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Inverse currents and electroantennographic responses elicited by eucalyptol
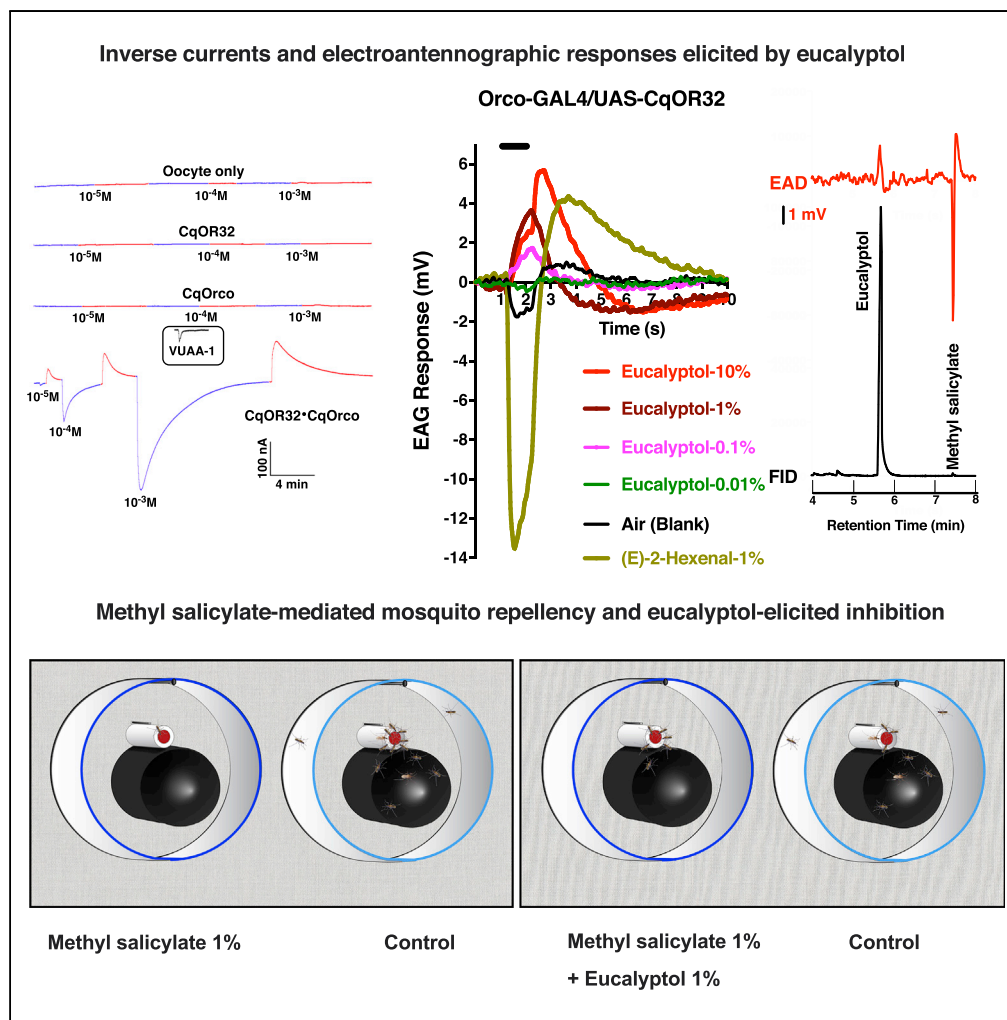
Pingxi Xu, Young-Moo Choo, Zhou Chen, ..., Anthony J. Cornel, Nannan Liu, Walter S. Leal

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HIGHLIGHTS
We found dual inhibitory/excitatory odorant receptors (ORs) in mosquitoes

Inhibitory and endogenous ORs coexpressed in flies showed lateral inhibition

The bipolar nature of these inhibitory ORs was displayed in electrophysiology

The duality excitation/inhibition was also manifested in mosquito behavior

Article

Odorant Inhibition in Mosquito Olfaction

Pingxi Xu,[1] Young-Moo Choo,[1,5] Zhou Chen,[2,5] Fangfang Zeng,[1,5] Kaiming Tan,[1,5] Tsung-Yu Chen,[3] Anthony J. Cornel,[4] Nannan Liu,[2] and Walter S. Leal[1,6,*]

SUMMARY

How chemical signals are integrated at the peripheral sensory system of insects is still an enigma. Here we show that when coexpressed with Orco in *Xenopus* oocytes, an odorant receptor from the southern house mosquito, CquiOR32, generated inward (regular) currents when challenged with cyclohexanone and methyl salicylate, whereas eucalyptol and fenchone elicited inhibitory (upward) currents. Responses of CquiOR32-CquiOrco-expressing oocytes to odorants were reduced in a dose-dependent fashion by coapplication of inhibitors. This intrareceptor inhibition was also manifested *in vivo* in fruit flies expressing the mosquito receptor CquiOR32, as well in neurons on the antennae of the southern house mosquito. Likewise, an orthologue from the yellow fever mosquito, AaegOR71, showed intrareceptor inhibition in the *Xenopus* oocyte recording system and corresponding inhibition in antennal neurons. Inhibition was also manifested in mosquito behavior. Blood-seeking females were repelled by methyl salicylate, but repellence was significantly reduced when methyl salicylate was coapplied with eucalyptol.

INTRODUCTION

Integration of chemical signals at the peripheral sensory system (antennae, maxillary palps, and proboscis) remains one of the least understood mechanisms of insect olfaction, particularly in mosquitoes. Despite the great progress made in the last 2 decades in understanding how receptors form the basis of chemosensory perception in insects, how olfactory (as well as taste) signals integrate at the periphery remains an enigma (Joseph and Carlson, 2015). "It is as if a new continent has been discovered but only the coastline has been mapped" (Joseph and Carlson, 2015). In the largest majority of reported cases (Hill et al., 2009; Syed and Leal, 2009; Liu et al., 2013; Ye et al., 2016), antennal neurons of *Cx. quinquefasciatus* displayed excitatory responses (increased spike frequency upon stimulus), but evidence for inhibitory responses (reduction in spontaneous activity upon stimulus), already known for *Ae. aegypti* (Ghaninia et al., 2007), is now emerging for *Cx. quinquefasciatus* (Ye et al., 2016). It has been observed in moths (Kaissling, 1996), beetles (Nikonov and Leal, 2002), the fruit (vineger) fly (Su et al., 2012), and mosquitoes (Tauxe et al., 2013) that activation (firing) of one neuron interferes with signaling of other olfactory receptor neurons (ORNs; also referred to as olfactory sensory neurons). It has also been reported that a single compound (iodobenzene) caused reduction of nerve impulse (inhibition) followed by a transient post-stimulus excitation (de Brito sanchez and Kaissling, 2005). Although Carlson and collaborators elegantly demonstrated that in the fruit fly lateral inhibition is most likely mediated by ephaptic coupling (Su et al., 2012), the complete ensemble of the molecular mechanism(s) of inhibition at the peripheral olfactory system of mosquitoes remains *terra incognita*. A simple explanation of the ephaptic coupling is that, upon (continuous) stimulation of an ORN, the (external) potential (of the sensillum lymph surrounding dendrites) declines. Consequently, per channel current generated by a cocompartmentalized neuron (when stimulated by its cognate ligand) is reduced (Van Naters, 2013). This scenario argues that the firing of a neuron causes reduced spike frequency by a colocated neuron because of the close apposition (ephaptic, Greek for "to touch") of their neuronal processes. Although ephaptic coupling could explain lateral inhibition, other mechanisms of intraneuron inhibition may exist. While de-orphanizing odorant receptors (ORs) expressed predominantly in *Cx. quinquefasciatus* female antennae, we serendipitously recorded currents from an OR that generate inhibition in response to certain odorants. Further studies unraveled a hitherto unknown mechanism of peripheral, intrareceptor inhibition in mosquito olfaction.

RESULTS AND DISCUSSION

Recordings of Inward Currents and Currents in Upward Direction

In our attempts to de-orphanize ORs from the southern house mosquito, *Cx. quinquefasciatus*, we challenged *Xenopus* oocytes coexpressing CquiOR32 along with the obligatory coreceptor Orco with a panel

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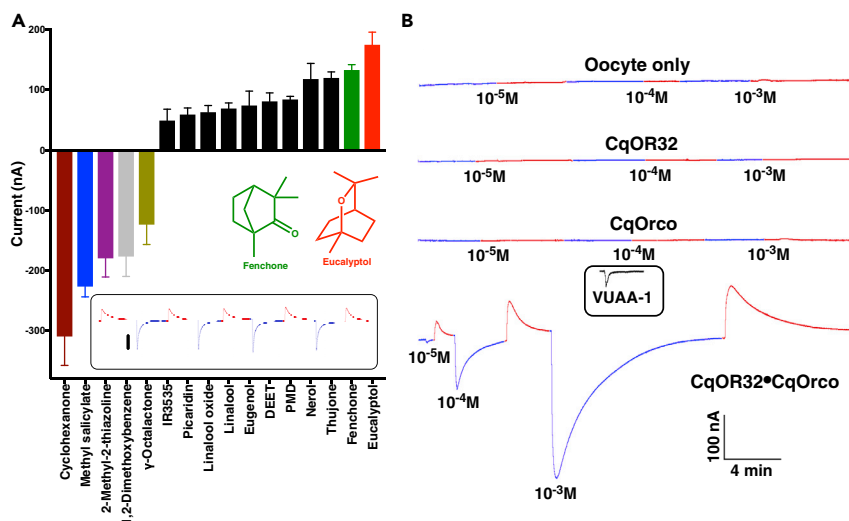[4]Department of Entomology and Nematology, University of California-Davis, Davis, CA 95616, USA

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Figure 1. Recordings and Quantification of Inward and Inhibitory Currents

(A) CquiOR32-CquiOrco-expressing oocytes responses to ligands at 1 mM. Error bars represent SEM (n = 3-4). For clarity, bars representing reverse and inward currents are displayed upward and downward, respectively. The following compounds are not displayed for conciseness. Inward current-eliciting compounds: γ-hexalactone, 115 ± 14 nA (all data in absolute values); guaiacol, 101 ± 11 nA; acetophenone, 58 ± 12 nA; 2-butanone, 45 ± 7 nA; 2-phenylethanol, 44 ± 9 nA. Inhibitory compounds: α-terpinene, 43 ± 5 nA; terpinolene, 38 ± 8 nA (for a complete list, see Table S1). *Inset:* Continuous recordings from a single oocyte expressing CquiOR32-CquiOrco and repeatedly stimulated with 1 mM eucalyptol (reverse peaks; traces in maraschino) and 0.1 mM methyl salicylate (inward peaks; traces in blueberry).

(B) Control experiments with oocytes only and oocytes expressing only CquiOR32, CquiOrco, or a combination of CquiOR32 and CquiOrco. Response of a CquiOrco-expressing oocyte to Orco agonist, VUAA-1, is displayed in an inset. For clarity, the traces before, during, and after stimulus are displayed with the same color as the bars in (A). Specifically, methyl salicylate in blueberry (CYMK, Cyan 87%, Magenta 61%, Yellow 0%, Black 0%) and eucalyptol in maraschino (CYMK, Cyan 0%, Magenta 81%, Yellow 94%, Black 1%). Stimuli were delivered for 2 s.

of more than 200 compounds, including mostly physiologically and behaviorally relevant compounds (Xu et al., 2014). Because CquiOR32 is predominantly expressed in female antennae (Figure S1), we reasoned that this receptor might be involved in the reception of attractants or repellents. CquiOR32-CquiOrco-expressing oocytes generated dose-dependent inward (regular) currents when challenged with various odorants, including cyclohexanone, methyl salicylate, and 2-methyl-2-thiazoline (Figure 1A, Table S1). Interestingly, however, eucalyptol, fenchone, DEET, picaridin, IR3535, PMD, and other compounds generated currents in reverse direction (Figure 1A and Table S1) thus resembling inverse agonists. These unusual currents of reverse direction were reproducible, and no indication was found of adaptation (Figure 1A, inset). No currents were recorded when oocytes alone or oocytes expressing only CquiOR32 or only CquiOrco were challenged either with methyl salicylate or eucalyptol (Figure 1B). However, CquiOR32-CquiOrco-expressing oocytes responded to both compounds. Methyl salicylate elicited inward currents, and eucalyptol generated currents in the reverse direction (Figure 1B) in a dose-dependent manner (Figures S2 and S3, respectively).

