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The sulcatone receptor of the strict nectar-feeding mosquito *Toxorhynchites amboinensis*

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ABSTRACT

Controlling *Ae. aegypti* populations and the prevention of mosquito bites includes the development of monitoring, repelling and attract-and-kill strategies that are based on understanding the chemical ecology of these pests. Olfactory-mediated attraction to mammals has recently been linked to the mosquito *Aedes aegypti* odorant receptor *Or4*, which is activated by animal-released 6-Methyl-5-hepten-2-one (sulcatone). This odorant is also a major component of flower scents and may play a role outside animal-host seeking. To explore the role of this chemical cue, we looked at the interaction between sulcatone and an *Or4* homolog expressed in the antennae of the strict nectar-feeding mosquito *Toxorhynchites amboinensis*. Using the two-electrode voltage clamp of *Xenopus* oocytes as a heterologous expression system, we show that this receptor is a high intensity sulcatone receptor comparable to its *Aedes* counterparts. We also show that OR4 is activated by other aliphatic ketones and is inhibited by DEET. This pharmacological characterization suggests that sulcatone may be operating in more than one context in the Culicidae family.

1. Introduction

The hematophagous mosquito *Aedes aegypti* transmits viruses causing dengue fever, yellow fever, Zika and chikungunya throughout the tropics and remains a major nuisance in developed countries. Different populations of *Ae. aegypti* with different animal-host preferences thrive in populated urban/suburban areas as well as in forest environments. The prevention of mosquito bites in urban/suburban areas using non-insecticide-based strategies is predicated on understanding the chemical ecology of these pests.

Acetone, aldehydes, ammonia, carbon dioxide, carboxylic acid, indoles, (*R*)-1-octen-3-ol have been associated with animal-host attraction. The molecular mechanisms involved in the detection of these odorants include members of three membrane bound receptor families known as the odorant receptors (ORs), gustatory receptors (GRs) and ionotropic receptors (IRs). Human host-seeking requires the obligate OR-coreceptor (Orco) (Degennaro et al., 2013), the IR co-receptors IR8a (Raji et al., 2019) and the CO_2 receptor GR3 (McMeniman et al., 2014). The (*R*)-1-octen-3-ol receptor OR8 (Dekel et al., 2016) and the indolergic receptors (Dekel et al., 2019) may also be involved in animal-host attraction but recent pharmacological characterization of their homologs in the non-hematohagous mosquito *Toxorhynchites amboinensis* suggest that they operate outside animal-host-seeking.

Recently, sulcatone has been linked to human preference in domestic populations of *Ae. aegypti aegypti* through the mediation of odorant receptor 4 (*AaegOr4*) (McBride et al., 2014). Forest-dwelling zoophilic populations of *Aedes aegypti formosus* exhibit various *Or4* alleles with relatively lower sulcatone sensitivity than its domestic counterparts. However, the role of sulcatone may be more complex due to its non-animal origins. Sulcatone is a common floral compound produced by over 400 plant species (Pherobase, El-Sayed, 2018) and a predominant volatile released by fruits and essential oils (Anet, 1972; Green et al., 2012). The biogenic origin of sulcatone associated with humans and cattle (Birkett et al., 2004) is likely fungal (Buśko et al., 2014; Rines et al., 1974; Van Lancker et al., 2008; Wilkins, 1996) and bacterial (Dickschat et al., 2005; Höckelmann and Jüttner, 2004). Thus, we surmised that this volatile compound may be detected by mosquitoes in various contexts, including plant-host seeking (Fig. 1a).

Despite their ecological success and protein-rich blood-feeding habits, a small proportion of mosquito species exhibit a strict vegetarian diet. The elephant mosquito *Toxorhynchites amboinensis* belongs to one of these obligatory nectar-feeding mosquito species that exclusively feeds on plant hosts during the adult stage. This atypical feeding habit among mosquitoes provides an opportunity to explore the functional evolution of olfactory traits and their ecological significance (Fig. 1a).

