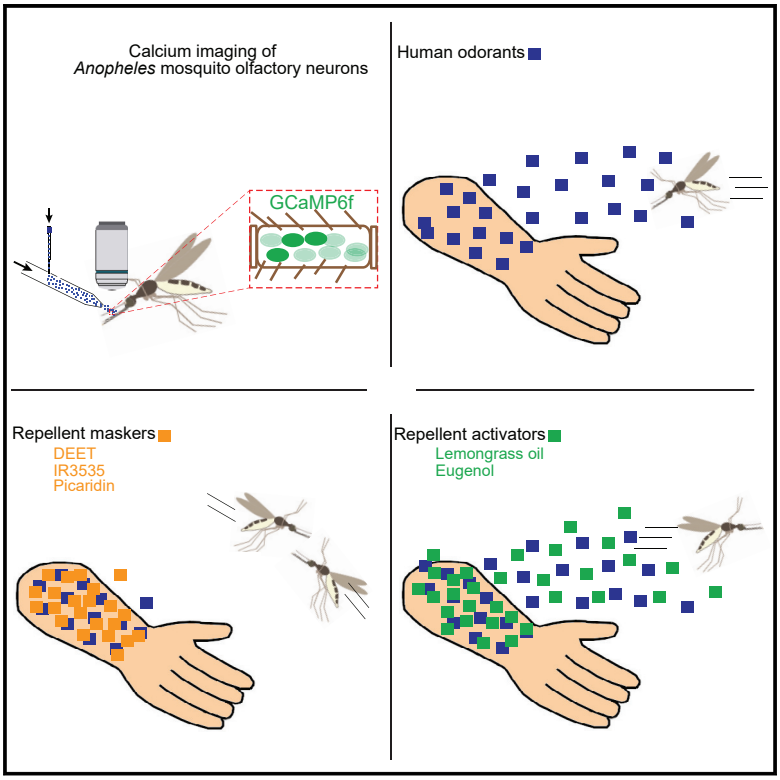
Article

Current Biology

Commonly Used Insect Repellents Hide Human Odors from *Anopheles* Mosquitoes

Graphical Abstract



Authors

Ali Afify, Joshua F. Betz, Olena Riabinina, Chloé Lahondère, Christopher J. Potter

Correspondence

cpotter@jhmi.edu

In Brief

The olfactory mode of action for mosquito repellents is unclear. Afify et al. genetically engineered *Anopheles* mosquitoes to reveal odor-induced activities in olfactory neurons. Natural repellents activated olfactory neurons, and synthetic repellents did not. Synthetic repellents instead likely reduce attraction to humans by decreasing the amount of odorants reaching the mosquito.

Highlights

- Odor responses of *An. coluzzii* ORNs revealed by a genetically encoded calcium sensor
- Natural repellents activate olfactory receptor neurons
- Synthetic repellents likely do not activate *Anopheles* olfactory receptor neurons
- Synthetic repellents reduce the amount of odorants reaching the antenna

Afify et al., 2019, Current Biology *29*, 3669–3680
November 4, 2019
https://doi.org/10.1016/j.cub.2019.09.007

CellPress

Current Biology
Article

Commonly Used Insect Repellents Hide Human Odors from *Anopheles* Mosquitoes

Ali Afify,[1] Joshua F. Betz,[2] Olena Riabinina,[1,4] Chloé Lahondère,[3] and Christopher J. Potter[1,5,*]
[1]The Solomon H. Snyder Department of Neuroscience, The Center for Sensory Biology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA
[2]Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA
[3]Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA
[4]Present address: Department of Biosciences, Durham University, Durham, UK
[5]Lead Contact
*Correspondence: cpotter@jhmi.edu
https://doi.org/10.1016/j.cub.2019.09.007

SUMMARY

The mode of action for most mosquito repellents is unknown. This is primarily due to the difficulty in monitoring how the mosquito olfactory system responds to repellent odors. Here, we used the Q-system of binary expression to enable activity-dependent Ca^{2+} imaging in olfactory neurons of the African malaria mosquito *Anopheles coluzzii*. This system allows neuronal responses to common insect repellents to be directly visualized in living mosquitoes from all olfactory organs, including the antenna. The synthetic repellents N,N-diethyl-meta-toluamide (DEET) and IR3535 did not activate *Anopheles* odorant receptor co-receptor (Orco)-expressing olfactory receptor neurons (ORNs) at any concentration, and picaridin weakly activated ORNs only at high concentrations. In contrast, natural repellents (i.e. lemongrass oil and eugenol) strongly activated small numbers of ORNs in the *Anopheles* mosquito antennae at low concentrations. We determined that DEET, IR3535, and picaridin decrease the response of Orco-expressing ORNs when these repellents are physically mixed with activating human-derived odorants. We present evidence that synthetic repellents may primarily exert their olfactory mode of action by decreasing the amount of volatile odorants reaching ORNs. These results suggest that synthetic repellents disruptively change the chemical profile of host scent signatures on the skin surface, rendering humans invisible to *Anopheles* mosquitoes.

INTRODUCTION

Mosquitoes are vectors for many debilitating diseases, such as malaria, Zika, dengue fever, and yellow fever. Malaria alone caused an estimated 435,000 deaths in 2017 [1]. Mosquitoes primarily depend on olfaction, in combination with other senses, to locate their hosts [2, 3]. Therefore, targeting the mosquito's sense of smell using repellent odorants is an effective strategy to prevent them from biting humans. The synthetic compound N,N-diethyl-meta-toluamide (DEET) is the most widely used mosquito repellent in public use since 1957 [4, 5]. However, DEET has some drawbacks, including high concentrations (~>30%) are required for it to be effective, an unpleasant odor and oily feeling to some people, and the ability to dissolve some plastics and synthetic rubber [4]. Commercially synthetized alternatives to DEET have been developed (IR3535 and picaridin), but these too have similar drawbacks, such as also requiring high concentrations to be effective. In order to improve or identify new repellents, a better understanding of how insect repellents affect a mosquito's olfactory system is needed. However, the olfactory mode of action of synthetic insect repellents, such as DEET, IR3535, and picaridin, as well as natural insect repellents, such as lemongrass oil and eugenol, is surprisingly not well understood.

The olfactory system of the *Anopheles gambiae* species of mosquitoes primarily consists of two organs: the antennae and maxillary palps [2, 6]. The labella is a third chemosensory organ on the head that might detect low volatile odorants [7]. Each of these organs is covered with sensory hairs called sensilla, and each sensillum houses olfactory sensory neurons that may contain one of three types of chemoreceptors: odorant receptors (ORs); gustatory receptors (Grs); and/or ionotropic receptors (IRs). ORs are expressed in the majority of olfactory neurons, and each OR is expressed along with the odorant receptor co-receptor (Orco) to form a receptor complex that is either narrowly or broadly tuned to a variety of host-derived odors [2, 6, 8].

A consensus for how DEET affects the mosquito olfactory system and alters host-seeking behavior has not yet emerged. Currently, there are three hypotheses of how DEET affects mosquitoes: (1) DEET directly activates chemoreceptors (ORs, Grs, and/or IRs) on the mosquito antennae, maxillary palps, or the labella to drive repellent behavior ("smell and avoid") [9–17]; (2) DEET modulates ("scrambles/confuses") OR activity in response to odorants [11, 12, 18–20]; and (3) DEET acts directly on the odorant to decrease its volatility and thereby reduces the amount of attractive odorants capable of activating mosquito olfactory receptors ("masking") [16]. These hypotheses are not necessarily mutually exclusive; DEET may have more than one mode of action.

The mode of action for DEET and other commonly used insect repellents toward *An. gambiae* mosquitoes, which kill more

people worldwide than all other mosquito species combined [1], is the most poorly understood. From studies in *Culex* [17] and *Aedes* [13], the olfactory functions of DEET have been reported to work directly through an Orco/OR pathway. However, *Culicinae* (e.g., *Culex* and *Aedes* mosquitoes) and *Anophelinae* (*Anopheles* mosquitoes) diverged about 190 mya [21] (for context, mice and humans diverged about 75 mya) [22]. So although *Culicinae* and *Anophelinae* are grouped together as mosquitoes, their divergence suggests their olfactory systems might respond differently to repellent odors. As such, although work in *Culicinae* mosquitoes offers a useful guide, it remains important to examine repellent responses directly in *Anophelinae* mosquitoes.

A lack of understanding for DEET's mode of action is primarily due to the lack of available methods for testing the simultaneous responses of individual olfactory neurons toward DEET or other repellents. Traditionally, insect repellents must be used to individually stimulate each of the ~750 sensilla using single sensillum recording (a high technical hurdle) or tested against each individual OR ectopically expressed in *Xenopus* oocytes or in the *Drosophila* empty neuron system [17, 23]. To address this technical challenge and examine endogenous responses to insect repellents, we generated transgenic *Anopheles coluzzii* (formerly *Anopheles gambiae* M form) [24] mosquitoes in which the calcium indicator GCaMP6f [25] was expressed in all Orco-expressing neurons (genotype: *Orco-QF2* and *QUAS-GCaMP6f*). We used these mosquitoes to directly visualize odor responses in olfactory neurons in the mosquito antenna, which to our knowledge is the first time this has been accomplished in any insect besides the vinegar fly *Drosophila melanogaster*. This allowed us to re-visit the three leading hypotheses of how DEET and other commonly used insect repellents may affect the *An. coluzzii* olfactory system. We found that the natural repellents eugenol and lemongrass oil strongly activate a subset of olfactory receptor neurons, and DEET, IR3535, and picaridin do not directly activate olfactory neurons. These three synthetic repellents instead function as "maskers," a term we use here to describe odors that decrease odor-evoked responses of olfactory neurons. Our data further support the hypothesis that the masking effect of DEET, IR3535, and picaridin in *Anopheles* mosquitoes is not due to direct inactivation of odorant receptors but instead results from chemical interactions that decrease the amount of activating odorant reaching olfactory receptor targets on the mosquito antennae.

RESULTS

To examine olfactory responses in all olfactory organs of *An. coluzzii*, we utilized the Q-system of binary expression by generating a mosquito line that contained a *QUAS-GCaMP6f* transgene and crossing this to the validated *Orco-QF2* driver line [26]. The combination of these transgenes directed the expression of the calcium indicator GCaMP6f to all Orco-expressing olfactory neurons. To validate this mosquito model for monitoring odorant-induced olfactory neuron activity, we directly visualized the antennal response to 1-s pulses of six human skin odorants previously shown to activate *An. gambiae* ORs in heterologous expression screens [23] (Figure S1A). All OR ligands (1-octen-3-ol, 2-acetylthiophene, benzaldehyde, *p*-cresol, 1-hepten-3-ol, and indole) at 1% concentrations elicited olfactory response across the entire antenna (Figure S1). This enabled a rapid method for linking odors to their induced olfactory responses throughout the *An. coluzzii* olfactory system with single-cell resolution. To achieve higher resolution for analysis, we focused on one antennal segment (11th segment) as a representative for antennal neural responses (Figure 1A; STAR Methods). Fine glass pipette tips were used to flatten down the antenna at basal (segment 1 and 2) and distal segments (12 and 13). Segment 11 was chosen for imaging, as it is the most stable distal segment not touched during the preparation. We found that each of the six odorants activated distinct olfactory receptor neurons (ORNs) at the 11th antennal segment (Figures 1B–1E). Together, our results indicated that calcium imaging of olfactory neurons provides a rapid method to interrogate olfactory responses directly in the peripheral olfactory organs of *An. coluzzii* mosquitoes.

