$). The response to offspring extracts was not significantly different from the response elicited by the positive control (dead bee extracts, $z=-1.42$, $P=0.61$), or from the response level observed after the injection of *Varroa* family extracts ($z=1.83$, $P=0.36$). The injection of founder female extracts (Fig. 1; 'Female') failed to trigger a hygienic response significantly greater than that elicited by the negative control ($z=1.97$, $P=0.28$).

To identify compounds potentially responsible for the *Varroa*-specific hygienic behavior triggered by mite extracts, gas chromatography (GC) profiles of extracts derived from the contents of parasitized cells were compared with extracts of non-parasitized cell contents. This approach aimed to identify the differential release of volatiles in parasitized and nonparasitized cells. Profiles from total extracts of nonparasitized cell contents versus parasitized cell contents were analyzed first. Both profile types were very similar (Fig. 2), but close examination of profile pairs revealed six peaks in the profiles of parasitized extracts that were absent in the profiles of nonparasitized extracts (Fig. 2 insets). The six compounds were present in 78, 100, 87, 100, 70 and 87% of the parasitized extracts, respectively, in increasing retention-time order ($n=23$). These compounds could not be identified in the profiles of nonparasitized extracts, with the exception of the fourth compound that was present in a very small amount in 29% of the nonparasitized samples ($n=23$). Analysis of *Varroa* family extracts, identified as active in the bioassay described above, revealed that all six compounds were present in 100% of the samples examined ($n=23$).

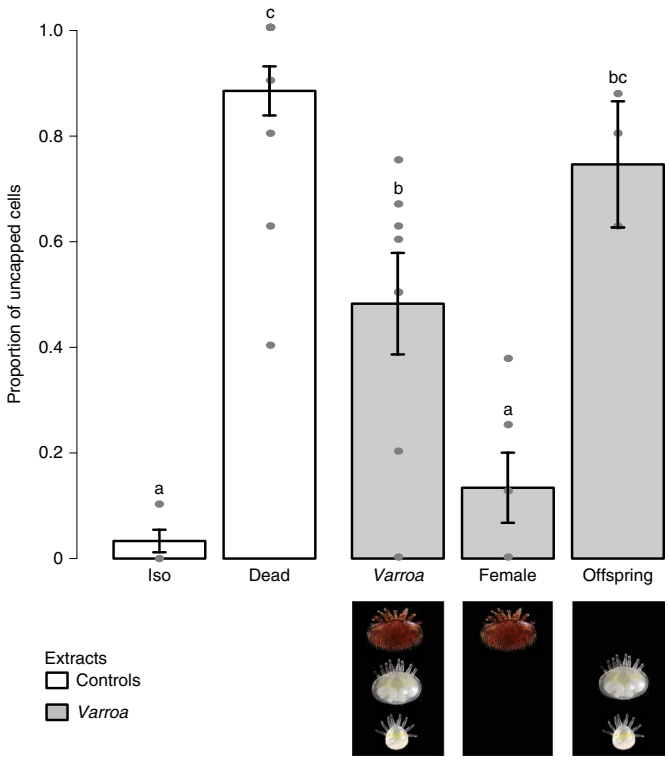

Fig. 1 | Field bioassay to assess the hygienic behavior of honey bees toward brood cells treated with different *Varroa* extracts. Mean proportion (±s.e.) of treated brood cells uncapped by bees performing hygienic behavior. Iso, isohexane (negative control); Dead, dead pupae extract (positive control); *Varroa*, *Varroa* family extract; Female, female *Varroa* extract; Offspring, *Varroa* offspring extract. Gray dots represent the data ($n=8$ colonies per treatment). Different letters above the bars indicate significantly different means at $\alpha=0.05$ (FDR-adjusted *P* values from pairwise comparisons of estimated marginal means: $P_{\text{Iso-Dead}}<0.0001$, $P_{\text{Iso-Female}}=0.28$, $P_{\text{Iso-Offspring}}<0.0001$, $P_{\text{Dead-}\textit{Varroa}}<0.0001$, $P_{\text{Dead-Female}}<0.0001$, $P_{\text{Dead-Offrspring}}=0.61$, $P_{\textit{Varroa}\text{-Female}}=0.015$, $P_{\textit{Varroa}\text{-Offspring}}=0.36$, $P_{\text{Female-Offspring}}=0.0006$).

The six compounds were absent from extracts of founder female *Varroa* sp., except for one compound, which was present in 17.4% of the samples ($n=23$), corroborating the field bioassay results. The six compounds were also detected in extracts of parasitized pupae in 87.7, 100, 83.3, 100, 66.7 and 83.3% of the samples ($n=24$).

Mass spectrometry (MS) analysis of fractions of *Varroa* family extracts, together with analysis of standards, identified the six compounds as four ketones and two acetates: tricosan-2-one (**1**; TrCO), pentacosan-2-one (**2**; PCO), tetracosyl acetate (**3**; TCA), heptacosan-2-one (**4**; HPCO), hexacosyl acetate (**5**; HCA) and nonacosan-2-one (**6**; NCO) (Fig. 2).

Quantitative analyses confirmed that the six compounds were specific to the parasitization status of the brood cells (Fig. 3a). All six compounds could be detected in notable amounts in extracts from both parasitized pupae (Ppu) and mite family (Mfam) sample types (Fig. 3a). Added together, amounts of each compound detected on parasitized pupae and mite families equaled the amounts measured in parasitized cells (Fig. 3a). When expressed per cell, the amount of the six compounds was significantly lower in mite families compared with parasitized pupae, with the exception of HCA and NCO (Fig. 3a).

The last set of analyses focused on quantification of the compounds in mites. We first quantified the compounds by extracting

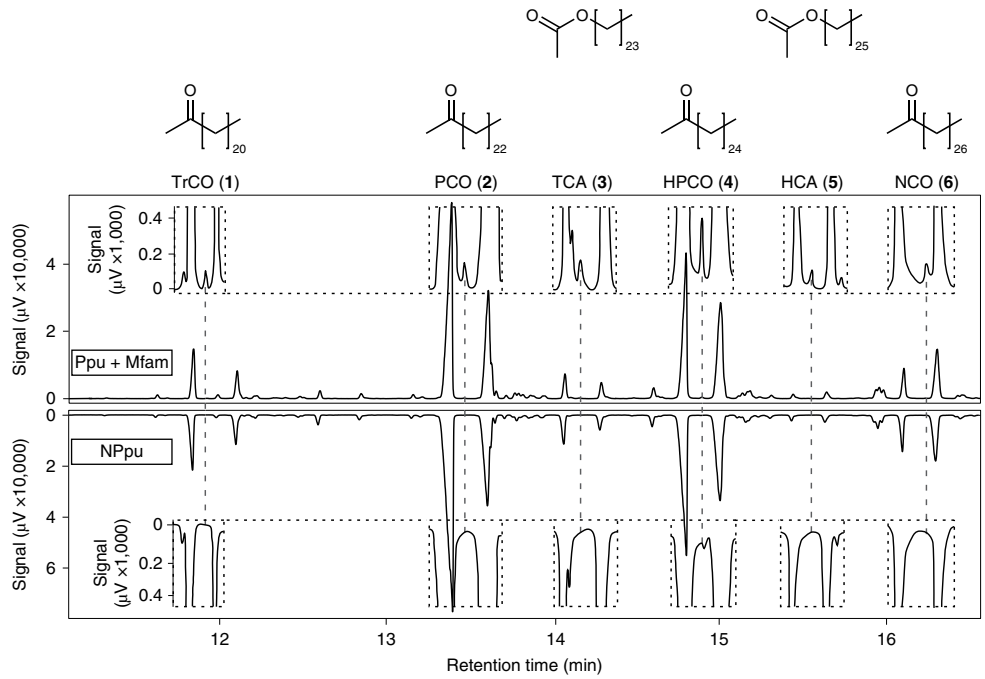

Fig. 2 | Identification of six cuticular compounds that are specific to the *Varroa*-parasitized status of brood cells (VPS compounds). Gas chromatograms of cuticular extracts of parasitized cell contents (Ppu + Mfam) and nonparasitized cell contents (NPpu), as well as chemical structures of the six VPS compounds.

separately the founder female from the offspring. All six compounds could be detected in offspring at similar levels to those detected in total mite families (Fig. 3b). Only HPCO was found in founder females but at a very low level, similar to the small amount found on nonparasitized pupae (NPpu) (Fig. 3b). To investigate the potential of mite offspring at different stages to present and/or produce each compound, each category of individuals (eggs, males, protonymphs, deutonymphs) was extracted separately. All six compounds were detected in every category of mite offspring examined, including in the eggs. The six compounds are referred to hereafter as VPS compounds.

