FELIPE ANDREAZZA

PHYSIOLOGICAL AND MOLECULAR BASIS OF SPATIAL REPELLENCY MEDIATED BY PYRETHRUM AND PYRETHROIDS IN *Aedes aegypti*

Thesis presented to the Universidade Federal de Viçosa, as part of the requeriments of the Entomology Graduate Program, to obtain title of *Doctor Scientiae*.

Advisor: Eugênio Eduardo de Oliveira
Co-advisor: Ke Dong

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Advisor

This thesis is entirely dedicated to my wife Tays P. da Rosa, who tireless supported me through this path. I also dedicate this thesis to my parents Carmem L. Z. Andreazza and Simão Andreazza, and to my brother Fernando Andreazza, who always inspired, believed, and encouraged me to pursue my dreams. Lastly, I want to dedicate this thesis to the memory of my lovely mother in law Flúvia P. da Rosa, who gave birth to who I love and departed while we were overseas developing this work.

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Thank you all!

Biography

Felipe Andreazza, son of Carmem L. Z. Andreazza and Simão Andreazza, was born in Paraí, State of Rio Grande do Sul, Brazil, on Sep. 24th, 1992. In 2007, in parallel with his high school, he joint a technical college at the **Federal Institute of Education, Science and Technology of Rio Grande do Sul** (IFRS), Bento Gonąlves, State of Rio Grande do Sul, Brazil, graduating as **Technician in Agriculture** in 2010. In the same year, Felipe started his undergraduate course in **Agronomy** at the **Federal University of Pelotas** (UFPel), Pelotas, State of Rio Grande do Sul, Brazil. During his undergraduate course, Felipe spent 14 months at **University of California Davis** (UCDavis) as part of the **Brazilian Without Borders** program, where he focused his learnings in Entomology, before returning to Brazil and graduating as **Agronomy Engineer** in January 2016. Weeks later Felipe joint the Graduate Program in Entomology of the **Federal University of Viçosa** (UFV), Viçosa, State of Minas Gerais, Brazil, to pursue his **Master degree in Entomology**, achieved in July 2017. In August 2017 he started his doctoral studies at the same graduate program at the Federal University of Viçosa. After becoming a Ph.D. candidate, Felipe moved from Viçosa to East Lansing, Michigan, USA, aiming to executing his doctoral research project in the laboratory of his co-adviser, Prof. Ke Dong, at the Department of Entomology, **Michigan State University**. Before Felipe finished his studies, in Sep. 2020 the laboratory of Prof. Ke Dong moved to **Duke University**, Department of Biology, Durham, North Carolina, USA. At Durham Felipe could finalize his studies, needed to complete his course at UFV, under the supervision of Prof. Dr. Eugênio Eduardo de Oliveira.

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less." (Marie Curie)

Abstract

ANDREAZZA, Felipe, D.Sc., Universidade Federal de Viçosa, February, 2021. **Physiological and molecular basis of spatial repellency mediated by pyrethrum and pyrethroids in *Aedes aegypti*.** Advisor: Eugênio Eduardo de Oliveira. Co-advisor: Ke Dong.

Aedes aegypti mosquitoes are vectors of several viruses, such the ones causing Dengue and Yellow fevers, Chikungunya and Zika. Thus, compounds that can repel mosquitoes are used to prevent human-mosquito contact, aiding the fight against vector-borne diseases. Pyrethrum and its synthetic analogs, pyrethroids, are widely used for this purpose. However, the physiological and molecular mechanism of their spatial repellency (i.e., non-contact repellency) have not yet being deciphered. In this thesis, I bring two major contributions to the field, divided into two chapters. In the chapter 1, I present a continuing effort for understanding pyrethrum spatial repellency. As previous studies had shown that the activation of the odorant receptor (Or) protein AaOr31 by a minor component of pyrethrum [i.e., *E*)-β-farnesene (EBF)] only partially explain the pyrethrum repellency, I showed that pyrethrins I and II, the major components of this extract, elicit repellency by acting in two targets, the voltage-gated sodium channels and an Or expressed in a specific sensilla (i.e., sst-1) in *Ae. aegypti* antenna. Interesting, I also discovered that pyrethrins action on the voltage-gated sodium channels are responsible for enhancing the EBF repellency, explaining why EBF plays a role in pyrethrum repellency even at minute concentrations. In the chapter 2, I bring a contribution toward understanding the spatial repellency by transfluthrin, one of the main pyrethroids being recently deployed as mosquito repellent. Similarly to pyrethrins, transfluthrin elicits repellency by acting on the voltage-gated sodium channel. However, transfluthrin could not activate any neuron in mosquito major sensing appendages, as well as its repellency does not involve activation of any Or. Transfluthrin also enhances repellency of other Or-dependent repellents, including the gold-standard repellent DEET. In fact, I discovery that samples of commercial transfluthrin-based mosquito repellents elicit Or-mediated repellency, even though transfluthrin does not act on Ors. Together, these chapters illustrate that humans have been unknowingly exploiting this dual target synergistic mechanism for insect repellency for centuries, and that single action on voltage-gated sodium channels is sufficient to elicit repellency in *Ae. aegypti*. These results should inspire new chemistries toward volatile compounds acting on sodium channels and development of potent multi-target mixtures. The reduction in the concentration of individual components when in a mixture is expected to decrease environment contamination, and allergies, such as caused by high concentrations of DEET. Therefore, this new framework to be exploited promotes environmental and social sustainability, and aid the control of deadly vector-born human diseases.