T. amboinensis and *Aedes aegypti* diverged about 40 My ago (Fig. 1a).

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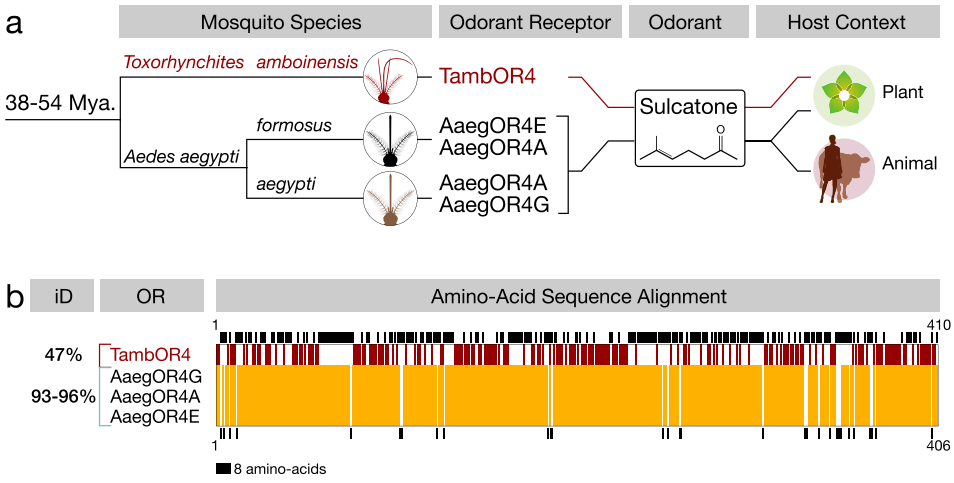

Fig. 1. Plant- and animal-host seeking mosquitoes express an OR4 homolog
a, Odorant receptor 4 from the nectar feeding mosquito *Toxorhynchites amboinensis* (TambOR4), the forest/zoophilic *Aedes aegypti formosus* A and E alleles (AaegOR4A & E) and domestic/anthropophilic *Aedes aegypti aegypti* A and G alleles (AaegOR4A & G) may be tuned to sulcatone, which is released by plants and animals. **b**, TambOR4 and AaegOR4A,E,G share 47% amino-acid identity. Amino-acid identity (iD) between TambOR4 and AaegOR4A,E,G are colored in red and within the AaegOR4 alleles are colored in orange. Amino-acid differences between TambOR4 and AaegOR4, and within AaegOR4 alleles are shown in black. The source alignment is available in Supplementary Fig. 2. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