Comparisons were made between the VPS compound profiles of samples originating from three types of brood cell: noninfested cells (NI), infested cells not targeted by bees performing VSH behavior (NT) and infested, targeted cells (TA) ($18 \leq n \leq 21$). Qualitative analysis indicated that five of the six VPS compounds (HPCO excepted) were never detected in samples belonging to the NI group (Fig. 3c). This confirms that the presence of five of the six compounds provides a reliable indicator of the *Varroa*-parasitization status of a cell. Quantitative analysis revealed significant amounts of all six VPS compounds in samples from the NT group (Fig. 3c). However, significant differences between NT and TA cells were revealed: amounts of five of the six VPS compounds (TrCO excepted) were significantly higher in TA cells than in NT cells (Fig. 3c). This difference between NT and TA groups disappeared when amounts of the six VPS compounds were normalized with the number of mite offspring found in each sampled cell, that is, expressed as a quantity per cell and per mite offspring. NT and TA cells displayed on average 17.1 ng ± 10.1 (s.e.) and 20.2 ± 6.6 (s.e.) per cell and per mite offspring, respectively.

The quantity of VPS compounds measured in NT and TA cells was investigated in relation to the number of mites present in each cell ($n_{NT} = 21$; $n_{TA} = 20$). A significant relationship was found between the total amount of the six VPS compounds per cell and the number of mite offspring per cell (Fig. 3d; $\chi^2 = 164.63$, degrees of freedom (d.f.) = 1, $P < 0.001$). A strong correlation was found (Spearman's $\rho_{NT} = 0.81$, $\rho_{TA} = 0.82$). This enabled a strong link to be made between the quantity of the six VPS compounds and the number of mites present in each brood cell.

Hygienic behavior toward synthetic VPS compounds. The field bioassay, designed to test bees' responses to extracts of cell contents (described above), was used to test the ability of synthetic VPS compounds to trigger hygienic behavior. The response of colonies was first tested with varying amounts of a mixture of the six VPS compounds generated synthetically, ranging from 0.5 equivalent to 50 equivalents (equivalents of the amounts of VPS compounds detected in TA cells; $n = 12$ colonies). Injected VPS compounds were found to be active and to trigger a dose-dependent hygienic response (Fig. 4a; first-order term: $\chi^2 = 32.20$, d.f. = 1, $P < 0.001$; second-order term: $\chi^2 = 7.01$, d.f. = 1, $P = 0.008$). Comparison of the response obtained for each dose indicates that a significant response was obtained when a dose of VPS compounds of ≥1 equivalent is used ($z = 3.33$, $P < 0.001$). The highest dose tested (50 equivalents) triggered a response only slightly lower than that obtained with the positive control (dead pupae extracts; $z = -2.26$, $P = 0.026$).

The VPS compounds were tested separately to analyze their activity further ($n = 33$ colonies). There was a significant compound effect ($\chi^2 = 269.98$, d.f. = 8, $P < 0.001$) but no single compound triggered a response as high as that triggered by the six VPS compounds applied together (Fig. 4b), suggesting that the mixture of the six VPS compounds is the best extract to use in tests evaluating the VSH activity of honey bee colonies.

The hygienic response elicited by VPS compounds was investigated further to ascertain whether the level of cell uncapping triggered by VPS compounds reflects the level of expression of VSH behavior occurring naturally within the *Varroa*-infested field colonies of different VSH responsiveness. The correlation between the response obtained in the reference VSH assay (VSH activity) and that obtained in the bioassay (uncapping rate) was significant for both VPS doses tested, but higher at 30 equivalents (Fig. 4c; $n = 16$, $S = 201.47$, d.f. = 14, $P = 0.0023$) than at 1 equivalent ($n = 10$,

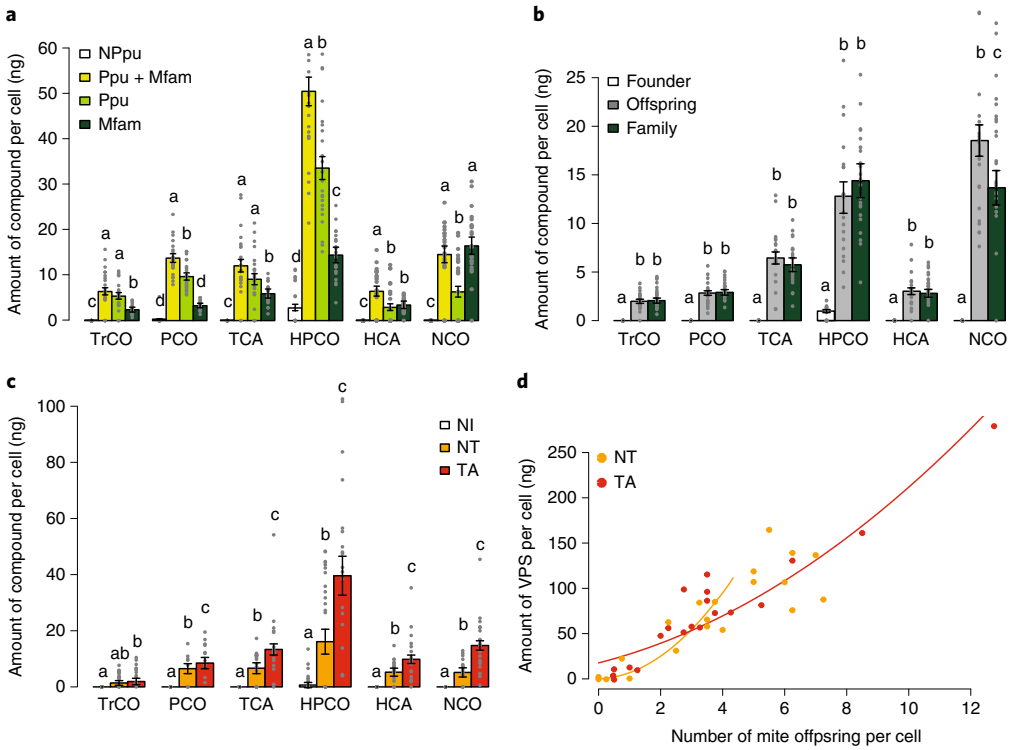

Fig. 3 | Quantification of the six VPS compounds in bee pupae and mites. a, Adjusted mean levels (±s.e.) of the six VPS compounds per cell in brood and mite samples, among NPpu, Ppu + Mfam, Ppu and Mfam types. Gray dots represent the data ($n_{NPpu} = 24$, $n_{Ppu+Mfam} = 23$, $n_{Ppu} = 24$, $n_{Mfam} = 23$ individual bees ± mite families; FDR-adjusted *P* values from pairwise comparisons of estimated marginal means: for each of the six VPS compounds, $P_{NPpu\text{-}Ppu} < 0.0001$, $P_{NPpu\text{-}Ppu+Mfam} < 0.0001$, $P_{NPpu\text{-}Mfam} < 0.0001$). **b**, Adjusted mean levels (±s.e.) of the six VPS compounds per cell in mite samples, across founder females, offspring and *Varroa* families (GLMM, $n = 20$ groups of 10 mite individuals/families; FDR-adjusted *P* values from pairwise comparisons of estimated marginal means: for each of the six compounds, $P_{Founder\text{-}Family} < 0.0001$, $P_{Founder\text{-}Offspring} < 0.0001$; TrCO, $P_{Family\text{-}Offspring} = 0.84$; PCO, $P_{Family\text{-}Offspring} = 0.86$; TCA, $P_{Family\text{-}Offspring} = 0.46$; HPCO, $P_{Family\text{-}Offspring} = 0.74$; HCA, $P_{Family\text{-}Offspring} = 0.7$; NCO, $P_{Family\text{-}Offspring} = 0.012$). **c**, Adjusted mean levels (±s.e.) of the six VPS compounds in developing bees from NI, NT and TA brood cells (GLMM, $n_{NI} = 20$, $n_{NT} = 21$, $n_{TA} = 18$ brood cell contents; FDR-adjusted *P* values from pairwise comparisons of estimated marginal means: TrCO, $P_{NI\text{-}NT} = 0.56$, $P_{NI\text{-}TA} < 0.0001$, $P_{NT\text{-}TA} = 0.073$; PCO, $P_{NI\text{-}NT} = 0.0002$, $P_{NI\text{-}TA} < 0.0001$, $P_{NT\text{-}TA} = 0.0012$; TCA, $P_{NI\text{-}NT} = 0.0015$, $P_{NI\text{-}TA} < 0.0001$, $P_{NT\text{-}TA} = 0.011$; HPCO, $P_{NI\text{-}NT} < 0.0001$, $P_{NI\text{-}TA} < 0.0001$, $P_{NT\text{-}TA} = 0.0030$; HCA, $P_{NI\text{-}NT} = 0.0008$, $P_{NI\text{-}TA} < 0.0001$, $P_{NT\text{-}TA} = 0.020$; NCO, $P_{NI\text{-}NT} = 0.0032$, $P_{NI\text{-}TA} < 0.0001$, $P_{NT\text{-}TA} = 0.0001$). **d**, Relationship between the total amount of the six VPS compounds and the number of mite offspring per cell, in TA and NT brood cells (LMM, $n = 41$ brood cell contents, Spearman's $\rho_{NT} = 0.81$, $\rho_{TA} = 0.82$). Different letters above the bars indicate significantly different mean amounts of a VPS compound between groups at $\alpha = 0.05$.