Keywords: Mosquitoes. Vector-borne diseases. Behavior. Voltage-gated sodium channels. Odorant receptors. Synergism.

Resumo

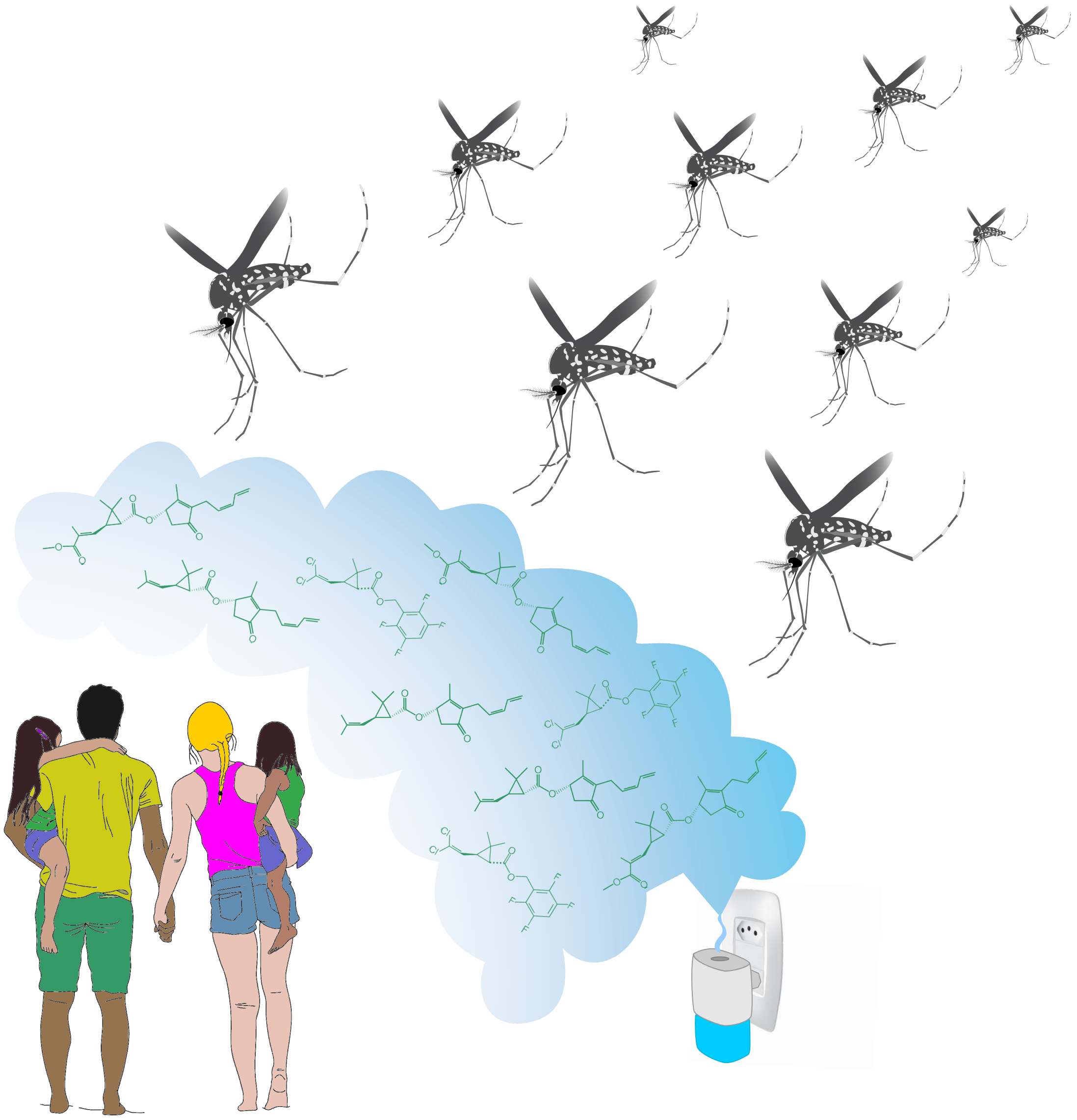
ANDREAZZA, Felipe, D.Sc., Universidade Federal de Viçosa, fevereiro de 2021. **Bases fisiológicas e moleculares da repelência mediada por piretro e piretróides em *Aedes aegypti***. Orientador: Eugênio Eduardo de Oliveira. Coorientadora: Ke Dong.