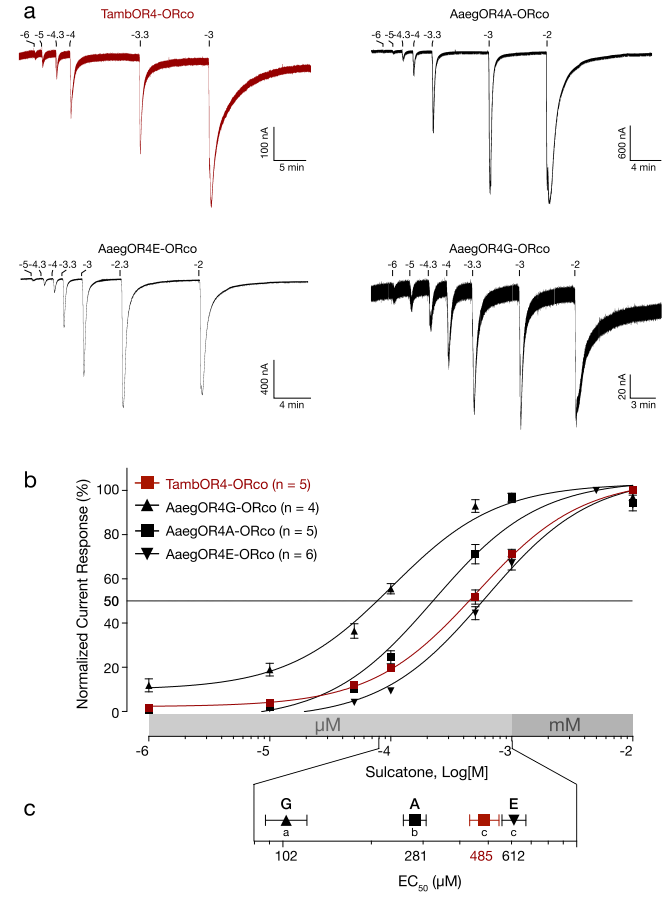

Fig. 2. TamOR4 is activated by high sulcatone concentrations
a, Example current traces of OR4 response to log concentrations of sulcatone (serial dilutions). Sulcatone concentrations are converted into Log values. **b**, Concentration-response relationships of TambOR4-ORco and AaegOR4-ORco complexes in response to increasing sulcatone concentrations. Current amplitudes were normalized to the maximum response. **c**, Interpolated half maximal effective concentration (EC_{50} in micromolar) are magnified below. Statistically significant differences are displayed by small letters (multiple comparison ordinary one-way ANOVA; $P < 0.001$). Odorant concentrations were plotted on a logarithmic scale. Each data point represents the mean and error bars indicate s.e.m.

They share a majority of their chemosensory genes including odorant receptors (Zhou et al., 2014). Recently, functional orthologs of the (*R*)-1-octen-3-ol receptor OR8 (Dekel et al., 2016) and indolergic receptors (Dekel et al., 2019) have been identified in this non-blood-feeding mosquito questioning the ecological role of these compounds in animal-host seeking. In addition to raising questions about the potential pleiotropy of mosquito chemical cues, understanding the context in which these mosquito attractants operate may provide new tools or optimize existing ones to manipulate mosquito behavior (attract and repel) and to monitor their populations.

To explore the potential ecological role of sulcatone in plant-host seeking, we have applied a pharmacological approach to study its functional relationship to the closest OR4 homolog in *T. amboinensis* (TambOR4). We show that TambOR4 is a sulcatone receptor based on two pharmacological criteria including selectivity towards ketones and a sulcatone sensitivity comparable to its *Aedes* counterparts, suggesting that this odorant cue is used in a non-animal host seeking context in *T. amboinensis*.

2. Materials and methods

2.1. In vitro transcription

TambOr4 was custom synthesized (Bio Basic Inc. Markham, Ontario, Canada) using published sequenced information (Zhou et al., 2014) available here (https://figshare.com/articles/Sequence_and_functional_annotation_of_T_amboinensis_genes/1092617; last accessed March 18, 2019). *AaegOr4* alleles A, E and G were kindly provided by Dr. Carolyn S. McBride (Princeton, NJ, USA). All four genes were subcloned into the *Xenopus laevis* expression destination vector, pSP64t RFA.

2.2. Electrophysiological recordings

Expression and pharmacological characterization of TambOR4-TambORco and AaegOR4-AaegORco alleles were performed as described previously (Dekel et al., 2016) using the two-electrode voltage clamp electrophysiological recording of *Xenopus* oocytes. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (NIH approval number: OPRR-A01-5011). All receptor genes were transcribed in vitro, injected into stage V-VI oocytes and responses were recorded 3–4 days later using a −80 mV holding potential. For the establishment of concentration-response curves, oocytes were exposed to sulcatone dilutions (10^{-6} M to 10^{-2} M) for 8 s. Effective concentrations eliciting 50% of the maximum response (EC_{50} values) and ordinary one-way ANOVA were performed using GraphPad Prism version 7.0c for Mac OS X, GraphPad Software,

