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1 # A death pheromone, oleic acid, triggers hygienic behavior in honey
2 # bees (*Apis mellifera* L.)

3 Alison McAfee,[1] Abigail Chapman,[1] Immacolata Iovinella,[2] Ylonna Gallagher-Kurtzke,[1] Troy F. Collins,[1]
4 Heather Higo,[1] Lufiani L. Madilao,[3] Paolo Pelosi,[4] and Leonard J. Foster[1*]

5 [1]Department of Biochemistry & Molecular Biology and Michael Smith Laboratories, University of British
6 Columbia, 2125 East Mall, Vancouver, British Columbia, Canada

7 [2]Dipartimento di Biologia Università degli Studi di Firenze Via Madonna del Piano 6, 50019 Sesto
8 Fiorentino, Italy

9 [3]Wine Research Center; Food, Nutrition and Health Building, University of British Columbia, 2205 East
10 Mall, Vancouver, British Columbia, Canada

11 [4]Austrian Institute of Technology GmbH, Biosensor Technologies, 24 Konrad-Lorenzstrasse, Tulln, Austria

12 *Corresponding author. Email: foster@msl.ubc.ca

13

14 Eusocial insects live in teeming societies with thousands of their kin. In this crowded environment,
15 workers combat disease by removing or burying their dead or diseased nestmates. For honey bees, we
16 found that hygienic brood-removal behavior is triggered by two odorants – β-ocimene and oleic acid –
17 which are released from brood upon freeze-killing. β-ocimene is a co-opted pheromone that normally
18 signals larval food-begging, whereas oleic acid is a conserved necromone across arthropod taxa.
19 Interestingly, the odorant blend can induce hygienic behavior more consistently than either odorant
20 alone. We suggest that the volatile β-ocimene flags hygienic workers' attention, while oleic acid is the
21 death cue, triggering removal. Bees with high hygienicity detect and remove brood with these odorants
22 faster than bees with low hygienicity, and both molecules are strong ligands for hygienic behavior-
23 associated odorant binding proteins (OBP16 and OBP18). Odorants that induce low levels of hygienic
24 behavior, however, are weak ligands for these OBPs. We are therefore beginning to paint a picture of
25 the molecular mechanism behind this complex behavior, using odorants associated with freeze-killed
26 brood as a model.

27 ## Introduction

28 Disease and parasite transmission is a constant threat in dense insect societies[1-3]. Ants[4-8], termites[9-11],
29 and honey bees[12-16] have evolved social mechanisms of disease resistance which mitigate this risk and
30 improve the collective health of their colonies. Ants transport dead nestmates to their midden heaps,
31 termites bury or entomb their dead in graves, and honey bees remove dead and diseased brood from

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1 the hive. E. O. Wilson described these processes as 'necrophoresis,'[4] or the movement of dead
2 individuals away from the colony. Necrophoresis reduces pathogen reservoirs, inhibiting the spread of
3 diseases and parasites from fallen nestmates to those who endure[1,2,4].

4 In honey bees (*Apis mellifera*), one dominant form of necrophoresis is hygienic behavior[13,14]. Hygienic
5 honey bee workers will identify and remove diseased, dead, and sometimes parasitized larvae,
6 prepupae, and pupae from the colony. This is an effective defense against major diseases, including
7 chalkbrood (*Ascosphaera apis*)[17,18], American foulbrood (*Paenibacillus larvae*)[14,19], and the devastating
8 varroa mite (*Varroa destructor*)[13,14,20,21]. When highly hygienic colonies are challenged with these pests
9 and pathogens, they are less likely to develop clinical symptoms than non-hygienic hives, and are more
10 likely to recover and survive[19,22,23].

11 The underlying mechanism of the behavior has only been partially deciphered. Like other social insects,
12 honey bees identify their diseased nestmates via chemical cues[24-27]; however, since partway through
13 development (late 5th instar larvae and older) the brood becomes capped and completes development
14 in the confines of a sealed wax cell, the workers have an added challenge. The physical barrier between
15 the bees who execute the behavior and the brood interferes with their ability to detect their targets.
16 Detecting the dead, diseased, or parasitized capped brood is thought to rely on volatile odorant signals
17 that permeate the wax cell cap[26], but very few hygienic behavior-inducing odorants have been identified
18 and confirmed behaviorally[27,28]. Swanson *et al.*[27] found that a volatile chalkbrood odorant (phenethyl
19 acetate) was a strong hygienic behavior-inducer, and Nazzi et al. showed that a volatile varroa-
20 associated odorant ((Z)-6-pentadecene) does the same[28]. Non-volatile cues have not yet been
21 investigated behaviorally in honey bees, despite including some of the most taxonomically conserved
22 necrophoretic and necrophobic compounds (*e.g.* oleic acid and linoleic acid)[1,6,9,10,29-34].

23 Hygienic honey bees have superior olfactory sensitivity compared to non-hygienic honey bees[24-27], which
24 likely depends in part on differences in antennal gene expression[23,35-38]. In a search for antennal
25 biomarkers for hygienic behavior, we previously identified two odorant binding proteins – OBP16 and
26 OBP18 – that significantly correlated with colony hygienic score[35]. Antennae are honey bees' main
27 olfactory appendages, and OBPs aid odorant signal detection by binding and transporting hydrophobic
28 odorant molecules from the antennal pores to the olfactory nerves[39]. Despite some tantalizing
29 inferences, OBP16 and 18 have not been mechanistically linked to hygienic behavior.

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1 Previously, we compared odorant profiles of freeze-killed pupae and healthy pupae to find candidate
2 hygienic behavior-inducing compounds[40]. Although freeze-killing is not a natural means of death, it is a
3 relevant system because the freeze-killed brood assay[41] is the main method for determining colonies'
4 level of hygiene. We identified two new candidate compounds that were significantly more abundant in
5 freeze-killed brood: oleic acid and β-ocimene. Oleic acid is a non-volatile, oily substance which acts as a
6 death cue in eusocial and non-eusocial insects[1,6,9,10,29-34]. For example, oleic acid stimulates
7 necrophoretic behavior in multiple ant species[6,31,32], as well as termites[9,10]. In isopods[29], caterpillars[29],
8 crickets[30], cockroaches[34], and bumble bees[33], it induces avoidance behavior, presumably as a mechanism
9 to avoid the risk associated with disease or predation indicated by other dead insects. β-ocimene, on the
10 other hand, is a volatile honey bee brood pheromone that is normally a larval food-begging signal[42]. β-
11 ocimene emitted from larvae is also known to inhibit worker ovary development[43,44], regulate the nurse-
12 to-forager transition[44], and stimulate foragers to forage[45,46]. The queen also produces β-ocimene, which
13 also contributes to inhibiting worker egg-laying[47]. Prior to our work in 2017[40], β-ocimene and oleic acid
14 had not been linked to hygienic behavior in honey bees.

15 In the present work, we investigate oleic acid and β-ocimene's roles in hygienic behavior using
16 behavioral assays, electrophysiology, and OBP ligand binding assays. Our behavioral assay overcomes a
17 major hurdle in testing the hygienic behavior-inducing capacity of odorants: by adding odorants through
18 the resealable cells of Jenter™ queen cages, we can add individual odorants to brood cells while
19 maintaining perfect integrity of the wax cell walls and cap. By this method, we show that even the non-
20 volatile oleic acid induces hygienic behavior, but the blend of β-ocimene and oleic acid induced hygienic
21 behavior most strongly and consistently. However, we have not ruled out the possibility of a brood
22 effect induced by odorant contact toxicity. Electroantennogram (EAG) recordings of bees from a
23 hygienic colony show that at a hive-realistic temperature and humidity, oleic acid induces only slightly
24 above-background antennal nerve responses, suggesting that it is only detectable upon contact or
25 extremely close proximity. *In vitro* ligand binding assays show that both β-ocimene and oleic acid are
26 strong ligands for at least one of OBP16 or OBP18 (which are upregulated in hygienic bees'
27 antennae)[23,35], and the two odorants we tested which do not substantially induce hygienic behavior
28 were poor ligands. Taken together, we propose a mechanistic model where the co-opted, volatile brood
29 pheromone (β-ocimene) works together with an evolutionarily conserved death cue (oleic acid) via
30 interactions with hygienic behavior-associated odorant binding proteins (OBP16 and OBP18) to induce
31 hygienic behavior.