Current-Voltage Curves Suggest that OR32-Orco Forms Nonselective Cation Channels

To gain better insights into possible mechanism(s) of these currents in reverse direction, we obtained current-voltage (I-V) curves from oocytes expressing wild-type (WT) CquiOR32-CquiOrco with voltage clamped at −80, −60, −40, −20, 0, +20, and +40 mV and using methyl salicylate and eucalyptol as stimuli (Figures 2A and 2D, respectively). Similar recordings were performed using N-methyl-D-glucamine chloride (NMG) as a source of bulky, impermeable mono cation (Figures 2B and 2E) or with sodium gluconate buffer (a source of bulky, impermeable anions) instead of NaCl (Figures 2C and 2F). These data corroborate that OR32-Orco forms nonselective cation channels (Wicher et al., 2008; Smart et al., 2008; Sato et al., 2008), as indicated by the reversal potential shift to more negative voltage upon replacement of Na^+ by less permeable NMG (Figures 2A and 2B). An examination of the first and third quadrants for each graphic in Figure 2 shows that I-V curves elicited by methyl salicylate (Figures 2A–2C) are above baseline and below control I-V curves for positive and negative voltages, respectively (a common pattern of inward currents), consistent
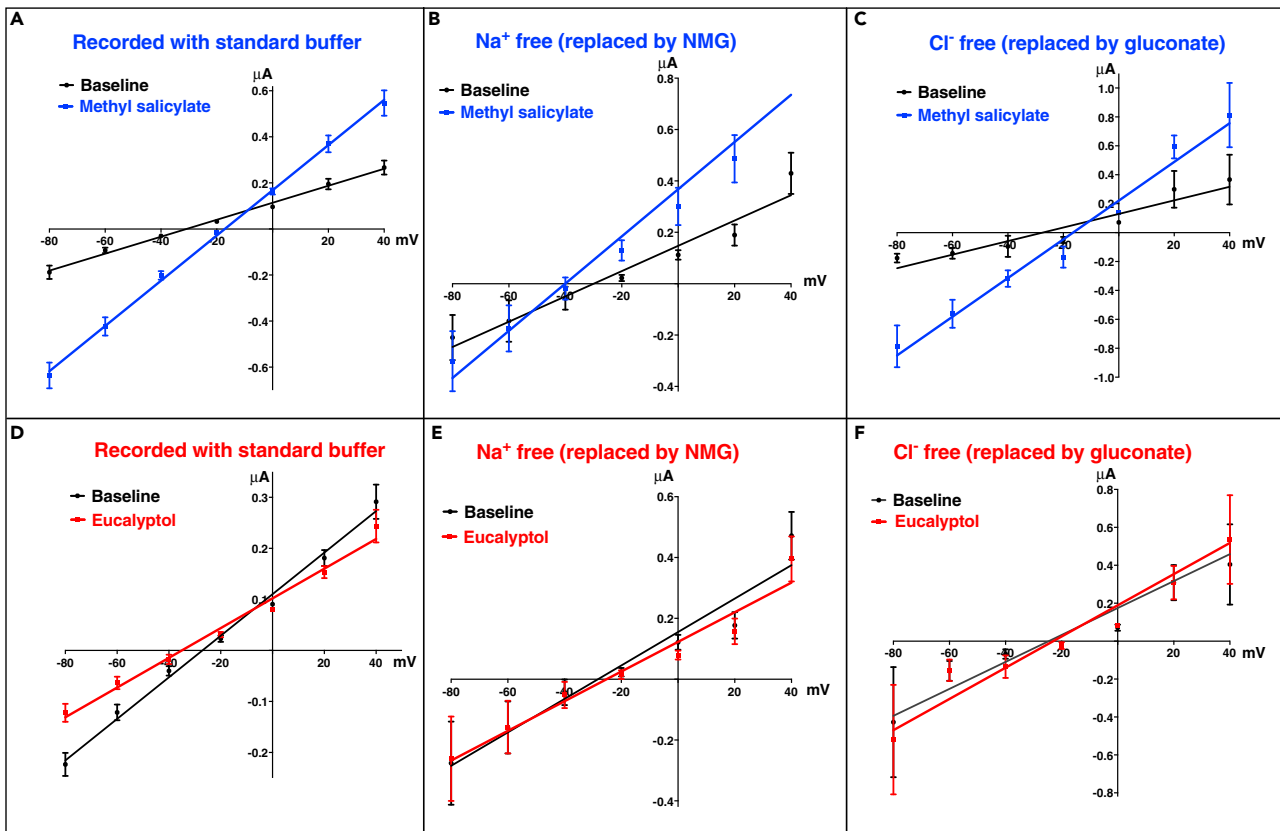
Figure 2. Current-Voltage (I-V) Curves for CquiOR32-CquiOrco Expressed in *Xenopus* Oocytes

Recordings with methyl salicylate (blueberry) and eucalyptol (maraschino) at 1 mM, with voltage clamped at −80, −60, −40, −20, 0, +20, and +40 mV and using regular and modified buffers. (A and D, n = 9) standard buffer; (B and E, n = 5) Na^+ free buffer; (C and F, n = 3) $Cl^−$ free buffer. Error bars represent SEM.

with a potentiation of the control current by methyl salicylate. By contrast, I-V curves generated with eucalyptol, albeit not robust, are below and above control I-V curves for positive and negative holding potentials, respectively (Figures 2D and 2E), suggesting an inhibition effect. In the presence of eucalyptol, replacing Na^+ with NMG had a minor effect on the I-V curves (Figure 2E), whereas replacing $Cl^−$ with gluconate seems to suggest that $Cl^−$ is implicated in this inhibition (Figure 2F).

Eucalyptol-Induced Inhibition in Oocytes

We then surmised that these "inhibitors" might modulate responses to odorants when these two types of stimuli are simultaneously delivered to receptors. First, we challenged a CquiOR32-CquiOrco-expressing oocyte with methyl salicylate (0.1 mM) and then eucalyptol (1 mM) and recorded regular and reverse currents, respectively (Figure S4). Then we challenged the same oocyte preparation with mixtures of methyl salicylate and eucalyptol. Binary mixtures with higher doses of eucalyptol elicited reverse currents. Inhibition was also observed with lower doses of eucalyptol, which generated dose-dependent reduced inward currents. A continuous trace displayed in Figure S4 shows no difference in the responses to methyl salicylate before and after these tests thus ruling out adaptation. Taking together, these findings suggest that "intrareceptor" inhibition occurred consistently with outward currents previously recorded from antennal neurons of the vinegar fly, *Drosophila melanogaster* (Cao et al., 2017).

Inhibition in the Antennae of Flies Expressing CquiOR32

To test whether the inhibitory responses were manifested *in vivo* at the periphery of the olfactory system, we generated transgenic flies, with CquiOR32 expression driven by DmelOrco promoter, and recorded electroantennogram (EAG) responses by using a standard method (Ueira-Vieira et al., 2014). Not surprisingly, control flies (w[1118]) gave strong response to (*E*)-2-hexenal and weak response to methyl salicylate (Figure 3A), whereas Orco-Gal4/UAS-CqOR32 flies gave robust response to methyl salicylate, with the
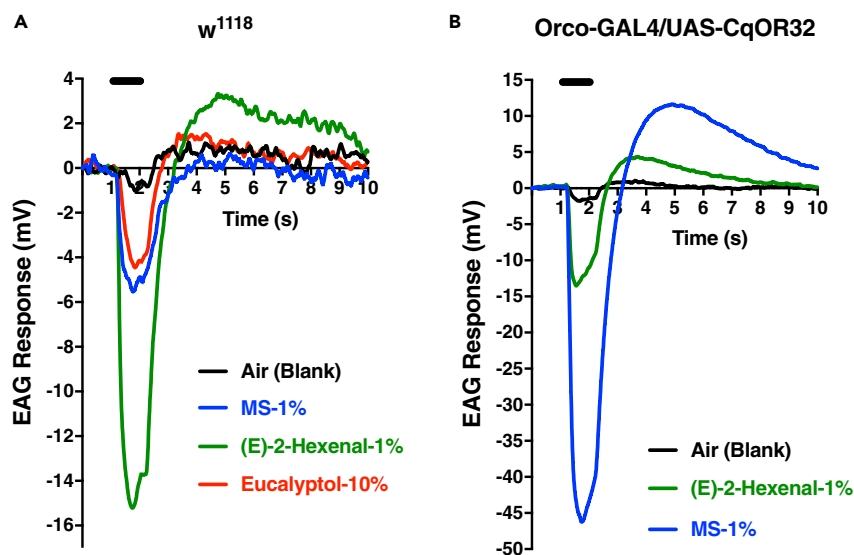
Figure 3. Electroantennogram (EAG) Obtained with Wild-Type and Transgenic Flies

Responses elicited by 1% methyl salicylate (MS) and 1% (E)-2-hexenal in the antennae of w[1118] (A) and Orco-GAL4/UAS-CqOR32 flies. Note the unusual robust response elicited by MS in transgenic flies (B) due to overexpression of CquiOR32.

strong response to (E)-2-hexenal unchanged (Figure 3B). w1118 flies gave very weak responses to eucalyptol at high doses, but interestingly Orco-Gal4/UAS-CqOR32 flies generated dose-dependent, inverse EAG responses (Figure 4). To further scrutinize this unusual reverse EAG responses, we used gas chromatography (GC) with electroantennographic detection (EAD). In GC-EAD analyses, injected mixtures (e.g., eucalyptol and methyl salicylate) are separated by GC and subjected to antennal preparations under the same condition thus ruling out any possibility of mechanical interference and minor sample contamination. Here, methyl salicylate responded with regular EAG responses, i.e., with the first phase (downward), which is referred to as rise of the receptor potential, and the second phase starting at the end of the stimulus, commonly referred to as the decline of the receptor potential (upward, return to the baseline). This is analogous to the depolarization, repolarization, and hyperpolarization of a nervous impulse. As opposed to methyl salicylate, eucalyptol consistently gave inverse EAD responses (upward then downward) (Figure 5) thus corroborating what we observed in EAG analyses (see earlier discussion).

Next, we recorded EAG responses when flies were challenged with odorants and an inhibitor. First, we compared the response of w[1118] and Orco-Gal4/UAS-CqOR32 flies to (E)-2-hexenal when it was delivered alone or in combination with eucalyptol. EAG responses from w[1118] flies to 0.1% (E)-2-hexenal alone or in combination with 10% eucalyptol did not differ significantly (Figure 6A). By contrast, EAG responses from Orco-Gal4/UAS-CqOR32 flies to 0.1% (E)-2-hexenal plus 10% eucalyptol were significantly lower than those elicited by 0.1% (E)-2-hexenal alone (Figure 6B). We then examined the dose-dependent effect of this inhibition by using Orco-Gal4/UAS-CqOR32 flies. Robust responses to 0.1% methyl salicylate were reduced in a dose-dependent manner with the addition of eucalyptol (Figure 6C) but remained unchanged at the end of the tests. Likewise, EAG responses to 0.01% (E)-2-hexenal were reduced when coappled with eucalyptol (0.1, 1, and 10%) (Figure 6D). Of note, (E)-2-hexenal does not activate CquiOR32 (Table S1, entry #136). Such inhibition presumably results from CquiOR32 indirectly inhibiting responses of the fly endogenous receptors (e.g., DmelOR7a) to (E)-2-hexenal. In these continuous experiments, a small difference between EAG responses before and after costimulus tests may be due to loss of this volatile semiochemical from the cartridge rather than adaptation. Similar inhibition was observed when 2-heptanone was applied alone or coapplied with eucalyptol (Figure S5). Taken together, these results further suggest that intrareceptor inhibition occurs *in vivo* as indicated by the inhibitory effect of eucalyptol on methyl salicylate responses. Additionally, the effect of eucalyptol on the response to (E)-2-hexenal suggests that intraneuronal inhibition occurred. A few lines of evidence support this hypothesis. First and foremost, eucalyptol does not cause inhibition in control flies (Figure 6A) and (E)-2-hexenal does not activate CquiOR32 (Table S1). The simplest explanation is that, in Orco-Gal4/UAS-CqOR32 flies, all endogenous receptors are coexpressed

Orco-GAL4/UAS-CqOR32
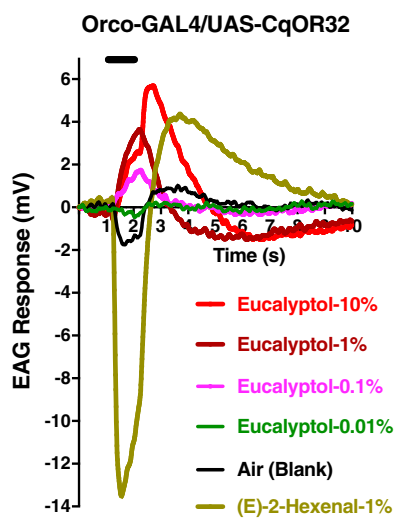
Figure 4. EAG Obtained by Challenging Orco-GAL4-CquiOR32 Flies with Eucalyptol

While blank generate a normal downward deflection, eucalyptol elicited reverse responses in a dose-dependent manner.

with CquiOR32. Thus, CquiOR32 response to eucalyptol interferes with the response of DmelOR7a (in ab4A neuron) to (E)-2-hexenal. In short, inhibitor and agonist are likely to be acting on different receptors in the same neurons, thus an intraneuron inhibition. To further test the notion of intraneuronal inhibition, we turned to single sensillum recordings (SSRs).

Inhibition Is Also Manifested in Single Sensillum Recordings

The best ligand for ab4A, the neuron in ab4 sensilla with a large spike amplitude, is (E)-2-hexenal (de Bruyne et al., 2001), although ab4A is also very sensitive to other ligands, including hexanal (Figure S6). Contrary to ab4B, ab4A houses only one OR, namely, DmelOr7a (Hallem et al., 2004). Because expression of CquiOR32 was driven by DmelOrco, ab4A neurons in our transgenic flies house both DmelOr7a and CquiOR32. Coexpression was confirmed by a significantly stronger response to methyl salicylate (p = 0.0001, unpaired t test) recorded from Orco-Gal4/UAS-CquiOR32 than from WT flies (Figures 7 and S6), while retaining response to hexanal (Figure S7). It is known that methyl salicylate is the best ligand for DmelOr10a in ab1D (Hallem et al., 2004) but elicits only very low response in ab4A (de Bruyne et al., 2001). The low response of WT flies to methyl salicylate did not differ significantly (p > 0.9999) when the odorant was delivered alone or codelivered with eucalyptol (Figure 7). By contrast, responses recorded from Orco-Gal4/UAS-CquiOR32 flies were significantly lower (p = 0.0003) when the two stimuli were delivered simultaneously from two different cartridges (Figures 7 and S6). Next, we tested whether CquiOR32 response to eucalyptol would affect DmelOR7a response to a cognate ligand, hexanal. Responses of WT flies to hexanal did not differ significantly when comparing hexanal alone with hexanal plus eucalyptol (Figures S7 and S8). Recordings from ab4 sensilla in the Orco-Gal4/UAS-CquiOR32 flies showed a slight, albeit not significant, increase in response to hexanal. This is unlikely to be due to hexanal activation of CquiOR32 (Table S1, entry #128). When hexanal and eucalyptol were delivered simultaneously firing of DmelOR7a was completely abolished (Figures S7 and S8). We also recorded from ab7 sensilla, which expresses DmelOR98a, in ab7A and for which butyl acetate is one of the best ligands (Munch and Galizia, 2016). Eucalyptol elicited inhibitory response in ab7A neurons of Orco-Gal4/UAS-CquiOR32 flies (Figure S9). In the transgenic flies both methyl salicylate and butyl acetate generated excitatory responses (Figure S9), which were inhibited by eucalyptol (Figures 8 and S9). Because methyl salicylate and eucalyptol elicit inward and reverse currents in CquiOR32, this in vivo inhibition is not surprising. However, the consistent observation that eucalyptol inhibits the response of an endogenous receptor to a cognate ligand supports the notion that intraneuronal inhibition occurs when receptors are colocated in a neuron. Specifically, the inhibitory responses of CquiOR32 interferes with the activation of a collocated receptor by a cognate ligand. For example, activation of DmelOR7a in ab4A neuron by hexanal and activation of DmelOR98a in ab7A neuron by butyl acetate were both inhibited by eucalyptol upon interaction with CquiOR32. Contrary to the fruit fly, which expresses only one receptor per neuron (with a few exceptions), mosquitoes (and possibly other insect species) can coexpress multiple ORs in the same neuron (Karner et al., 2015).
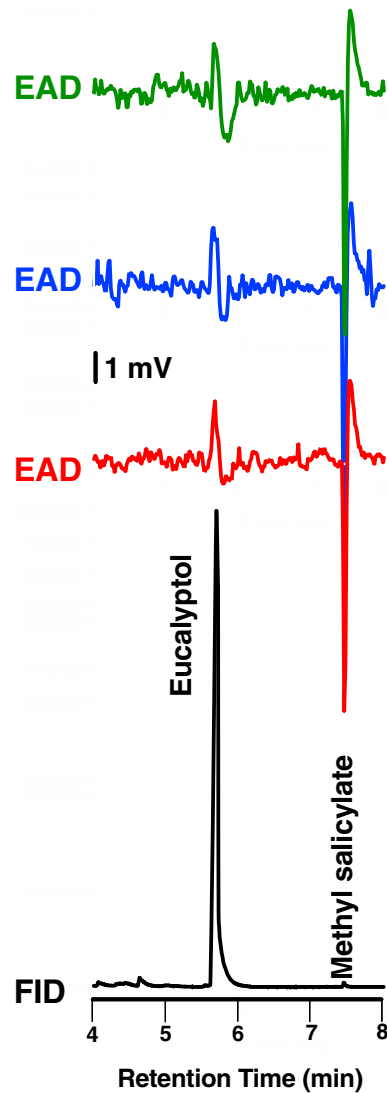
Figure 5. Antennal Response Obtained with Gas Chromatography (GC) Linked to Electroantennographic (EAD) Detection

Response of the flame ionization detector (FID) was synchronized with EAD responses. For clarity only one trace of FID response is provided in these three replicates. Each EAD replicate is displayed in a different color. Methyl salicylate, 10 ng; eucalyptol 0.5 μg.