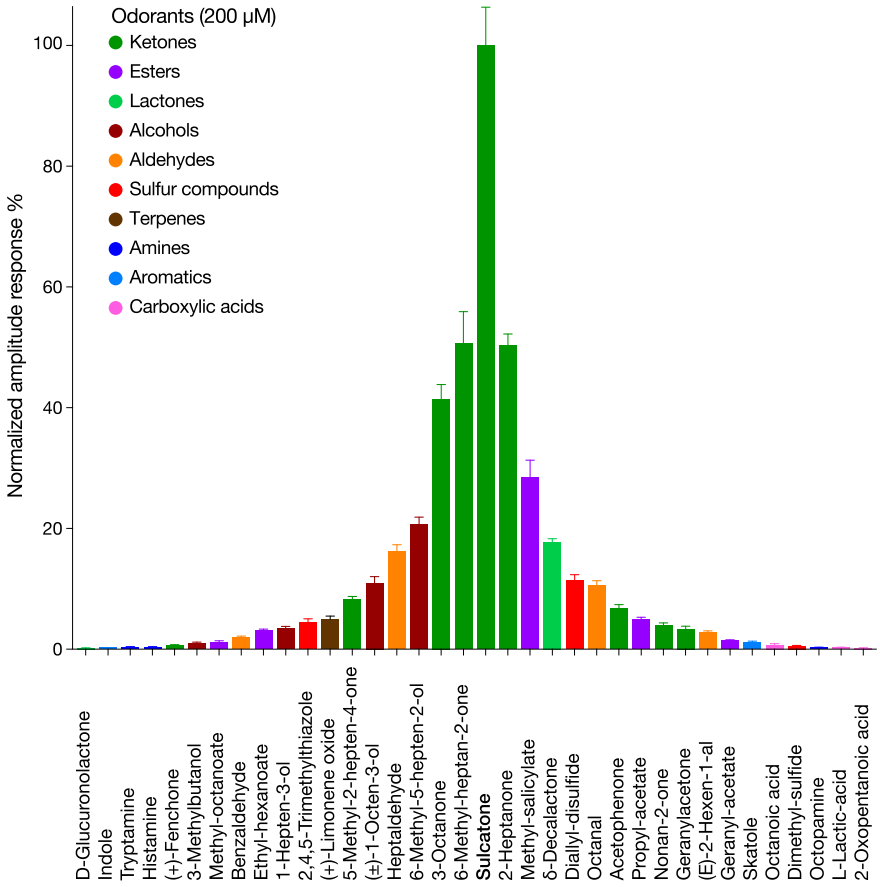

Fig. 3. OR4 is tuned to aliphatic ketones
AaegOR4A is activated by high concentrations (200 µM) of aliphatic ketones, including a subset of sulcatone chemical analogs. Methyl salicylate (magenta background) is an insect repellent of plant origin.

La Jolla California USA, www.graphpad.com. The response profile of OR4 was conducted using the AaegOR4A allele. A panel of 36 odorants belonging to a wide variety of chemical structures as well as close chemical analogs of sulcatone were applied at 200 µM, which corresponds to the EC_{50} value of this receptor for sulcatone. The amplitude of each response for any given compound was normalized to an internal sulcatone response set at 100%. Current was allowed to return to baseline between drug administrations.

2.3. Chemical reagents

For establishing the tuning curve, we used 36 chemicals (Supplementary Fig. 1) available commercially from Sigma-Aldrich (Milwaukee, WI, USA), Merck (Darmstadt, Germany), Alfa-Aesar (Ward Hill, MA, USA), Fluorochem (Graphite Way, Hadfield, UK) and Acros Organics (Thermo Fisher Scientific, Waltham, MA, USA). *N*,*N*-Diethyl-*m*-toluamide (DEET; CAS number 134-62-3) was obtained from Sigma-Aldrich (Milwaukee, WI, USA).