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1 **Results**

2 Previously, we found that β-ocimene and oleic acid were both emitted more strongly from freeze-killed
3 brood compared to live brood, making them promising candidates as putative hygienic behavior-
4 inducers. To test if these odorants are sufficient to induce brood removal, we developed a front-way
5 odorant assay (**Figure 1A**), which involves uncapping patches of brood (30 cells each, in two technical
6 replicates per colony) and dispensing 1 µl of either neat (100%) or diluted (1%) odorant standards on the
7 brood.

8 First, we confirmed that hexane was an appropriate negative control by recording the recapping
9 frequencies following the treatments (N = 9 colonies). We found that after just three hours, an average
10 of 44% of the hexane-treated cells were recapped, which was significantly higher than for all other
11 odorants (**Figure 1B;** one-way ANOVA followed by Tukey HSD; β-ocimene: $p = 2e-7$; oleic acid: $p = 1e-8$;
12 mix: $p = 1e-8$; phenethyl acetate: $p = 1e-8$). The next highest was β-ocimene, with 5.4% recapped. The
13 others all had recapping frequencies of 1% or less, indicating that the brood were no longer accepted by
14 the workers.

15 Next, we sought to confirm that there was no effect of patch proximity on brood removal. To test this,
16 we treated patches of ~30 cells with β-ocimene or oleic acid, and separated the patches by either one
17 band of untreated cells ('near' treatments) or located the patches on two different frames, with two
18 untreated brood frames separating them ('far' treatments). We did this for N = 5 colonies, and found no
19 effect of patch proximity on brood removal rates (**Figure 1C**; two-way ANOVA; levels: odorant,
20 proximity; $F = 0.025$, $p = 0.88$). In another test, we found that workers removed treated pupae and
21 prepupae at similar rates (**Figure 1D**; four-factor ANOVA; levels: dose, odorant, hygienicity, age; $F = 0.84$;
22 $p = 0.36$; see **Table 1** for sample sizes). Therefore, we combined data for the two ages and used the
23 front-way assay to test if colonies with higher hygienicity responded to the odorants differently than
24 colonies with lower hygienicity. We tested N = 5 colonies with high hygienicity (freeze-killed brood score
25 > 80%) and N = 5 colonies with low hygienicity (freeze-killed brood score < 80%) (**Figure 2A**), and found
26 significant effects of dose, odorant, and hygienicity (**Figure 2B**; three-factor ANOVA; dose: $F = 61.2$, $p =$
27 $4.3e-11$; odorant: $F = 19.8$; $p = 7.1e-11$; hygienicity: $F = 20.2$, $p = 2.7e-5$).

28 As expected, brood treated with neat odorants were removed significantly more frequently compared
29 to those treated with diluted odorants. We had intended phenethyl acetate to be a positive control
30 odorant, but surprisingly, we found that it induced similar brood removal as the negative control

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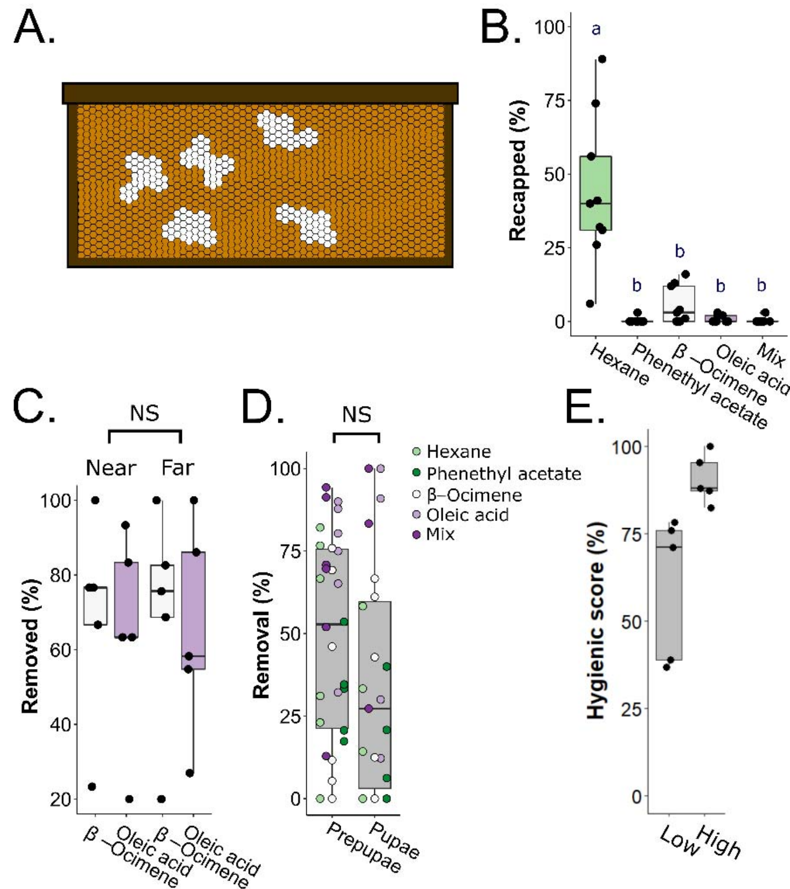

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2 **Figure 1**. *Front-way odorant assay preliminary tests.* **A)** Schematic of the front-way assay. Patches of
3 capped brood (~30 cells in technical duplicate per colony) developing naturally in a standard frame were
4 uncapped (white patches) and 1 μl of odorants (β-ocimene, oleic acid, a 1:1 v/v mix of the two,
5 phenethyl acetate or hexane) at either 1% or 100% concentrations (v/v in hexane) were dispensed onto
6 the brood. Frames were incubated in the colony's brood box for 3 hours before recording removal rates.
7 **B)** Post-front-way assay recapping frequencies. Data from N = 9 colonies were analyzed with a one-way
8 ANOVA (level: odorant; F = 13.3, p = 2.4e-8) followed by a Tukey HSD test. Letters indicate groups that
9 are significantly different from one another Tukey HSD p < 0.05). **C)** Preliminary test for a patch
10 proximity effect. N = 5 colonies were tested, varying the distance between β-ocimene and oleic acid
11 patches (near = patches on the same frame, separated by one band of untreated capped brood; far =
12 patches on different frames separated by two untreated brood frames). We analyzed the data by a two-
13 way ANOVA and found no effect of patch (F = 0.025, p = 0.88) nor interactive effect between patch and
14 odorant (F = 0, p = 1.0). **D)** Preliminary test for a brood age effect. We performed the front-way assay on
15 N = 9 colonies and calculated the percent prepupa and pupa removal. Due to variability in patch
16 composition, not every colony had the same number of replicates for each stage and dose (see Table 1
17 for all sample sizes). Data were analyzed with a four-way ANOVA (levels: odorant, age, hygienicity,
18 dose), which identified no significant effect of age nor interactions with any other factors, followed by a
19 Tukey HSD test. 1% and 100% refer to odorant concentrations. All boxes depict the interquartile range
20 (IQR) and the whiskers span 1.5*IQR.