Inhibition in *Culex* Mosquito Antennae

We investigated by using SSR whether inhibition would occur in antennae of the southern house mosquito. Of 240 contacts, 33 recordings from ORN-A in short sharp-tipped sensilla 2 (SST-2) showed that this neuron responded to cyclohexanone with dose-dependent excitatory responses (Figure 9A) and to eucalyptol (Figure 9B) and fenchone (Figure 9C) with dose-dependent inhibitory responses, thus resembling what has been observed in the *Xenopus* oocyte recording system (Figure 1). Additionally, the responses to cyclohexanone were modulated by both eucalyptol (Figure 9D) and fenchone (Figure 9E) in a dose-dependent manner.

CquiOR32 Orthologue in *Aedes aegypti* and Intrareceptor Inhibition

CquiOR32 has an orthologue in the genome of the yellow fever mosquito, AaegOR71 (AAEL017564), with 55.5% identity. We sequenced 20 clones and obtained 19 AaegOR71 sequences. Five clones showed sequences identical to the sequence in VectorBase and were, therefore, considered the WT. We expressed
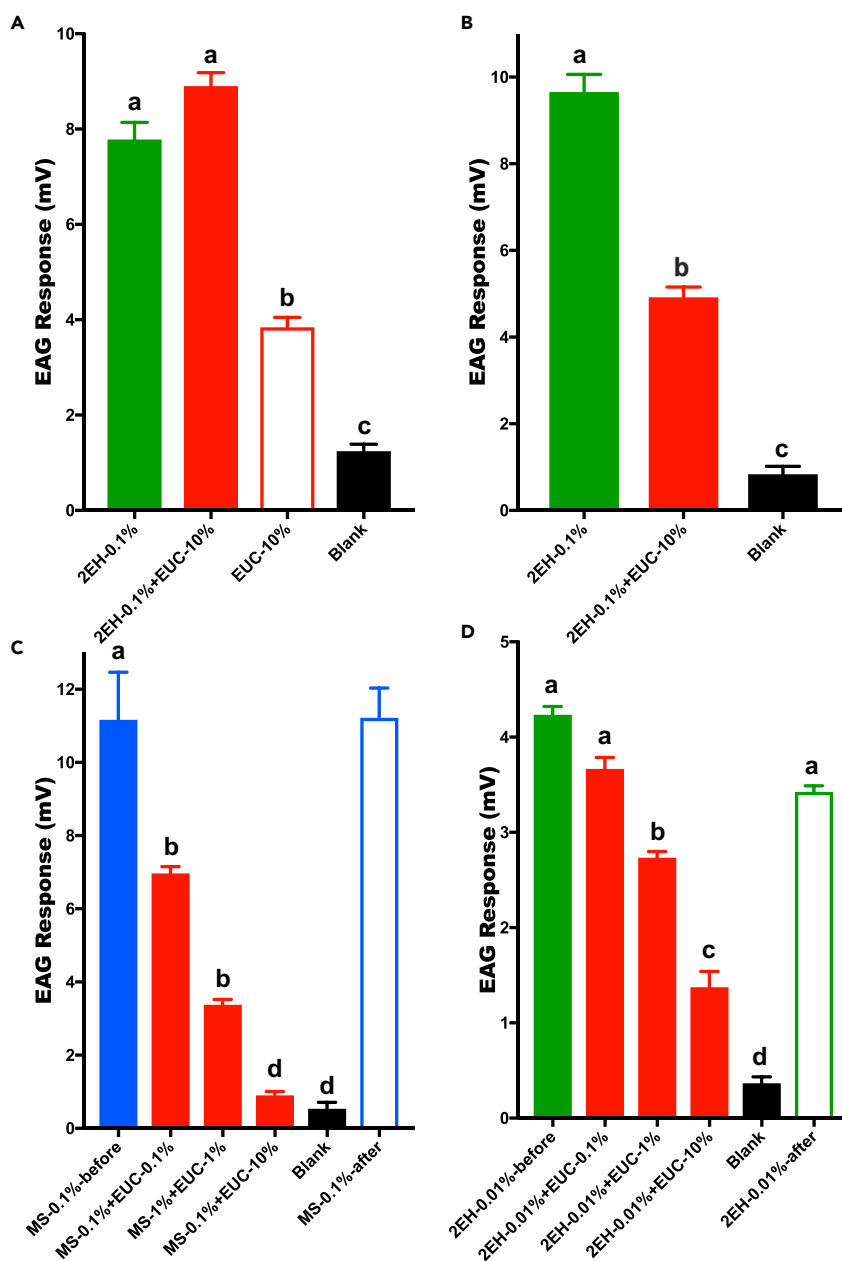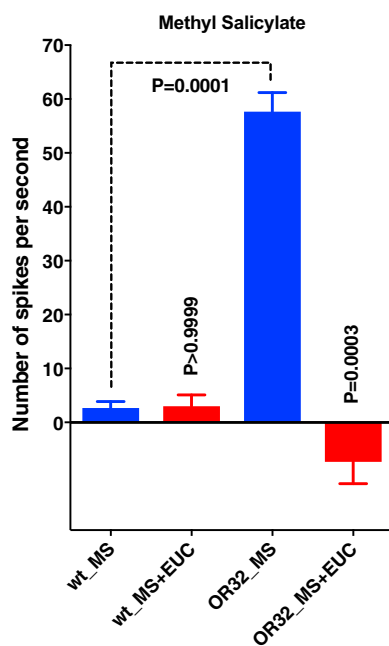
Figure 6. Quantification of EAG Responses

Effect of eucalyptol (EUC) on the responses of (A) w[1118] and (B–D) Orco-GAL4/UAS-CqOR32 flies to (E)-2-hexenal (2EH) and methyl salicylate (MS). Error bars represent SEM.

AaegOR71-WT in the *Xenopus* oocyte recording system (along with AaegOrco) and challenged the oocytes with compounds that elicited inward and inhibitory currents in CquiOR32. Cyclohexanone elicited inward currents, but the compounds generating the largest inward currents were 4,5-dimethylthiazole (DMT) and 2-methyl-2-thiazoline (2MT) (Figure S10); no response was observed with methyl salicylate. Although eucalyptol and fenchone did not elicit measurable inhibitory currents, these two compounds reduced AaegOR71 responses to cyclohexanone, DMT, and 2MT (Figure S10).

Four clones (AaegOR71-V2, GenBank MG593069) differed from WT in 7 amino acid residues, and 3 clones (AaegOR71-V1, MG593068) differed from WT in 11 amino acid residues. They both showed weak responses to odorants when tested in the *Xenopus* oocyte recording system. The other 7 clones differed from the WT

Figure 7. Quantification of Single Sensillum Recordings from ab4 sensilla in Wild-Type and Transgenic Flies

Basal response to methyl salicylate (MS) recorded from WT flies did not differ when this odorant (0.1%) was delivered alone or codelivered with eucalyptol (EUC, 1%) (p > 0.9999). Spike frequency recorded from CquiOR32 flies were significantly higher than those obtained with WT flies (p = 0.0001). When codelivered with EUC (1%), the spike frequency was significantly reduced (p = 0.0003) to a level below the spontaneous activity. Error bars represent SEM.

in 6–12 amino acid residues. AaegOR71-V5 (MG593071), AaegOR71-V14 (MG593074), and AaegOR71-V15 (MG593075) differed in 12, 9, and 11 amino acid residues, respectively, and none of them responded to odorants. AaegOR71-V8 (MG593072) differed in 10 amino acid residues and showed very weak response only to the Orco agonist VUAA-1. AaegOR71-V4 (MG593070), AaegOR71-V9 (MG593073), AaegOR71-V17 (MG593076) differed in 8, 10, and 6 amino acid residues but gave weak to moderate responses to odorants. None of these variants elicited detectable inhibitory currents when challenged with eucalyptol or fenchone.

Peripheral Inhibition in the Yellow Fever Mosquito Antennae

Next, we tested whether intrareceptor inhibition might be manifested *in vivo* in the antennae of the yellow fever mosquito. With 350 contacts, 69 recordings were made from SST-2 sensilla. SSR showed that cyclo-hexanone, 2-methyl-2-thiazoline, and 2,4-dimethylthiazole elicited dose-dependent excitatory responses in neuron-A in SST-2, whereas eucalyptol and fenchone showed inhibitory responses (Figure S11). When costimulated with 2-methyl-2-thiazoline and eucalyptol, the response to the odorant decreased markedly (Figure S12). We then analyzed the effect of inhibitors on the responses to the three odorants that caused excitatory responses. Both eucalyptol and fenchone inhibited the responses of ORN-A in SST-2 to cyclo-hexanone (Figures 10A and 10B), 2-methyl-2-thiazoline (Figures 10C and 10D), and 4,5-dimethylthiazole (Figures 10E and 10F) in a dose-dependent manner.

Intrareceptor Inhibition Manifested in Mosquito Behavior

Evidence in the literature suggests that eucalyptol is an oviposition deterrent (Klocke et al., 1987). We then tested in our surface landing and feeding assay (Leal et al., 2017) (Figure 11A) whether this inhibitory compound or other odorants would repel blood-seeking *Cx. quinquefasciatus* mosquitoes. In preliminary experiments, eucalyptol at 0.1%, 47.57 ± 3.15% mosquitoes responded to the treatment side of the arena, whereas 52.43 ± 3.15% responded to the control (n = 10, p = 0.3276; eucalyptol, 7.5 ± 0.4 mosquitoes in treatment and 8.5 ± 0.8 mosquitoes in the control side). With a higher dose (1%), 44.3 ± 5.7% and 55.7 ± 5.7% mosquitoes responded to treatment and control, respectively (n = 10, p = 0.3330; treatment, 8.1 ± 1.1; control 10.1 ± 1.0 mosquitoes), thus showing that eucalyptol per se is not a potent spatial repellent. Likewise, cyclohexanone did not show repellence activity at 0.1% (47.7 ± 4.2% treatment versus 52.3 ± 4.2% control, n = 4, p = 0.7027; 7 ± 1.1 and 7.5 ± 0.5 in treatment and control, respectively) or 1% (52.7 ± 2.7% treatment versus 47.3 ± 2.7% control, n = 4, p = 0.4950; 8.25 ± 0.9 and 7.75 ± 1.4 in treatment and control, respectively).
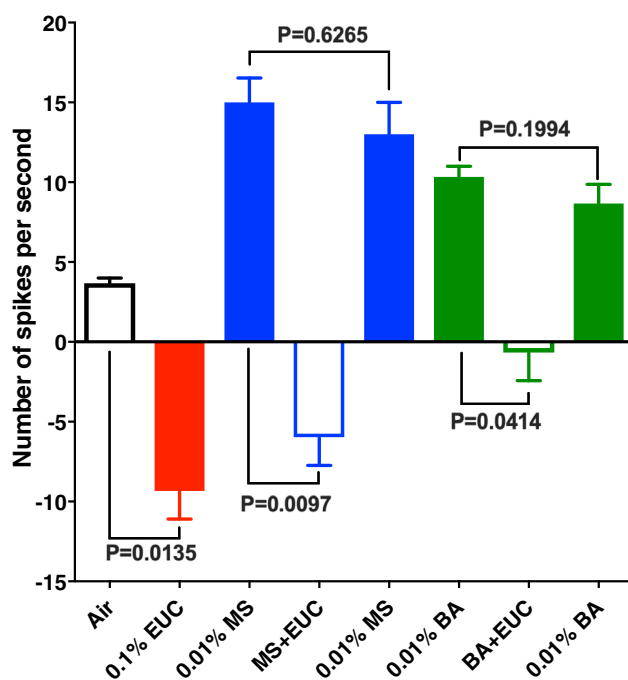
**Figure 8. Quantification of Single Sensillum Recordings from ab7 Sensilla in Orco-GAL4/UAS-CqOR32 Flies**

Responses to 0.1% eucalyptol (EUC), 0.01% methyl salicylate (MS) before and after stimulus with a mixture of 0.01% MS plus 0.1% EUC, and 0.01% butyl acetate (BA) before and after stimulus with 0.01% BA plus 0.1% EUC. Error bars represent SEM.

Contrary to eucalyptol, methyl salicylate showed repellence activity. At a dose of 0.1%, the number of mosquitoes responding to the treatment side of the arena was significantly lower than the number responding to control (39.9 ± 4.0% treatment versus 60.1 ± 4.0% control, n = 10, p = 0.0303; 8 ± 0.8 and 12.3 ± 1.1 in treatment and control, respectively). At a higher dose (1%), the difference was highly significant (15.3 ± 3.7% treatment versus 84.5 ± 3.7% control, n = 10, p < 0.0001; 1.9 ± 0.8 and 8.3 ± 0.9 in treatment and control, respectively).