Mosquitos *Aedes aegypti* são vetores de vários vírus, como os que causam Dengue, Febre Amarela, Chikungunya e Zika. Assim, compostos que podem repelir mosquitos são usados para reduzir o contato dos mosquito com os humanos, auxiliando no combate à doenças transmitidas por vetores. O piretro e seus análogos sintéticos, os piretróides, são amplamente utilizados com essa finalidade. No entanto, o mecanismo fisiológico e molecular de sua repelência espacial (i.e., sem-contato) ainda não foi decifrado. Nesta tese, eu trago duas contribuições importantes para a área, divididas em dois capítulos. No capítulo 1, apresento um esforço contínuo para compreender a repelência espacial do piretro. Como estudos anteriores mostraram que a ativação do receptor odorante (Or) da proteína AaOr31 por um componente minoritário do piretro [i.e., (E)-β-farnesene (EBF)] explica a repelência do piretro apenas parcialmente, eu mostrei que as piretrinas I e II, os principais componentes desse extrato, provocam repelência agindo em dois alvos, os canais de sódio dependentes de voltagem e um Or expresso em uma sensila específica (i.e., sst-1) na antena de *Ae. aegypti*. Curiosamente, eu também descobri que a ação das piretrinas nos canais de sódio dependentes de voltagem são responsáveis por aumentar a repelência do EBF, explicando por que o EBF desempenha um papel na repelência do piretro, mesmo em concentrações mínimas. No capítulo 2, trago uma contribuição para a compreensão da repelência espacial pela transflutrina, um dos principais piretróides recentemente implementados como repelente de mosquitos. Igualmente às piretrinas, a transflutrina provoca repelência ao agir nos canais de sódio dependentes de voltagem. No entanto, a transflutrina não conseguiu ativar nenhum neurônio nos principais apêndices dos mosquitos, e sua repelência não envolve a ativação de nenhum Or. A transflutrina também aumenta a repelência de outros repelentes que agem em Or, incluindo o conhecido repelente DEET. De fato, descobri que amostras comerciais de repelentes de mosquitos à base de transflutrina provocam repelência mediada por Or, mesmo que a transflutrina não atue sobre Ors. Juntos, esses capítulos ilustram que os humanos têm explorado sem saber esse mecanismo sinérgico com alvo duplo para repelência a insetos por séculos, e que uma ação apenas nos canais de sódio dependentes de voltagem já é suficiente para provocar repelência em *Ae. aegypti*. Esses resultados devem inspirar novas químicas de compostos voláteis que atuem nos canais de sódio, e o desenvolvimento de misturas mais potentes. Espera-se que a redução na concentração de componentes individuais quando em uma mistura diminua a contaminação do meio ambiente e as alergias, como as causadas por altas concentrações de DEET. Portanto, esta nova estrutura a ser explorada promove a sustentabilidade ambiental e social e auxilia no controle de doenças humanas transmitidas por vetores.

Palavras-chave: Mosquitos. Doenças transmitidas por vetores. Comportamento. Canais de sódio dependentes de voltagem. Receptores de odor. Sinergismo.