3. Results

3.1. Toxorhynchites expresses a divergent Or4 homolog

To explore the ecological context and functional evolution of *Or4*, we compared its homolog in the strict nectar-feeding mosquito *T. amboinensis* (Zhou et al., 2014) with its allelic counterparts from domestic and forest forms of *Ae. aegypti* (Fig. 1b). While AaegOR4 alleles share 93–96% amino-acid sequence identity, the closest *T. amboinensis Or4* homolog (m.36212; named here *TambOr4*) encodes a divergent protein (47%; Fig. 1b), which is expressed in the antennae of this insect (Zhou et al., 2014). Considering the low sequence similarity of OR4 between these two species and the nectar-feeding habit of *T. amboinensis*, we expected that TambOR4 would be less or not sensitive to sulcatone than its *Aedes* counterparts.

3.2. TambOR4 is a high intensity sulcatone receptor

Using the two-electrode voltage clamp of *Xenopus* oocytes, we tested this hypothesis by comparing the responses of TambOR4 to three of the 7 previously identified AaegOR4 alleles (alleles A, E and G) (McBride et al., 2014) (Fig. 2). For this analysis, we chose the least sensitive allele (allele E), the most sensitive allele (allele G) and an allele with intermediate sensitivity (allele A). The relative sensitivity between the *Ae. aegypti* alleles was consistent with the physiological response of *Or4*-expressing neurons (McBride et al., 2014). In our repeated recording sessions, we noticed that while AaegOR4G exhibited the highest sensitivity, the response amplitudes were consistently lower than the other two alleles (Fig. 2a). Sulcatone was equally potent towards TambOR4 and AaegOR4E, the least sensitive allele belonging to the forest form of *Ae. aegypti* (Fig. 2b). TambOR4 was activated by sulcatone within the same upper micromolar range exhibited by all three tested AaegOR4 alleles (Fig. 2b) indicating that TambOR4 and AaegOR4 are functional orthologs.

3.3. Sulcatone is a potent OR4 activator

Based on the sulcatone sensitivity exhibited by AaegOR4 alleles (McBride et al., 2014), we surmised that OR4 may be activated by other ketones. We challenged OR4 with a panel of 36 odorants belonging to a wide variety of chemical structures and to a subset of commercially