Activator and Non-activator Repellents

The ability to monitor all olfactory receptor neuron responses across olfactory tissues enabled us to investigate how common insect repellents might affect *An. coluzzii* Orco-expressing olfactory neurons. We tested two natural repellents (lemongrass oil and eugenol) at 1% concentrations and three synthetic repellents (DEET, IR3535, and picaridin) at 10% concentrations. We initially tested all odorants at the whole antenna (Figure S2). Natural repellents lemongrass oil and eugenol elicited strong olfactory responses, and the three synthetic repellents DEET, IR3535, and picaridin did not elicit any olfactory responses across the entire antenna (Figure S2A). For more robust analyses of the responses, we tested all five repellents again with higher resolution imaging at the 11th antennal segment. Lemongrass oil and eugenol at a concentration of 1% strongly activated a subset of ORNs (Figure 2A), and 10% DEET, IR3535, and picaridin did not activate any ORNs at the 11th segment (Figure S2B).

The solvent used for odor mixtures could affect the emission rates of odorants. To rule out that the lack of response toward the three synthetic repellents was due to the use of paraffin oil as the solvent, we tested the activity of the three repellents (at 30%) dissolved in ethanol (a more volatile solvent). 1-octen-3-ol dissolved in ethanol (1%) elicited a weak response (data not shown). The three repellents also elicited weak antennal olfactory neuron responses similar to the antennal neuron responses elicited by ethanol alone (data not shown).

We next asked whether higher concentrations of DEET, IR3535, and picaridin would elicit olfactory response in any of the olfactory organs (the antennae, maxillary palps, or labella). There were no olfactory responses to DEET or IR3535 at 100% concentrations across the entire olfactory organs (Figures 2B and 2C). Picaridin at 30% (data not shown) and 100% concentrations elicited a weak response at the antennae, maxillary palps, and proboscis (Figures 2B and 2C). We further tested whether DEET, IR3535, or picaridin would activate olfactory neurons from a close distance. We decreased the distance between the stimulant Pasteur pipette and the mosquito antenna from 20 cm to 0.5 cm (Figure S3A). At this close range, picaridin at 100% elicited a response in the antenna olfactory neurons that was weaker than the response to 1% 1-octen-3-ol (Figures S3B and S3C). Also, at this close range, a similarly weak

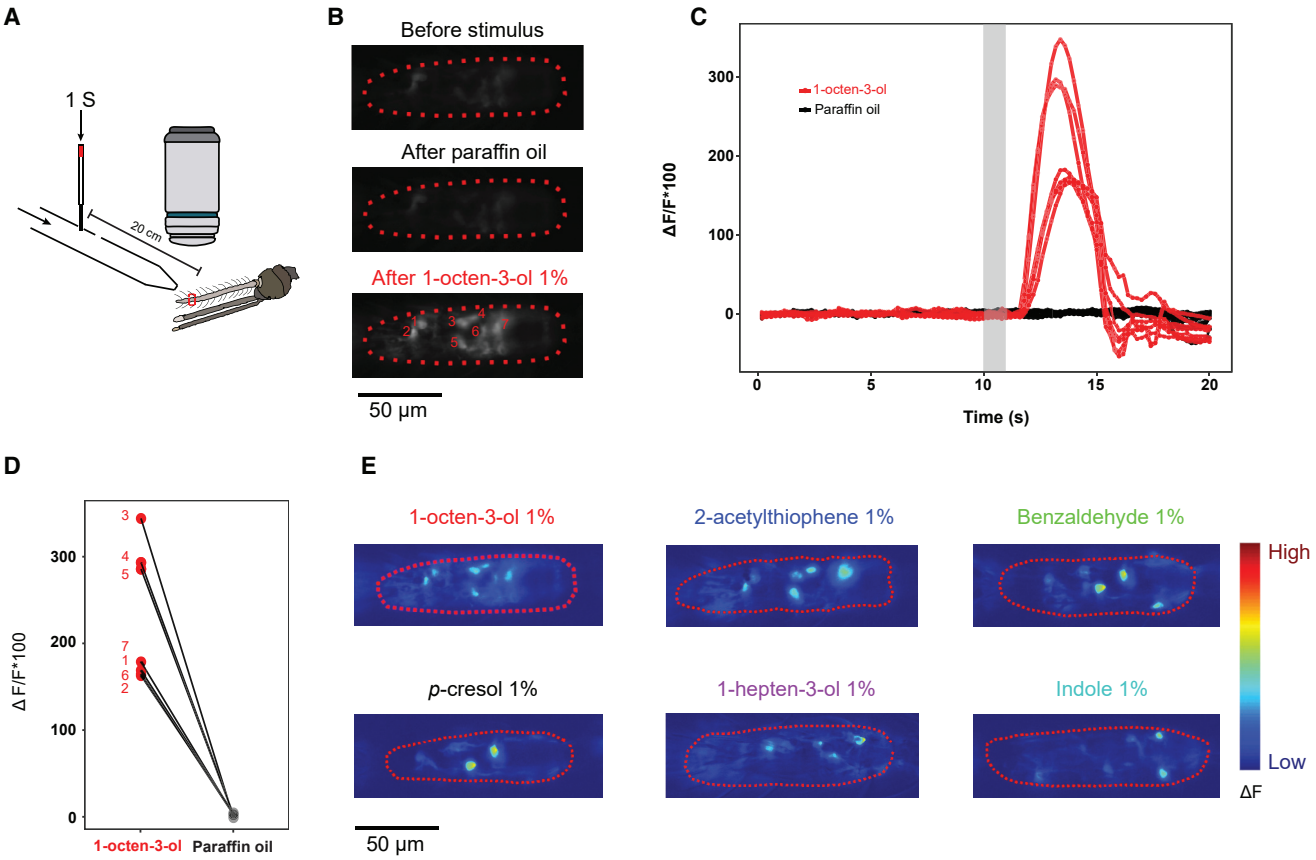


Figure 1. Visualizing Odor-Dependent Activation of *An. coluzzii* Antennal Olfactory Neurons

(A) Schematic of the calcium imaging setup. The distance between the antenna and the Pasteur pipette is 20 cm. A 50× microscope objective images the 11th antennal segment (dashed red rectangle). Arrows indicate the direction of air flow (continuous air and 1-s air pulse).

(B) Video frames from calcium imaging recordings. Dashed red lines indicate the border of the 11th antennal segment. Numbers identify neurons responding to 1-octen-3-ol at 1%.

(C) Traces from the calcium imaging recordings in (B).

(D) ΔF/F*100 values for the neuron responses from the recordings in (B).

(E) Example heatmaps of the responses toward OR ligands at 1%. Dashed red lines indicate the borders of the 11th antennal segment. The heatmap represents arbitrary units. Responses for the full antennae are shown in Figure S1.

response was visible both by DEET at 100% and by water (Figures S3B and S3C). IR3535 did not elicit responses to the antenna olfactory receptor neurons (Figures S3B and S3C).

The current calcium imaging method only allows visualization of odor-induced activity for Orco+ olfactory neurons and thus would not be able to detect whether the 3 synthetic repellents activated non-Orco+ neurons, such as ionotropic receptor neurons [26, 27]. To address this, we performed electroantennography experiments (EAGs) to monitor global response of the antennae to stimuli. First, we asked whether EAGs could detect non-Orco olfactory neuron activities not visualizable by the Orco-dependent calcium imaging experiments. To do this, we performed calcium imaging (Figures S4A–S4C) and EAG experiments (Figures S4D–S4F) using acid odors known to elicit olfactory ionotropic receptor responses in *Aedes* mosquitoes [28]. Calcium imaging in Orco neurons showed strong antennal responses to butyric acid only. Heptanoic acid and hexanoic acid elicited weak/medium responses, and lactic acid, nonanoic acid, and octanoic acid elicited very weak responses similar to the paraffin oil elicited response (Figures S4B and S4C). On the other hand, acids elicited stronger responses in EAG experiments. More specifically, butyric acid and hexanoic acid elicited strong antennal responses, similar to responses obtained with 1-octen-3-ol, and nonanoic acid elicited a medium response that is significantly stronger than paraffin oil (Figures S4E and S4F). We then tested the three synthetic repellents in EAG experiments (Figure 3). DEET and IR3535 (30% and 100%) elicited weak responses that were not significantly different than paraffin oil. However, consistent to our calcium imaging results, picaridin elicited stronger responses than paraffin oil (Figures 3B and 3C) but were significantly weaker than the response to 1-octen-3-ol (Figures 3B and 3C).

Synthetic Repellents Mask Odorant-Induced Responses

Insect repellents are typically applied directly to human skin and result in a mixture of repellent and human odorants. In this context, DEET might function by altering the olfactory responses to host odorants. Indeed, DEET has been reported

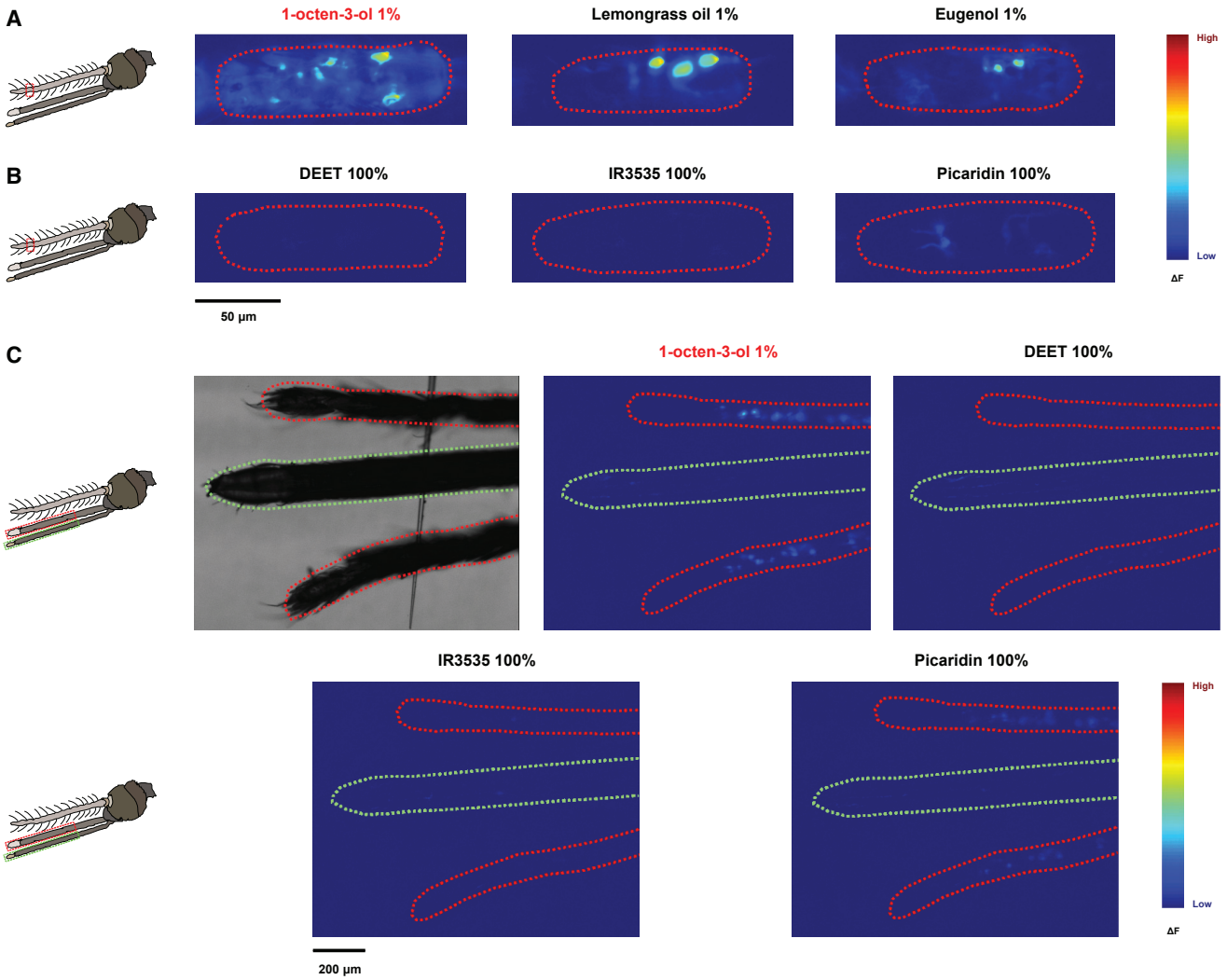


Figure 2. Natural Repellents, but Not Synthetic Repellents, Strongly Activate *Anopheles* Olfactory Neurons

(A) Example heatmaps showing responses at the 11th antennal segment (dashed red line) toward 1% natural repellents lemongrass oil and eugenol. Responses toward 1-octen-3-ol serve as a control stimulus. The heatmap represents a.u. Responses for the full antennae are shown in Figure S2.