Contents

General introduction



Every year several animal species are together responsible for causing death or serious illness in millions of people worldwide. Among then, blood sucking mosquitoes have their stage as the deadliest ones (Kamerow, 2014), not because their bite itself, which a mosquito female will make for acquire its blood meal, necessary for maturation and development of its ovaries (Clements et al., 1999), but because the deadly disease-causing pathogens it can transmit during the biting process (WHO, 2020b,a).

Several pathogens can be transmitted by a handful of mosquito species, mainly from three major mosquito genus, *Culex*, *Anopheles* and *Aedes*. *Anopheles* species are well known for transmitting protozoans from genus *Plasmodium*, the cause of Malaria disease, which heavily occur in sub-Saharan tropical Africa (WHO, 2020b; Raghavendra et al., 2011). *Culex* mosquitoes, along with some *Anopheles*, *Aedes* and *Mansonia* species, are both known for transmitting the worms that causes lymphatic filariasis (Bockarie et al., 2008). *Aedes* mosquitoes on the other hand, transmit several viruses, such as the ones causing Yellow and Dengue fevers, Chikungunya and Zika (Bowman et al., 2014; Chouin-Carneiro et al., 2016), and occurs from occident to orient tropical and subtropical regions of the globe.

Aedes aegypti, one of the most known species from *Aedes* genus, is considered an urbanized mosquito which adapted to lay eggs in small clear and quite water bodies, either naturally occurring, such as in bromeliad plants, or anthropologically made, such as small plastic container left uncovered and filled with water (Clements et al., 1999). Together the fact this species has specialized to occupy urban environment, it has also evolved to be specifically attracted to humans rather than other warm blood animals (McBride et al., 2014; Raji et al., 2019). These characteristics make *Ae. aegypti* one of the most widely occurring species, presents in over 128 countries, and responsible for infecting hundreds of millions of people annually (WHO2020b). The lack of proper and efficient preventive or curative treatment for most of the diseases these mosquitoes transmit, makes mosquito population management/control a widely used approach to reduce the global health burden caused by them (Roiz et al., 2018).

A continuous search for better approach and technique to reduce disease transmission risk is in place among the scientific community. Several different strategies have been used or proposed, alone or in combination. Two common approaches to manage *Ae. aegypti* and other mosquito species have been long used: 1^{st}, reduce mosquito population size/occurrence; 2^{nd}, reduce mosquito-human interaction/bites (Fouet and Kamdem, 2019; Gatton et al., 2013; Liu, 2015). To reduce mosquito populations, actions such as reducing the habitats female mosquitoes lay eggs and their larvae develop into adults can be taken by householders, but the lack of interest and/or cooperation, especially in socially and economically needy communities is a challenge. Therefore, the use of chemical insecticides targeting both pre-imaginal and imaginal stages is most commonly used specially in locations where the pathogen of interest is present and leading to disease outbreaks (Chareonviriyaphap et al., 2013; Liu, 2015; Roiz et al., 2018).

Considering several health safety concerns on the widely spray of chemicals into urban environment and that public authorities many times cannot reach the interior of the houses where mosquitoes are present, prevent mosquito-human contact is an effective way to actively prevent vector-born disease outbreaks. The prevention of mosquitoes contact with humans can be achieved by using physical barriers, such as simple cost-effective insecticide treated nets on the house entrances, or over bed for dusk and night biting species (Kampango et al., 2013; Che-Mendoza et al., 2018; Roiz et al., 2018). However, in communities with simply build houses/cabins which harbor multiple other entrances into the house, and also for any open-space environment, or daytime biting mosquito such as *Ae. aegypti*, the use of chemicals that can repel mosquitoes is the ultimate method been used to prevent mosquito bites (Azeem et al., 2019; Norris and Coats, 2017; Roiz et al., 2018).