$S = 52.32$, d.f. = 8, $P = 0.03$) and the degree of correlation quite high ($\rho_{1eq} = 0.50$, $\rho_{30eq} = 0.47$). This result confirms that the VPS compounds identified in the present study are involved in VSH behavior.

VPS compound perception of VSH and NVS bees. Recordings of the summed activity of antennal olfactory sensilla (electroantennograms (EAGs)) were used to determine whether or not bees could detect the VPS compounds. Antennal responses of individual bees observed performing VSH tasks (VSH bees) were compared with those of individuals not performing such tasks (NVS bees). Eight different odorant cues were tested: each of the six VPS compounds presented individually, all six VPS compounds combined (VPS mix) and a floral volatile compound (linalol, as a positive control). All eight odorant cues generated reproducible electrophysiological responses (Fig. 5a,b). For each compound or mixture tested, the responses of VSH bees and NVS bees were very similar (Fig. 5a; $\chi^2 = 0.032$, d.f. = 1, $P = 0.86$). Of the VPS compounds tested, TCA elicited the strongest response in both sets of bees, a response identical in magnitude to the response obtained with linalol (positive control; Fig. 5a). The global response to odors was identical in the left and right antennae of NVS bees, whereas a stronger global response was detected in the left antennae than in the right antennae of VSH bees (Fig. 5b, $t_{VSH} = -3.07$, d.f. = 411, $P = 0.012$; $t_{NVS} = 1.47$, d.f. = 409, $P = 0.46$). This trend was seen for TrCO, PCO, TCA, HCA, VPS and linalol; however, a significant difference in the amplitude of the responses of left and right antennae was observed only for linalol ($t_{Lin} = 4.07$, d.f. = 180, $P = 0.007$). These results indicate that, at the level of the antennae, responses to VPS compounds in VSH and NVS bees are similar, suggesting that both VSH bees and NVS bees are able to perceive the VPS compounds.

A differential conditioning protocol was used to determine whether bees could differentiate the smell of a healthy (nonparasitized) brood from the smell of a healthy brood to which VPS compounds had been added. We found no difference between VSH and NVS bees in their responsiveness to sucrose (95% confidence interval (CI) of model parameters: asymptote VSH: 95% CI = 0.56,1.27; NVS: 95% CI = 0.53,0.83; proportion of bees responding at a null sucrose concentration VSH: 95% CI = −0.016,0.097; NVS: 95% CI = −0.038,0.070; natural logarithm of the rate constant VSH: 95% CI = 1.13,2.88, NVS: 95% CI = 1.73,2.94). Bees were trained to respond with proboscis extension to the smell of VPS compounds combined with healthy brood odors, but not to respond to the smell of healthy brood odor alone. The percentage of bees displaying proboscis extension increased significantly over successive conditioning trials for all groups, showing that both VSH and NVS bees are able to learn odors (Fig. 5c; $\chi^2 = 56.13$, d.f. = 1, $P < 0.001$). However,

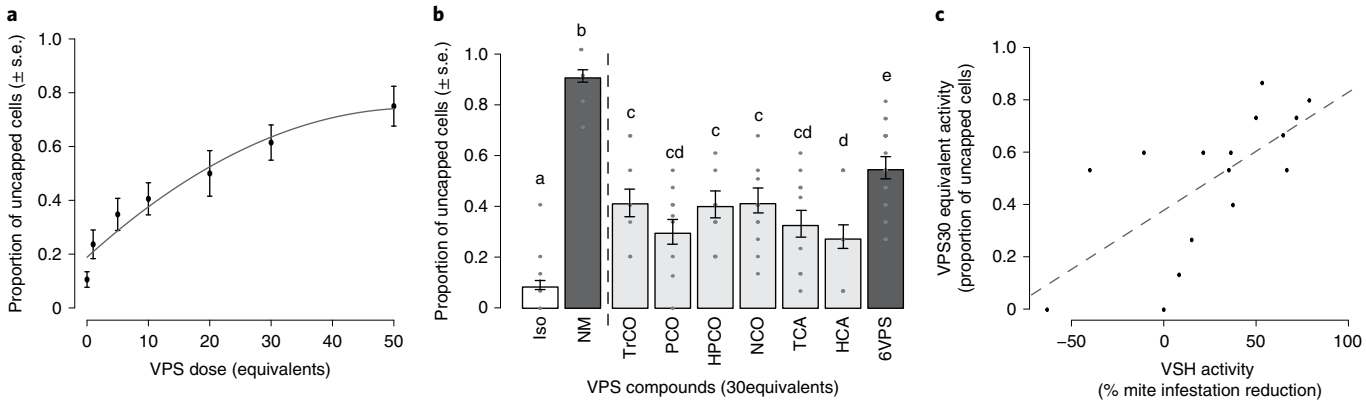

Fig. 4 | Biological activity of VPS compounds in the VSH field bioassay. a, Uncapping activity (±s.e.) in relation to the dose of VPS compounds injected (LMM, $n=12$ colonies). **b**, Uncapping activity (±s.e.) in response to application of single or all VPS compounds at 30 equivalents (GLMM, $n=33$ colonies; FDR-adjusted P values from pairwise comparisons of estimated marginal means: $P_{Iso\text{-}NM}<0.001$, $P_{Iso\text{-}TrCO}<0.001$, $P_{Iso\text{-}PCO}<0.001$, $P_{Iso\text{-}HPCO}<0.001$, $P_{Iso\text{-}NCO}<0.001$, $P_{Iso\text{-}TCA}<0.001$, $P_{Iso\text{-}HCA}<0.001$, $P_{Iso\text{-}6VPS}<0.001$, $P_{TrCO\text{-}PCO}=0.69$, $P_{TrCO\text{-}HPCO}=1$, $P_{TrCO\text{-}NCO}=1$, $P_{TrCO\text{-}TCA}=0.95$, $P_{TrCO\text{-}HCA}=0.41$, $P_{PCO\text{-}HPCO}=0.72$, $P_{PCO\text{-}NCO}=0.50$, $P_{PCO\text{-}TCA}=1$, $P_{PCO\text{-}HCA}=1$, $P_{HPCO\text{-}NCO}=1$, $P_{HPCO\text{-}TCA}=0.96$, $P_{HPCO\text{-}HCA}=0.43$, $P_{NCO\text{-}TCA}=0.83$, $P_{NCO\text{-}HCA}=0.23$, $P_{6VPS\text{-}TrCO}=0.013$, $P_{6VPS\text{-}PCO}=0.001$, $P_{6VPS\text{-}HPCO}=0.0067$, $P_{6VPS\text{-}NCO}=0.027$, $P_{6VPS\text{-}TCA}=0.0017$, $P_{6VPS\text{-}HCA}<0.0001$). **c**, Relationship between the colony response to VPS compounds in the field bioassay and the VSH score (Spearman's correlation, $n=16$ colonies, $P=0.0023$, $\rho=0.47$). Different letters above the bars indicate significantly different mean responses between treatments at $\alpha=0.05$. Iso, Isohexane; NM, dead pupae extract.