(B) Example heatmaps showing responses at the 11th antennal segment (dashed red line) toward 100% synthetic repellents DEET, IR3535, and picaridin (n = 5 animals).

(C) A still image and example heatmaps of the maxillary palps (dashed red line) and proboscis (dashed green line) showing responses toward 1% 1-octen-3-ol, 100% DEET, IR3535, and picaridin (n = 5 animals).

See also Figures S2 and S3.

to modulate antennal responses toward other odorants in single sensillum recording experiments in *Drosophila*, *Aedes*, and *Culex* [18–20]. In addition, *A. aegypti* olfactory receptors expressed in *Xenopus* oocytes showed an inhibited response toward odorant ligands when mixed with DEET, IR3535, or picaridin [11, 12]. We therefore asked whether mixing these three repellents individually with known mosquito OR ligands would alter the *An. coluzzii* ORN responses. Using electroantennography, we found that the mixtures of odorant 1-octen-3-ol with each of the 3 synthetic repellents led to a significant decrease in the EAG responses (Figures 3B and 3C). Similarly, calcium imaging showed that mixing DEET, IR3535, or picaridin with OR ligands decreased or "masked" the olfactory neuronal response (Figures 4, S5A, S5C, and S5D). In these experiments, each mosquito antenna was tested sequentially with several odorants (OR ligands alone and mixtures of OR ligands with repellents). These repeated measurements might be correlated within the same animal, which violates two assumptions common to many statistical models: independence and constant variance of outcomes. In addition, there could be an order effect whereby early measurements might affect subsequent measurements. Therefore, we randomized the order of odorants tested and paired each OR ligand with its respective mixture; e.g., OR ligand X was always paired with (precedes or follows) the mixture of OR ligand X + repellent. In order to account for potential correlation due to repeated measurements and non-constant residual variation, linear mixed effects regression models were used to model olfactory responses.

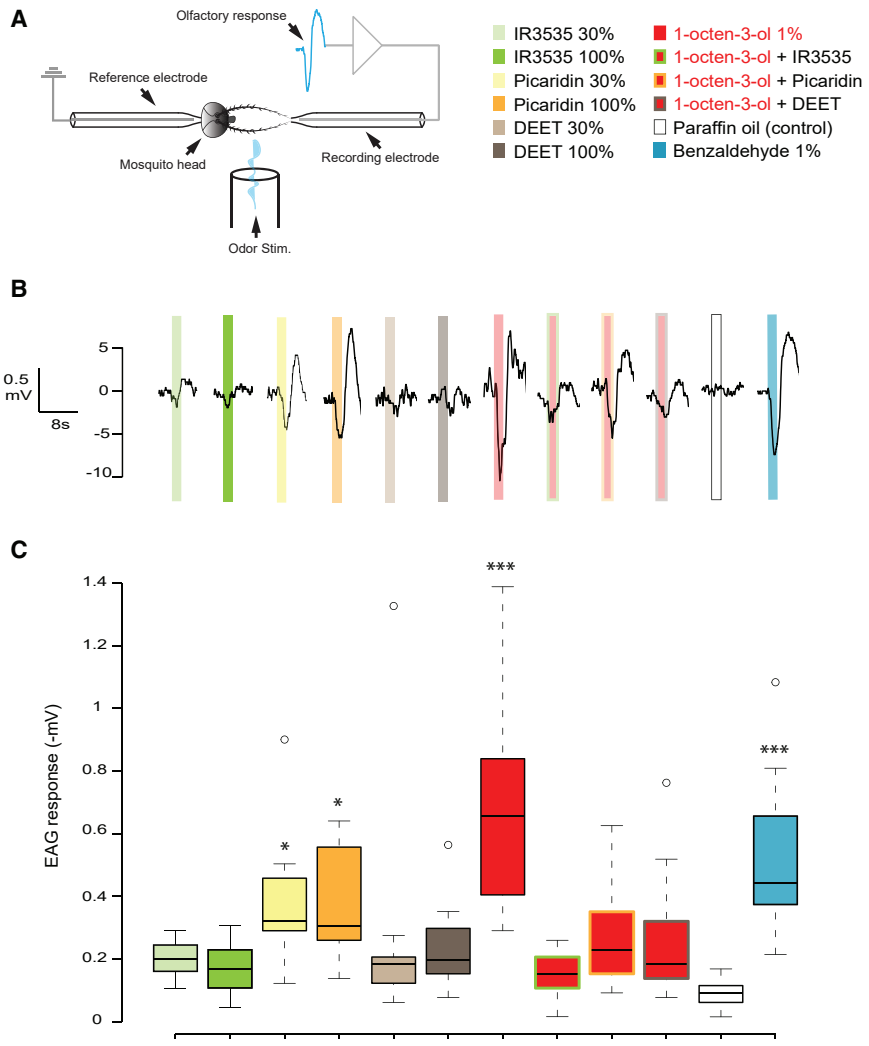


Figure 3. Whole Antennal Response to Repellents

(A) Schematic of the electroantennogram (EAG) setup. The head is mounted between two electrodes and both antennae inserted into the recording electrode. An odorant plume is added to the continuous clean air stimulation. The proboscis and palps are not represented for clarity.

(B) Representative EAG traces for the tested odorants. The colored bar represents the pulse. Note the typical EAG shape of the signal (deflection first) as well as the absence of response to the control.

(C) Boxplots of the EAG responses to repellents at different concentrations and in combination with 1-octen-3-ol. The bar inside the box represents the median, and the upper and lower parts of the box represent the 25th and 75th percentiles of the data. Circles represent outliers. n = 11 females. Asterisks indicate responses that were significantly different than the paraffin oil response (pairwise Wilcoxon rank sum test with a Bonferroni correction), picaridin 30% ($p = 0.01$), picaridin 100% ($p = 0.009$), 1-octen-3-ol, and benzaldehyde at 1% ($p < 0.001$).

See also Figure S4.

We found that the masking effect is concentration dependent, where 10% of each repellent showed a significantly stronger masking effect than 1% (Figures 4B–4D; statistics shown in Figure S5C). Additionally, DEET at 30% masked the response to OR ligands significantly more than 10% (Figures 4B and S5C). However, there were no differences between the effects of 30% and 10% for both IR3535 and picaridin (Figures 4C, 4D, and S5C). In addition, there were no differences between the effects of the three repellents when used at the same concentration, except at 10% of DEET and IR3535; DEET showed a significantly weaker masking effect than IR3535 at 10% (Figure S5D). Together, these data indicate that synthetic repellents mask the olfactory responses of OR ligands in a dose-dependent manner.

We also asked whether a potentially more effective repellent could be produced by mixing activator and masker repellents. We found the ability of activator repellents to stimulate olfactory neurons could also be suppressed by masker repellents; mixing eugenol with DEET, IR3535, or picaridin strongly decreased the eugenol-alone olfactory response. However, the response to the complex odorant mixture of lemongrass oil was only partially decreased (Figure S5B). If olfactory neuron activities could be linked to repellent behaviors, potentially more effective repellent odor mixtures could be identified by calcium imaging of olfactory neuron responses.

Olfactory Masking Requires Chemical Interactions

We sought to understand the mechanism by which repellent masking might occur in *An. coluzzii*. We hypothesized it might occur by one of two potentially overlapping mechanisms. First, olfactory masking could occur at the odorant receptor level, whereby the repellent binds to an odorant receptor complex and prevents its activation by other odorants [11, 12, 18–20]. Second, olfactory masking might occur at the chemical level by which the repellent reduces the volatility of an odor, resulting in decreased neuronal responses [16]. To determine whether masking occurs at the odorant receptor level, we modified how the repellents and OR ligands were delivered to the mosquito antenna in our calcium imaging system. Instead of delivering a 1-s pulse of either the OR ligands or the repellent and OR ligands mixture, we first delivered a 3-s pulse of the repellent. This allowed the repellent to arrive at the antenna before the OR ligands and potentially inhibit olfactory receptor complexes. During the last second of repellent odor delivery, we separately delivered a 1-s pulse of 1-octen-3-ol into the repellent odor stream (pre-stimulation with repellents; Figure 5A). If masking occurs at the odorant receptor level, we predicted the repellent would bind to the odorant receptor and inhibit its response toward the delayed OR ligand stimulus. This was not observed. Instead, we found no difference between the olfactory response to 1-octen-3-ol when delivered after a pre-stimulation with each of the three masker repellents and the response when delivered

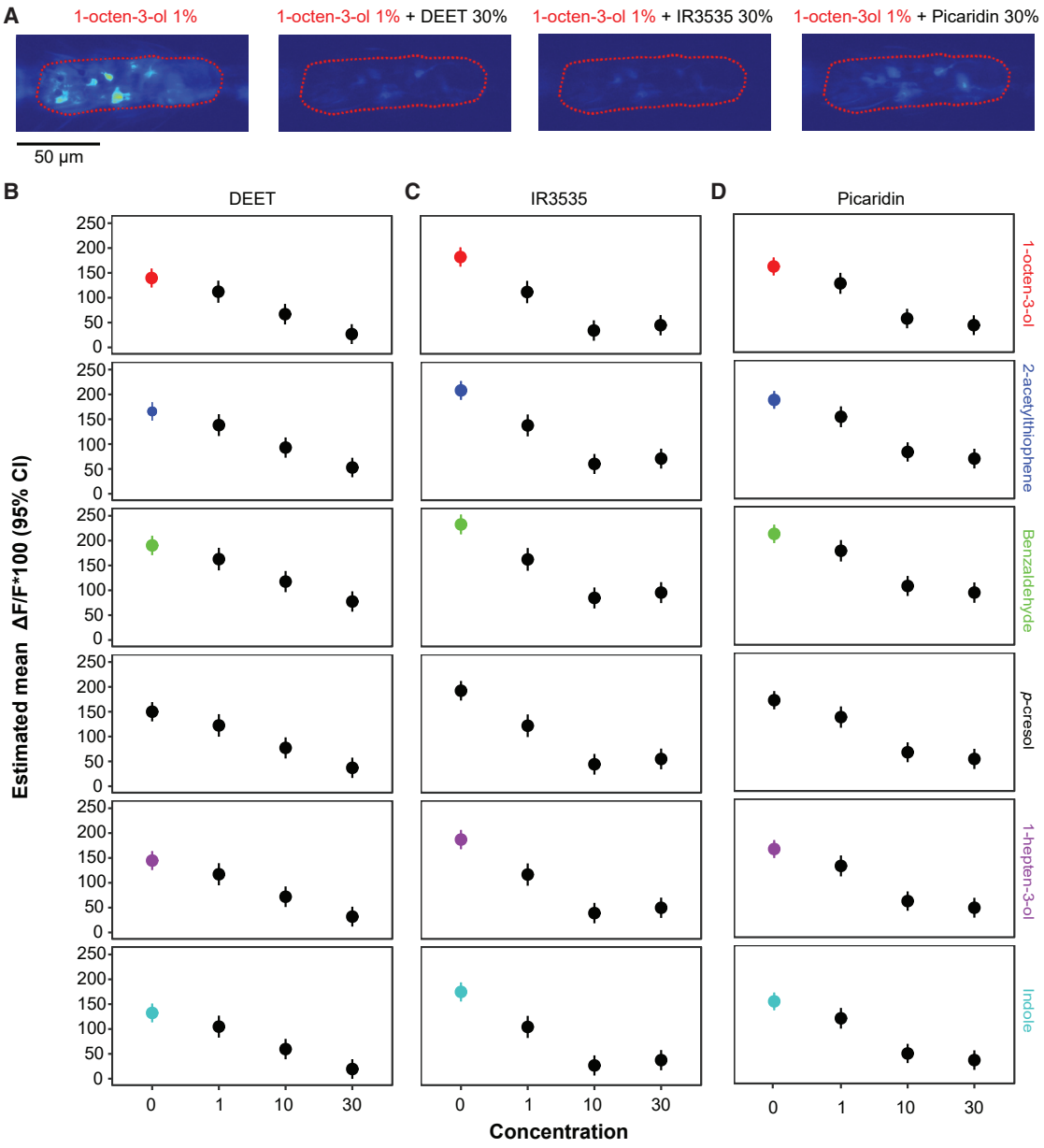


Figure 4. DEET, IR3535, and Picaridin Mask Olfactory Responses toward OR Ligands

(A) Example heatmaps of the responses toward 1% 1-octen-3-ol and its mixtures with 30% DEET, 30% IR3535, and 30% picaridin.