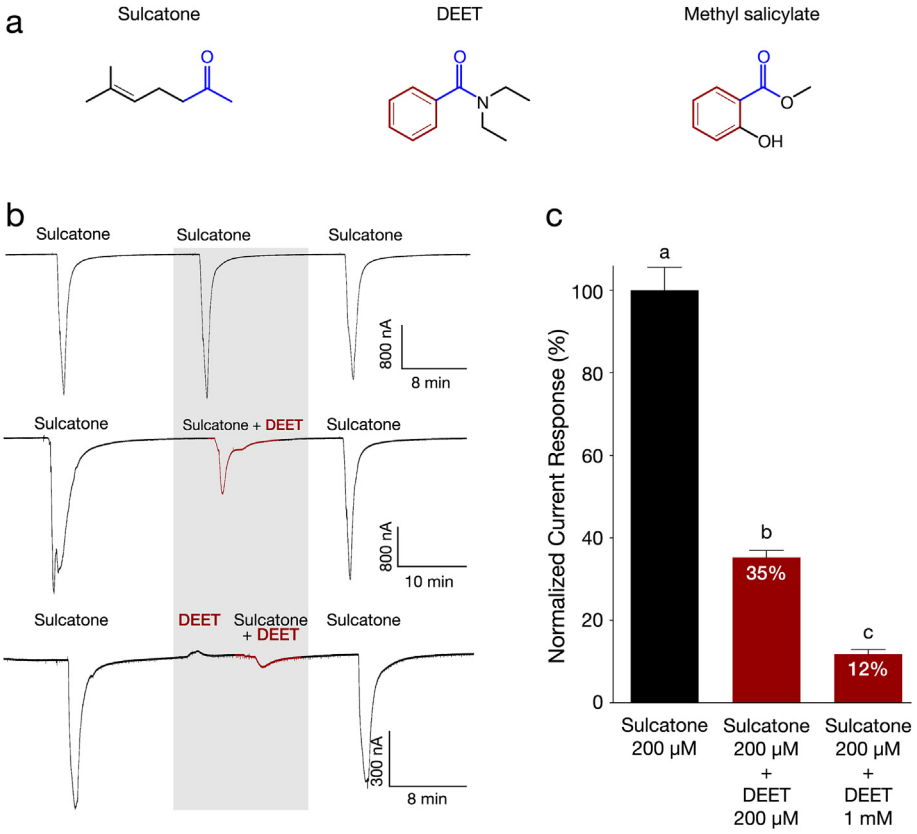

Fig. 4. OR4 is inhibited by the insect repellent DEET **a**, Structural formulas of sulcatone (6-methyl-5-hepten-2-one), DEET and methyl-salicylate. Carbonyl and benzene groups are shown in blue and red, respectively. **b**, Example current traces of AaegOR4A-ORco responses to sulcatone alone (200 µM) or to a mixture of sulcatone (200 µM) and DEET (middle trace 200 µM; bottom trace, 1 mM). **c**, The synthetic insect repellent DEET inhibits sulcatone-activated OR4. Methyl salicylate and DEET share a benzene moiety (red) and a carbonyl group (blue). . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

available sulcatone analogs. For this screen, we used allele A of AaegOR4 due to its ability to generate high current amplitudes in response to sulcatone (Fig. 3). We used 200 µM (corresponding to the EC_{50} of OR4-sulcatone) for all tested odorants. Overall, OR4 exhibited a response profile tuned towards ketones. Sulcatone elicited the strongest amplitude followed by 6-methylheptan-2-one, 2-heptanone, 3-octanone and methyl salicylate (Fig. 3).

3.4. TambOR4 is inhibited by the insect repellent DEET

Interestingly, methyl salicylate and the insect repellent DEET share similar moieties, including a benzene ring and a carbonyl group (Fig. 4a) suggesting that the latter may also activate AaegOR4A. We thus challenged OR4 with DEET alone or in combination with sulcatone using equimolar concentrations of both compounds. DEET alone did activate OR4 by eliciting a small membrane hyperpolarization (Fig. 4b). Surprisingly, DEET significantly inhibited the OR4 response to sulcatone, an effect that appeared to be dose-dependent (Fig. 4c).

4. Discussion

We provide evidence that the strict nectar-feeding mosquito *T. amboinensis* expresses a high intensity (Hallem et al., 2004) sulcatone receptor functionally orthologous to its *Aedes* counterparts. This finding suggests that sulcatone is used by a non-blood feeding mosquito in a non-animal host-seeking context. Because sulcatone is a ubiquitous compound produced by microbes and a major component of flower scents, we suggest that it may act as a foraging or oviposition cue in this species. Our findings do not exclude the possibility that sulcatone may be used in multiple contexts in *Ae. aegypti*, outside animal host-seeking. While both species may detect sulcatone using the same OR machinery, this information may be processed differently by the brain leading to different outcomes, i.e., one species interpreting sulcatone in a non-animal host context and the other to identify a potential blood meal. Further behavioral experiments are warranted to test these hypotheses.

The OR4-sulcatone interaction is an adaptation to animal-attraction in the forest and domestic forms of *Ae. aegypti*, and with a more recent modification of sensitivity, an adaptation to anthropophily in *Ae. aegypti aegypti* (McBride et al., 2014). In this scenario, the OR4-sulcatone interaction, which predates the 38–54 million year old *Aedes-Toxorhynchites* split, operates at a sulcatone concentration reflecting its elevated emission levels in humans (McBride et al., 2014). Our results suggest that OR4 is a sulcatone receptor that registers high odorant intensity. We have found that the relative sensitivity of the *Aedes* OR4 alleles towards sulcatone is consistent with what has been shown at the physiological level (McBride et al., 2014). The EC_{50} values reported here clustered within one log unit of the upper micromolar concentration range (Fig. 2) indicating that OR4 is activated by high sulcatone concentrations, consistent with the high level of this compound found in human odor.