Because eucalyptol per se is not a repellent, we then tested the effect of eucalyptol on repellence elicited by methyl salicylate. A mixture of 1% methyl salicylate and 1% eucalyptol had significantly lower protection than methyl salicylate alone (Video S1). We repeated these experiments and tested whether eucalyptol would inhibit DEET repellency (Figure 11B). Here we expressed the results in protection so as to infer the potency of each repellent alone as well as in combination with eucalyptol (Figure 11B). Again, eucalyptol at 1% showed no protection, whereas methyl salicylate and DEET at 1% had comparable protection when freshly applied. Addition of eucalyptol at 1% to methyl salicylate led to a loss of activity. By contrast, DEET repellency was not affected when DEET 1% was mixed with eucalyptol either at 1% or 10% (Figure 11B). These behavioral measurements suggest that eucalyptol modulates the mosquito olfactory response to methyl salicylate (but not DEET), thus reducing the behavioral response. Eucalyptol showed no effect on DEET, which is detected by CquiOR136 (Xu et al., 2014). These findings support the notion that the above-described inhibition is manifested in mosquito behavior.

Conclusions

We provided evidence showing that the same receptor (CquiOR32) is activated by some ligands and inhibited by others (intrareceptor inhibition). When this "inhibitory receptor" was coexpressed with another OR we found evidence of intraneuron inhibition. Are these inhibitory compounds merely inverse agonists? If receptors are constitutively open as previously suggested for a case in the vinegar fly (Cao et al., 2017), binding to eucalyptol (or another inhibitor) could shift the equilibrium toward the closed, inactive form of the receptor. However, CquiOR32 agonist and inhibitors are chemically so dissimilar that is hard to conceive, albeit possible, that both types of compound bind to the same orthosteric binding site as in the case of inverse agonists. It is, therefore, more likely that these are negative allosteric modulators. Of note, there is already evidence in the literature

Single sensillum recordings-*Cx. quinquefasciatus*
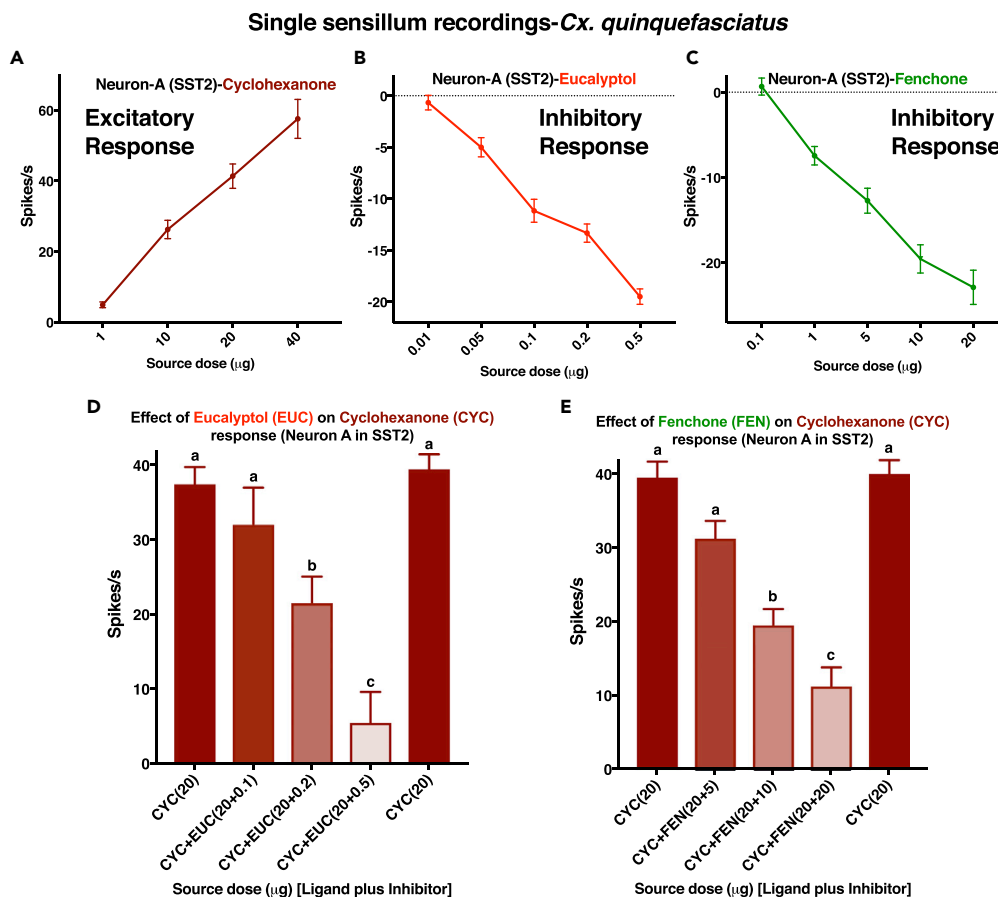
Figure 9. Single Sensillum Recordings from Short Sharp-Tipped-2 (SST-2) Sensilla on *Cx. quinquefasciatus* Antennae
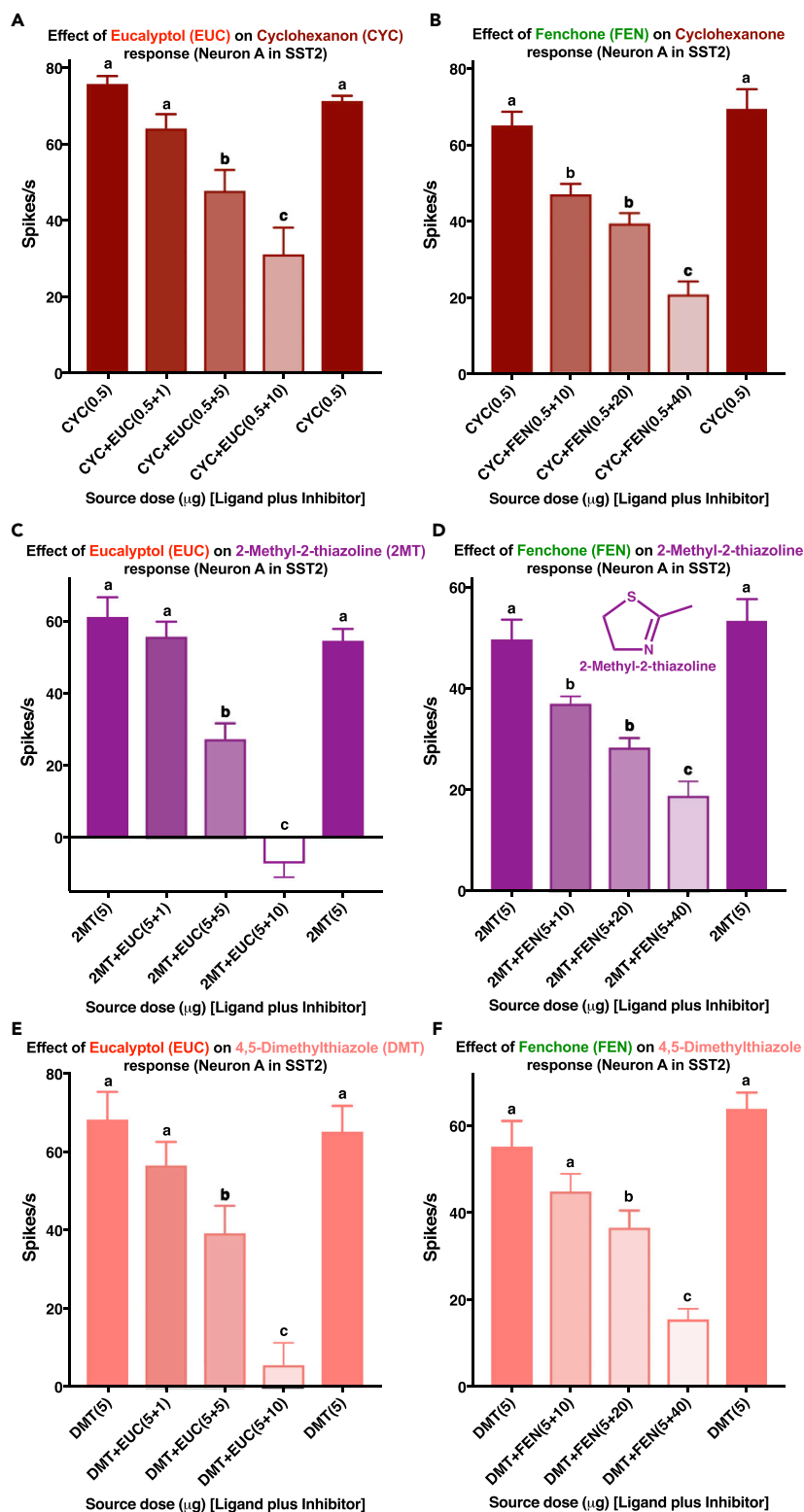
(A–C) Dose-dependent curves for excitatory and inhibitory compounds: (A) cyclohexanone, (B) eucalyptol, and (C) fenchone. (D and E) Effect of eucalyptol (EUC) (D) and fenchone (FEN) (E) on the excitatory responses elicited by cyclohexanone (CYC). Stimuli were delivered in sequence as displayed from left to right. Firing rates observed during 500 ms post-stimulus period were subtracted from spontaneous activities observed in the 500 ms pre-stimulus period, and the outcome was multiplied by 2 to obtain the number of spikes per second. The recording points in x axis are not drawn to scale. Error bars represent SEM. n = 6–12. Bars with different letters are considered statistically different at the 0.05 level, according to Tukey's test.

suggesting that there is positive allosteric modulation of mosquito ORs when expressed in insect cells (Tsitoura and Iatrou, 2016). Here we found *in vitro* and *in vivo* evidence suggesting that negative allosteric modulation may occur. It is known that insect ORx-Orco complexes form agonist-activated cation channels (Wicher et al., 2008; Smart et al., 2008; Sato et al., 2008). Upon binding, cation channels are open and an influx of cations generates inward currents. Theoretically, outward currents could be elicited by chloride influx. In insects, the difference in chemical composition of the sensillum (=receptor) lymph and hemolymph generates the standing electrical potential, i.e., the transepithelial potential, and it favors a possible Cl^- influx (Steinbrecht, 1992): sensillum lymph, K^+ 200 mM; Na^+ 25 mM; Cl^-, 25 mM; hemolymph, K^+, 36 mM; Na^+ 12 mM; Cl^-, 12mM; organic anions make the balance (Kaissling and Thorson, 1980). In the mammalian olfactory system in which the Cl^- gradient is reversed, Cl^- efflux (inward current) depolarizes the membrane further (Hartzell et al., 2005), thus providing the most pronounced amplification of the odor signal within the whole signal transduction cascade (Wicher, 2015). In summary, the exact mode of action of these putative negative allosteric modulators are yet to be elucidated, but apparently Cl^- currents are involved.

Limitations of the Study

Our dataset provided strong evidence for intrareceptor and intraneuron inhibition, but to unambiguously elucidate the mode of action of these inhibitory responses that lead to (outward) reverse currents in

Single sensillum recordings-*Ae. aegypti*

A Effect of Eucalyptol (EUC) on Cyclohexanon (CYC) response (Neuron A in SST2)

B Effect of Fenchone (FEN) on Cyclohexanone response (Neuron A in SST2)

C Effect of Eucalyptol (EUC) on 2-Methyl-2-thiazoline (2MT) response (Neuron A in SST2)

D Effect of Fenchone (FEN) on 2-Methyl-2-thiazoline response (Neuron A in SST2)

E Effect of Eucalyptol (EUC) on 4,5-Dimethylthiazole (DMT) response (Neuron A in SST2)

F Effect of Fenchone (FEN) on 4,5-Dimethylthiazole response (Neuron A in SST2)

Figure 10. Quantification of Responses from Neuron-A in the Short Sharp-Tipped-2 (SST-2) Sensilla on *Ae. aegypti* Antennae

The sensilla were challenges with mixtures of (A and B) cyclohexanone, (C and D) 2-methyl-2-thiazoline, or (E and F) 4,5-dimethylthiazole in combination with either eucalyptol (A, C, and E) or fenchone (B, D, and F). Stimuli were delivered in sequence as displayed from left to right. Firing rates observed during 500 ms post-stimulus period were subtracted from spontaneous activities observed in the 500 ms pre-stimulus period, and the outcome was multiplied by 2 to obtain the number of spikes per second. Error bars represent SEM. n = 6.

oocytes and "inverse" EAG responses we must wait for the elucidation of the structures of CquiOR-CquiOrco or other OR-Orco complexes with similar features.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.
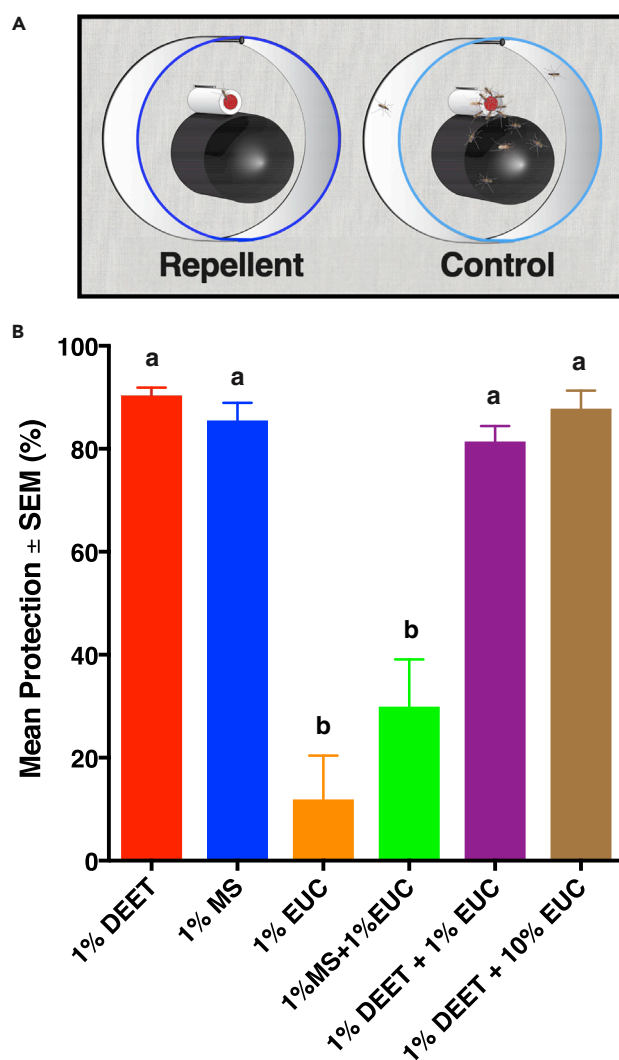
Figure 11. Effect of Eucalyptol on Methyl Salicylate-Elicited Repellency

(A) Diagram of the surface landing and feeding assay, (B) protection data. Eucalyptol (EUC) at 1% showed no repellency, but methyl salicylate (MS) or DEET at 1% repelled blood-seeking *Culex* mosquitoes. MS-elicited repellency was significantly reduced when tested in a mixture of 1% MS and 1% EUC, but EUC at 1% or 10% had no effect on DEET repellency. Error bars represent SEM. n for each bar from left to right: 35, 16, 12, 31, 28, and 11, respectively.