(B–D) Estimated responses (means and 95% confidence intervals [CIs]) from linear mixed effect model (LME) toward mixtures of the six OR ligands at 1% with repellents (B) DEET, (C) IR3535, and (D) picaridin at 0% (OR ligand alone), 1%, 10%, and 30% (n = 15–17 animals for each condition of 0% repellent; n = 5–7 animals for all other conditions; 1–7 responding olfactory neurons/animal).

All raw data are reported in Figure S5A.

after the control odor paraffin oil (Figures 5A, S6A, and S6B). All olfactory responses remained higher than the response to the 1-octen-3-ol mixed with the repellent (Figures 5A, S6A, and S6B). This suggests that olfactory masking in *An. coluzzii* does not occur at the receptor level but more likely at a chemical level.

We next asked whether repellent masking occurs only to odorants mixed with repellents in the liquid phase (as when on human skin) or might also occur during mixing as volatiles. To answer this question, we delivered the two odorants separately and simultaneously through a Y-tube to allow their molecules to mix in the headspace inside a long pipette directed at the antenna (simultaneous odorant delivery; Figure 5B). In this setup, there was no difference between the responses to 1-octen-3-ol when delivered separately from the repellent and when 1-octen-3-ol was delivered with the control odor paraffin oil; the position of the stimulus pipette relative to the repellent pipette likewise had no effect on altering odorant responses (Figures 5B, S6C, and S6D). The olfactory responses were significantly higher

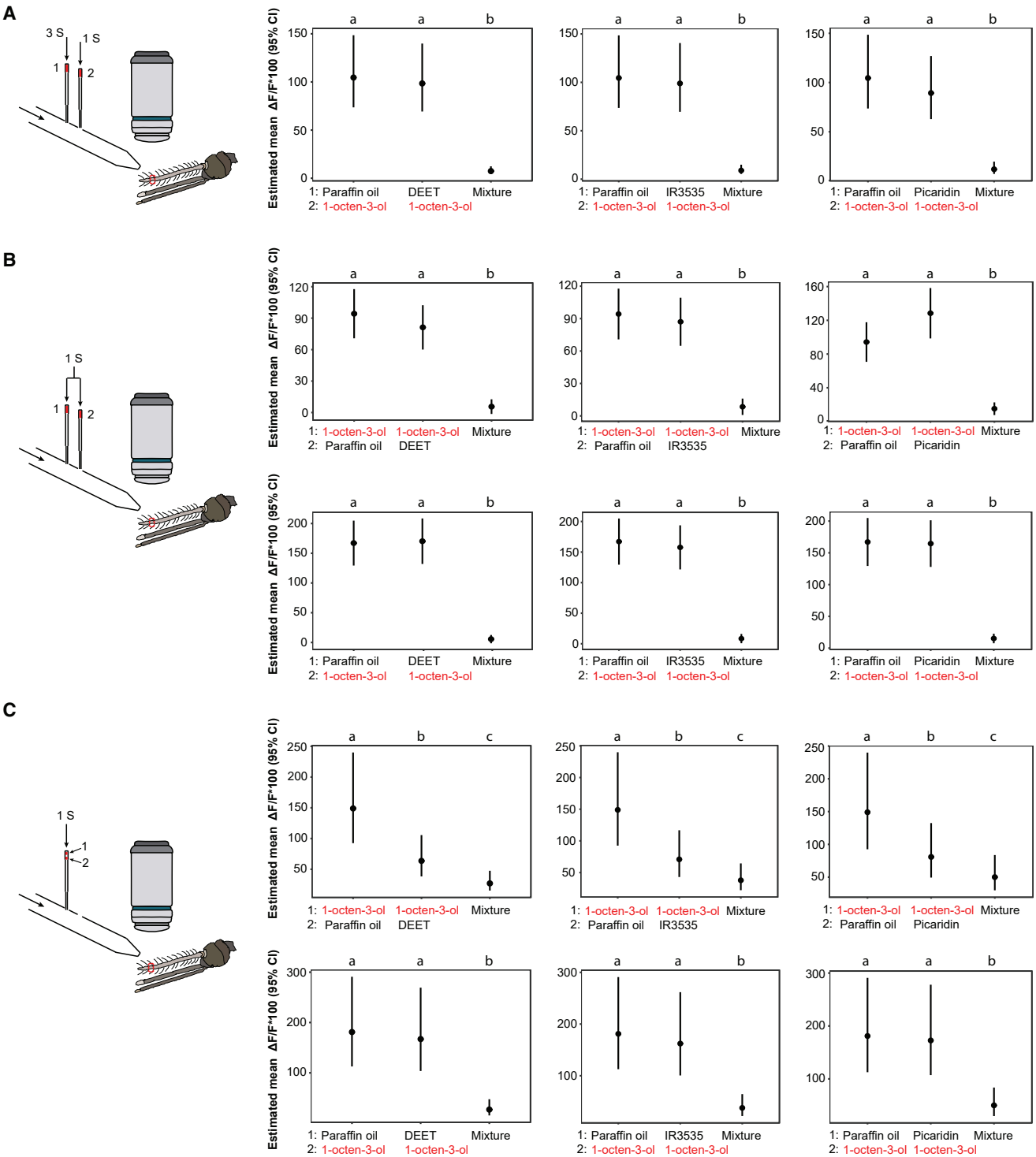


Figure 5. Repellent Olfactory Masking Requires Chemical Interactions with OR Ligands

(A) Estimated responses (means and 95% CIs) from LME toward a 1-s pulse of 1% 1-octen-3-ol occurring during the last second of a 3-s pulse of paraffin oil, 30% DEET, 30% IR3535, or 30% picaridin, compared to the response toward physical mixtures of 1% 1-octen-3-ol with 30% DEET, 30% IR3535, or 30% picaridin. The numbers next to odorant names indicate the position of the odorants in the Pasteur pipette(s) as shown in the schematic.

(B) Estimated responses (means and 95% CIs) from LME toward a 1-s pulse of 1% of 1-octen-3-ol in the first position or the second position simultaneously delivered with a 1-s pulse of paraffin oil, 30% DEET, 30% IR3535, or 30% picaridin, compared to the response toward physical mixtures of 1% 1-octen-3-ol with 30% DEET, 30% IR3535, or 30% picaridin.

(legend continued on next page)

than the response to 1-octen-3-ol when it was physically mixed with a repellent (Figures 5B, S6C, and S6D). To confirm that physical mixing is required for masking, we applied 1-octen-3-ol and a repellent on two separate filter papers inside the same Pasteur pipette (same pipette delivery; Figure 5C). In this setup, the odorants from the upper filter paper would pass by the lower filter paper as they travel toward the antennae. We found no repellent masking effect when the repellent was on the upper filter paper, but the response to 1-octen-3-ol was significantly reduced when DEET, IR3535, or picaridin were applied to the lower filter paper (Figures 5C, S6E, and S6F). This second setup mimics situations in which a masker repellent is applied to clothing, which may allow the activating OR ligand to mix with the repellent on their way toward the mosquito antenna. Nonetheless, the olfactory response in the non-mixed condition remained significantly higher than the response to 1-octen-3-ol when it was physically mixed with DEET, IR3535, or picaridin (Figures 5C, S6E, and S6F). Altogether, these data suggest that masking occurs most effectively when the OR ligand and synthetic repellent are physically mixed but can also occur to lesser degrees when such ligands travel over a repellent solution that might trap these molecules.

Masker Repellents Reduce the Concentrations of Odorants Reaching the Antenna

The calcium imaging experiments indicate that masker repellents reduce neuronal responses to the panel of OR ligands we have tested. We hypothesized this neuronal effect occurs due to a reduction in the volatility of the odorants we tested, which results in fewer ligand molecules reaching the antennae capable of activating olfactory neurons [16]. To test this hypothesis, we initially used a gas chromatography-mass spectrometry (GC-MS) method to measure the amount of odorants released from the stimulus Pasteur pipettes. However, after the initial use of a DEET sample, we detected DEET in all subsequent samples, including samples that should not contain DEET (e.g., 1-octen-3-ol by itself; data not shown). This suggested DEET contaminated the GC-MS system. Therefore, we stopped using GC-MS and instead used a photoionization detector (PID) to measure the concentrations of odorants that reached the antenna during the different imaging experiments (Figures 6A–6G). The PID measures the total concentration of odorant molecules in air but does not identify these odorants. We found that DEET and IR3535 were likely not detectable by the 10.6 eV PID (Figures 6A and 6B). The mixtures of 1-octen-3-ol with 30% DEET or 30% IR3535 showed significantly lower concentrations of odorant molecules than 1-octen-3-ol alone (Figures 6A and 6B). This supported the hypothesis that physically mixing the OR ligand with DEET or IR3535 resulted in a lower concentration of that test odorant reaching the antenna. On the other hand, picaridin was strongly detected by the PID, and when 1-octen-3-ol was mixed with picaridin, the mixture showed a concentration that was higher than 1-octen-3-ol alone (Figure 6C), but not significantly different than picaridin alone (Figure 6C). Nonetheless, the concentration detected from the picaridin/1-octen-3-ol mixture was lower than the expected sum of the mean concentrations of the two individual odorants (Figure 6C), suggesting that picaridin was likely decreasing the levels of volatile 1-octen-3-ol reaching the PID. As a control, we tested 1-octen-3-ol mixed with an activator repellent (lemongrass oil) and found the lemongrass oil/1-octen-3-ol mixture showed odorant concentrations equal to the expected sum of the individual components (Figure 6D).

Finally, we used the PID to determine whether decreased volatility might also underlie the results obtained under the three modified odorant delivery methods (Figures 6E–6G). We found the concentration of 1-octen-3-ol was unchanged when delivered after a pre-stimulation with DEET or paraffin oil (Figure 6E). The concentration of 1-octen-3-ol similarly did not change when delivered simultaneously (but not mixed) with DEET (Figure 6F). The concentration of 1-octen-3-ol significantly decreased when applied on the upper filter paper in the same Pasteur pipette with DEET on the lower filter paper (Figure 6G). These PID experiments support our hypothesis that the masking effect observed during calcium imaging experiments was due to a lower concentration of the OR ligand we screened reaching the antenna when the OR ligand was physically mixed with or trapped by a masker repellent. The differential effects of the three masker repellents on olfactory responses likely reflects their chemical differences in altering OR ligand volatilities.