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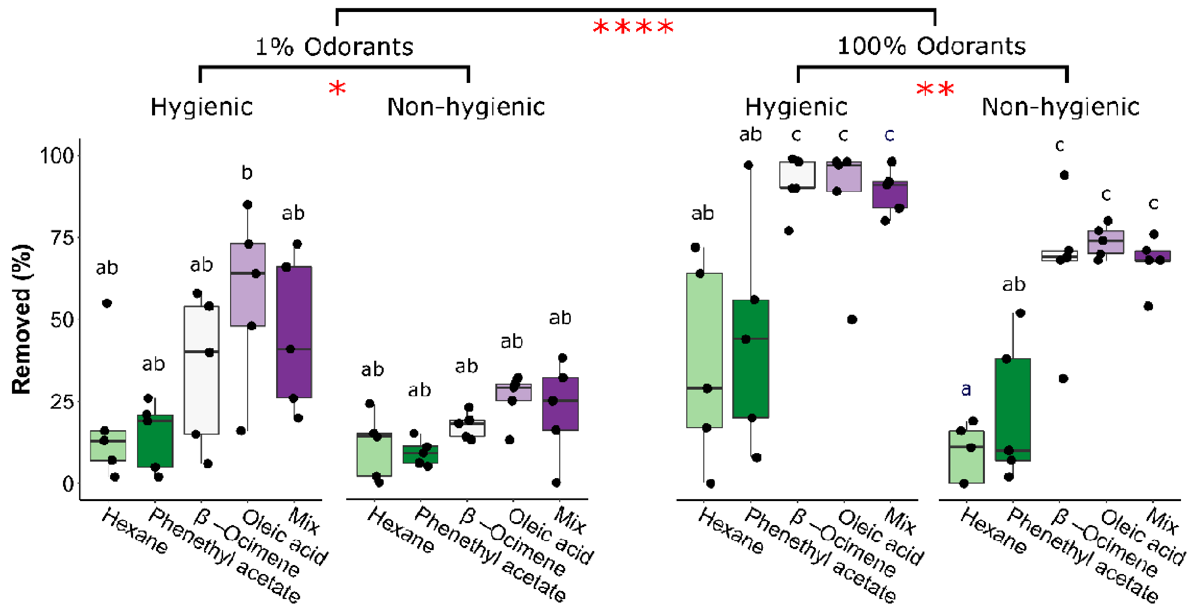

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2 **Figure 2.** *Front-way odorant assays to investigate effects of hygienicity.* **A)** Distribution of hygienic scores
3 for the tested colonies. 10 colonies were tested in total. The lowest-scoring 5 were assigned to the 'low
4 hygienicity' group (scores < 80%) and the highest-scoring 5 were assigned to the 'high hygienicity' group
5 (scores > 80%)**. B).** Post-front-way assay removal frequencies. Hexane is the negative control and
6 phenethyl acetate (a chalkbrood odorant) was meant to be the positive control. Data from 5 low
7 hygienicity and 5 high hygienicity hives were analyzed with a three-factor ANOVA (levels: dose, odorant,
8 hygienicity; dose: F = 61.2, p = 4.3e-11; odorant: F = 19.8; p = 7.1e-11; hygienicity: F = 20.2, p = 2.7e-5),
9 followed by a Tukey HSD post-hoc test. Significance code (Tukey HSD): * p < 0.05, ** p < 0.01, **** p <
10 0.0001. Boxes depict the interquartile range (IQR) and the whiskers span 1.5*IQR. Letters indicate
11 groups that are significantly different from one another at Tukey HSD p < 0.05).

12

13 (hexane), both of which were the lowest of all those we tested. In the neat odorant treatments, β-
14 ocimene, oleic acid and their blend all induced significantly higher brood removal relative to hexane
15 (Tukey HSD; p = 0.0034, p = 0.0075, and p = 0.0049 respectively), but in the diluted odorant treatments,
16 none of the odorants induced significantly different brood removal. However, their relative patterns still
17 reflect what's observed in the neat odorant treatments.

18 We expected colonies with higher hygienicity to respond more strongly to the odorant stimuli than
19 colonies with lower hygienicity. We found that indeed, the higher hygienicity colonies removed
20 significantly more treated brood overall in both the neat odorant treatments (Tukey HSD; p = 0.0084), as
21 well as the diluted treatments (p = 0.011). This agrees with previous electroantennography studies

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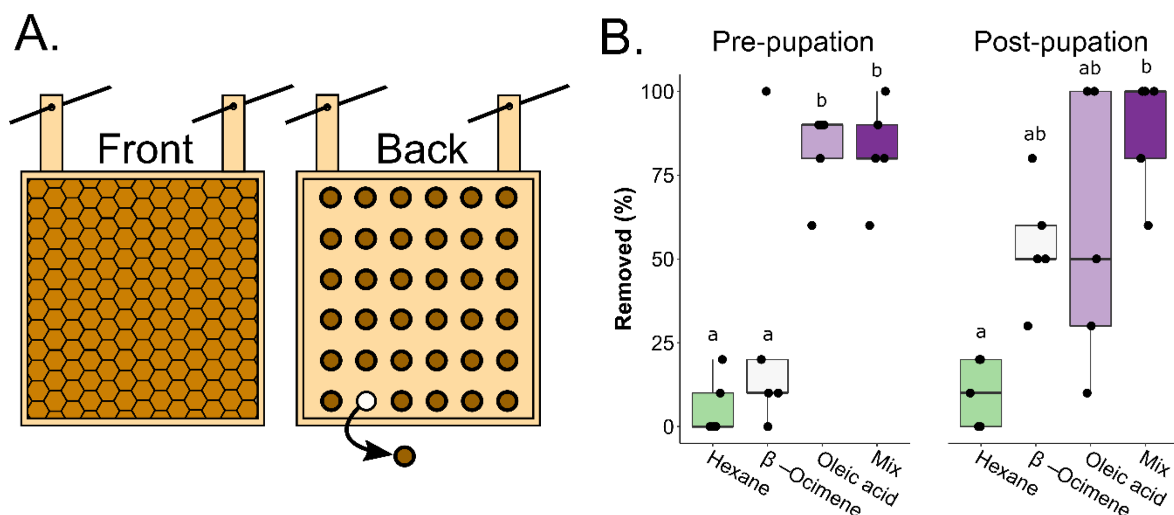
1 showing that hygienic bees' antennae are more sensitive to disease odorants than non-hygienic
2 bees[24,25].

3 The front-way odorant assay is a quick method of gauging if odorants can induce brood removal, but it
4 cannot test for odorant transmission through the physical barrier of the wax cap. To investigate the
5 odorants in a more realistic scenario, we devised a new assay using the Jenter™ system that allows us to
6 treat brood with odorants while maintaining the integrity of the brood cells. We call this the back-way
7 odorant assay (**Figure 3A**), since we add the odorants through the back of the brood cell. Briefly, we
8 place a queen in a Jenter™ cage until she lays eggs in the comb of the cage, then release her and allow
9 the workers to rear the brood until it is capped. The back of the Jenter™ cage is equipped with
10 removable plugs that enable odorants to be added inside the cell without disturbing the delicate wax
11 cell cap, and plugged again to close the brood cell. We used this method to add neat hexane, β-ocimene,
12 oleic acid and the odorant blend to 9-10 brood cells each, before and after pupation (N = 5 colonies for
13 each age). We found that after incubating in the hive for 20 h, β-ocimene did not induce significantly
14 more brood removal relative to hexane (**Figure 3B**; two-factor ANOVA followed by Tukey HSD; p = 0.82
15 for pre-pupal brood and p = 0.10 for post-pupal brood). However, oleic acid strongly induced pre-pupal
16 removal (p = 0.0004) and marginally non-significant post-pupal removal (p = 0.057). The odorant blend
17 induced the most consistently high brood removal of them all, which was significant for both brood ages
18 (p = 0.0004 for pre-pupal and p = 0.0003 for post-pupal).



19

20 **Figure 3**. *Back-way odorant addition assays.* **A)** Schematic of the back-way assay. Queens were caged in
21 a Jenter™ queen rearing cage (a hanging square of artificial comb) until she populated the cells with
22 eggs. The queens were released and brood were allowed to develop until capping (front view). We

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treated brood cells with neat odorants in a semi-random design through the cell plugs (back view, brown circles), then the odorant-impregnated brood was incubated in the colony for 20 h to allow time for odorant diffusion, uncapping, and removal. Diagrams are not to scale. The actual Jenter™ cage has ~ 100 removable plugs (one every 3rd cell). **B.)** We treated pre-pupal and post-pupal brood with each odorant (9-10 brood cells for each age and odorant, N = 5 colonies). Data was analyzed using a two-factor ANOVA (levels: age and odorant) followed by a Tukey HSD post hoc test. There was a significant effect of odorant ($F = 20.3$, $p = 1.51e-7$), no significant effect of age ($F = 0.16$, $p = 0.694$), and no significant interactive effect ($F = 1.9$, $p = 0.157$). Letters indicate groups that are significantly different from one another (Tukey HSD $p < 0.05$). Boxes depict the interquartile range (IQR) and whiskers span 1.5*IQR.