SUPPLEMENTAL INFORMATION

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

P.X., Y.-M.C., Z.C., F.Z., and K.T. performed research; T.-Y.C., A.J.C., and N.L. contributed material and analytical tools; W.S.L. designed research, interpreted the data, and wrote the paper; all authors analyzed the data.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

REFERENCES

Cao, L.H., Yang, D., Wu, W., Zeng, X., Jing, B.Y., Li, M.T., Qin, S., Tang, C., Tu, Y., and Luo, D.G. (2017). Odor-evoked inhibition of olfactory sensory neurons drives olfactory perception in Drosophila. Nat. Commun. *8*, 1357.

de Brito sanchez, M.G., and Kaissling, K.E. (2005). Inhibitory and excitatory effects of iodobenzene on the antennal benzoic acid receptor cells of the female silk moth *Bombyx mori* L. Chem. Senses *30*, 435–442.

de Bruyne, M., Foster, K., and Carlson, J.R. (2001). Odor coding in the Drosophila antenna. Neuron *30*, 537–552.

Ghaninia, M., Ignell, R., and Hansson, B.S. (2007). Functional classification and central nervous projections of olfactory receptor neurons housed in antennal trichoid sensilla of female yellow fever mosquitoes, *Aedes aegypti*. Eur. J. Neurosci. *26*, 1611–1623.

Hallem, E.A., Ho, M.G., and Carlson, J.R. (2004). The molecular basis of odor coding in the *Drosophila* antenna. Cell *117*, 965–979.

Hartzell, C., Putzier, I., and Arreola, J. (2005). Calcium-activated chloride channels. Annu. Rev. Physiol. *67*, 719–758.

Hill, S.R., Hansson, B.S., and Ignell, R. (2009). Characterization of antennal trichoid sensilla from female southern house mosquito, *Culex quinquefasciatus* Say. Chem. Senses *34*, 231–252.

Joseph, R.M., and Carlson, J.R. (2015). *Drosophila* chemoreceptors: a molecular interface between the chemical world and the brain. Trends Genet. *31*, 683–695.

Kaissling, K.E. (1996). Peripheral mechanisms of pheromone reception in moths. Chem. Senses *21*, 257–268.

Kaissling, K.E., and Thorson, J. (1980). Insect olfactory sensilla: structural, chemical and electrical aspects of the functional organization. In Receptors for Neurotransmitters, Hormones and Pheromones in Insects, D.B. Sattelle, L.M. Hall, and J.G. Hildebrand, eds. (Elsevier/North-Holland), pp. 261–282.

Karner, T., Schneider, I., Schultze, A., Breer, H., and Krieger, J. (2015). Co-expression of six tightly clustered odorant receptor genes in the antenna of the malaria mosquito *Anopheles gambiae*. Front. Ecol. Evol. *3*, https://doi.org/10.3389/fevo.2015.00026.

Klocke, J.A., Darlington, M.V., and Balandrin, M.F. (1987). 1,8-Cineole (Eucalyptol), a mosquito feeding and ovipositional repellent from volatile oil of *Hemizonia fitchii* (Asteraceae). J. Chem. Ecol. *13*, 2131–2141.

Leal, W.S., Barbosa, R.M., Zeng, F., Faierstein, G.B., Tan, K., Paiva, M.H., Guedes, D.R., Crespo, M.M., and Ayres, C.F. (2017). Does Zika virus infection affect mosquito response to repellents? Sci. Rep. *7*, 42826.

Liu, F., Chen, L., Appel, A.G., and Liu, N. (2013). Olfactory responses of the antennal trichoid sensilla to chemical repellents in the mosquito, *Culex quinquefasciatus*. J. Insect Physiol. *59*, 1169–1177.

Munch, D., and Galizia, C.G. (2016). DoOR 2.0–Comprehensive mapping of *Drosophila melanogaster* odorant responses. Sci. Rep. *6*, 21841.

Nikonov, A.A., and Leal, W.S. (2002). Peripheral coding of sex pheromone and a behavioral antagonist in the Japanese beetle, *Popillia japonica*. J. Chem. Ecol. *28*, 1075–1089.

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L.B., and Touhara, K. (2008). Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature *452*, 1002–1006.

Smart, R., Kiely, A., Beale, M., Vargas, E., Carraher, C., Kralicek, A.V., Christie, D.L., Chen, C., Newcomb, R.D., and Warr, C.G. (2008). *Drosophila* odorant receptors are novel seven transmembrane domain proteins that can signal independently of heterotrimeric G proteins. Insect Biochem. Mol. Biol. *38*, 770–780.

Steinbrecht, R.A. (1992). Experimental morphology of insect olfaction: tracer studies, X-ray microanalysis, autoradiography, and immunocytochemistry with silkmoth antennae. Microsc. Res. Tech. *22*, 336–350.

Su, C.Y., Menuz, K., Reisert, J., and Carlson, J.R. (2012). Non-synaptic inhibition between grouped neurons in an olfactory circuit. Nature *492*, 66–71.

Syed, Z., and Leal, W.S. (2009). Acute olfactory response of *Culex* mosquitoes to a human- and bird-derived attractant. Proc. Natl. Acad. Sci. U S A *106*, 18803–18808.

Tauxe, G.M., Macwilliam, D., Boyle, S.M., Guda, T., and Ray, A. (2013). Targeting a dual detector of

skin and CO2 to modify mosquito host seeking. Cell *155*, 1365–1379.

Tsitoura, P., and Iatrou, K. (2016). Positive allosteric modulation of insect olfactory receptor function by ORco agonists. Front. Cell. Neurosci. *10*, 275.

Ueira-Vieira, C., Kimbrell, D.A., De Carvalho, W.J., and Leal, W.S. (2014). Facile functional analysis of insect odorant receptors expressed in the fruit fly: validation with receptors from taxonomically distant and

closely related species. Cell. Mol. Life Sci. *71*, 4675–4680.

Van Naters, W.V. (2013). Inhibition among olfactory receptor neurons. Front. Hum. Neurosci. *7*, 690.

Wicher, D. (2015). Olfactory signaling in insects. Prog. Mol. Biol. Transl. Sci. *130*, 37–54.

Wicher, D., Schafer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., Heinemann, S.H., and Hansson, B.S. (2008). *Drosophila* odorant receptors are

both ligand-gated and cyclic-nucleotide-activated cation channels. Nature *452*, 1007–1011.

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. U S A *111*, 16592–16597.

Ye, Z., Liu, F., and Liu, N. (2016). Olfactory responses of southern house mosquito, *Culex quinquefasciatus*, to human odorants. Chem. Senses *41*, 441–447.
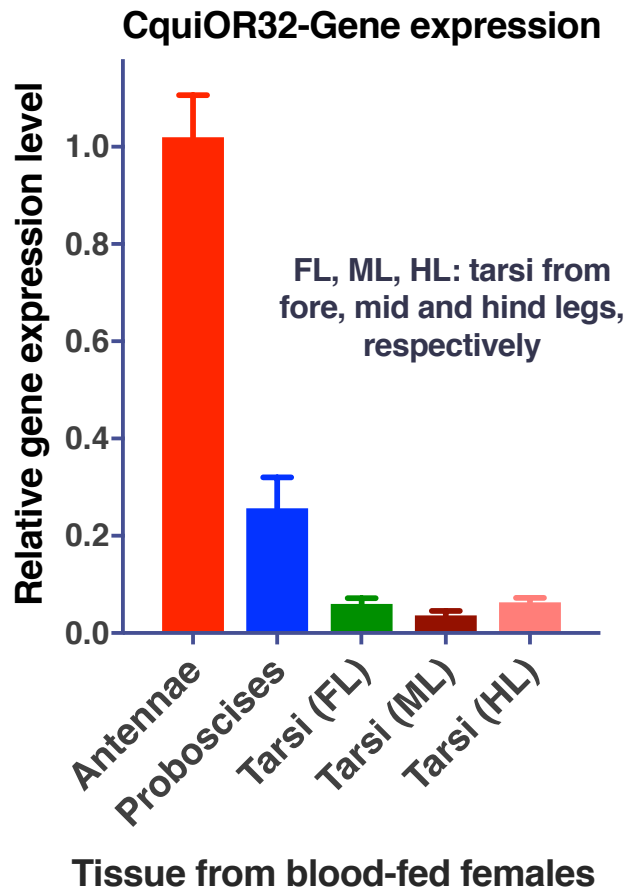
Supplemental Information

Odorant Inhibition in Mosquito Olfaction

Pingxi Xu, Young-Moo Choo, Zhou Chen, Fangfang Zeng, Kaiming Tan, Tsung-Yu Chen, Anthony J. Cornel, Nannan Liu, and Walter S. Leal

CquiOR32-Gene expression

FL, ML, HL: tarsi from fore, mid and hind legs, respectively

Figure S1. Quantitative PCR data for CquiOR32. This receptor is predominantly expressed in female antennae. Error bars represent SEM. n = 3 biological samples, each with 3 biological replicates.
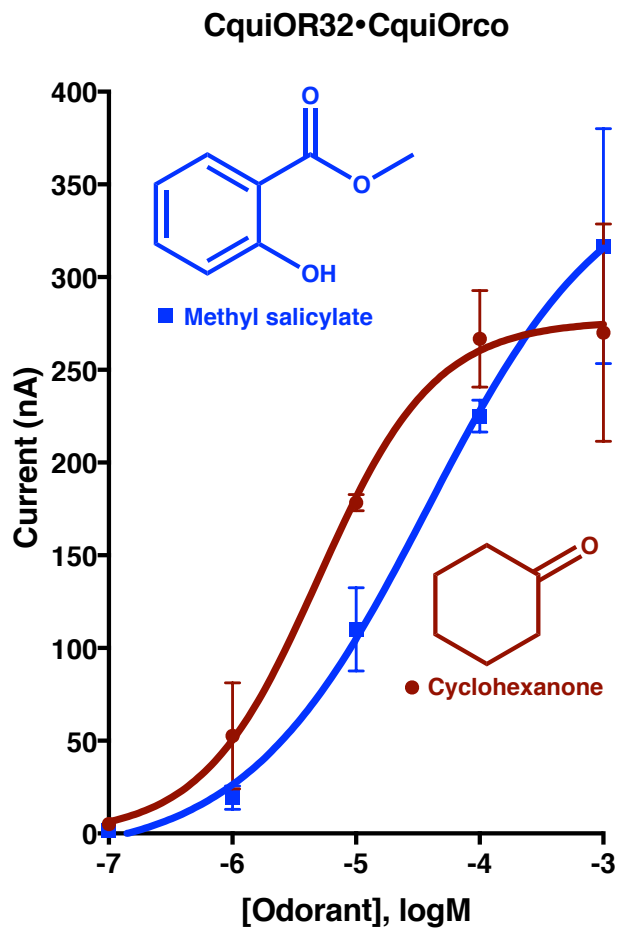
Figure S2. Concentration-response relationships, Related to Figure 1. EC_{50} for methyl salicylate and cyclohexanone: 3.7×10^{-5}M and 4.9×10^{-6}M, respectively. N = 3 for each data point, each from a different oocyte of the same batch of eggs. Error bars represent SEM.
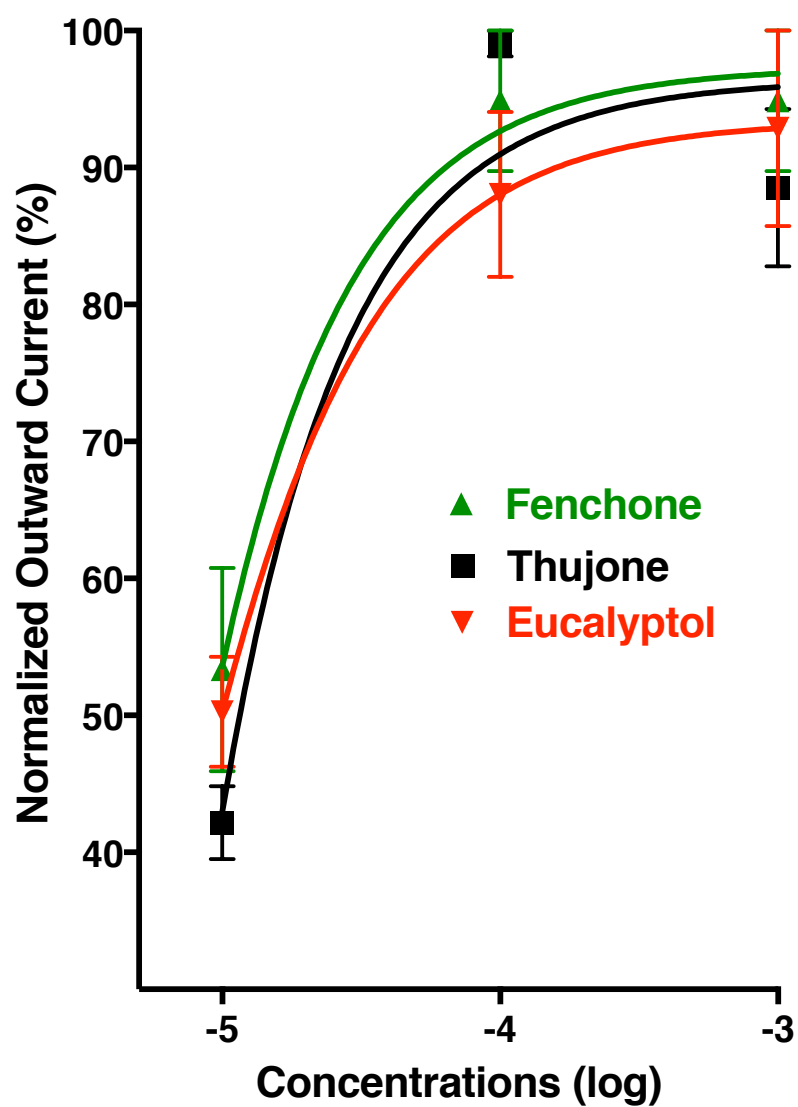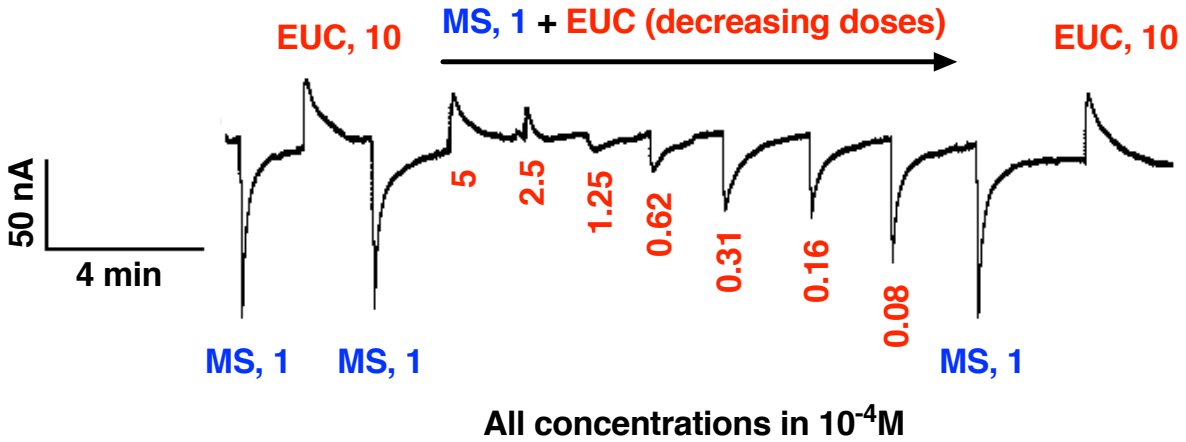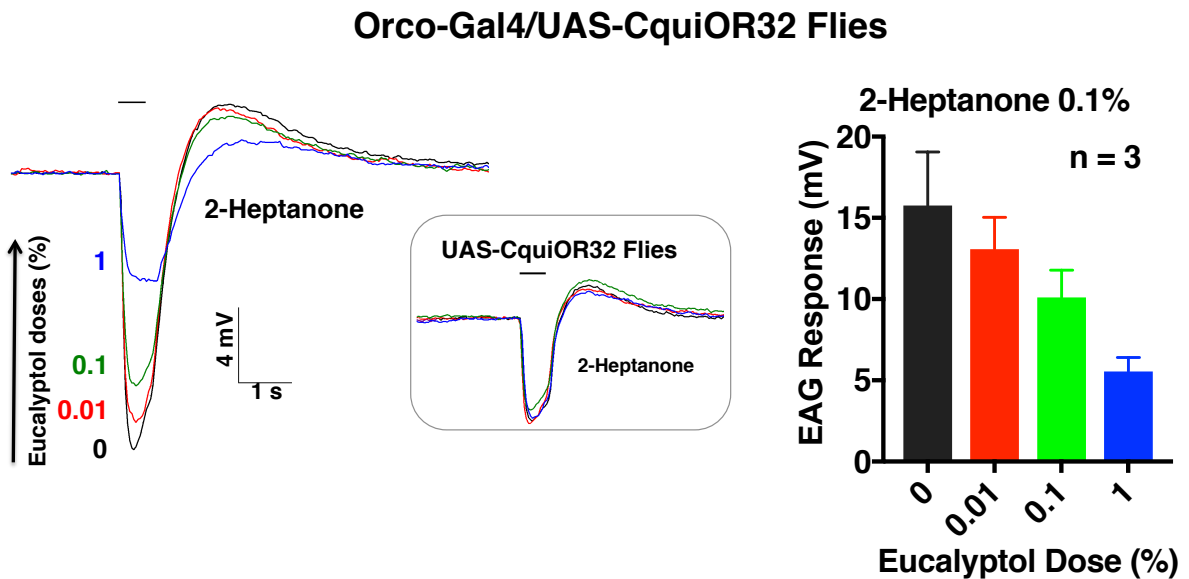
Figure S3. **Dose-dependence curves for inhibitory compounds, Related to Figure 1.**