The chemical nature by which DEET (and the other synthetic repellents) chemically masks odors requires future investigation. Nonetheless, the low volatility of DEET (vapor pressure 0.0017 mmHg at 25°C) suggests it may contribute to this mechanism, as mixtures with a low volatile odorant can reduce the overall volatility of the mixture (Raoult's Law). To test this, we used three compounds with low vapor pressures similar to DEET (nerolidol, 0.001 mmHg at 25°C; α-humulene, 0.008 mmHg at 25°C; and farnesene, 0.01 mmHg at 25°C; http://thegoodscentscompany.com) in mixtures with 1-octen-3-ol (vapor pressure 0.531 mmHg at 25°C; http://thegoodscentscompany.com). The three compounds (at 30%) masked the response to 1-octen-3-ol to differing levels (data not shown). Interestingly, farnesene by itself elicited strong neuronal responses in some antennal neurons and yet acted as a masker for 1-octen-3-ol responsive neurons (data not shown). This suggests that low volatile odorants can elicit antennal neuronal responses detectable by calcium imaging. In addition, these results suggest that low vapor pressure chemicals can generally mask odors and can be considered candidates for new masker repellents.

We hypothesized that the primary olfactory function of DEET was to mask attractant odors without direct activation of olfactory neurons. This suggested that DEET would not act directly

(C) Estimated responses (means and 95% CIs) from LME toward a 1-s pulse of 1% 1-octen-3-ol when applied on the upper filter paper or the lower filter paper with paraffin oil, 30% DEET, 30% IR3535, or 30% picaridin in the same Pasteur pipette, compared to the response toward physical mixtures of 1% 1-octen-3-ol with 30% DEET, 30% IR3535, or 30% picaridin.

For (A)–(C), n = 5 animals for each condition (1–6 responding neurons/animal); conditions denoted with the same letter were not significantly different ($p > 0.05$; LME model with Wald approximation). Pairwise comparisons between subsequent concentrations are shown in Figures S6B, S6D, and S6F. Corresponding raw data for (A)–(C) are reported in Figures S6A, S6C, and S6E.

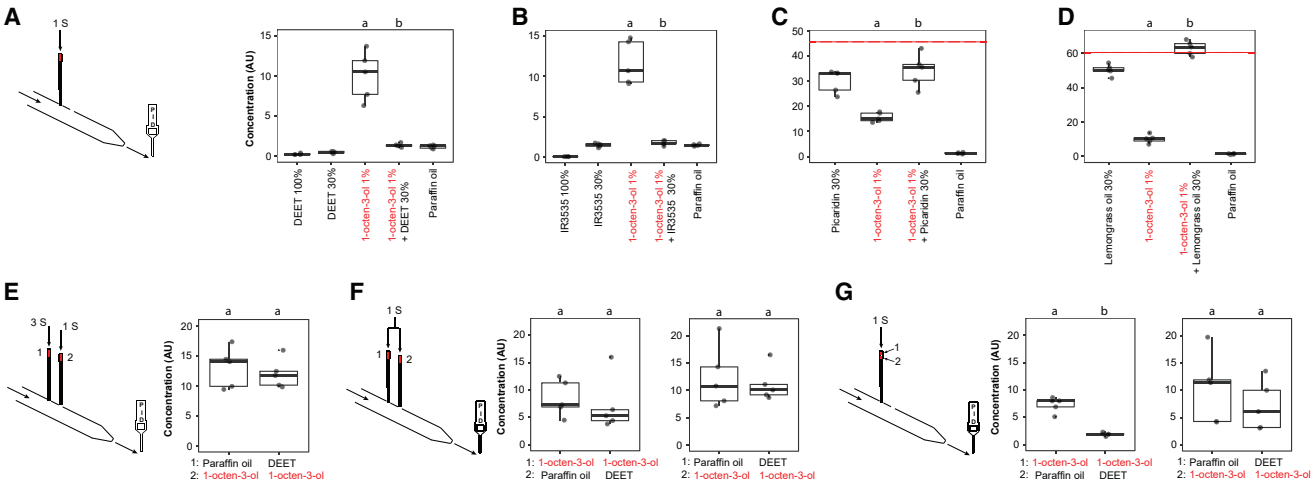


Figure 6. Repellent Maskers Reduce the Volatility of Odorants

(A–D) Total concentrations (tested by the PID) of odorants released from Pasteur pipettes containing single odorants or their mixtures with the repellents (A) DEET, (B) IR3535, (C) picaridin, or (D) lemongrass oil (n = 5 Pasteur pipettes for each odorant). Boxplots represent the median and 25th–75th percentiles. Dashed red line in (C) indicates the calculated sum of the mean concentrations released from the 1-octen-3-ol and picaridin pipettes. Dashed red line in (D) indicates the calculated sum of the mean concentrations released from the 1-octen-3-ol and lemongrass oil pipettes. The 10.6-eV PID did not detect DEET or IR3535.

(E) Total concentrations released from the 1% 1-octen-3-ol pipette following a 3-s pulse of 30% DEET or paraffin oil (n = 5 Pasteur pipettes for each odorant).

(F) Total concentrations released from the 1% 1-octen-3-ol pipette in the first position or the second position when a 1-s pulse of 30% DEET or paraffin oil were used simultaneously (n = 5 Pasteur pipettes for each odorant).

(G) Total concentrations released from 1% 1-octen-3-ol applied on the upper filter paper or the lower filter paper, and 30% DEET or paraffin oil are applied in the same pipette (n = 5 Pasteur pipettes for each odorant pair). The PID was calibrated to a reference gas (ethyl acetate). Concentrations are PID measurements reported here as a.u. Concentrations denoted with different letters were significantly different (Welsh two-sample t test; $p < 0.05$).

as a spatial olfactory repellent. To experimentally address this, we performed a close proximity response assay in which a female mosquito resting on a cage mesh wall was slowly approached by a pipette tip containing a piece of filter paper soaked with an odorant (Figure 7A). The distance between the mosquito and the filter paper was approximately 0.5 cm (Figure 7A). The mosquito was observed for 30 s, and the time it flew away was scored. When using paraffin oil as the odorant, 5 mosquitoes flew away (out of 30 mosquitoes) within the 30-s window (Figure 7B). When lemongrass oil (100%) was used as the odor, all 30 mosquitoes flew away within 30 s, and the duration on the net was 26-fold shorter than paraffin oil (Figure 7B). When DEET at 100% was used as the odor, only 6 mosquitoes flew away (out of 30 mosquitoes) within the 30-s window (Figure 7B). The duration mosquitoes took to fly away after encountering DEET was not significantly different than when encountering paraffin oil. Together, these experiments suggest that DEET does not act as a short-range olfactory repellent to *Anopheles* mosquitoes.

Our calcium imaging and behavioral experiments support two modes of action for olfactory repellents in *An. coluzzii* (Figures 7C and 7D): (1) natural repellents, such as eugenol and lemongrass oil, activate subsets of Orco/OR-expressing olfactory neurons to guide mosquito repulsion (Figure 7C), and (2) synthetic repellents do not activate Orco/ORs directly but instead chemically interact with OR ligands to prevent them from reaching the mosquito antenna (Figure 7D). Chemical masking by synthetic repellents may therefore act directly on the skin surface to dramatically alter the chemical profile of human volatiles released into the environment, potently disrupting mosquito olfactory attraction.

DISCUSSION

By monitoring olfactory receptor neuron responses to odors, we present evidence that adult *An. coluzzii* Orco-expressing olfactory neurons do not directly respond to three of the most commonly used synthetic repellents (DEET, IR3535, and picaridin). These findings differ from studies exploring DEET perception in *Culex* and *Aedes* mosquito species. *Culex quinquefasciatus* mosquitoes encode an odorant receptor (*CqOR136*) activated by DEET, IR3535, and picaridin when expressed with *CqOrco* in *Xenopus* oocytes [16, 17]. Although a DEET receptor remains to be identified in *Aedes aegypti* mosquitoes, *orco* mutant behavioral studies suggest that Orco-expressing olfactory neurons are likely necessary for DEET-based responses in the presence of human odor [13]. Interestingly, *An. coluzzii* larvae behaviorally respond to DEET in water [29]; however, DEET detection in this context might be mediated by a larval-specific OR or via non-olfactory neurons.

Calcium imaging is a powerful approach to simultaneously visualize the odor-induced activity of many olfactory neurons, but it does have technical limitations. For example, calcium imaging studies may not be able to detect olfactory neurons only weakly activated by DEET or other repellents; however, in the current study, even 100% DEET (a concentration 3-fold higher than commonly effective) failed to activate olfactory neurons. DEET elicited weak neural activation in antennal ORs when used at a close distance (0.5 cm). However, water elicited a similar response at a close distance, suggesting that this atypical stimulation might have a non-olfactory effect. In addition, in our current work, GCaMP6f is expressed specifically in

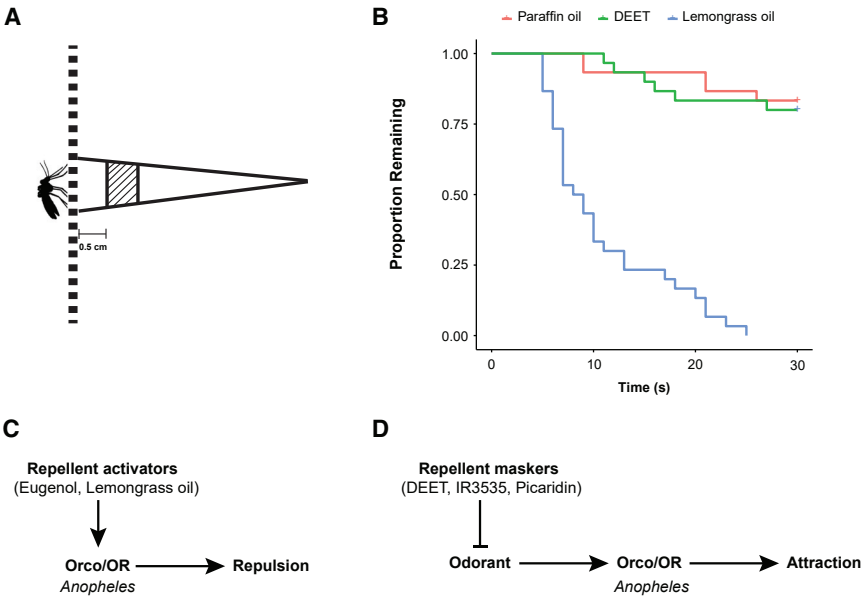


Figure 7. Activator Repellents, but Not Masker Repellents, Trigger Mosquito Repulsion

(A) Schematic of the close proximity response assay. A mosquito is resting on the mesh wall of a cage while a pipette tip containing a piece of filter paper soaked with an odorant is placed on the other side of the mesh. The filter paper is 0.5 cm away from the mosquito.

(B) Kaplan-Meier estimate shows the proportion of mosquitoes that remained on the cage wall over time (n = 30 mosquitos). The effect of DEET is not significantly different than paraffin oil (Cox proportional hazard model; $p > 0.05$).

(C and D) Our models for the effects of insect repellents on olfactory responses in *Anopheles* mosquitoes.

(C) Natural repellents (eugenol and lemongrass oil) activate a subset of ORs leading to repulsion of *Anopheles* mosquitoes.

(D) Synthetic repellents (DEET, IR3535, and picaridin) interact with odorants to mask the attraction of *Anopheles* mosquitoes toward humans.