To try to explain the patterns of pre-pupation and post-pupation brood removal, we investigated changes in the background volatile and non-volatile odorant profiles that could confound with our odorant treatments. To do this, we performed solid-phase micro-extraction gas chromatography-mass spectrometry (SPME GC-MS) on extracts from 5th instar larvae, prepupae, and pupae. We analyzed N = 5 independent brood, from 5 different colonies, for each stage. We also used a hexane wash (with the same replicate structure as before) to extract cuticle compounds from these life stages and analyzed them by GC-MS as well, capturing the less volatile signals. We found that β-ocimene abundance changed most significantly according to age (one-way ANOVA, Benjamini-Hochberg corrected 1% FDR; $p = 0.0010$, $q = 0.01$), with relatively high amounts emitted in 5th instar larvae and prepupae, and low amounts in pupae (**Figure 4A and B**). Two other minor chromatogram components were also differentially emitted (compounds 2 and 4, corresponding to isopropanol and 2-pentanone, respectively). Other volatile compound identifications are reported in **Table S1**. The hexane wash identified many branched chain hydrocarbons which were differentially emitted with age but importantly, oleic acid was not among the identified molecules for any of the three developmental stages (**Table S2**).

Previously, we reported that stimulating honey bee antennae with oleic acid yielded no measurable nerve depolarization signal above the background stimulus of air alone[40]. Since we clearly observe that oleic acid can induce hygienic behavior in brood removal assays (including when the brood cell cap remains in-tact), we questioned if the workers were detecting oleic acid-treated cells by olfaction or some other sense (*e.g.* gustation). To investigate this further, we replicated the electroantennography experiment (N = 13 left antennae and N = 14 right antennae) comparing oleic acid to background stimulation, but at a temperature that better-matches in-hive conditions. When we administered

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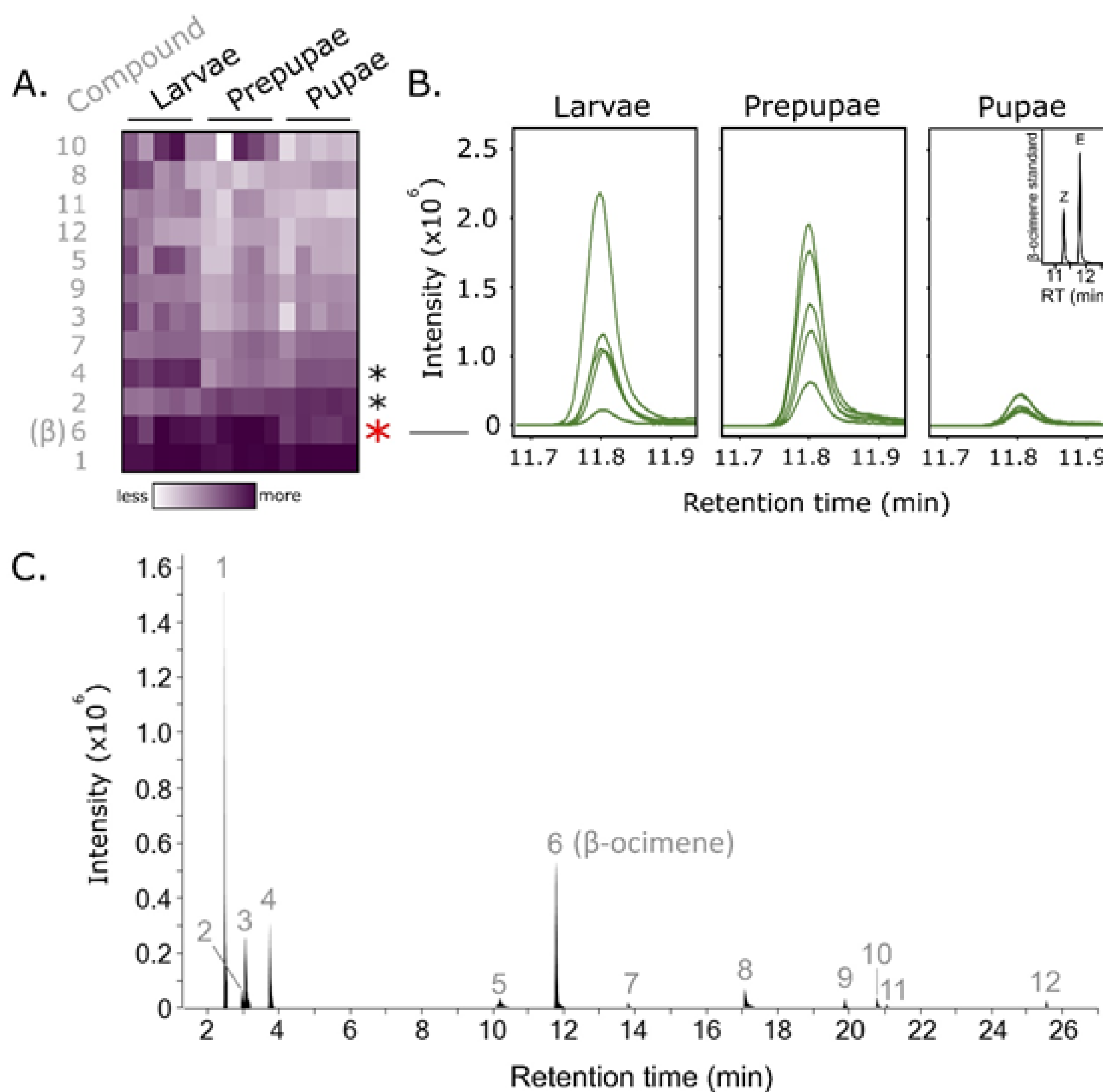

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2 **Figure 4**. *β-ocimene abundance in larvae, prepupae and pupae.* We performed solid phase micro-
3 extraction gas chromatography mass spectrometry (SPME-GC-MS) on extracts from 5th instar larvae,
4 prepupae and pupae (N = 5 colonies each). **A)** Heatmap showing intensities of all integrated peaks. Areas
5 under the curve were compared between ages using a one-way ANOVA and Benjamini-Hochberg
6 correction (5% FDR). Each row corresponds to peak intensities belonging to a different compound. β-
7 ocimene, the most significantly different compound, is indicated with a red asterisk, while two other
8 significantly different compounds (matching to isopropanol (2) and 2-pentanone (4)) are indicated with
9 black asterisks. Raw GC-MS data is available at http://github.com/AlisonMcAfee/test. **B)** Chromatogram
10 traces of the β-ocimene peak. Its identity was confirmed with a synthetic standard (inset
11 chromatogram). Based on its retention time, only the E isomer was identified in the brood. **C)** Example
12 SPME-GC-MS total ion chromatogram. Numbers correspond to compounds labelled in **3A**. Further
13 compound identity and abundance information is available in Table S1.