NVS bees responded as if the two forms of stimulation were identical, whereas VSH bees responded to CS+ and CS− differently (Fig. 5c). After the third conditioning trial, the percentage of VSH bees responding to CS+ (brood odor + VPS compounds) continued to increase. Their responses to CS− (brood odor alone), on the other hand, declined to a level similar to that observed in NVS bees. By the sixth conditioning trial, the percentage of VSH bees responding to CS+ was significantly higher than the percentage of the same bees responding to CS− ($z=4.14$, $P<0.001$). There was no significant difference overall between the CS+ and CS− response curves obtained for NVS bees and the CS− response curve of VSH bees (Fig. 5c). In memory tests conducted 1 h after the last conditioning trial, VSH bees responded significantly more to CS+ than to CS− (Extended Data Fig. 1; $z=2.97$, $P=0.035$), whereas NVS bees showed a similar level of response to both the reinforced odor, CS+ and the nonreinforced odor, CS− ($z=1.46$, $P=0.69$). Moreover, as shown by the proportion of response to the CS+ only, VSH bees showed a higher level of memory specificity than NVS bees (Fig. 5d; $\chi^2=3.89$, d.f. = 1, $P=0.049$). These results indicate that VSH bees can differentiate VPS odors from healthy brood odors, whereas NVS bees cannot; NVS bees behaved in this learning assay as though the two odorant cues they were detecting (CS+ and CS−) were identical. In memory tests, NVS bees tended to respond more frequently to the new odor than VSH bees ($t=3.33$, $P=0.0015$).

Discussion

Chemical cues, identified in the present study, that are unique to *Varroa*-parasitized brood cells trigger hygiene behavior in the honey bee *A. mellifera*, and mirror intrinsic levels of VSH defense in field colonies. The six VPS compounds identified include four ketones and two acetates. As bees performing VSH behavior need to be able to detect the presence of a *Varroa*-infested brood through the cap of a brood cell, it has been assumed that compounds involved in VSH initiation must be highly volatile. However, the compounds identified in the present study do not fall into this category (24–29 carbon apolar chains). Based on our findings, we propose that the six VPS compounds diffuse through the caps similar to hydrocarbons, esters and alcohols[28,36,37].

Only one VPS compound, HPCO, has been described previously in honey bees as a secretion that accumulates in the mandibular glands of foragers[38]. In insects, higher methyl ketones used in defense and trail marking persist for appreciable periods of time before they evaporate or degrade by oxidation. Given their chain length, this is highly likely to be true also of the four ketones described in the present study.

In the field bioassay, extracts of *Varroa* mite offspring elicited strong hygienic behavior (Fig. 1), suggesting that VSH may involve kairomonal communication, where hygienic bees detect signals emitted by *Varroa* sp. This assumes, however, that mites display and/or synthesize the VPS compounds. Consistent with this possibility, some VPS compounds were detected in equal (HCA) or significantly higher (NCO) levels in mites than in pupae (Fig. 3b). However, the six compounds associated with VSH behavior were also identified on parasitized pupae, suggesting that pheromonal communication could also be used by hygienic bees[13,18,39]. The further possibility that mites synthesize the VPS compounds and pupae are 'contaminated' through their close contact with mites seems unlikely, because VSH can start soon after capping, when contacts and feeding of mite offspring are limited[16,40].

Earlier studies searching for odorants associated with *Varroa*-infested brood cells have identified a variety of candidate compounds, including CHCs (primarily alkenes), aliphatic acids, esters and alcohols[13,16,18,21,29,32]. So far, only two studies have performed direct testing of candidate compounds to confirm correlational results[16,21]. They found that hygienic responses could be elicited by (*Z*)-pentadec-6-ene and/or (*Z*)-10-tritiacontene, two compounds not identified in our study. It would be interesting in the future to test all candidate compounds in combination to examine their efficacy. Although the first phase of post-capping development examined in an earlier study[16] is no longer thought to be the main target of VSH behavior[27,40], it is possible that different signals may be emitted depending on the stage of the targeted brood. Early detection of an infested brood that cannot be linked to the presence of mite offspring may be due to viral infection of the brood, an indirect effect of mite infestation[13,32].

The VPS compounds identified in the present study are linked with the VSH behavior of honey bee colonies, making them primary candidates as triggers for VSH. Two further studies have confirmed the role of olfactory cues in the triggering of hygienic behavior, but targeted at a dead brood[17,41]. The compounds tested in these studies also differ from those shown in our study to elicit VSH behavior, suggesting that compounds that signal severe *Varroa* infestation