Orco-expressing neurons, and will not label olfactory neurons that express ionotropic or gustatory receptors. EAG, on the other hand, can detect responses from all antennal neurons, and our EAG experiments showed very weak responses to DEET and IR3535 that were not significantly different from the paraffin-oil-induced response. This suggests that any neurons missed by our calcium imaging recordings would likely, at best, express only low-affinity DEET receptors. In addition, our behavioral data suggest that DEET by itself is not sufficient to drive mosquito repulsion, suggesting that even if low-affinity DEET receptors are present, they are not sufficient to drive olfactory behaviors. Calcium imaging may also poorly detect neuronal inhibition (potentially visualized as a decrease in basal GCaMP6f fluorescence); nonetheless, the effects of neuronal inhibition on odor-induced activities would have been easily detectable (Figure 5), and their absence suggests any direct inhibitory effect is negligible.

DEET, IR3535, and picaridin likely exhibit multiple overlapping modes of action in preventing mosquito bites. Their ability to function as chemical maskers undoubtedly translates into their function in masking attraction of humans to other insects, but they may also act as activator repellent in *Aedes* or *Culex* mosquitoes that can detect these odors. It has been proposed that DEET may also "confuse" the olfactory system; this could be tied to its masking effects if its ability to affect volatility varies across odors. Although DEET masked all 6 OR ligands we tested, there may be others that are less susceptible to DEET's effects. This might contribute to olfactory confusion in host-seeking mosquitoes by disrupting sensory input into olfactory circuits underlying mosquito behavioral attraction or host preference [30].

Our data support the hypothesis that, for *An. coluzzii*, synthetic repellents reduce the volatility of OR ligands. This olfactory mode of action may further synergize with effects of these synthetic compounds on other sensory modalities. For instance, recent data in *Aedes aegypti* mosquitoes suggest a non-olfactory-based function for DEET as a contact repellent [31]. *Aedes* mosquitoes contain sensory neurons on their tarsi that mediate DEET repulsion. Although the DEET receptor and sensory neurons on the tarsi remain to be identified, they may share a conserved function across many insects. For example, DEET is effective against ticks [32–35], which do not express Orco or ORs [36]. Interestingly, high concentrations of DEET need to be applied (typically >30%) for it to be effective. Our data suggest this may have two effects. First, we found chemical masking by DEET is most effective at concentrations >30%. Second, as mosquito tarsi are exposed during landing, sufficiently high concentrations of DEET or other insect repellents may be able to trigger contact repellent receptors to elicit repellent behaviors. As such, the effectiveness of DEET against mosquito biting could be due to two overlapping characteristics: its olfactory effect in reducing host attraction and its contact effect as a repellent.

Our data suggest that chemicals that reduce the volatility of key host odorants might be effective as host-seeking protectants. In addition, low volatile odorants could be a good candidate for a screening study to identify new masker repellents. An ideal mosquito repellent or repellent mixture might be one that combines three modes of action: active odor-based repellency; odor masking; and contact repellency. Repellents like lemongrass oil were less affected by chemical masking, and their combinational use may increase the potency of DEET-based products. Future studies monitoring neural responses directly in the mosquito could yield insights into the function of new repellents as they are identified as well as streamline the discovery of improved insect repellents.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Mosquitoes

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.09.007.

ACKNOWLEDGMENTS

We thank C. McMeniman, D. Task, S. Maguire, and K. Robinson for mosquito technical support and for comments on the manuscript; Sophia Hager for assistance with imaging experiments; and Mark Wu for the use of his imaging camera. This work was supported by grants from the Department of Defense to C.J.P. (W81XWH-17-PRMRP) and from the NIH to C.J.P. (NIAID R01AI137078), and by a Johns Hopkins 2018 Catalyst Award to C.J.P., a Johns Hopkins Malaria Research Institute Pilot Fund to C.J.P., and a Johns Hopkins Malaria Research Institute Postdoctoral Fellowship to A.A. We thank the Johns Hopkins Malaria Research Institute, Bloomberg Philanthropies, and Virginia Tech Department of Biochemistry for their support.

AUTHOR CONTRIBUTIONS

Conceptualization and Methodology, A.A. and C.J.P.; Investigation, A.A. and C.L.; Resources, O.R.; Formal Analysis and Visualization, A.A., J.F.B., and C.L.; Writing – Original Draft, A.A., C.L., and C.J.P.; Writing – Review & Editing, A.A., J.F.B., O.R., C.L., and C.J.P.; Supervision, C.J.P.; Funding Acquisition, C.J.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: March 2, 2019
Revised: July 1, 2019
Accepted: September 4, 2019
Published: October 17, 2019

REFERENCES

1. World Health Organization (2018). World malaria report. https://www.who.int/malaria/publications/world-malaria-report-2018/en/.
2. Potter, C.J. (2014). Stop the biting: targeting a mosquito's sense of smell. Cell *156*, 878–881.
3. McMeniman, C.J., Corfas, R.A., Matthews, B.J., Ritchie, S.A., and Vosshall, L.B. (2014). Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. Cell *156*, 1060–1071.
4. Debboun, M., Frances, S., and Strickman, D. (2014). Insect Repellents Handbook, Second Edition (CRC Press).
5. Brown, M., and Hebert, A.A. (1997). Insect repellents: an overview. J. Am. Acad. Dermatol. *36*, 243–249.
6. Carey, A.F., and Carlson, J.R. (2011). Insect olfaction from model systems to disease control. Proc. Natl. Acad. Sci. USA *108*, 12987–12995.
7. Saveer, A.M., Pitts, R.J., Ferguson, S.T., and Zwiebel, L.J. (2018). Characterization of chemosensory responses on the labellum of the malaria vector mosquito, *Anopheles coluzzii*. Sci. Rep. *8*, 5656.
8. McIver, S.B. (1982). Sensilla mosquitoes (Diptera: Culicidae). J. Med. Entomol. *19*, 489–535.
9. Boeckh, J., Breer, H., Geier, M., Hoever, F.P., Krüger, B.W., Nentwig, G., and Sass, H. (1996). Acylated 1,3-aminopropanols as repellents against bloodsucking arthropods. Pestic. Sci. *48*, 359–373.
10. Stanczyk, N.M., Brookfield, J.F.Y., Ignell, R., Logan, J.G., and Field, L.M. (2010). Behavioral insensitivity to DEET in *Aedes aegypti* is a genetically determined trait residing in changes in sensillum function. Proc. Natl. Acad. Sci. USA *107*, 8575–8580.
11. Bohbot, J.D., and Dickens, J.C. (2010). Insect repellents: modulators of mosquito odorant receptor activity. PLoS ONE *5*, e12138.
12. Bohbot, J.D., Fu, L., Le, T.C., Chauhan, K.R., Cantrell, C.L., and Dickens, J.C. (2011). Multiple activities of insect repellents on odorant receptors in mosquitoes. Med. Vet. Entomol. *25*, 436–444.
13. DeGennaro, M., McBride, C.S., Seeholzer, L., Nakagawa, T., Dennis, E.J., Goldman, C., Jasinskiene, N., James, A.A., and Vosshall, L.B. (2013). *orco* mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET. Nature *498*, 487–491.
14. Lee, Y., Kim, S.H., and Montell, C. (2010). Avoiding DEET through insect gustatory receptors. Neuron *67*, 555–561.
15. Leal, W.S., and Uchida, K. (1998). Application of GC-EAD to the determination of mosquito repellents derived from a plant, *Cymbopogon citratus*. J. Asia Pac. Entomol. *1*, 217–221.
16. Syed, Z., and Leal, W.S. (2008). Mosquitoes smell and avoid the insect repellent DEET. Proc. Natl. Acad. Sci. USA *105*, 13598–13603.
17. Xu, P., Choo, Y.M., De La Rosa, A., and Leal, W.S. (2014). Mosquito odorant receptor for DEET and methyl jasmonate. Proc. Natl. Acad. Sci. USA *111*, 16592–16597.
18. Ditzen, M., Pellegrino, M., and Vosshall, L.B. (2008). Insect odorant receptors are molecular targets of the insect repellent DEET. Science *319*, 1838–1842.
19. Davis, E.E., and Sokolove, P.G. (1976). Lactic acid-sensitive receptors on the antennae of the mosquito, *Aedes aegypti*. J. Comp. Physiol. *105*, 43–54.
20. Pellegrino, M., Steinbach, N., Stensmyr, M.C., Hansson, B.S., and Vosshall, L.B. (2011). A natural polymorphism alters odour and DEET sensitivity in an insect odorant receptor. Nature *478*, 511–514.
21. Moreno, M., Marinotti, O., Krzywinski, J., Tadei, W.P., James, A.A., Achee, N.L., and Conn, J.E. (2010). Complete mtDNA genomes of *Anopheles darlingi* and an approach to anopheline divergence time. Malar. J. *9*, 127.
22. Waterston, R.H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J.F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P., et al.; Mouse Genome Sequencing Consortium (2002). Initial sequencing and comparative analysis of the mouse genome. Nature *420*, 520–562.
23. Carey, A.F., Wang, G., Su, C.Y., Zwiebel, L.J., and Carlson, J.R. (2010). Odorant reception in the malaria mosquito *Anopheles gambiae*. Nature *464*, 66–71.
24. Coetzee, M., Hunt, R.H., Wilkerson, R., Della Torre, A., Coulibaly, M.B., and Besansky, N.J. (2013). *Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex. Zootaxa *3619*, 246–274.
25. Chen, T.W., Wardill, T.J., Sun, Y., Pulver, S.R., Renninger, S.L., Baohan, A., Schreiter, E.R., Kerr, R.A., Orger, M.B., Jayaraman, V., et al. (2013). Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature *499*, 295–300.
26. Riabinina, O., Task, D., Marr, E., Lin, C.C., Alford, R., O'Brochta, D.A., and Potter, C.J. (2016). Organization of olfactory centres in the malaria mosquito Anopheles gambiae. Nat. Commun. *7*, 13010.
27. Pitts, R.J., Derryberry, S.L., Zhang, Z., and Zwiebel, L.J. (2017). Variant ionotropic receptors in the malaria vector mosquito *Anopheles gambiae* tuned to amines and carboxylic acids. Sci. Rep. *7*, 40297.

28. Raji, J.I., Melo, N., Castillo, J.S., Gonzalez, S., Saldana, V., Stensmyr, M.C., and DeGennaro, M. (2019). *Aedes aegypti* mosquitoes detect acidic volatiles found in human odor using the IR8a pathway. Curr. Biol. *29*, 1253–1262.e7.

29. Xia, Y., Wang, G., Buscariollo, D., Pitts, R.J., Wenger, H., and Zwiebel, L.J. (2008). The molecular and cellular basis of olfactory-driven behavior in *Anopheles gambiae* larvae. Proc. Natl. Acad. Sci. USA *105*, 6433–6438.

30. McBride, C.S., Baier, F., Omondi, A.B., Spitzer, S.A., Lutomiah, J., Sang, R., Ignell, R., and Vosshall, L.B. (2014). Evolution of mosquito preference for humans linked to an odorant receptor. Nature *515*, 222–227.

31. Dennis, E.J., Goldman, O.V., and Vosshall, L.B. (2019). *Aedes aegypti* mosquitoes use their legs to sense DEET on contact. Curr. Biol. *29*, 1551–1556.e5.

32. Carroll, J.F., Klun, J.A., and Debboun, M. (2005). Repellency of deet and SS220 applied to skin involves olfactory sensing by two species of ticks. Med. Vet. Entomol. *19*, 101–106.

33. Carroll, J.F., Solberg, V.B., Klun, J.A., Kramer, M., and Debboun, M. (2004). Comparative activity of deet and AI3-37220 repellents against the ticks *Ixodes scapularis* and *Amblyomma americanum* (Acari: Ixodidae) in laboratory bioassays. J. Med. Entomol. *41*, 249–254.