OR4 is another mosquito receptor shown to be modulated by DEET. High concentrations of insect repellents such as the synthetic DEET compound have been shown to exert multiple effects on insect ORs (Bohbot and Dickens, 2012, 2010; Bohbot et al., 2011). We have shown that an equimolar concentration of DEET inhibits sulcatone-activated OR4. We also show that other aliphatic ketones (e.g., 6-methylheptan-2-one, 2-heptanone) and methyl salicylate activate OR4 at concentrations comparable to sulcatone. The latter has been shown to repel *Ae. aegypti* (Dekker et al., 2011) while the former appears to repel insects in general (Farrar and Kennedy, 1987). The study of non-blood-feeding mosquitoes is a valuable approach to explore the ecological context(s) in which candidate semiochemicals are used. A better understanding of when and how these insects use olfactory cues to identify specific resources will help develop improved vector control and personal protection tools.

Conflicts of interest

The authors declare no conflict of interest.

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Author contribution statement

J.D.B planned and designed the study. A.D. and E.Y. performed experiments. J.D. B. and A.D. analyzed the data. J.D.B. wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.05.009.

References

Anet, E.F.L.J., 1972. Superficial scald, a functional disorder of stored apples VIII. Volatile products from the autoxidation of α-farnesen. J. Sci. Food Agric. 23, 605–608. https://doi.org/10.1002/jsfa.2740230508.

Birkett, M.A., Agelopoulos, N., Jensen, K., 2004. The role of volatile semiochemicals in mediating host location and selection by nuisance and disease-transmitting cattle flies. Med. Vet. Entomol. 18, 313–322.

Bohbot, J.D., Dickens, J.C., 2012. Odorant receptor modulation: ternary paradigm for mode of action of insect repellents. Neuropharmacology 62, 2086–2095. https://doi.org/10.1016/j.neuropharm.2012.01.004.

Bohbot, J.D., Dickens, J.C., 2010. Insect repellents: modulators of mosquito odorant receptor activity. PLoS One 5, e12138. https://doi.org/10.1371/journal.pone.0012138.

Bohbot, J.D., Fu, L., LE, T.C., Chauhan, K.R., Cantrell, C.L., Dickens, J.C., 2011. Multiple activities of insect repellents on odorant receptors in mosquitoes. Med. Vet. Entomol. 25, 436–444. https://doi.org/10.1111/j.1365-2915.2011.00949.x.

Buśko, M., Kulik, T., Ostrowska, A., Góral, T., Perkowski, J., 2014. Quantitative volatile compound profiles in fungal cultures of three different *Fusarium graminearum* chemotypes. FEMS Microbiol. Lett. 359, 85–93. https://doi.org/10.1111/1574-6968.12569.

Degennaro, M., McBride, C.S., Seeholzer, L., Nakagawa, T., Dennis, E.J., Goldman, C., Jasinskiene, N., James, A.A., Vosshall, L.B., 2013. Orco mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET. Nature 498, 487–491. https://doi.org/10.1038/nature12206.

Dekel, A., Pitts, R.J., Yakir, E., Bohbot, J.D., 2016. Evolutionarily conserved odorant receptor function questions ecological context of octenol role in mosquitoes. Sci. Rep. 6, 37330. https://doi.org/10.1038/srep37330.

Dekel, A., Yakir, E., Bohbot, J.D., 2019. The evolutionarily conserved Indolergic receptors of the non-hematophagous elephant mosquito Toxorhynchites amboinensis. Insect Biochem. Mol. Biol (in press), Accepted Manuscript).