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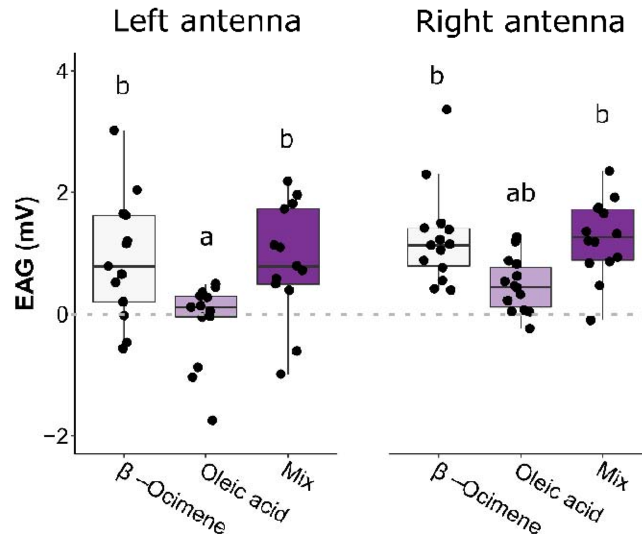
1 warmed oleic acid (at approximately 33°C) we found that it stimulates worker antennae only slightly
2 more than blank stimuli (**Figure 5**). There was also a significant effect of odorant (two-way ANOVA;
3 levels: odorant, side; F = 12.4; p = 2.3e-5), with β-ocimene and the odorant blend inducing significantly
4 higher antennal nerve depolarizations than oleic acid in left antennae (p = 0.011 and p = 0.016,
5 respectively). The same comparisons yielded a marginally non-significant response in the right antennae
6 (p = 0.085 and p = 0.086, respectively).

7 Recently, several antennal protein biomarkers for hygienic behavior have emerged, including two
8 odorant binding proteins (OBP16 and OBP18) which are up-regulated in hygienic bees. To test if β-
9 ocimene and oleic acid are strong ligands for these proteins, we performed *in vitro* binding assays with
10 OBP16 and OBP18. Like our front-way behavioral assays, we used hexane as the odorant negative
11 control and we included phenethyl acetate despite the surprising outcomes of behavioral tests. We
12 found that of the four tested odorants, hexane and phenethyl acetate consistently had the lowest
13 binding affinity (**Figure 6, Table S3**), which mirrors the behavioral response to these compounds (**Figure**
14 **2B**). β-ocimene bound OBP16 strongly, but not OBP18. Oleic acid, however, bound both OBPs strongly,
15 with OBP18 being the strongest.



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17 **Figure 5**. *Electroantennography (EAG) responses to odorants.* We excised left (N = 13) and right (N = 14)
18 antennae from honey bees in a single highly hygienic colony (score = 95%) and measured the EAG
19 response to neat odorants (Syntech™ CS-55) at hive-realistic temperatures (around 33°C). The EAG
20 response represents blank-subtracted odorant stimuli. We found a significant effect of odorant (two-
21 way ANOVA; levels: side, odorant; F = 12.4, p = 2.3e-5), and letters indicate groups that are significantly
22 different from one another (Tukey HSD p < 0.05). Boxes depict the interquartile range (IQR) and
23 whiskers span 1.5*IQR.

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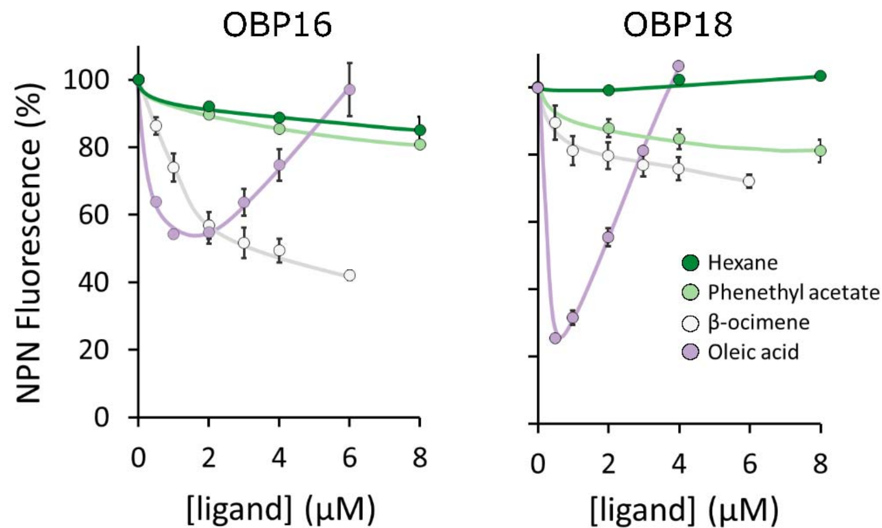

1

2 **Figure 6**. *Affinity curves for OBP16 and OBP18.* We used an NPN (N-Phenyl-1-naphthylamine)
3 competitive binding assay to measure affinities of β-ocimene, oleic acid, phenethyl acetate, and hexane
4 (negative control). Assays were performed in technical duplicate with 2 µM protein and 2 µM NPN in all
5 cases. Lower NPN fluorescence intensity indicates stronger ligand binding. The high NPN fluorescence
6 intensity for the high oleic acid concentrations is due to the formation of micelles at higher
7 concentrations of the ligands[55,56]. A 1% solution of β-ocimene, oleic acid, phenethyl acetate, and hexane
8 corresponds to approximately 60 mM, 32 mM, and 63 mM, and 76 mM, respectively. Error bars are
9 standard error of the mean.

10

11 **Discussion**

12 Hygienic behavior has been studied in honey bees since at least the 1960s[15], but our knowledge of the
13 molecular mechanism behind it is incomplete. In the present work, we investigate two candidate
14 hygienic behavior inducers that are emitted from freeze-killed brood – β-ocimene (a co-opted
15 pheromone emitted by brood and queens[43,44,46-48]) and oleic acid (a well-known necromone and
16 necrophobic compound in other arthropods[1,6,9,10,29-34]) – using *in vivo*, *ex vivo*, and *in vitro* techniques.
17 We demonstrated 1) that treating brood with the odorants is sufficient to induce hygienic behavior in
18 realistic behavioral assays (**Figure 2B and 3B**), 2) despite being a viscous compound, oleic acid can
19 stimulate nerve depolarizations worker antennae at hive temperatures (**Figure 5**), and 3) oleic acid and
20 β-ocimene have high affinities to odorant binding proteins that are upregulated in hygienic honey bees
21 (**Figure 6)**. Although these specific compounds are from freeze-killed brood and do not extrapolate to all
22 brood diseases, it is a relevant model with which to investigate some molecular interactions governing
23 this complex behavioral process. This is not the first time that a brood pheromone has been implicated

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1 in social immunity; Mondet *et al*.[49] found that *Varroa*-infested brood produced elevated levels of brood
2 ester pheromone. Other researchers have found that oleic acid is both contained and emitted by
3 *Varroa,* on top of it being generally associated with insect death[50-53].

4 β-ocimene and oleic acid have very different chemical properties: β-ocimene is a volatile alkene (boiling
5 point: 65-66°C) and oleic acid is a viscous, mono-unsaturated carboxylic acid (boiling point: 360°C). Both
6 are emitted more strongly in freeze-killed honey bee brood compared to live brood[40], but based on their
7 differences in volatility, we expect them to permeate the brood cell cap at different rates. In a
8 biologically relevant scenario, this spatial diffusion should be necessary for adult workers to detect
9 odorant signals evolving under the cap. Since the odorant blend induces brood removal most
10 consistently in the back-way odorant assays (**Figure 3B**), but not the front-way odorant assays (**Figure**
11 **2B**), we suggest that β-ocimene and oleic acid may be acting in a cooperative manner when they have to
12 diffuse through the cap. Since our electroantennogram recordings show that there is no synergistic
13 effect at the level of antennal odorant detection, we suggest they could instead be cooperating via
14 volatility mechanics. For example, a potential mechanism is that β-ocimene diffuses rapidly and attracts
15 worker visits (as it is already known to do for larval feeding[48]) and after subsequent cell inspection, oleic
16 acid acts as the determinant death cue that stimulates brood removal. In the front-way odorant assay,
17 however, the workers are in constant contact with the odorants (since there is no cap acting as a
18 barrier); therefore, oleic acid is readily detectable even in the absence of an attractant.