34. Pretorius, A.M., Jensenius, M., Clarke, F., and Ringertz, S.H. (2003). Repellent efficacy of DEET and KBR 3023 against *Amblyomma hebraeum* (Acari: Ixodidae). J. Med. Entomol. *40*, 245–248.

35. Kumar, S., Prakash, S., Kaushik, M.P., and Rao, K.M. (1992). Comparative activity of three repellents against the ticks *Rhipicephalus sanguineus* and *Argas persicus*. Med. Vet. Entomol. *6*, 47–50.

36. Carr, A.L., Mitchell, R.D.I., III, Dhammi, A., Bissinger, B.W., Sonenshine, D.E., and Roe, R.M. (2017). Tick Haller's Organ, a new paradigm for arthropod olfaction: how ticks differ from insects. Int. J. Mol. Sci. *18*, 1563–1597.

37. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682.

38. R Core Team (2018). R: A language and environment for statistical computing (R Foundation for Statistical Computing).

39. Beyenbach, K.W., and Masia, R. (2002). Membrane conductances of principal cells in Malpighian tubules of *Aedes aegypti*. J. Insect Physiol. *48*, 375–386.

40. Pinheiro, J., Bates, D., DebRoy, S., and Sarkar, D.; R Core Team (2018). nlme: Linear and nonlinear mixed effects models. R package version 3.1-137. https://cran.r-project.org/web/packages/nlme/index.html.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Halocarbon oil	Sigma-Aldrich	Series 27
Paraffin oil	Sigma-Aldrich	Product# 18512
Ethanol	Fisher Scientific	Product# BP2818500
1-octen-3-ol	SAFC	Product# W280518
2-acetylthiophene	Sigma-Aldrich	Product# W503509
Benzaldehyde	Aldrich	Product# 418099
p-cresol	Sigma-Aldrich	Product# C85751
1-hepten-3-ol	SAFC	Product# W412901
Indole	Aldrich	Product# I3408
Lemongrass oil	SAFC	Product# W262404
Eugenol	Aldrich	Product# E51791
DEET	Sigma-Aldrich	Product# 36542
IR3535	EMD Chemicals	Product# 111887
Picaridin	Cayman Chemical	Product# 16458
Lactic acid	Sigma-Aldrich	Product# A6283
Nonanoic acid	Sigma-Aldrich	Product# 73982
Octanoic acid	Sigma	Product# C2875
Heptanoic acid	Sigma-Aldrich	Product# 43858
Hexanoic acid	Aldrich	Product# 153745
Butyric acid	Sigma-Aldrich	Product# 19215
Nerolidol	Aldrich	Product# H59605
α- Humulene	Aldrich	Product# 53675
Farnesene	Sigma-Aldrich	Product# W383902
Critical Commercial Assays		
InFusion cloning kit	Clontech	Catalogue# 639645
Experimental Models: Organisms/Strains		
Anopheles coluzzii (genotype: Orco-QF2 [26], QUAS-GCaMP6f	This study	N/A
D. melanogaster carrying a QUAS-GCamp6f transgene	Ya-Hui Chou	N/A
Oligonucleotides		
Primer: pBac-TATA-GCamp-SV40-Inf-FOR (5′-gcg gcc gcg gct cga gat ggg ttc tca tca tca tca tc-3′)	Integrated DNA Technologies	N/A
Primer: pBac-TATA-GCamp-SV40-Inf-REV (5′-ttc aca aag atc gac gtc taa gat aca ttg atg agt ttg gac aaa c-3′)	Integrated DNA Technologies	N/A
Recombinant DNA		
pBAC-ECFP-15xQUAS-TATA-SV40 plasmid	[26]	Addgene #104875
Software and Algorithms		
Fiji	[37]	https://imagej.net/Fiji
R version 3.5.1	[38]	https://www.r-project.org/
MATLAB	The MathWorks Inc.	https://www.mathworks.com/products/matlab.html
NIS Elements Advanced Research	Nikon instruments	https://www.microscope.healthcare.nikon.com/products/software/nis-elements/nis-elements-advanced-research

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Andor Solis (i)	Oxford Instruments	https://andor.oxinst.com/products/solis-software/solis-i
WinEDR	Strathclyde Electrophysiology Software	http://spider.science.strath.ac.uk/sipbs/software_ses.htm
Other		
TetraMin® tropical flakes fish food	Tetra GMBH	Model# 16106
Glass capillary tubes	Harvard Apparatus	Product# 30-0108
Stimulus controller	Syntech	Model CS-55
Pasteur pipettes	Fisher Scientific	Cat# 13-678-6A
Plastic pipette	Denville Scientific Inc	Product# 1158R03
Spectra® 360 Electrode gel Electrode gel	Parker Laboratories	Product# 12-08
Silver wire 0.01"	A-M Systems	Cat# 782500
Borosilicate pulled capillary	Sutter Instrument Company	Cat# B100-75-10
Saline solution	[39]	NA
Poulten Graf Fortuna Optima All Glass Luer-Tip Syringe	MilliporeSigma	Product# 7.102-27
PrecisionGlide, 21G disposable needle	BD	Cat# 305165
Whatman filter paper	GE Healthcare Bio-Sciences	Product# 1001 090
3-way solenoid valve	The Lee Company	5VDC, Vac*45 psig (0-30 psid) Soft Tube Ported Style Solenoid Valves #LHDA0533115H
BugDorm-1 insect rearing cage	BugDorm store	https://shop.bugdorm.com/bugdorm-1-insect-rearing-cage-p-1.html
Photoionization detector	Honeywell RAE Systems	Model: MiniRAE 3000
Microelectrode AC Amplifier	A-M Systems	Model: 1800
Analog-to-digital board	National Instruments	BNC-2090A
Humbug noise eliminator	Quest Scientific	http://www.quest-sci.com/

LEAD CONTACT AND MATERIALS AVAILABILITY

Requests for resources and reagents should be directed to the Lead Contact, Christopher J. Potter (cpotter@jhmi.edu). Plasmids generated in this study are available upon request or from Addgene. *Anopheles* mosquito strains used in this study are available upon request or from BEI Resources (https://www.beiresources.org/AnophelesProgram/Anopheles.aspx).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mosquitoes

Anopheles coluzzii mosquitoes (genotype: *Orco-QF2* [26], *QUAS-GCaMP6f, this study*) were raised in a climate chamber maintained at 26-28°C, 70%–80% RH and L14:D10 cycle. After hatching, mosquito larvae were fed on fish food (TetraMin®), added every day. Cotton rolls soaked with sugar solution (10%, w/vol) were provided to feed adult mosquitoes as a source of carbohydrates. Mosquito females were blood fed on mice for egg laying. The blood feeding protocol was approved by the Johns Hopkins University Animal Care and Use Committee. For all experiments, we used non blood-fed female mosquitoes that were allowed to mate freely.

METHOD DETAILS

Generation of transgenic QUAS-GCaMP6f mosquitoes

Cloning of pXL-BACII-ECFP-15xQUAS-TATA-Gcamp6f-SV40

The *GCamp6f-SV40-terminator* sequence was PCR amplified from genomic DNA of transgenic *Drosophila* carrying a *QUAS-GCamp6f* transgene (gift from Ya-Hui Chou, unpublished) with primers pBac-TATA-GCamp-SV40-Inf-FOR (5′-gcg gcc gcg gct cga gat ggg ttc tca tca tca tca tc-3′) and pBac-TATA-GCamp-SV40-Inf-REV (5′-ttc aca aag atc gac gtc taa gat aca ttg atg agt ttg gac aaa c-3′). The PCR product was InFusion-cloned (Clontech, catalog number 639645) into the *pBAC-ECFP-15xQUAS-TATA-SV40* plasmid [26] (Addgene #104875), digested with ZraI and XhoI. The cloning product was verified by DNA sequencing.

Embryo injection

Injections were performed into *Anopheles coluzzii* N'Gousso strain embryos by the Insect Transformation Facility (Rockville, MD) using standard procedures as previously described [26]. Gravid females were provided with wet filter paper for 15-20 minutes, after which the eggs were collected and arranged side-by-side on a double-sided tape fixed to a coverslip. Eggs were covered with halocarbon oil (Sigma, series 27) and injected with an injection cocktail at their posterior pole. Injection cocktails consisted of a mixture of two plasmids, one with a piggyBac vector carrying the transgene of interest with a dominant visible marker gene (ECFP) under the regulatory control of the 3xP3 promoter, and a piggyBac transposase-expressing plasmid consisting of the transposase open reading frame under the regulatory control of the promoter from the *Anopheles stephensi vasa* gene. Vector concentrations were at either 35, 75 or 150 ng/μl while the transposase-expressing plasmid was at 300 ng/μl in 5 mM KCl, 0.1 mM sodium phosphate pH 6.8. Halocarbon oil was immediately removed and coverslips with injected embryos were placed in trays of water at 28°C where first instar larvae hatched approximately 24hrs later. Adults developing from injected embryos were separated by sex prior to mating and small groups of 5-10 injected adult males and females were mixed with wild-type Ngousso adults of the opposite sex. The progeny from these matings were screened during the third or fourth larval instar for the presence of vector-specific marker gene expression. Transgenic larvae were saved and were backcrossed as adults to wild-type.

Two transgenic lines were established, CP-04-15-M2 and CP-04-15-M3. In functional pilot experiments in crosses to *Orco-QF2* transgenic mosquitoes, both showed similar levels of induced expression and olfactory-directed calcium responses. CP-04-15-M2 was used for all subsequent experiments.

Odorants

All odorants were purchased at the highest purity available. Details on the source and purity of all odorants are included in the key resource table. Odorants were used undiluted, diluted in paraffin oil (to 1%, 10%, or 30%), in ethanol (to 30%), or in mixtures with odorants.

Calcium Imaging

Mosquito preparation

3-10 day old female mosquitoes were immobilized on ice for 1 min. A mosquito was then carefully inserted into a pipette tip. The mosquito was pushed so only the antennae extended outside the pipette tip. The pipette tip was then attached to a glass slide using modeling clay. For imaging, an antenna was placed forward and flattened on a glass coverslip using two pulled glass capillary tubes (Harvard Apparatus, 1 OD x 0.5 ID x 100 L mm). One tube was used to flatten the 3rd-4th antennal segment, and the other to flatten the 12th-13th segment (the most distal segments). Preliminary recordings were performed to visualize responses from the whole antenna. Olfactory responses were similar in each segment but could vary in the number of responding neurons. To achieve higher resolution imaging for analyses, all subsequent recordings were done at one antennal segment (11th antennal segment). Based on pilot experiments examining multiple segments, the responses in one segment (11th segment) were representative of responses in all segments.

Imaging system

Antennae were imaged through a 10x (Zeiss EC Epiplan-Neofluar 10x/0.25) or a 50x (LD EC Epiplan-Neofluar 50x/0.55 DIC) objective mounted on a Zeiss Axio Examiner D1 microscope. For fluorescence, a light source (Zeiss Illuminator HXP 200C) and eGFP filter cube (FL Filter Set 38 HE GFP shift free) were used.

For image acquisition, an EMCCD camera (Andor iXon Ultra, Oxford Instruments), NIS Elements Advanced Research software (Nikon instruments), and Andor Solis software (Oxford Instruments) were used. Recordings were for 20 s, at a resolution of 512x512 pixels, and an exposure time of 200 ms (5 Hz).