Responses were normalized (n = 3 for each compound and dose). Error bars represent SEM.

EUC, 10   MS, 1 + EUC (decreasing doses)   EUC, 10

50 nA

4 min

5   2.5   1.25   0.62   0.31   0.16   0.08

MS, 1   MS, 1   MS, 1

All concentrations in 10^{-4}M

Figure S4. **Eucalyptol (EUC)-elicited, dose-dependent inhibition of responses of CquiOR32-CquiOrco-expressing oocytes to methyl salicylate (MS).** Continuous trace from the same oocyte.

Orco-Gal4/UAS-CquiOR32 Flies

2-Heptanone

Eucalyptol doses (%)

1
0.1
0.01
0

4 mV
1 s

UAS-CquiOR32 Flies

2-Heptanone

2-Heptanone 0.1%

n = 3

EAG Response (mV)

Eucalyptol Dose (%)
0   0.01   0.1   1

Figure S5. Inhibitory effect of eucalyptol on odorant reception in transgenic flies, Related to Figure 6. Odorant (0.1%) was codelivered onto fly antennae with eucalyptol at various doses. Error bars represent SEM. *Inset:* EAG recordings from a control line stimulated with 2-heptanone alone or coapplied with eucalyptol (0.01-1%).

SSR from ab4 sensilla

WT Flies **Orco-Gal4/UAS-CquiOR32 Flies**

Eucalyptol alone

MS alone

MS plus Eucalyptol

MS alone

MS plus Eucalyptol

MS alone

MS plus Eucalyptol

Eucalyptol alone

MS alone

MS plus Eucalyptol

MS alone

MS plus Eucalyptol

MS alone

MS plus Eucalyptol

3 mV | 1 s

Methyl salicylate 0.1%
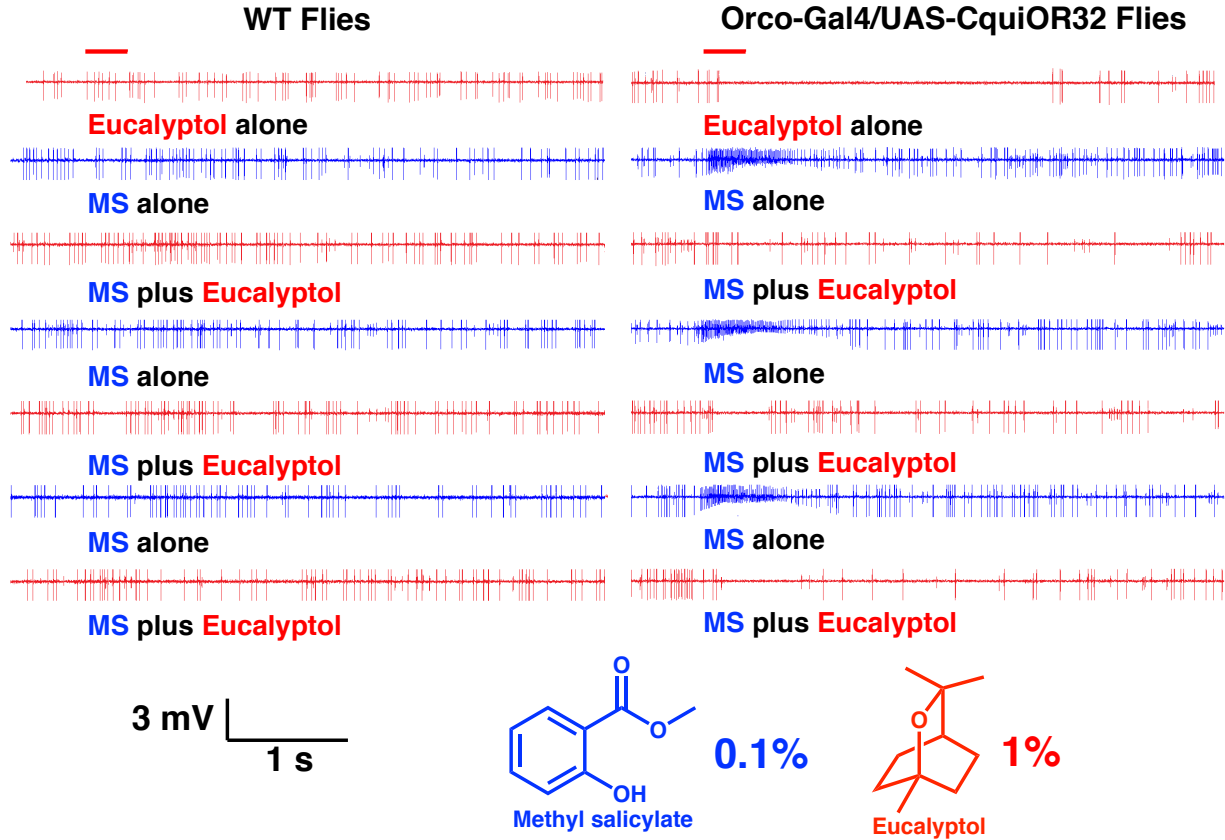
Eucalyptol 1%

Figure S6. **Single sensillum recordings from the ab4 sensilla in the antennae of WT and transgenic flies, Related to Figures 6 and 7.** Traces obtained by challenging the sensilla with methyl salicylate (MS) at 0.1% and eucalyptol at 1%.
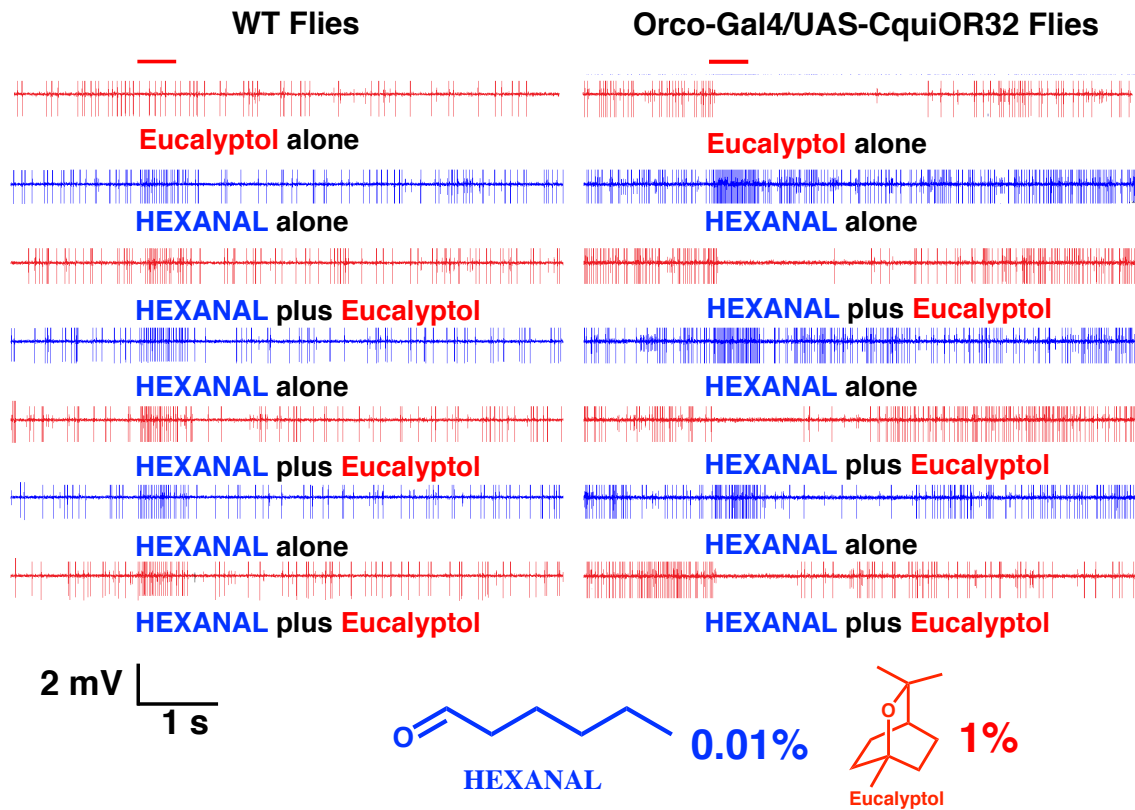
SSR from ab4 sensilla