19 The back-way odorant assay we describe here is the most biologically relevant assay for testing different
20 odorants' abilities to induce hygienic behavior. Unlike other behavioral assays where cells are either
21 uncapped (as in our front-way odorant assay) or filled with odorant-impregnated brood dummies[27,28],
22 this assay fully maintains comb integrity and allows the workers to perform the complete behavior
23 (uncapping and removal). While the odorant blend was the most consistently high inducer of brood
24 removal, oleic acid alone also induced significant brood removal for young (pre-pupal) brood, but not
25 post-pupal brood (**Figure 3B**). Based on our analysis of the background brood odorant profile, this could
26 be because of naturally released β-ocimene (**Figure 4**) interfering with the synthetic odorant treatments.
27 Since the younger brood emitted significantly more natural β-ocimene compared to the older brood, the
28 young brood treated with oleic acid was, in a way, also a blend, which could explain why this treatment
29 induced similar removal to the synthetic blend for the pre-pupal brood but not post-pupal. Very few
30 pre-pupal β-ocimene-treated brood were removed (28%), which is consistent with young brood emitting

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1 their own β-ocimene already. Post-pupal β-ocimene-treated brood, which emit very little natural β-
2 ocimene, were removed at higher rates (54%), although this was not statistically significant (p = 0.10).

3 With a large body of research showing that olfaction is important for hygienic behavior, combined with
4 two odorant binding proteins (OBP16 and OBP18) emerging as protein biomarkers for hygienic behavior,
5 a tempting hypothesis is that the OBPs are aiding the detection of odorants associated with disease or
6 death. After showing that bees with higher hygienicity remove more β-ocimene- and oleic acid-treated
7 brood compared to bees with lower hygienicity, we performed ligand binding assays with OBP16 and
8 OBP18 to test if the OBPs linked to hygienic behavior have a high affinity to these odorants (**Figure 6**).
9 Interestingly, both hexane and phenethyl acetate had a low affinity to both OBPs, which is consistent
10 with both odorants being poor inducers of hygienic behavior (as demonstrated in our behavioral assays;
11 **Figure 2B**). β-ocimene, however, displayed strong affinity for OBP16. Oleic acid was a strong ligand for
12 both OBPs, and bound OBP18 the strongest of all those we tested. Since β-ocimene and the odorant
13 blend induced significantly higher antennal nerve depolarizations than oleic acid (**Figure 5**), this suggests
14 that either the worker bees must be very close to the emanating cell (or possibly even contacting the
15 source) to sense it, or the odorant treatment induces the brood to emit a different, more volatile signal.

16 Swanson *et al.*[27] originally identified phenethyl acetate as a strong hygienic behavior-inducing
17 compound emitted from chalkbrood-infected larvae; however, in our experiments, we found that it
18 induces similar levels of hygienic behavior relative to the negative control in both the diluted (p = 0.99)
19 and neat (p = 0.97) odorant treatments, which is less than both oleic acid and β-ocimene. In fact,
20 Swanson *et al.* found that phenethyl acetate induced 40-100% brood removal using just 5% of the
21 odorant amount we used. One reason why we did not observe high phenethyl acetate removal rates
22 could simply be because the colonies used by Swanson *et al.*[27] were from a genetic lineage that was
23 more sensitive to chalkbrood odorants than ours. Indeed, the two populations of colonies are
24 geographically isolated and are likely adapted to different climates, conditions, and disease challenges.
25 Furthermore, the surprisingly low degree of overlap between differential expression studies comparing
26 hygienic and non-hygienic bees suggests that there are many adaptive routes for bees to become
27 hygienic[54]. It could simply be that the hygienic bees in Swanson *et al.*[27] possess different molecular
28 machinery that allows them to be sensitive to different disease odorants than the colonies used in the
29 present study.

30 Based on our data, we cannot yet rule out the possibility that some of the behavioral response toward
31 odorant-treated brood was a result of toxicity of the odorant itself. In an acute toxicity assay we found

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1 that 1 µl of oleic acid was sufficient to cause contact toxicity when dispensed on the abdomen of pupae,
2 halting the development of 40% of treated individuals and inducing a prophenoloxidase immune
3 response after 2.5 d (**Table S4**). However, 100% of hexane- and β-ocimene-treated brood developed
4 normally, and the odorant blend caused a response midway between these extremes. While the pattern
5 of removal rates for some of the front-way assay treatments is similar to the toxicity treatments, these
6 differences were not significant. Furthermore, the removal rates for the back-way assays – particularly
7 those post-pupation, which is directly comparable to the toxicity assays in terms of application site and
8 brood age – does not mirror the outcome of the toxicity assay. That being said, the only toxicity
9 outcomes we measured was the prophenoloxidase response and developmental delay. There could be
10 other cues that odorant contact stimulates the brood to emit, which we did not measure. In addition,
11 we only investigated abdominal contact toxicity, which is the application site for the back-way assays,
12 whereas in the front-way assays, we applied odorants to the head, which could yield a different
13 response. Other limitations include that the toxicity outcome was measured after 2.5 d, when other
14 developmental effects could take longer to appear. We note, however, that 2.5 d is much longer than
15 the duration of any of our behavioral assays here. In addition, we tested only pupae in the toxicity assay,
16 and not 5th instar larvae or prepupae, which could respond differently to the odorants. These are all
17 important caveats to this work, and warrant further investigation.

18 One way these concerns can be addressed in the future is by developing an assay utilizing brood
19 dummies instead of real brood to eliminate the brood effect. Swanson *et al.*[27] developed a similar assay
20 using brood ester pheromone- and odorant-impregnated paraffin brood dummies in open cells,
21 measuring cell capping (non-hygienic activity) and capping refrainment (hygienic activity) as a proxy for
22 hygienic behavior, since worker bees cannot physically remove the paraffin brood dummies from the
23 cells. This eliminates the brood effect, but has the caveat that leaving a cell uncapped is not the same as
24 performing hygienic behavior. In our front-way experiments, we noticed that cells were frequently left
25 both uncapped and uncannibalized – an outcome which would count as hygienic activity if using paraffin
26 brood dummies. We are currently developing a broodless hygienic test that still allows the object to be
27 removed, for example, by removing developing brood through the back of a Jenter™ set and replacing it
28 with a small odorant-treated object (e.g. a ball of paper or cotton).

29 On one hand, our 100% odorant treatments (1 µl) could be criticized as not being biologically relevant
30 because the signal is too strong; however, this may work to our advantage to overcome the brood
31 effect. By using such a strong odorant signal in the front-way assays, and measuring the behavior

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response after a short period of time (3 h, compared to 24 h for the standard freeze-killed brood assay to measure hygienicity), this should a) minimize the amount of time the brood has to produce a strong response, and b) the experimental treatment should be the dominant signal. For the back-way assays, a longer incubation period (20 h) was utilized since in preliminary tests the behavioral response after 3 h was too low to be useful. This means that there was more time for a potential brood effect to evolve, which may have impacted our results.

Regardless of the caveats to the behavioral assays, the ligand binding assays provide a clear picture: β-ocimene and oleic acid each strongly bind at least one of the two odorant binding proteins (OBP16 and OBP18) whose expression is more strongly correlated with hygienic behavior[23,35]. While oleic acid produces high fluorescence intensities (which normally indicates weak binding) at higher ligand concentrations (*i.e.* > 1 μM), this is a well-known phenomenon for amphipathic ligands[55,56]. The very low fluorescence intensity < 1 μM indicates that it is indeed a strong ligand for OBP18, which agrees with previous binding assays[35]. Conversely, the two odorants which induced low rates of hygienic behavior in our assays also were poor ligands for these OBPs. Therefore, the results of this *in vitro* binding assay can explain the behavioral observations surprisingly well. Despite this evidence, it's difficult to know how well the OBP and ligand concentrations reflect reality. For example, the absolute concentration of OBPs in the hemolymph of honey bee antennae is currently unknown, as is the effective ligand concentration at the antennal pore (the interface between the hemolymph and the surrounding air). While a 1% solution of β-ocimene corresponds to approximately a 60 mM solution, which is much higher than the concentrations in the ligand binding assays (<10 μM), with volatility mechanics and spatial diffusion, the airborne concentration is likely much lower (but unknown).