Odorant preparation and delivery

For testing neural responses toward OR ligands, repellents, acids, and low volatile odorants, 20 μl of the solution was pipetted onto a piece of filter paper (1X2 cm) placed in a Pasteur pipette (Fisher Scientific). For mixtures, 10 μl of an OR ligand was pipetted along with 10 μl of repellent on the same filter paper. Each odorant was prepared at double the final concentration to reach the desired final concentration when mixed. The Pasteur pipette was then inserted into a hole in a plastic pipette (Denville Scientific Inc, 10ml pipette) that carried a purified continuous air stream (8.3 ml/s) directed at the antenna. A stimulus controller (Syntech) was used to divert a 1 s pulse of charcoal-filtered air (5 ml/s) into the Pasteur pipette starting 10 s after the beginning of each recording. Each animal was tested with 6 odorant pairs (6 OR ligands and their respective mixtures). Four animals out of a total of 45 animals stopped responding before testing all odorants, and the remaining odorant pairs were tested in new animals. The sequence of odorants was randomized, and recordings from a mosquito were discarded if a response to a positive control odorant (usually 1-octen-3-ol) was absent. New Pasteur pipettes were prepared for each recording day.

Close range odorant delivery

To test the three synthetic repellents at a closer distance, a small hole was made at the tip of the long pipette used to deliver continuous air to the antenna (Figure S3A). The stimulus Pasteur pipette was then inserted into the small hole so that the tip of the Pasteur pipette is 0.5 cm away from the mosquito antenna. A Pasteur pipette containing a dry piece of filter paper (blank), a Pasteur pipette containing paraffin oil soaked filter paper, and a Pasteur pipette containing water soaked filter paper were used as negative controls.

Modified odorant delivery

To test whether masking occurs at the receptor or the chemical level, the odorant delivery described above was modified as described below.

Pre-stimulation with repellents

An OR ligand (1-octen-3-ol) and a repellent (DEET, IR3535, picaridin, or paraffin oil for control) were prepared in two separate Pasteur pipettes as previously described. Each Pasteur pipette contained 10 μl of either 2% 1-octen-3-ol or 60% repellent to reach a final concentration of 1 and 30%, respectively. The two Pasteur pipettes were inserted into two holes in the plastic pipette that carried a purified continuous air stream directed at the antenna. One branch of a polyethylene Y-tube was used to deliver a 3 s pulse of charcoal-filtered air into the Pasteur pipette that contains the repellent. At the third second, the other branch of the Y-tube was attached to the 1-octen-3-ol Pasteur pipette to deliver 1 s pulse of 1-octen-3-ol. For comparison, a mixture of the repellent and 1-octen-3-ol was also tested with each animal as previously described. Each animal was tested with 7 odorant conditions.

Simultaneous odorant delivery

An OR ligand (1-octen-3-ol) and a repellent (or paraffin oil for control) were prepared in two separate Pasteur pipettes as previously described. The two Pasteur pipettes were inserted into two holes in the plastic pipette that carried a purified continuous air stream directed at the antenna. A 1 s pulse of charcoal-filtered air (5 ml/s) was diverted into the two Pasteur pipettes using a polyethylene Y-tube in order to deliver the two odorants at the same time into the continuous air stream. Afterward, the two Pasteur pipettes were switched between the two holes in the long plastic pipette to rule out any position bias. For comparison, a mixture of the repellent and 1-octen-3-ol was also tested with each animal as previously described. Each animal was tested with 11 odorant conditions.

Same pipette delivery

An OR ligand (1-octen-3-ol) and a repellent (or paraffin oil for control) were applied on two separate filter papers (0.5X1 cm) within the same Pasteur pipette. We made certain the two filter papers were not touching and therefore the odorants were never physically mixed. To deliver the odorants, a 1 s pulse of charcoal-filtered air was diverted into the Pasteur pipette. Afterward, we used another Pasteur pipette, in which the position of the repellent and 1-octen-3-ol was swapped, to rule out any position bias. For comparison, a mixture of the repellent and 1-octen-3-ol was also tested with each animal as previously described. Each animal was tested with 11 odorant conditions.

Electroantennography

Mosquito head preparation

4-7 day old females *Anopheles coluzzii* mosquitoes were used for the electroantennography (EAG) experiments. A female mosquito was briefly placed on ice and immobilized on a cool aluminum block. The rear tip of each antenna (i.e., about half one segment) was cut off with fine scissors under a binocular microscope and the head was excised. The tips of the antennae were then dipped into electrode gel (Spectra® 360 Electrode gel, Parker Laboratories, Fairfield, NJ, USA) and gently pushed against each other so they stick together when coming out of the electrode gel. The head was then mounted by the neck on an electrode (i.e., reference) composed of a oxidized silver wire 0.01" (A-M Systems, Carlsbord, WA, USA) and a borosilicate pulled capillary (Sutter Instrument Company, Novato, CA, USA) filled with saline solution (adapted from Beyenbach and Masia, 2002 [39]). The mounted head preparation was transferred to the EAG setup and the tips of the antennae were inserted into the recording electrode, which was identical to the reference electrode, under the microscope using micromanipulators. The head was oriented at 90° from the main airline which was carrying medical grade air (Praxair, Danbury, CT, USA) at a constant rate of 15 cm.s-1 for the whole duration of the experiment along with volatiles from the syringe during the stimulation to the preparation (Figures 3A and S4D).

Odorant preparation and stimulation

Twenty microliters of each chemical were loaded onto a piece of Whatman filter paper (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) placed in a glass syringe (Poulten Graf Fortuna Optima All Glass Luer-Tip Syringe, MilliporeSigma, St Louis, MO, USA) before the experiment started. Mixtures were prepared by physically mixing 1-octen-3-ol with DEET, IR3535, or picaridin to reach a final concentration of 1% 1-octen-3-ol and 30% of the repellent. The disposable needle (BD PrecisionGlide, 21G, BD, Franklin Lakes, NJ, USA) of the glass syringe was inserted in the main airline through a small hole to allow the molecules to mix with clean air and create an odor plume before reaching the mosquito antennae. Odor pulses were triggered using a 3-way solenoid valve (The Lee Company, Westbrook, CT, USA) controlled by a custom-written MATLAB script (The MathWorks Inc., Natick, MA, USA). The stimuli consisted of two 1 s. long pulses (2.3 cm.s-1) separated by 10 s. The recordings for each set of 2 pulses lasted 45 s. total. Then, the odor syringe was removed to test the following odorant. Single chemicals and mixture of chemicals were loaded in a specific glass syringe to avoid any contamination. Prior to starting to deliver the odor stimuli, two pulses of clean air (empty syringe containing a clear filter paper) were used as a control to ensure that no mechanical perturbation of the antennae due to air movements was occurring. As a negative control, two paraffin oil pulses were presented randomly during the experiment. As a positive control, two pulses of 1% benzaldehyde were delivered at the end of the experiment to ensure that the preparation was still responsive. Odor stimuli were randomly generated using MATLAB while making sure that the 1% octenol and the combination of octenol and repellents were presented in a randomized sequence but without being separated by the 30% and 100% dilutions of repellents to allow for comparisons.

Close proximity response assay

Wild-type female mosquitoes (N'Gousso strain) were tested individually (30 mosquitoes total). Each mosquito was transferred to a cage (BugDorm, 30 X 30 X 30 cm) and given $\geq$ 5 minutes to rest on one of the cage mesh walls. The mosquito was then approached by a 1000 μL pipette tip containing a piece of filter paper soaked with an odorant. The pipette tip was rested on the outer side of the

cage wall so that the mosquito was at a 0.5 cm distance from the filter paper. The mosquito was observed for 30 s and the time it took to fly away was scored. Each mosquito was exposed to three consecutive odorants (lemongrass oil, DEET, and paraffin oil) and the sequence of the odorants was randomized. The mosquito was given $\geq$ 2 minutes between odorants. If the mosquito flew off, it was allowed to land and rest for $\geq$ 2 minutes before the next odorant was used.

Photoionization detector

The MiniRAE 3000 photoionization detector (Honeywell RAE Systems) was used to calculate concentrations of odorants delivered to the mosquito antenna in different experiments. The photoionization detector was calibrated to a reference gas (ethyl acetate) and was attached to the tip of the plastic pipette used to deliver odorants in calcium imaging experiments. The maximum reading (arbitrary units, AU) following each odorant delivery was reported.

QUANTIFICATION AND STATISTICAL ANALYSIS

Analysis of Calcium imaging recordings

To make the heatmap ΔF images, Fiji software [37] was used with a custom-built macro. This Macro uses the ''Image stabilizer'' plug-in to correct for movements in the recording, followed by the ''Z project'' function to calculate the mean baseline fluorescence (mean intensity in the first 9 s of recording, before stimulus delivery). Then, the ''Image calculator'' function was used to subtract the mean baseline fluorescence from the image of maximum fluorescence after odorant delivery (this image was manually chosen). Afterward, this ΔF image was used to produce heatmaps.

To produce intensity time traces, the ''ROI manager'' tool in Fiji was used to manually select ROIs. ROIs were drawn around cells that showed increased fluorescence in response to odorants (based on the heatmap ΔF images). Then the ''multi-measure'' function in the ''ROI manager'' was used to produce intensity values for those ROIs across time. Finally, these values were saved into Excel and used to calculate $\Delta F/F$*100. $\Delta F/F$*100 = F_i- F_0/F_0*100, where F_i is the fluorescence intensity value at frame $_i$, while F_0 is the mean fluorescence intensity before odorant delivery (first 9 s, 45 frames). Sample traces for each experiment are available upon request.

For analysis, each odorant response was represented by the maximum $\Delta F/F$*100 value following that odorant (the single frame at the peak of the response).

Linear Mixed Effects (LME) regression was used to model the average value of the outcome under an experimental condition, accounting for both correlation due to repeated-measurements and non-constant residual variation. In all experiments, fixed effects were used to model the average value of the outcome at each experimental condition, and a linear term was used to model the average change in the outcome over repeated-measurements. Within-subject correlation was accounted for using random intercepts, and heteroskedasticity was accounted for by modeling the residual variance.

For odorant delivery and pre-stimulation experiments, the residual variance was modeled as a power of the fitted values. In the simultaneous odorant delivery experiments, the outcome was log transformed and a separate residual variance term was estimated for conditions where repellents were physically mixed with the OR ligands. In the same pipette delivery experiment, the outcome was log transformed and the residual variance was modeled as an exponential function of fitted values.

Model assumptions, such as linearity of relationships, normally distributed scaled residuals, and normally distributed random effects, were assessed using residual diagnostic plots. Confidence intervals and p values provided use the Wald approximation. No multiple comparisons corrections were performed. All analyses were performed using R version 3.5.1 [38] using the nlme package version 3.1-137 [40].

Data acquisition and analysis of EAG recordings

The electrophysiological signals were amplified 100X and filtered (0.1-500 Hz) (A-M Systems Model 1800, Sequim, WA, USA), recorded and digitized at 20 Hz using WinEDR software (Strathclyde Electrophysiology Software, Glasgow, UK) and a BNC-2090A analog-to-digital board (National Instruments, Austin, TX, USA) on a computer. A Humbug noise eliminator (Quest Scientific, Vancouver, Canada) was used to decrease electrical noise (50-60 Hz). The responses (i.e., deflection in mV) of female mosquito antennae to the different odorants were filtered. Each response was individually inspected to ensure that the observed response had the typical EAG shape and was measured for each mosquito preparation and averaged for each chemical. The data were then compared using a Pairwise Wilcoxon Rank Sum test with a Bonferroni correction using R [38]. Normality was assessed using a Shapiro Wilk test.

Analysis of Close proximity response assay

To plot the time mosquitoes took to fly in response to odorants, a Kaplan-Meier survival Estimates was used. A cox Proportional Hazard Model was used to assess the relationship between the time to fly and odorants, and account for the number of previous odorant exposures. The plot and analysis was performed using R [38].

All statistical details (for calcium imaging, EAG, and behavioral experiments) are included in the figure legends

DATA AND CODE AVAILABILITY

The imaging files and datasets generated and/or analyzed during the current study are available from the Lead Contact on request.