WT Flies **Orco-Gal4/UAS-CquiOR32 Flies**

Eucalyptol alone   Eucalyptol alone

HEXANAL alone   HEXANAL alone

HEXANAL plus Eucalyptol   HEXANAL plus Eucalyptol

HEXANAL alone   HEXANAL alone

HEXANAL plus Eucalyptol   HEXANAL plus Eucalyptol

HEXANAL alone   HEXANAL alone

HEXANAL plus Eucalyptol   HEXANAL plus Eucalyptol

2 mV

1 s

HEXANAL 0.01%

Eucalyptol 1%

Figure S7. **Single sensillum recordings from the ab4 sensilla in the antennae of WT and transgenic flies, Related to Figure 6.** Traces obtained by challenging the sensilla with hexanal at 0.01% and eucalyptol at 1%.
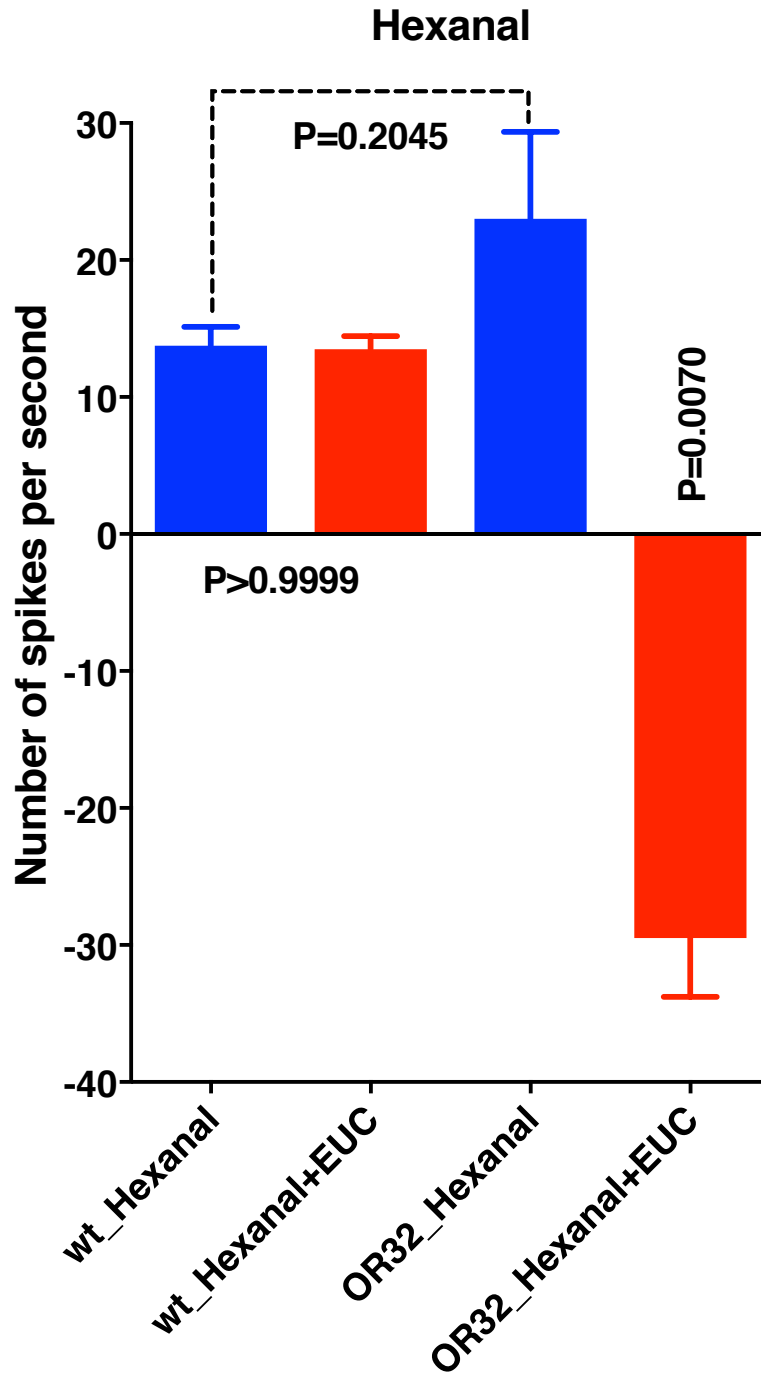
Figure S8. **Quantification of responses of WT and transgenic flies to hexanal alone or in combination with eucalyptol, Related to Figure 6.** Costimulation with eucalyptol (EUC) did not affect the response of WT flies to hexanal. By contrast, the response of Orco-GAL4/UAS-CquiOR32 flies to hexanal was completely abolished by costimulation with eucalyptol.
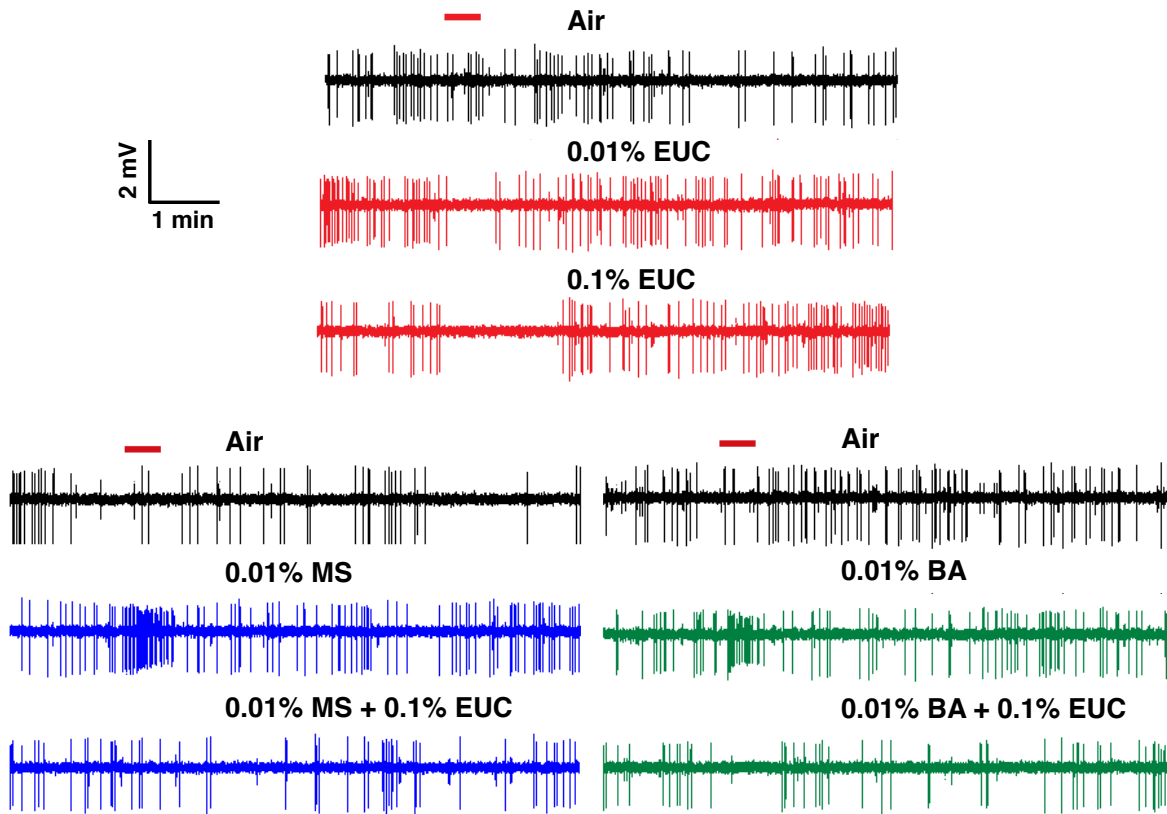
Figure S9. **Single sensillum recordings from the ab7 sensilla in the antennae of Orco-GAL4/UAS-CqOR32 flies, Related to Figure 8.** Inhibitory responses elicited by 0.01 and 0.1% eucalyptol (EUC), excitatory responses generated by 0.01% methyl salicylate (MS) or 0.01% butyl acetate (BA), and inhibition caused by costimulation with 0.1% EUC.
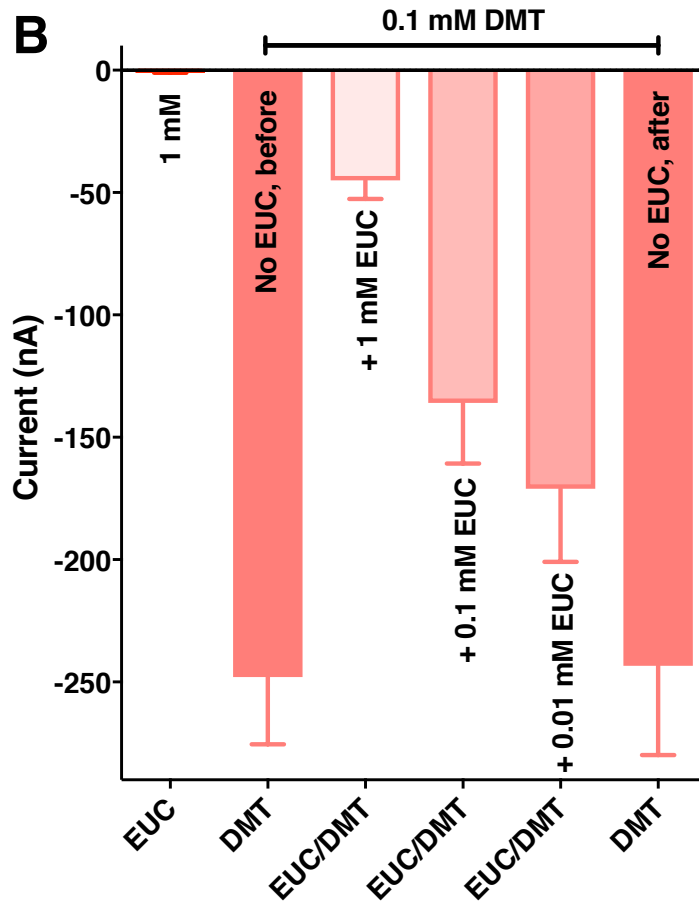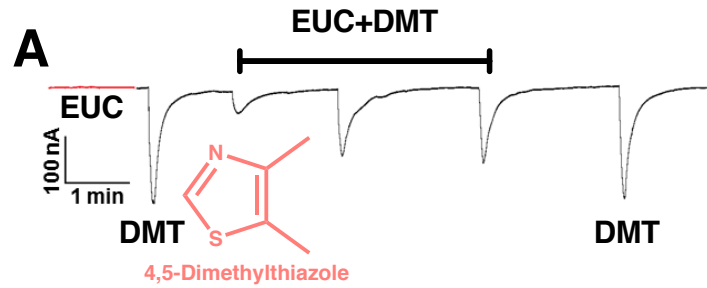
Figure S10. Eucalyptol inhibition of responses elicited by 4,5-dimethylthiazole (DMT) on oocytes coexpressing AaegOR71 and AaegOrco, Related to Figure 9. Although eucalyptol (EUC) did not elicit detectable inhibitory currents, it caused a dose-dependence reduction in DMT-elicited responses. Error bars represent SEM. n = 5. Bars with different letters are considered statistically different at the 0.05 level, according to Tukey's test.
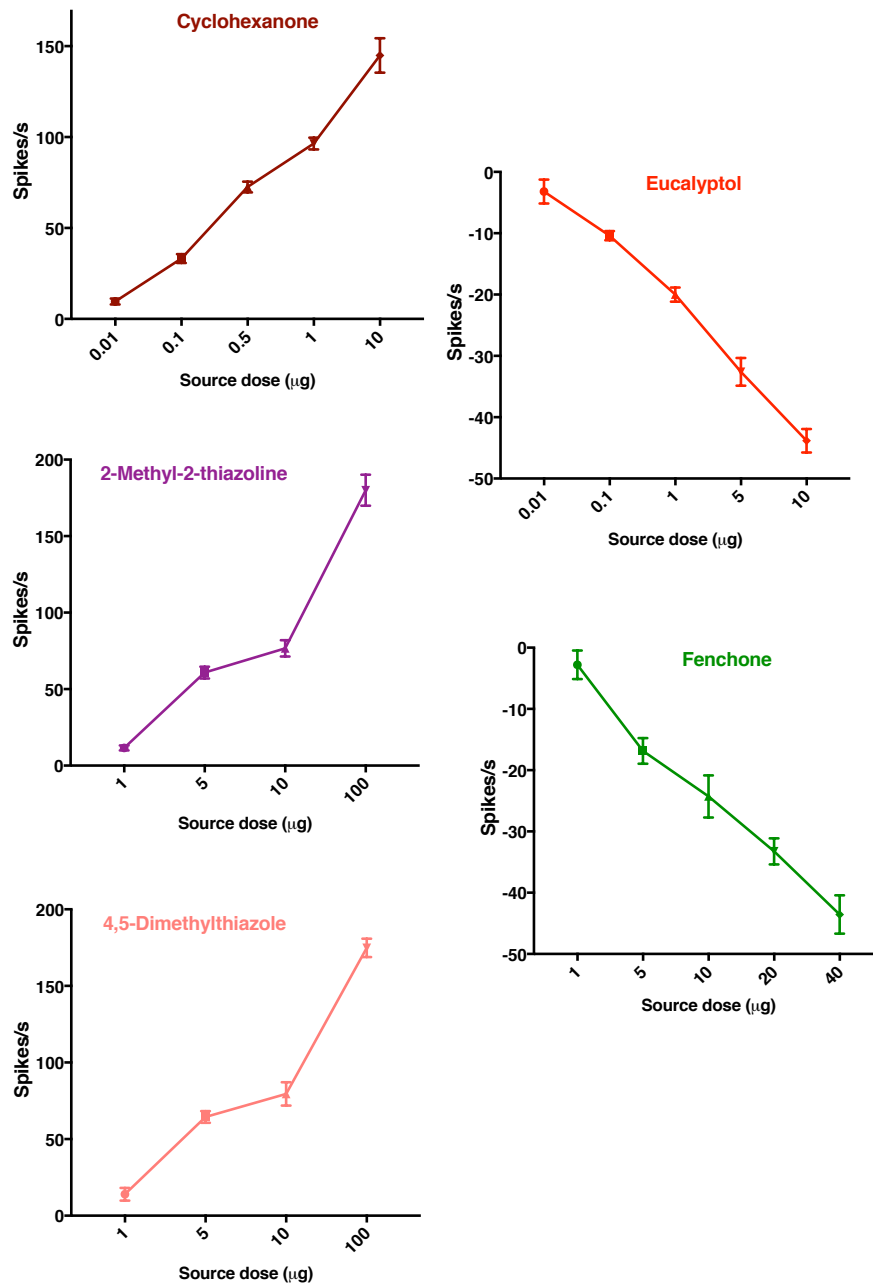
Figure S11. **Dose-dependent curves recorded from *Ae. aegypti* antennae by SSR, Related to Figure 10.** Firing rates observed during 500 ms post-stimulus period were subtracted from spontaneous activities observed in the 500 ms pre-stimulus period and the outcome was multiplied by 2 to obtain the number of spikes per second. The recording points in X-axis are not drawn to scale. Error bars represent SEM. n = 5-20.

2-Methyl-2-thiazoline (before)

2-Methyl-2-thiazoline plus Eucalyptol

2-Methyl-2-thiazoline (after)
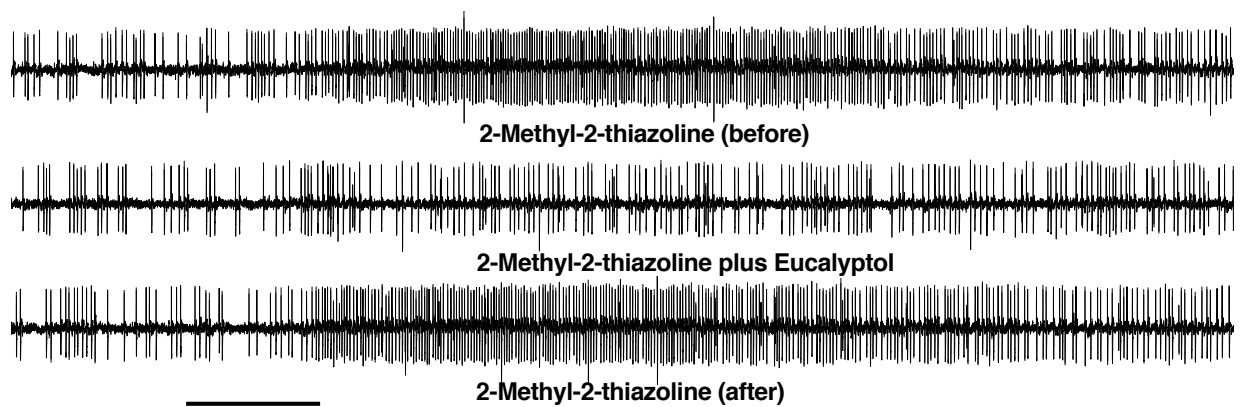
Figure S12. **SSR traces from SST-2 sensilla on *Ae. aegypti* antennae, Related to Figure 10.**

A bar beneath the traces indicates the duration of the stimulus (500 ms). Horizontal line after the

bottom trace, 4 mV.