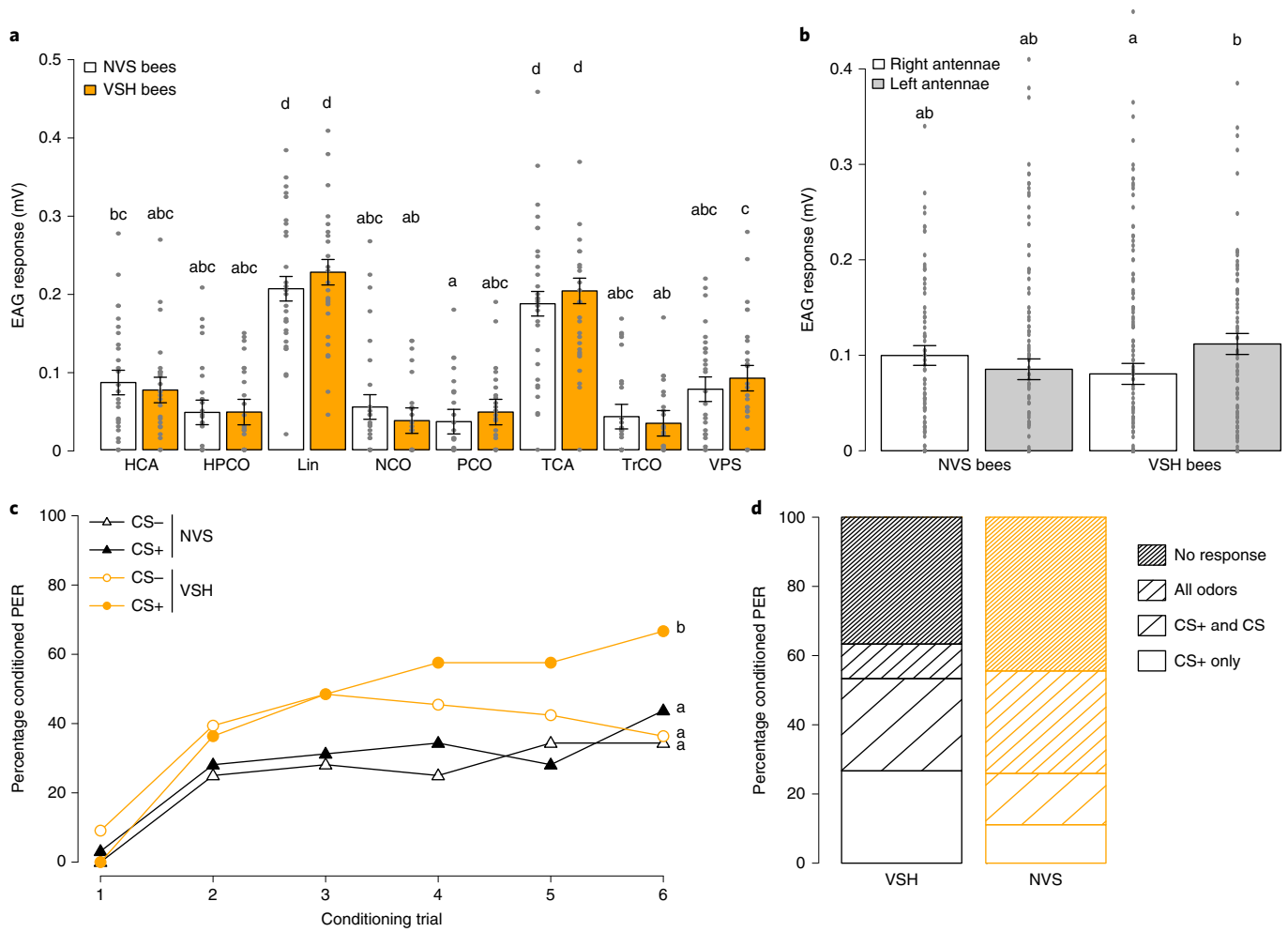

Fig. 5 | Adult bee perception and integration of the VPS compounds. a, EAG response (±s.e.) of NVS and VSH bees toward each of the six VPS compounds, the VPS blend and linalol (LMM, $n=34$ antennae per group; for each of the tested compounds, $P_{VSH\text{-}NVS}=1$; for all Lin-TCA comparisons, $P_{VSH\text{-}NVS}=1$). **b**, EAG response (±s.e.) of the right or left antenna of NVS and VSH bees, toward the six VPS compounds, the VPS blend and linalol (LMM, $n=17$ antennae per group, $P_{NVSright\text{-}NVSleft}=0.45$, $P_{NVSright\text{-}VSHright}=0.58$, $P_{NVSright\text{-}VSHleft}=0.85$, $P_{NVSleft\text{-}VSHright}=0.98$, $P_{NVSlest\text{-}VSHleft}=0.32$, $P_{VSHright\text{-}VSHleft}=0.012$). **c**, Conditioned PER of VSH bees compared with NVS bees. Bees were trained over six trials (Test) to discriminate between an odorant paired with a sugar reward (CS+, healthy brood extract + VPS compounds) and nonreinforced odorant (CS−, healthy brood extract) (LMM, $n_{VSH}=32$, $n_{NVS}=28$, $P_{NVS/CS\text{-}NVS/CS+}=1$, $P_{NVS/CS\text{-}VSH/CS-}=0.49$, $p_{NVS/CS\text{-}VSH/CS+}=0.045$, $P_{NVS/CS+\text{-}VSH/CS-}=0.47$, $P_{VSH/CS\text{-}VSH/CS+}=0.002$, $P_{NVS/CS+\text{-}VSH/CS+}=0.042$). **d**, The short-term memory of bees was tested 1 h after training (Test), with the CS+, the CS− and a third odor not presented during the learning phase (nonanol). The specificity of the memory is shown by the white bars (CS+ only). Different letters above the bars indicate significantly different means between groups at $\alpha=0.05$.

status may differ from the signals that emanate from a dead brood. However, all the studies outlined above, including the present study, suggest that a combination of molecules, rather than one single compound, is probably responsible for initiating hygienic behavior. Hygienic behavior may also rely on the redundancy or synergy of the signal, a mechanism shown to provide evolutionarily relevant stability and accuracy of behavioral responses[42].

VSH involves multiple steps: the identification of a compromised brood, the selective uncapping of *Varroa*-infested cells and, finally, the sacrifice of a targeted brood. It is possible that each step is triggered by one or more different compounds. A first step, for example, might involve the detection of highly volatile compounds, such as ocimene, which could help bees locate areas containing a damaged brood[43]. The detection of less-volatile compounds, such as the VPS compounds, alkenes or oleic acid, may then allow the precise targeting of only the most damaged brood. Arguably the most challenging step, however, involves the selective sacrifice of an infested brood. Importantly, VPS compounds disperse slowly and provide a highly accurate indicator for determining whether a developing pupa should be killed. VPS compounds seem to be specific to the sacrifice of a brood, the most evolutionarily constrained step of VSH behavior.

Although all worker bees can detect the VPS compounds at the level of the antennae, only VSH bees can distinguish these compounds from the smell of healthy brood odors (Fig. 5). As we could find no difference between VSH bees and NVS bees in signals generated at the level of the antennae in response to the delivery of VPS compounds, the ability of VSH bees to recognize and discriminate VPS compounds from other chemical cues would appear to occur centrally, most probably in antennal lobes and mushroom bodies of the brain[44]. Unlike NVS bees, VSH bees also showed an asymmetrical odorant response between the left and right antennae, with the left antennae producing stronger EAG signals. Lateralization of olfactory sensitivity has been described elsewhere, with the same bias toward left antennae, between hygienic and nonhygienic bee EAG responses[17,45].

The present study reveals that VPS compounds play a primary role in the triggering of VSH, a response that arms honey bees with

their most important defense against *Varroa* sp. It is hoped that the bioassay developed in the present study will have direct practical applications for the beekeeping industry. VPS compounds used to assess honey bee colonies for their performance of VSH behavior could potentially support breeding programs selecting bees for resistance to *Varroa* sp., and help the implementation of integrated pest management strategies for the control of the *Varroa* mite in honey bee populations throughout the world.

Online content

Any methods, additional references, Nature Research reporting summaries, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41589-020-00720-3.

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Methods

Field bioassay. *Bioassay design and preliminary validations.* The field bioassay tested the hypothesis that VSH behavior can be elicited in honey bee colonies by experimental exposure of healthy pupae to appropriate stimuli. The assays were conducted during the summers and falls (June to October) of 2012, 2013 and 2014 on the experimental apiary located at INRAE (National Institute for Agricultural Research) research center of Avignon, France.

The odorant stimuli were extracted in isohexane (Sigma-Aldrich) and exposed to the brood by injection of 1 µl of the extract through the cap of white- to pink-eyed pupae using a Hamilton syringe. Preliminary laboratory assays demonstrated that the injection of solvent through capped cells does not alter the survival of the developing pupae ($n = 9$ frames, with 30 cells per frame). The survival rate was estimated by measuring the number of cells from which bees emerged at the end of metamorphosis. Frames were kept in an incubator (34 °C) on injection and until adult emergence. Control cells (no injection) had an emergence success of 92 ± 6% and pierced cells (sham group where a hole was pierced through the cap with the syringe) of 93 ± 7%; 91 ± 8% of cells injected with 1 µl of isohexane emerged, whereas only 15 ± 12% of cells injected with 5 µl of isohexane emerged. Field bioassays were therefore performed with the injection of 1 µl of extract. This preliminary validation also ensured that, if hygienic behavior activity was recorded in our field bioassay, it did not target a dead brood.

To control for the age of the brood in cells that received treatments and to ensure homogeneity between cell and colony replicates, the queen of each tested colony was caged on an empty frame, and kept isolated for 24 h after she started laying eggs. After 11 d, the brood frame was brought back to the laboratory to perform injection of the extracts. The experiment was performed on eight frames belonging to eight different colonies.

On each frame, eight to ten capped cells were injected for each treatment condition. The position of the injected cells was marked on a transparent plastic sheet, to enable a follow-up of the hygienic behavior activity. Frames were kept for 1 h in an incubator maintained at 34 °C to allow the release of the solvent vapors, before being reintroduced into their colony of origin. Then, 48 h after reintroduction, frames were checked for any hygienic behavior response. Cells that were uncapped were included in the hygienic behavior-targeted group. At the end of the observation period, the total proportion of cells targeted by hygienic behavior was estimated.

Negative and positive control validations. This field bioassay was designed to assess the possibility for adult honey bees to detect odorant stimuli and perform VSH behavior tasks.

The first step consisted of validating the bioassay. Treatments were used as negative controls if, after injection, they failed to trigger hygienic behavior at a level greater than that directed toward cells left untouched. Three different negative controls were tested: pierced cells (cells that were injected with 1 µl of air—'pierced'), isohexane (cells injected with 1 µl of isohexane—'Iso') and nonparasitized pupa extracts (cells injected with 1 µl of purple-eyed pupae extracts—'NP'). None of the three controls triggered hygienic activity significantly above that directed toward cells that were left untouched ($z_{\text{pierced versus untouched}} = 0.007$, $z_{\text{Iso versus untouched}} = 0.008$, $z_{\text{NP versus untouched}} = 0.007$, $P > 0.05$). However, as isohexane is used in the extraction process, injection of cells with 1 µl of isohexane was considered the most appropriate negative control to use for comparison when analyzing hygienic behavior induced by *Varroa* extracts.

Validation of the bioassay also required finding a biological extract containing odors to which bees can be naturally exposed in a colony, and that—once injected through capped cells—would trigger a high level of hygienic behavior activity. Preliminary experiments showed that the injection of extract containing 20 dead bee equivalents prepared in isohexane triggered a quasi-systematic hygienic behavior activity, with targeting of 85% of the injected cells, on average. This level of hygienic activity was significantly higher than that obtained for the negative control isohexane ($z = 6.90$, $P < 0.001$). Dead bee extracts at 20 equivalents were used as a positive control in the field bioassay. This procedure is important to validate our behavioral assay set-up and to ensure that tested colonies were in the right conditions to exhibit hygienic behavior.

Varroa extract preparation for the bioassays. Different *Varroa* mite extracts were prepared to test their ability to elicit a hygienic response in the field bioassay. Mites were obtained from heavily infested *A. mellifera* colonies, and sampled at the reproductive stage. Mites were collected in cells containing pupae with purple eyes but a white head and body. In such cells, a female mite can be found as well as a variable number of offspring[35]. 'Mite families' were collected only if they comprised at least one reproducing founder female, one male and one deutonymph. Three types of extracts were tested: '*Varroa*' extracts containing founder females and their offspring, 'female' extracts containing founder females only and 'offspring' extracts containing offspring only (eggs, protonymphs, deutonymphs, males).