In summary, this data suggests that oleic acid and β-ocimene induce brood removal in honey bees. Bees with higher hygienicity respond to the odorants more strongly than bees with lower hygienicity, and the blend induces brood removal most consistently in the most biologically realistic brood removal assay. Despite being non-volatile, oleic acid appears to be detectable even beneath a brood cell cap; however, it's possible that the bees are detecting the brood's reaction to the odorant rather than the odorant alone. Our electrophysiology tests show that oleic acid only marginally stimulates antennal nerve responses in environmental conditions similar to those inside a hive, suggesting that if they are detecting the odorant alone, extremely close proximity would be necessary for bees to detect it. Both odorants are strong ligands for at least one of the OBPs linked to hygienic behavior, whereas hexane and phenethyl acetate (which induced the lowest levels of hygienic behavior) are weak ligands for both

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OBPs. These molecular interactions between the odorant ligands and the OBPs mirror the results of our behavioral assay surprisingly well. Furthermore, oleic acid elicits necrophoretic and necrophobic behavior across phylum Arthropoda[1,6,9,10,29-34], and these data piece its activity in honey bees into the phylogenetic puzzle. To the best of our knowledge, our data shows for the first time that this 'death cue' function is evolutionarily conserved in honey bees, and that oleic acid may be working in concert with β-ocimene as an attractant. Future experiments will be necessary to eliminate the possibility of an odorant-induced brood effect contributing to these results.

Methods

Honey bee colonies and hygienic testing

We kept honey bee colonies at four separate apiaries in Greater Vancouver, Canada, and performed hygienic testing as previously described[23]. Briefly, for each test, polyvinyl chloride pipes (5 cm inner diameter, ~25 cm length) were pressed into capped brood comb in two areas containing white-eyed to red-eyed pupae, then filled with approximately 250 ml of liquid nitrogen to freeze. Frames were returned to the colony and assessed 24 h later for percent removal of the frozen brood cells. One week later, the test was repeated, and the average of the two tests (four 5 cm brood patches in total) yielded the FKB score. All hygienic testing, sampling and odorant assays were completed during the summer of 2017.

Front-way odorant assays

To perform the front-way odorant assays, we retrieved two brood frames from each colony, uncapped patches of brood with tweezers and dispensed 1 µl of odorant treatments onto the exposed brood (**Figure 1A**). Wax caps were not replaced after odorant addition. We tested the odorants β-ocimene, oleic acid, a 1:1 v/v blend of the two, phenethyl acetate (positive control), and hexane (negative control) at concentrations of 100% and 1% (v/v in hexane). Phenethyl acetate was not included in the blend because it is not known to co-occur with the other odorants (phenethyl acetate is from chalkbrood, while β-ocimene and oleic acid are associated with freeze-killed brood). For each odorant and concentration, we performed two technical replicates (2 patches of 30 brood cells each, one on each frame). We tested the different concentrations on different days. After treating the brood patches with odorants, we photographed, traced, and labelled each patch on a transparency and replaced the brood frame in the hive. After 3 h, we returned to the hive and recorded the number of brood cells that were cannibalized and partially cannibalized (cumulatively yielding the number 'removed') or recapped.

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1 Brood patches were composed of variable developmental stages (mostly prepupae and pupae, but some
2 5th instar larvae; **Table S5**), so we used the photographs from pre- and post-incubation to assess the
3 fraction of each developmental stage that were removed and/or recapped by the workers. With a clear
4 anterior view, the prepupae can be distinguished from 5th instar larvae based on their upright, elongated
5 body and a 'crook-neck' appearance. Due to variable patch composition, we did not obtain the same
6 number of biological replicates for every developmental stage and odorant (see **Table 1** for complete
7 replicate information for each stage and odorant concentration). Data for 5th instar larvae are not shown
8 because too few patches contained them to reliably test if there was a differential response to larvae
9 (they made up < 10% of tested brood cells overall). This is because the time between cell capping and
10 transforming to a prepupa is very short – in the order of hours – so catching this stage in a naturally laid
11 comb is infrequent. These sparse data were therefore excluded from subsequent analyses. In a
12 preliminary test, brood removal data were analyzed with a four-factor ANOVA (levels: dose, odorant,
13 age, hygienicity) followed by a Tukey HSD to determine if there was an effect of age between prepupae
14 and pupae. Since there was no significant effect of age alone ($F = 0.87$; $p = 0.36$) nor in combination with
15 any other factors (odorant*age: $p = 0.61$, dose*age: $p = 0.15$, hygienicity*age: $p = 0.79$,
16 odorant*dose*age: $p = 0.58$, odorant*hygienicity*age: $p = 0.73$, dose*hygienicity*age: $p = 0.17$,
17 odorant*dose*hygienicity*age: $p = 0.71$), we pooled the pupa and prepupa data for subsequent
18 analyses. All statistical analyses were performed in R unless otherwise specified.

19 In a second preliminary experiment, we confirmed that there was no effect of patch proximity in the
20 front-way odorant assay. We varied proximity by testing two patches of brood per colony that were
21 either separated by a single capped cell-width on the same side of a frame ('near'), or on different
22 frames with two brood frames located between them ('far,' N = 5 colonies each). One microliter of oleic
23 acid (the least volatile odorant tested) or β-ocimene (the most volatile odorant tested) was added to the
24 cells of each patch. The data were analyzed with a two-factor ANOVA (levels: proximity, odorant).

25 To assess the relationship between hygienicity and odorant-treated brood removal, we performed the
26 front-way odorant assay on 10 colonies (and two technical replicates per colony, which were averaged
27 to produce one biological replicate) with varying hygienic score (39% to 100%). We grouped the colonies
28 into N = 5 with higher hygienicity (scoring > 80%), and N = 5 with lower hygienicity (scoring < 80%)
29 (**Figure 2A**). As before, we removed the larval cells from the analysis (~10% overall) and since we
30 previously determined that there was no effect of brood age between prepupae and pupae in the front-
31 way assay, we did not distinguish between these stages statistically. These data were analyzed using a

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1 three-factor ANOVA (levels: dose, odorant, hygienicity) followed by a Tukey HSD post hoc test. Brood
2 recapping data was derived from the same assays (N = 9 for each odorant (data was unavailable for one
3 colony)) using a one-way ANOVA (level: odorant).

4 **Table 1:** Replicate information for age-related brood removal measurements

	Prepupae		Pupae	
	1%	100%	1%	100%
Hexane	6	6	6	4
β-ocimene	6	7	5	6
Oleic acid	6	6	4	5
Blend	6	7	3	5
Phenethyl acetate	6	7	5	6

5

6 *Back-way odorant assays*

7 To test the effects of β-ocimene, oleic acid and their 1:1 v/v blend in a more biologically realistic
8 scenario, we developed the back-way odorant assay (**Figure 3A**). This assay adapts artificial comb cages
9 of the Jenter™ queen rearing system to instead rear worker brood *in situ*. The Jenter™ set features
10 removable plastic plugs from the rear of the comb – usually used to harvest eggs/larvae for queen
11 rearing – which provide convenient access points for odorant addition without damaging the wax brood
12 cell caps or the brood itself.

13 We conditioned the Jenter™ comb cages by placing them in a colony for several days, allowing the bees
14 to draw out full-height comb cells. We then caged the queens and allowed them sufficient time to
15 populate the combs with eggs (typically overnight). We released the queens and allowed the workers to
16 rear the brood *in situ*. Once capped, we inspected the brood via the removable plugs to confirm the
17 developmental stage. Through this small posterior window, 5th instar larvae and prepupae are
18 indistinguishable, but pupae are easily recognized by their clearly developed abdomen and hind feet.
19 This is in contrast to the front-way odorant assay, where 5th instar larvae and prepupae are
20 distinguishable due to the clear anterior view of the head.