Transparent Methods

Insect preparations

Cx. quinquefasciatus used in this study were from a laboratory colony originating from adult

mosquitoes collected in Merced, CA in the 1950s (Syed and Leal, 2008) and kept at the Kearney

Agricultural Research Center, University of California, Parlier, CA. Specifically, we used

mosquitoes from the UC Davis colony, which was initiated about 7 years ago with mosquitoes

from the Kearney colony. In Davis, mosquitoes were maintained at 27±1°C, 75±5% relative

humidity, and under a photoperiod of 12:12 h. Two colonies of *Ae. aegypti* were used in this

study, namely, the Orlando strain, kept at Auburn University, and a colony established in 2016

from eggs that were laid by females collected in BG-sentinel traps (Biogents, Regensburgs,

Germany) in the City of Clovis, California. These colonies were maintained at 25±2°C, 75±5%

relative humidity, and under a photoperiod of 12:12 h.

Behavioral studies

Repellency was measured using a previously reported surface landing and feeding assay (Leal et

al., 2017). The arena (30.5 × 30.5 × 30.5 cm) housing test mosquitoes was attached to a frame

that supported a wood board (30 × 30 × 2.5 cm) and held two Dudley bubbling tubes and two

syringe needles separated from each other by 17 cm. The Dudley tubes were painted internally

with black glass ink and dried before use. They were placed on a transverse plane at the

middle line of the wooden board. One side of the mosquito cage was prepared with a red

cardstock having openings to allow the Dudley tubes and syringe needles to protrude inside of

the mosquito cage by 5.5 and 4 cm, respectively. Each syringe was placed 8 mm above a

Dudley tube so to have enough room to tightly hold dental cotton rolls. Insect pins were placed 1.8 cm above the syringe needles to hold filter paper rings (width 4 cm; 25 cm; overlapped 1 cm for stapling), which were loaded with test repellent or solvent (hexane, control). For tests with two compounds (eg, methyl salicylate and eucalyptol), each compound was applied to one filter paper ring and the two rings were placed concentrically in the test side of the arena. Once defibrinated sheep blood (100 μl) was loaded on dental cotton rolls, carbon dioxide started to flow at 50 ml/min from each needle, and water at 38 °C circulated inside the Dudley tubes. The two choices differed only by a curtain of repellent at the side with the treatment filter paper ring. Assays were recorded with a camcorder equipped with Super NightShot Plus infrared system (Sony Digital Handycam, DCR-DVD 810) and the number of mosquitoes on each side of the arena were counted at the end of each test. Behavioral responses are expressed in protection rate, according to WHO and EPA recommendations: P%=(1-[T/C]) X 100, where T and C represent the number of mosquitoes in treatment and control sides of the arena.

OR cloning

Total RNA samples were extracted from 1 thousand 4-7 day-old *Culex* female antennae and 800 *Aedes* female antennae with TRIzol reagent (Invitrogen, Carlsbad, CA). Antennal cDNA was synthesized from 1 μg of antennal total RNA from each species using a SMARTer™ RACE cDNA amplification kit according to manufacturer's instructions (Clontech, Mountain View, CA). To obtain full-length coding sequences of CquiOR32, PCRs were performed using gene-specific primers containing restriction endonuclease sites and Kozak motif (acc): CquiOR32 Fwd-XmaI (underlined) primer, 5'-

TCCC<u>CCCGGG</u>GGGAaccATGTTCACCACTCAAAACAGTCACC-3'and Rev-XbaI

(underlined) primer, 5'-GC<u>TCTAGA</u>GCTTATATCATAGTCAACGCTTCCTTCAGCA-3'.

PCR products were purified by a QIAquick gel extraction kit (Qiagen) and then cloned into

pGEM-T vector (Promega). Plasmids were extracted using a QIAprep spin mini prep kit

(Qiagen) and sequenced (Davis Sequencing). To subclone CqOR32 into pGEMHE, pGEM-T-

CquiOR32 was digested by XmaI and XbaI (BioLabs) before being subcloned. After

transformation, plasmids were extracted using the QIAprep Spin Miniprep kit (Qiagen) and

sequenced by ABI 3730 automated DNA sequencer at Davis Sequencing (Davis, CA) for

confirmation. Likewise, AaedOR71 was cloned with a pair of cloning primers: AaeagOR71-

Fwd: AGATCAATTCCCCGGGaccATGGAACTGTCCTACCATCGAAGTC

AaegOR71-Rev: TCAAGCTTGCTCTAGACTAAAGCCGATCCAGAACATCTTTG

Quantitative PCR, qPCR.

For qRT-PCR, each type of tissue (antennae, maxillary palps, proboscis, and legs) from 300

blood-fed female mosquitoes (4-7 days old) was dissected and collected in TRIzol reagent

(Invitrogen, Carlsbad, CA) on ice using a stereo microscope (Zeiss, Stemi DR 1663, Germany).

Total RNA was extracted using TRIzol reagent. After RNA was quantified on NanoDrop Lite

spectrometer (Thermo Fisher Scientific, Rockford, IL), cDNA was synthesized from 200 ng of

equal amount RNA using iScript™ Reverse Transcription Supermix for RT-qPCR according to

the manufacturer's instructions (Bio-Rad, Hercules, CA). Real-time quantitative PCR (qPCR)

was carried out by using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) and

SsoAdvanced SYBR Green Supermix (Bio-Rad). *CquiRPS7* gene was used as the reference. The

following primers, designed by Primer 3 program ([http://frodo.wi.mit.edu/)](http://frodo.wi.mit.edu/), were used:

CquiOR32-Fw: 5'-GCGATTTTTGCTTCGAAAAG-3'

CquiOR32-Rv: 5'-GTGCGTCCAATACCGAAAGT-3'.

qPCR was performed with 3 biological replicates, and each of them was replicated 3 times (3 technical replicates per biological replicate); data were analyzed using the $2-\Delta\Delta CT$ method.

Electrophysiology

D. melanogaster w[1118] and Orco-GAL4/UAS-CquiOR32 homozygous flies were used for EAG (Ueira-Vieira et al., 2014) and other electrophysiological studies. The EAG apparatus (Syntech Ltd., Hilversum, The Netherlands) was linked to a computer with an EAG2000 data acquisition interface. Recording and indifferent electrodes were made of Ag/AgCl wires enclosed in drawn glass capillary needles, which were filled with 1 M potassium chloride in 1% polyvinylpyrrolidone. The reference electrode was inserted in the eye of an immobilized insect and the recording electrode was placed on the third segment of a fruit fly antenna by using a micromanipulator MP-12 (Syntech). Single sensillum recording from flies were obtained as previously reported (Syed et al., 2010). Compounds used as stimuli were freshly dissolved in paraffin oil and loaded on a filter paper strip (1 cm^2), which were placed into Pasteur pipettes as cartridges. The preparation was bathed in a high-humidity air stream flowing from a Stimulus Controller CS-55 (Syntech) at 610 mL/min to which compensatory flow or stimulus pulse (125 mL/min, 300 ms) was added. For dual delivery, the stimulus flow was split and passed through 2 cartridges, each one having a filter paper strip laden with one of the tested compounds. The outlets of these cartridges merged in the continuous flow and placed 1 cm away from the antennal preparation. Signal from the antenna induced by stimulus or control puff was recorded for 10 s. Gas chromatography with electroantennographic detection (GC-

EAD) was done with a gas chromatograph (HP 5890 Series II Plus, Agilent Technologies, Palo Alto, CA) equipped with transfer line and temperature control units (Syntech, Kirchzarten, Germany). The effluent from the capillary column was split into EAD and flame ionization detector (FID) in a 3:1 ratio. The analog signal was fed into an A/D 35900E interface (Agilent Technologies) and acquired simultaneously with FID signal on an Agilent Chemstation. The gas chromatograph was equipped with a capillary column (HP-5MS, 30 m×0.25 mm; 0.25 μm; Agilent Technologies). The temperature program started at 70°C for 1 min, increased at a rate of 10°C/min to 110°C then increased at a rate of 20°C/min to 290°C and finally held at this final temperature for 1 min. After identifying the retention times of eucalyptol and methyl salicylate, data were acquired from 4 to 8 min. Both injection port and detector were operated at 250°C.

For mosquito SSR, 4- to 5-day-old females were used after being anesthetized on ice and fixed with a 200-μL pipette tip (6). Mosquitoes were fixed with dental wax and using a cover slip (22 X 22 mm) with double-sided tape. The reference tungsten electrode was inserted into one eye of a test mosquito, whereas the recording electrode was inserted into the shaft of a test sensillum under a microscope (Leica Z6 Apo) by using a micromanipulator (Leica, Cat #: 115378). Chemical compounds used as stimuli were freshly prepared with dimethyl sulfoxide (DMSO) at desired concentrations and 1 μL of each chemical solution was dispersed onto a piece of filter paper (3 × 45 mm), which was inserted into a glass Pasteur pipette to create a stimulus cartridge. The preparation was bathed in a high-humidity air stream flowing from a Stimulus Controller CS-55 (Syntech) at 20 mL/s to which compensatory flow or stimulus pulse (0.5 L/min, 500 ms) was added. The signal acquired by a preamplifier (Universal AC/DC Probe Gain 10X, Syntech) was digitized by using an IDAC 4 (Syntech). Action potential evoked by stimulus or control puff was recorded for 10 s, starting 1 s before the stimulation.

Action potentials were counted off-line over a 500-ms period before and after the stimulation (Liu et al., 2013). Specifically, firing rates observed during 500 ms post-stimulus were subtracted from spontaneous activities observed in the 500 ms pre-stimulus period and the outcome was multiplied by 2 to obtain the number of spikes per second (Liu et al., 2013).

The 2-electrode voltage-clamp technique (TEVC) was performed as previously described (Leal et al., 2013, Xu et al., 2014, Xu et al., 2012a, Xu et al., 2012b, Zhu et al., 2013). Briefly, the capped cRNAs were synthesized using pGEMHE vectors and mMESSAGE mMACHINE T7 Kit (Ambion). Purified OR cRNAs were resuspended in nuclease-free water at 200 ng/mL and 9.2 nl aliquots were microinjected with the same amount of CquiOrco cRNA into *Xenopus laevis* oocytes in stage V or VI (purchased from EcoCyte Bioscience, Austin, TX). Then, the oocytes were kept at 18°C for 3-7 days in modified Barth's solution (NaCl 88 mM, KCl 1 mM, NaHCO$_3$ 2.4 mM, MgSO$_4$ 0.82 mM, Ca(NO$_3$)$_2$ 0.33 mM, CaCl$_2$ 0.41 mM, HEPES 10 mM, pH 7.4) supplemented with 10 mg/mL of gentamycin, 10 mg/mL of streptomycin. Odorant-induced currents at holding potential of -80 mV were collected from oocytes bathed in perfusion Ringer (NaCl 96 mM, KCl 2 mM, CaCl$_2$ 1.8 mM, MgCl$_2$ 1 mM, HEPES 5 mM. pH 7.6) flowing at 3.2 ml/min. For I-V curves, the holding potentials were held at -80, -60, -40, -20, 0, +20 and +40 mV. Stimulus were injected (100 µl in 2 s) at 1 cm upstream of the flow. After each stimulus, oocytes were thoroughly washed until a steady baseline was recovered. Source doses rather than the actual doses reaching oocytes are reported. Currents were amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT), low-pass filtered at 50 Hz and digitized at 1 kHz. Data acquisition and analysis were carried out with Digidata 1440A and pCLAMP 10 software (Molecular Devices, LLC, Sunnyvale, CA). The panel of odorants is detailed in Table 1. N-(4-

ethylphenyl)-2-[(4-ethyl-5-pyridin-3-yl-1,2,4-triazol-3-yl)sulfanyl]acetamide (VUAA-1) was used as an Orco agonist.

REFERENCES

LEAL, W. S., BARBOSA, R. M., ZENG, F., FAIERSTEIN, G. B., TAN, K., PAIVA, M. H., GUEDES, D. R., CRESPO, M. M. & AYRES, C. F. 2017. Does Zika virus infection affect mosquito response to repellents? *Sci Rep,* 7**,** 42826.

LEAL, W. S., CHOO, Y. M., XU, P., DA SILVA, C. S. & UEIRA-VIEIRA, C. 2013. Differential expression of olfactory genes in the southern house mosquito and insights into unique odorant receptor gene isoforms. *Proc Natl Acad Sci U S A,* 110**,** 18704-9.

LIU, F., CHEN, L., APPEL, A. G. & LIU, N. 2013. Olfactory responses of the antennal trichoid sensilla to chemical repellents in the mosquito, *Culex quinquefasciatus*. *J Insect Physiol,* 59**,** 1169-77.

SYED, Z., KOPP, A., KIMBRELL, D. A. & LEAL, W. S. 2010. Bombykol receptors in the silkworm moth and the fruit fly. *Proc Natl Acad Sci U S A,* 107**,** 9436-9.

SYED, Z. & LEAL, W. S. 2008. Mosquitoes smell and avoid the insect repellent DEET. *Proc Natl Acad Sci U S A,* 105**,** 13598-603.

UEIRA-VIEIRA, C., KIMBRELL, D. A., DE CARVALHO, W. J. & LEAL, W. S. 2014. Facile functional analysis of insect odorant receptors expressed in the fruit fly: validation with receptors from taxonomically distant and closely related species. *Cell Mol Life Sci,* 71**,** 4675-80.

XU, P., CHOO, Y. M., DE LA ROSA, A. & LEAL, W. S. 2014. Mosquito odorant receptor for DEET and methyl jasmonate. *Proc Natl Acad Sci U S A,* 111**,** 16592-7.

XU, P., GARCZYNSKI, S. F., ATUNGULU, E., SYED, Z., CHOO, Y. M., VIDAL, D. M., ZITELLI, C. H. & LEAL, W. S. 2012a. Moth sex pheromone receptors and deceitful parapheromones. *PLoS One,* 7**,** e41653.

XU, P., HOOPER, A. M., PICKETT, J. A. & LEAL, W. S. 2012b. Specificity determinants of the silkworm moth sex pheromone. *PLoS One,* 7**,** e44190.

ZHU, F., XU, P., BARBOSA, R. M., CHOO, Y. M. & LEAL, W. S. 2013. RNAi-based demonstration of direct link between specific odorant receptors and mosquito oviposition behavior. *Insect Biochem Mol Biol,* 43**,** 916-23.