To collect mites, brood cells were opened with fine forceps, the pupa was removed and the cell content inspected. Mature and immature mites were collected using a fine brush, and placed in a 1-ml glass vial. Tools were cleaned with 70% ethanol and distilled water before use, and the glassware was baked for 4 h at 400 °C. After 10 'mite families' were placed in the vial, 200 µl of isohexane was added. Soaking was allowed for 10 min; the extract was then placed in a new tube. The extracts were tested at a concentration of 20 'mite-family equivalents' in 1 µl, by concentration of the extracts under a nitrogen stream. A total of 2,880 mite families were collected to prepare the extracts.

The exact amounts of identified compounds actually released in the capped cells, and potentially perceived by the adult bees, could not be determined. Therefore, the dose of extracts chosen for the bioassay had to take into account possible losses at the different steps involved in preparing and applying the extracts (for example, losses during extraction, concentration, injection, natural evaporation or absorption of the extracts by the wax). The dose applied for the negative biological control (nonparasitized pupae) was determined by comparing the total peak areas of 20 mite-family extracts analyzed by GC (see below) to the total peak area of a nonparasitized pupa extract. To apply the same total amount of compounds in the negative biological control and the testing extracts, nonparasitized pupae extracts were applied at a concentration of 0.77 equivalents.

Synthetic extract preparation. Different extracts were prepared using various mixtures of the six candidate synthetic compounds. Compounds from the VPS blend were synthesized by Omega-Cat System. The compound purity varied from 94% to 99%. The identity of all compounds was checked by GC–MS (see below).

Compounds were solubilized and diluted in isohexane. Compounds and mixtures were tested in different concentrations, expressed in 'equivalents'. One equivalent represents the average amount of each compound that has been measured from VSH-targeted brood cells (for details on the methodology, check Mondet et al.[32]). As compounds can be lost during the extraction process, as well as through evaporation and absorption into the hive matrix (see above), 1 equivalent should be considered a conservative estimate of the actual amount of each compound present in *Varroa*-infested brood cells.

Evaluation of the VSH trait. The assays were conducted during the summer of 2017 (July and August) on the experimental apiary located at INRAE. Sixteen colonies were evaluated concomitantly for their response to the VPS blend in the bioassay and their VSH activity. Assessment of VSH cells followed the protocol developed by McAfee et al.[46]. Capped brood frames containing sealed larvae and pre-pupae were collected from mite donor colonies and 150 cells were dissected along transects across the brood patch to determine the initial level of infestation (only frames containing 10–35% of infested cells were used). The frames were incubated for 1 week in the colonies to test, in the center of the brood nest. After 7 d, the frames were retrieved and another 150 cells, located on transects close to the ones examined on day 1, were dissected to determine the final level of infestation. The reduction in mite infestation level between day 1 and day 7 was used as the estimate of VSH activity[46].

Chemical analysis. *Biological material and extract preparation for chemical analyses.* Brood frames sampled from ten different colonies were brought to the laboratory. Capped brood cells were opened using fine forceps. Cells containing purple-eyed pupae with white heads and bodies were sampled. To do so, the pupa was removed using a soft forceps. Care was taken not to damage and/or pierce the pupa. If the cell was parasitized by *Varroa* sp., the pupa was inspected and any immature white *Varroa* sp. were gently removed using a fine brush. Pupae were then placed individually in 4-ml glass vials. Mite families, mite offspring, founder females, eggs, protonymphs, deutonymphs and males were collected using a fine brush and placed in 1-ml glass vials. Mite extracts were prepared using four individuals (or four groups of individuals in the case of mite families and offspring), except in the case of eggs, which were analyzed by grouping ten entities. The composition of each extracted mite family was recorded.

Comparison of the candidate compound amounts in VSH-targeted versus nontargeted cells was performed on cell contents, as described in Mondet et al.[32]. Briefly, the samples correspond to three groups of capped brood cells: TA, NT and NI cells. The TA cell contents were collected from cells identified as partially or totally uncapped but containing intact brood. Untouched cells located near TA cells were used as control cells. Control cells contained broods at an identical stage and were either NT or NI. For each cell, the pre-pupa/pupa, as well as the mite family, was carefully removed using a soft forceps and a fine brush, respectively. The stage of the developing bee and the composition of the mite family were recorded. The contents of four cells were sequentially placed in a 4-ml glass tube, weighed and stored at −30 °C until further processing.

All extracts were prepared by immersing the samples for 10 min at room temperature in 2 ml (four pupae samples), 600 µl (individual pupae samples) or 200 µl (mite samples) of isohexane (Sigma-Aldrich); 6 µl of eicosane (C20—Sigma-Aldrich) at 5 ng µl^{-1} was added as an internal standard (30 µl at 20 µg ml^{-1} for the four pupae samples). Each sample was fully evaporated under a nitrogen stream, and then resuspended in 30 µl of isohexane. Samples were stored at −20 °C until further analysis.

GC analysis. Qualitative and quantitative analysis of molecules specific to the parasitized status were performed on a fast gas chromatograph (Shimazu GC-2014) equipped with a split/splitless inlet, a flame ionization detector and a capillary Equity 5 column (15 m × 0.10 mm, 0.10-µm film thickness). Then, 1 µl of each sample was injected with a 10-ratio split mode, and a column flow of 0.55 ml min^{-1}.

The carrier gas was hydrogen, and the temperatures of the injector and detector were both set at 330 °C. The oven temperature was programmed with the following conditions: 70 °C held for 30 s, followed by temperature increases at a rate of 40 °C min^{-1} up to 150 °C, and at a rate of 10 °C min^{-1} up to 320 °C. The oven was finally held at 320 °C for 4 min. The peak corresponding to the internal standard was determined by injection of pure eicosane diluted in isohexane at the end of each batch of ten samples.

Data transformation and quantification. Analysis of the GC profiles was performed in two steps: first, profiles of nonparasitized cell extracts (pupae) were compared with profiles of parasitized cell extracts (pupae and corresponding mite families) to identify differences in peaks detected between the two conditions.

Quantification series were then performed on the molecules that appeared to be specific to the parasitized status; 20–24 samples were analyzed for each category (pupae, mites). Calibration of each molecule's peak area was performed using the internal standard to deduce an amount of compound in nanograms per cell, or nanograms per milligram of individual.

MS analysis. The structures of the six VPS compounds found to be specific to the parasitized status were determined after fractionation of extracts of 'mite families'. Combined *Varroa* extracts of 80 'mite families' in total, prepared as described above, were applied to a silica column (silica gel 60, particle size 40–63 mm, 230–400 mesh). The column was first rinsed with a solvent mix (isohexane:diethyl ether, 98.5:1.5, Sigma-Aldrich) until 3 ml of the mix was collected. Samples were added to the column and eluted in a final volume of 3 ml of the solvent mix containing 1.5% of diethyl ether. The second fraction was eluted in a final volume of 3 ml of a second solvent mix (isohexane:diethyl ether, 94:6). Then, 3 ml of each fraction was concentrated to 30 µl under a nitrogen stream. GC analysis of the fractions using the program described above showed that the molecules of interest are present in the second fraction only.

Extracts of the first and second fractions of 'mite families', as well as nonparasitized pupae extracts, were analyzed by GC–MS; 1 µl of sample was injected into a GC–MS (Shimazu QP2010 plus) equipped with an electronic impact ion source and a Macherey–Nagel Optima 5 MS column (30 m × 0.25 µm). A split ratio was set to 5, the column flow at 0.89 ml min^{-1} and the carrier gas was helium. The injector temperature was at 180 °C and the oven temperature program as follows: 40 °C isothermal held for 1 min, followed by temperature increases at a rate of 20 °C min^{-1} up to 150 °C and at a rate of 6 °C min^{-1} up to 320 °C. The oven was finally held at 320 °C for 8 min. The ion source was set at 200 °C, and *m*/*z* scanned from 30 to 350.

Comparison of the mass spectra of the six VPS compounds with those available in the NIST 2011 and Wiley 9ed libraries allowed the chemical identity of the compounds to be determined. For compounds absent in the library, first identification was made using the fragmentation pattern of homologs with different chain lengths and calculation of the expected retention indices (P408–409)[47,48]. This identification was further confirmed by injection of the reference compound after synthesis (Omega-Cat System).