21 We removed the plugs for 9-10 semi-randomly located brood cells (each group of 9-10 cells in a
22 different colony = 1 biological replicate) and dispensed odorants (1 µl of neat solutions) onto the brood
23 through the back of the comb and re-plugged each cell. The number of brood in these patches is smaller
24 than for the front-way odorant assays because the size of the Jenter™ cage limits the total brood area.
25 We traced a map of the odorant-treated cells and placed the combs in colonies for 20 h to allow workers

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1 to detect the odorant signals through the cap and respond. We performed five biological replicates (i.e.
2 repeated the test in five colonies) for each odorant and developmental stage (pre-pupation and post-
3 pupation). Since the 5th instar larvae and pre-pupae are indistinguishable (as described above), the 'pre-
4 pupation' group contains both stages. After incubation, we removed the comb and counted the number
5 of brood cells from each odorant treatment that were removed and/or partially cannibalized. Removal
6 data was analyzed as described above except we used a two-factor ANOVA (levels: odorant, age). Due to
7 spontaneous re-queening events and subsequent worker turn-over, the hygienic scores are not known
8 for all of the colonies in this experiment.

9 *Gas chromatography mass spectrometry (GC-MS)*

10 We performed GC-MS on extracts from larvae, prepupae and pupae to detect differences in their
11 natural odorant profiles. Here, the three stages are distinguishable because by removing the brood from
12 the cell, we can clearly differentiate the features of a prepupae compared to a 5th instar larva (the
13 elongated body and 'crook-neck' appearance). We collected capped 5th instar larvae, prepupae and
14 pupae from five different colonies and performed solid-phase micro-extraction (SPME) GC-MS as well as
15 cuticle hexane wash GC-MS as previously described[40]. Briefly, for the SPME analysis, we sealed individual
16 brood in 10 mL glass vials (having previously confirmed that this is sufficient air to prevent suffocation)
17 for 24 h prior to GC-MS analysis to allow volatiles to equilibrate in the headspace. We analyzed N = 5
18 brood for each stage, with all 5 coming from different colonies, in a sample order randomized by colony
19 and stage to avoid batch effects. The extracted compounds were analyzed by low-resolution GC-MS
20 (Agilent 7890A/5975C Inert XL MSD) with a DB-wax analytical column (J&W 122–7032) and a 45 min
21 temperature gradient spanning from 50°C to 230°C. For the hexane wash analysis, we soaked individual
22 brood (also N = 5 brood for each developmental stage, from 5 different colonies) in 300 µl of HPLC-grade
23 hexane for 5 min with gentle agitation. We injected 1 µl of each sample on to the analytical column
24 (same as above) connected to a Agilent 6890N/5975C Inert XL MSD mass spectrometer. The
25 temperature gradient spanned the same temperatures but was 30 min long.

26 Spectral data was searched using Mass Hunter Qualitative Analysis software (vB.06.00) and the Wiley
27 Chemical Compound Library (W9N08.L). Since the automatic integration algorithm within Mass Hunter
28 often applies erroneous peak baselines, peak areas were integrated manually. Only peaks with apex
29 intensities exceeding 4,000 cts were integrated, since less intense peaks rarely yielded confident
30 spectral matches to known compounds. Raw data is available for download at
31 http://github.com/AlisonMcAfee/test. Peak areas were log10 transformed and compound profiles were

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1 compared between developmental stages using a one-way ANOVA followed by a Benjamini-Hochberg
2 correction (5% FDR) performed in Perseus (v1.5.5.3). We confirmed the identity of β-ocimene,
3 isopropanol, and 2-pentanone against synthetic standards (Sigma).

4 *Electroantennography (EAG) recordings*

5 We obtained EAG recordings on bees' antennae collected from a single highly hygienic colony (freeze-
6 killed brood score = 95%) maintained at the University of British Columbia. For *ex vivo* EAG analysis, we
7 sampled adult nurses from an open brood frame and kept them in a humid incubator (35°C) with access
8 to sucrose water (1:1) until antennal excision. Immediately prior to EAG testing, either the left or right
9 antenna was removed from individual bees (according to *a priori* randomization) by cutting at the base
10 of the scape. We trimmed the last flagellum segment with dissection scissors, then connected the ends
11 to recording electrodes via glass capillary tubes filled with insect saline solution (210 mM NaCl, 3.1 mM
12 KCl, 10 mM $CaCl_2$, 2.1 mM $NaCO_3$, and 0.1 mM NaH_2PO_4, pH 7.2) as previously described[57]. After data
13 was acquired for the first antenna, the second was collected and the bee was euthanized. In total we
14 acquired data for N = 13 left antennae and N = 14 right antennae.

15 During EAG acquisition, we used a Syntech™ CS-55 stimulus controller to continuously pass humidified
16 air over the antenna and to deliver 1 s pulses of odorized air. To produce odorized air, we cut 1 cm^2 slips
17 of No. 1 Whatman filter paper and inserted them into glass Pasteur pipette cartridges. We heated the
18 cartridges to 37°C using a flexible chromatography column heater, at which time we dispensed onto the
19 filter paper 5 µl of distilled water (blank), β-ocimene, oleic acid, or a 1:1 v/v blend of β-ocimene and
20 oleic acid (mix). After allowing 30 s of initial evaporation and slight cooling for the cartridge to reach
21 approximately 33°C, we aimed away from the antenna and passed a 1 s burst of room-temperature air
22 through the pipette before stimulating the antennae with the odorants. We then exposed the antennae
23 to a set of 3 consecutive 1 s bursts for each odorant in a randomly-determined order. Between 0.5 and 1
24 min was allowed between each presentation to allow antennal electrical activity to return to baseline.
25 Blank stimuli (also 3 consecutive 1 s bursts each) were performed at two randomly determined times
26 during acquisition. For each antenna, we subtracted the average blank intensity from the odorant EAG
27 intensities, then compared odorant groups with a two-way ANOVA (levels: odorant, side).

28 *Ligand affinity assays for odorant binding proteins (OBPs)*

29 Recombinant OBP16 and OBP18 were cloned, expressed, and purified exactly as previously described[35].
30 Briefly, the OBP genes were PCR amplified from honey bee cDNA and cloned into a PET-5b bacterial

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expression vector. Plasmids were transformed into BL21(DE3)Rosetta-gami (OBP16) and BL21(DE3)pLysS *E. coli* strains and protein expression was induced via IPTG. The recombinant proteins were then purified by a series of chromatographic elutions, including anion exchange (DE-52, QFF, or Mono-Q) and gel filtration (Sephacryl-100 or Superose-12) as well as other standard purification protocols[56,58].

We then used an NPN (N-Phenyl-1-naphthylamine) competitive binding assay to measure relative affinities of β-ocimene, oleic acid, phenethyl acetate, and hexane (negative control). Binding assays were also conducted as previously described(31), except they were performed in technical duplicate with 2 µM protein, 2 µM NPN, and between 0 and 8 µM of hexane and phenethyl acetate or between 0 and 6 µM of β-ocimene and oleic acid. Dissociation constants of the ligands were calculated from the corresponding IC_{50} values (concentrations of ligands halving the initial fluorescence value of 1-NPN), using the equation: $K_D = [IC_{50}]/(1 + [1 - NPN]/K_{(1 - NPN)})$ where [1-NPN] is the free concentration of 1-NPN and $K_{(1 - NPN)}$ is the dissociation constant of the complex protein/[1-NPN].

Odorant toxicity assays

To test the toxicity of the odorants, we retrieved 60 purple-eyed, white body pupae and applied 1 µl of neat odorant (phenethyl acetate was not included) to the dorsal abdominal area (n = 15 each). We placed the pupae in tissue-lined petri dishes and incubated them at 33°C for 2.5 d. We then scored the pupae for whether their development was halted (*i.e.* their cuticle did not begin to brown or harden and their eye pigment did not change colour) and whether a prophenoloxidase response had initiated (*i.e.* the dorsal abdominal region became black). All pupae with halted development also had a prophenoloxidase response.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. The raw GC-MS data is available for download at http://www.github.com/AlisonMcAfee/test.

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Author contributions

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1 AM wrote the first draft of the manuscript, designed the experiments and collected behavioral data with
2 the help of AC, HH, and LJF. YGK performed the EAG experiments, with assistance from TFC. II and PP
3 performed the in vitro binding assays. LLM acquired the GC-MS data.

4 **Additional information**

5 The authors declare no competing interests.

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