Dekker, T., Ignell, R., Ghebru, M., Glinwood, R., Hopkins, R., 2011. Identification of mosquito repellent odours from *Ocimum forskolei*. Parasites Vectors 4, 183. https://doi.org/10.1186/1756-3305-4-183.

Dickschat, J.S., Wagner-Döbler, I., Schulz, S., 2005. The chafer pheromone buibuilactone and ant pyrazines are also produced by marine bacteria. J. Chem. Ecol. 31, 925–947.

El-Sayed, AM., 2018. The Pherobase: database of pheromones and semiochemicals. http://www.pherobase.com.

Farrar, R.R., Kennedy, G.G., 1987. 2-Undecanone, a constituent of the glandular trichomes of *Lycopersicon hirsutum* f. *glabratum*: effects on *Heliothis zea* and *Manduca sexta* growth and survival. Entomologia Experimentalis et Apllicata 43, 17–23.

Green, S.A., Chen, X., Nieuwenhuizen, N.J., Matich, A.J., Wang, M.Y., Bunn, B.J., Yauk, Y.-K., Atkinson, R.G., 2012. Identification, functional characterization, and regulation of the enzyme responsible for floral (E)-nerolidol biosynthesis in kiwifruit (Actinidia chinensis). J. Exp. Bot. 63, 1951–1967. https://doi.org/10.1093/jxb/err393.

Hallem, E.A., Ho, M.G., Carlson, J.R., 2004. The molecular basis of odor coding in the *Drosophila* antenna. Cell 117, 965–979. https://doi.org/10.1016/j.cell.2004.05.012.

Höckelmann, C., Jüttner, F., 2004. Volatile organic compound (VOC) analysis and sources of limonene, cyclohexanone and straight chain aldehydes in axenic cultures of *Calothrix* and *Plectonema*. Water Sci. Technol. 49, 47–54.

McBride, C.S., Baier, F., Omondi, A.B., Spitzer, S.A., Lutomiah, J., Sang, R., Ignell, R., Vosshall, L.B., 2014. Evolution of mosquito preference for humans linked to an odorant receptor. Nature 515, 222–227. https://doi.org/10.1038/nature13964.

McMeniman, C.J., Corfas, R.A., Matthews, B.J., Ritchie, S.A., Vosshall, L.B., 2014. Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. Cell 156, 1060–1071. https://doi.org/10.1016/j.cell.2013.12.044.

Raji, J.I., Melo, N., Castillo, J.S., Gonzalez, S., Saldana, V., Stensmyr, M.C., Degennaro, M., 2019. *Aedes aegypti* mosquitoes detect acidic volatiles found in human odor using the IR8a pathway. Curr. Biol. https://doi.org/10.1016/j.cub.2019.02.045.

Rines, H.W., French, R.C., Daasch, L.W., 1974. Nonanal and 6-methyl-5-hepten-2-one: endogenous germination stimulators of uredospores of *Puccinia graminis* var. *tritici* and other rusts. J. Agric. Food Chem. 22, 96–100.

Van Lancker, F., Adams, A., Delmulle, B., De Saeger, S., Moretti, A., Van Peteghem, C., De Kimpe, N., 2008. Use of headspace SPME-GC-MS for the analysis of the volatiles produced by indoor molds grown on different substrates. J. Environ. Monit. 10, 1127–1133. https://doi.org/10.1039/b808608g.

Wilkins, K., 1996. Volatile metabolites from actinomycetes. Chemosphere 32, 1427–1434. https://doi.org/10.1016/0045-6535(96)00051-3.

Zhou, X., Rinker, D.C., Pitts, R.J., Rokas, A., Zwiebel, L.J., 2014. Divergent and conserved elements comprise the chemoreceptive repertoire of the non-blood feeding mosquito *Toxorhynchites amboinensis*. Genome Biol Evol 6, 2883–2896. https://doi.org/10.1093/gbe/evu231.