EAG recordings on VSH and NVS bees. *VSH and NVS bee collection.* This experiment was conducted in the summer of 2017 (August and September). A behavioral assay was set up to identify and sample adult honey bees performing VSH tasks, as well as bees not performing such tasks (non-VSH or NVS bees). An observation hive consisting of a six-frame nucleus was set up with a mated queen and about 1,500 adult bees collected from a colony showing low signs of VSH activity and low suppressed mite reproduction scores (which can be linked to VSH). The observation hive was maintained in an indoor apiary with the temperature controlled at 28 °C. The bees had free access to outside activities during the whole experiment. A wooden insulated extension was placed at the back of the observation hive to hold an infrared camera (Dalsa Genie Nano-M1280-NIR, Teledyne Dalsa) and six infrared lights. The back panel of the hive was replaced by a Plexiglass panel to allow filming of bees inside the colony. The organization of the colony was modified so that a frame containing a capped brood was visible to the camera. Every 3–4 d, a frame containing late instar pupae was collected from one of three colonies selected for their good VSH performances. The frame was maintained in an incubator (34 °C) and newly emerged bees were individually marked with an identity tag, including a shape and a number, together with a paint mark (Posca) on the thorax. A total of 2,300 newly emerged bees were marked and introduced into the observation colony, in groups of 150–300 bees at a time.

Observations began when the oldest marked bee was aged 7 d (known onset of hygienic behavior[49]), and the bees were exposed to a frame containing artificially *Varroa*-infested brood cells[50]. The frame was collected from a colony with low levels of *Varroa* sp. Early morning, a frame with cells containing ready-to-be-capped L5 larvae was identified, and their location was marked on a transparent sheet. After 6 h, the brood-donor frame was retrieved and cells that had been capped in the 6-h time interval were marked. In parallel, the same procedure was applied to a colony with high levels of *Varroa* sp. In this *Varroa* donor colony, cells capped within the 6-h interval were opened and mites infesting these cells were collected with a fine brush. On the brood donor frame, fine incisions were performed on the sides of the freshly capped cells using a scalpel, and a mite was introduced in each cell before gently closing the cap by applying gentle pressure to the edges of the cells. Then, 60 cells were artificially infested using this protocol and their positions on the frame carefully recorded. This artificially infested frame was placed back overnight in its original colony to be cleaned (none of the artificially infested cells were destroyed). The next morning the frame was placed in the observation colony behind the Plexiglass sheet and the video recordings started. Video recordings were performed 24 h a day for 18 d, and the infested brood frame was replaced after 9 d. Video files were retrieved regularly and analyzed in almost real time by watching the behaviors of marked bees. VSH bees were identified as bees performing VSH-related tasks, that is, opening a capped infested cell and/or enlarging a preopened infested cell and/or starting to remove the content of an infested cell. For removers, only bees that were the first to start removing a developing bee were included in the analysis; bees removing a brood that had already started to be eaten were discarded in an effort to avoid confusion between *Varroa* parasitism and cues of deadness for removal. Marked bees that were never seen to perform VSH-related tasks (or even scanning the brood) were designated as NVS bees. These bees were included as negative controls. To ensure that the performance of VSH-related tasks by controls was not missed, control bees were observed for a minimum of 7 d before they were identified as NVS bees. Sampling occurred twice a week for 2 weeks and, each time a VSH bee was sampled, one NVS bee of the same age and the same colony of origin was also collected. Bees were placed in a cage, with water and candy, and kept in an incubator (28 °C) until EAG recording occurred.

EAG recordings. Immediately before EAG testing, bees were cold anesthetized. The left or right antenna was removed by transecting it at the base of the scape. The tip of the distal-most flagellum segment was also cut. The antenna was connected at both ends to recording electrodes via glass capillary tubes prepared from 76-mm long and 1.12-mm diameter (World Precision Instrument) tubes pulled and cut using a vertical micropipette-puller (P-30 model, World Precision Instruments) and filled with insect Ringer's solution ($NaCl/KCl/CaCl_2/NaHCO_3$, Na^+ 131 mmol l^{-1}, K^+ 5 mmol l^{-1}, Cl^- 111 mmol l^{-1}, $C_3H_5O_3^-$ 29 mmol l^{-1}). Data were acquired from both antennae, one after another, in a randomized order.

EAG acquisition occurred with a specific detector set-up (Syntech IDAC-2), using a CS-55 stimulus controller (Syntech) to continuously pass purified and humidified air over the antenna and to deliver 0.5-s pulses of odorized air (continuous flow: 435 ml min^{-1}, pulse flow: 890 ml min^{-1}). The odorant stimuli were prepared in Pasteur pipettes filled with 1 cm^2 of No. 1 Whatman filter paper impregnated with 4 µl of the odorant solution of interest. Pipettes were prepared 30 min before testing, to allow evaporation of the solvent (isohexane). Each odor was puffed at the antenna for 0.5 s, and we waited for 1 min between each stimulus. Each antenna was tested first with a puff of air (control for living antenna) and second (and last) with solvent alone (negative control for the mecanoreceptor response). The eight odorant stimuli, which included the six individual VPS compounds (10,000 ng $µl^{-1}$), the VPS mixture (10,000 ng $µl^{-1}$) and a floral volatile compound (*R*-linalol) as a positive olfactory detection control (1 per 1,000), were presented in a randomized order to each antenna. The olfactory response (in mV) for each of the eight odorant stimuli was calibrated using the mean value of the two mecanoreceptor responses obtained using solvent alone.

VPS odor discrimination assay. An associative learning protocol based on the proboscis extension reflex (PER) was used to determine whether VSH bees and NVS bees can detect and respond to the presence of VPS compounds added to healthy brood extracts. Using a standardized differential conditioning protocol[51], bees were trained to respond with proboscis extension to the presence of VPS compounds, and not to respond if presented with brood extract odors alone. The learning performances of VSH bees and NVS bees were compared during the fall of 2016 (February and March) in research facilities in the Department of Zoology at the University of Otago, New Zealand. Bees were sampled after a behavioral assay in an observation hive using bees marked on emergence, as described by Mondet et al.[27].

VSH bees were designated as marked bees performing VSH-related tasks for more than 2 min, that is, opening a capped cell and/or enlarging a preopened cell and/or removing the contents of a cell. NVS bees were designated as marked bees that were never seen to perform VSH-related tasks. To ensure the absence of VSH-related tasks, control bees were observed for a minimum of 7 d before they were identified as NVS bees. For each sampled VSH bee, an effort was made to sample one NVS bee, of similar age.

For every learning session, 12 bees (6 VSH bees, 6 age-matched NVS bees) were collected from the observation colony in the late afternoon and cold anesthetized before being individually harnessed in metal tubes. The training protocol uses sucrose to elicit the PER and reward the bee. As appetitive learning performance can be affected by variations in levels of sucrose sensitivity between individuals, the sucrose responsiveness of VSH bees and NVS bees was tested first ($n = 40$ per group). To compare sucrose response thresholds in VSH and NVS bees, solutions containing 0, 0.1, 0.3, 1, 10 and 30% sucrose were touched to the antennae of each bee, each interspersed with water stimulations to avoid sensitization. A sucrose sensitivity score was generated for each individual, as described by Scheiner et al.[52].

After sucrose responsiveness testing, bees were fed and kept overnight in the dark at 28 °C (very little mortality occurred, $n_{VSH} = 1$, $n_{NVS} = 3$). The following morning, the PER was retested by stimulating the antennae with 50% sucrose

solution. Any bee that failed to respond reflexively to sucrose stimulation at that time, or during the conditioning phase, was discarded ($n_{VSH}=8$, $n_{NVS}=9$). Conditioning was undertaken on 32 VSH and 28 NVS bees. Odorant syringes used to present olfactory stimuli to the antennae of each bee were prepared by placing a 1 cm^2 piece of No. 1 Whatman filter paper impregnated with 4 µl of odorant solution into the syringe. Two odorant mixtures were used: healthy brood extract alone (2 equivalents), and healthy brood extract (2 equivalents) containing 50 equivalents of the VPS mixture. Odorant syringes were prepared 30 min before the conditioning started to allow evaporation of the solvent. The syringes were kept at 50 °C for the total duration of the session to ensure volatility of the tested compounds.

Conditioning protocol. Each bee underwent six conditioning trials with healthy brood odor alone and six trials with brood odor containing VPS compounds, presented in a random order with a 10-min interval between each trial. During conditioning, bees were placed in front of an air exhaust system to avoid odor contamination. During each conditioning trial, odor was presented to the bee for 4 s. When healthy brood odor plus VPS compounds were presented as the conditioned stimulus (CS+), proboscis extension was elicited (by touching the antennae with sucrose) and bees were allowed to drink for 3 s from the sucrose droplet. When healthy brood odor alone was presented to bees (CS−), the odor stimulus was not reinforced. As a bee learns to associate a particular odor stimulus with food, it begins extending its proboscis to the odor alone, in expectation of a food reward. In this challenging task, bees are expected after differential conditioning to respond to a healthy brood odor containing VPS compounds, but not to respond to a healthy brood odor alone. This was tested 1 h after the last conditioning trial.

Short-term memory recall. An hour after the last conditioning trial, bees were exposed to the CS+, the CS− and a new odor stimulus (nonanol) to determine whether they responded selectively. Bees were placed individually in the learning arena and presented with the three odors in a random order, with an interval of 10 min between each stimulus, and without any sucrose reinforcement. At the end of these tests, sucrose stimulation of the antennae was used to confirm that bees still exhibited the PER. The percentages of bees responding with proboscis extension to each stimulus were recorded and the performance of VSH bees and NVS bees compared.

Statistical analysis. All statistical analyses and figures were generated in the R environment (v.3.5.3).

Wald's tests were systematically used to assess the effect of independent variables in mixed models. Pairwise comparisons of estimated marginal means were systematically performed when needed (R package 'emmeans'; https://cran.r-project.org/web/packages/emmeans/index.html). *P* values of pairwise comparisons were always adjusted using the false discovery rate (FDR) correction[53]. Data from the field bioassay were analyzed using a generalized linear mixed model (GLMM, distribution law: binomial, link function: logit) in which the proportion of cells targeted by hygienic behavior was explained by the treatment (fixed factor) and the bee colony (random factor) (R packages 'lme4': https://cran.r-project.org/web/packages/lme4/index.html; and 'car': https://cran.r-project.org/web/packages/car/index.html). The quantity of each of the six VPS compounds was compared between mite samples or brood + mite samples using *F* tests applied on separate generalized linear models (GLMs) accounting for the nonhomoscedasticity of the residuals (GLM, link function: identity, custom relationship between μ and V: $V=\mu$). The treatment and the bee colony were included as independent variables in all models. The quantity of each of the six VPS compounds was compared between types of developing bees using GLMMs (distribution law: Gaussian, link function: identity, square root or log depending on the compound) which included the type of bee (fixed factor) and the sample (random factor). The relationship between the total amount of the six VPS compounds and the number of mite offspring per cell was assessed using a GLMM (distribution law: gamma, link function: log) which included as independent variables the number of mite offspring per cell, the type of cell (fixed factor) and their interaction, and the sample (random factor). The strength of the relationship was also assessed for each type of cell using Spearman's correlation coefficient. The relationship between the uncapping activity and the dose of VPS compounds injected was assessed using a quadratic linear mixed model (LMM) including the dose and the bee colony (random factor). The effect of synthetic compounds on the uncapping activity was tested using a GLMM (distribution law: binomial, link: logit) including the treatment (fixed factor) and the bee colony (random factor). A Spearman's correlation test was used to assess the relationship between the colony response to VPS compounds in the field bioassay and the VSH score. The EAG responses were analyzed using an LMM, which included the compound tested (fixed factor), the type of bee (fixed factor), the side of the antenna (fixed factor), and the interaction between the type of bee and the side of the antenna, as well as the individual bee (random factor) and the bee colony (random factor). The sucrose responsiveness was analyzed using an asymptotic model relating sucrose concentration to the proportion of bees responding. Models were fitted for NVS and VSH bees, and 95% confidence intervals of parameters (asymptote, proportion of bees responding at a null sucrose concentration, natural logarithm of the rate constant) were compared for overlapping as significance tests. The relationship between the conditioned PER and the conditioning trial was analyzed using a GLMM (distribution law: binomial, link function: logit) including the number of the trial, the type of bee (fixed factor), the type of odorant (fixed factor), and all two- and three-way interactions, as well as the individual bee (random factor). Finally, the short-term memory of the bees was assessed using a GLMM (distribution law: binomial, link function: logit) in which the proportion of bees showing proboscis extension was explained by the type of bee (fixed factor), the type of odorant (fixed factor) and their interaction, with the individual bee included as a random factor. Memory specificity was assessed using Wald's test applied on a GLM (distribution law: binomial, link function: logit) in which the proportion of bees responding to CS+ only was compared between NVS and VSH bees. Data and codes can be made fully available on request to the corresponding author.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated for the present study are available upon request to the corresponding author.

Code availability

All computer codes generated for the present study are available upon request to the corresponding author.

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Author contributions

F.M., A.R.M. and Y.L.C. designed the project. F.M., S.B., D.B., N.B., B.L., C.B., S.H.K., B.B. and G.C. performed the experiments with contributions as follows. F.M., S.B., C.B., B.B. did the field assays. F.M., S.B. and D.B. did the GC analyses. F.M., N.B. and G.C. did the MS analyses. F.M. and S.H.K. performed the sucrose responsiveness assay. F.M. performed the learning assay. F.M., C.B. and B.L. did the EAGs. F.M. and M.H. analyzed the data. F.M., A.R.M. and Y.L.C. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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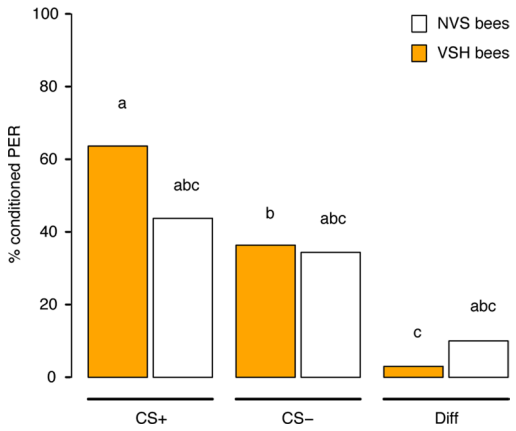

Extended Data Fig. 1 | One-hour memory recall in VSH bees and NVS bees. Percentages of VSH bees and NVS bees displaying conditioned proboscis extension responses (PER) 1 hour after the final (6th) conditioning trial. All bees were trained to discriminate between an odorant paired with a sugar reward (CS+, healthy brood extract + VPS compounds) and a non-reinforced odorant (CS-, healthy brood extract). The figure shows the percentages of bees responding to the CS+, the CS- and to a novel odour not presented during the learning phase (Diff, Nonanol), (GLMM, $n_{VSH} = 32$, $n_{NVS} = 28$). Different letters indicate significant results in the analyses.

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Sample size	Sample sizes were chosen to ensure that the statistical tests performed allowed to detect effect of the tested factors. Sample size was also chosen depending on the number of fixed factor and random factor tested, as well as their interactions.
Data exclusions	No data were excluded, except in our behavioural tests for colonies that did not respond to the positive controls (see appropriate description and n-values in the M&M section)
Replication	All results of the field study and compound quantification could be replicated at least twice on different seasons, apiaries and years. Some of the experiments (quantification of the VPS compounds) were also replicated in two different countries, with different honey bee genetics. All attempts at replication were successful.
Randomization	The observer was always consistent and treatments were coded with colours. Spatial organisation of the treatments on the frames was assigned randomly (random assignation of the order of the treatments using R). Order of the treatments in the EAG was also assigned randomly for each antenna; bee order (VSH, non -VSH) was also randomised. For all other studies, the different treatment groups were analysed on each batch, and their order was randomized.
Blinding	For the learning and EAG experiments, sample group (VSH, non-VSH) was blind for the observer (assigned by a second person). For field assays involving injection of different doses or mixtures of VPS compounds, composition/dose of the extracts was blind to the person performing the injections. For odour extractions from biological samples, the presence of the biological sample (mite, pupae) did not allow for a blind procedure. For later chemical analysis through GC-MS, it was kept blind.

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Laboratory animals	Apis mellifera (female workers) and Varroa destructor (female founders and male and female offspring), insect species. No specific line used. Bees were collected from our experimental apiaries at the University of Otago and INRAE
Wild animals	No wild animals were used in this study
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