The use of chemicals to repel insects is reported to be an ancient practice. Several plant species are naturally producers of a wide range of chemicals that serves as a plant defense mechanism against herbivores (Schoonhoven et al., 2005). Certainly, many plants-derived repellents are still to be discovered, some are just being discovered recently, and some others are been used for thousands of years (Nerio et al., 2010; Moore and Debboun, 2007; Peterson and Coats, 2001). An example of long used plant extracts to repel mosquitoes is the pyrethrum, which is extracted from daisy flowers buds of *Chrysanthemum* (*Tanacetum cinerariifolium*) (Casida and Quistad, 1995). Pyrethrum extract already shed lights on insect control chemistry decades ago, by inspiring the synthesis of pyrethroids insecticides, one of the most commonly used insecticide class until nowadays, including to control mosquitoes (Bibbs and Kaufman, 2017; Casida and Quistad, 1995; Elliott, 1977; Ogoma et al., 2017; Kawasaki et al., 2017). More precisely, pyrethroids are based on the structure of six related major components of pyrethrum extract known as pyrethrins (Elliott, 1977; Ujihara, 2019). Both pyrethroids and pyrethrins are able to bind to inset voltage-gated sodium channels, eliciting hyper activation of the nervous system, followed by paralysis and death (Dong et al., 2014; Du et al., 2013; Narahashi, 2000; Smith et al., 1997; Soderlund, 2005; Vais et al., 2000). Pyrethroids however, often present stronger effects, and improved chemical stability properties, in addition to be produced at low costs (Casida and Quistad, 1998; Ujihara, 2019; Yamamoto, 1970).

While pyrethroids are widely used for killing insects, similar to its predecessor pyrethrum extract, they are both also widely used to repel mosquitoes (Bibbs and Kaufman, 2017; Casida and Quistad, 1995; Hill et al., 2014; Kawada et al., 2005; Kawasaki et al., 2017; Ogoma et al., 2012; Masalu et al., 2020; Moore and Debboun, 2007; Riotte, 1998; Xue et al., 2012). However, how both pyrethrum extract and pyrethroids can elicit spatial (i.e., non-contact) repellency remains unknown. Several hypotheses have been raised on how these compounds could elicit repellent behavior in mosquitoes, including: activation of olfactory neurons, general toxicity, confusion, and inhibition of insect olfactory system (Bohbot and Dickens, 2010; Bohbot et al., 2011; Wagman et al., 2015; Yang et al., 2020). However, those hypotheses remain to be confirmed, and controversies are still present in the field.

Aiming to understand the long time sought mechanism of pyrethrum and pyrethroids spatial repellency, starting 2014-2015, Prof. Ke Dong (formally at Michigan State University, now at Duke University), used her extensive understanding of pyrethroids action on insect nervous system to build a team seeking to decipher the molecular mechanism underlining those sublethal, repellent effects in mosquitoes. The research approaches used by her team include the use of both mosquitoes and fly species, molecular genetics, behavior and electrophysiological assays, both *in vivo* and *in vitro*. Multiple compounds were tested, and the research project overall is still running on its several ramifications.

During my doctoral studies, I had the opportunity to join Prof. Ke Dong's team and collaborating with other colleagues in the laboratory (mainly Wilson R. Valbon), or former laboratory members, I contributed to or led several projects. In my thesis I bring two major contributions for the understand of spatial repellency mechanism in *Ae. aegypti* elicited by pyrethrum and pyrethroids. 1^{st}, a continued contribution to the exciting project toward deep understanding of pyrethrum repellency (which results are partially presented in Chapter 1 on page 26, in this thesis, but additional results from several other researchers will compose a single publication in a scientific journal); and 2^{nd}, a comprehensive deciphering of transfluthrin spatial repellency (in Chapter 2 on page 58), one of the main pyrethroids used currently as mosquito spatial repellent worldwide. Other projects I was involved at different stages are listed on Appendix B on page 112, but briefly includes understanding the repellency of other pyrethroids and essential oils in *Ae. aegypti*, as well several essential oils and pyrethrum in *Drosophila melanogaster* and *Drosophila suzukii* flies. Those are collaborative works that are currently at multiple stages, and therefore, were not brought into this thesis.

As an overall approach, in both chapters, it was assessed the possibility of repellency behavior being elicited by activation of specifics proteins in the olfactory receptor neurons, located mainly at the mosquito antennae. Those proteins could be either from the large gene family known as Odorant receptor (Benton, 2006; Hallem et al., 2005), or the insect voltage-gated sodium channels. The latter is also expressed in all neurons in the insect, and as mentioned earlier in this introduction, is a well-known target to both pyrethrum and pyrethroids (Chen et al., 2018; Dong et al., 2014). In short, we discovery that while pyrethrins can activate both odorant receptors and the insect voltage-gated sodium channels to elicit repellency in mosquitoes, synthetic pyrethroids such as transfluthrin only target the voltage-gated sodium channels to elicit repellency. Specific details for each case are presented and discussed within each of the two individual chapters of this thesis, and general conclusions, remaining questions, and future directions are latter discussed in brief at the Final considerations and future directions section on page 101.

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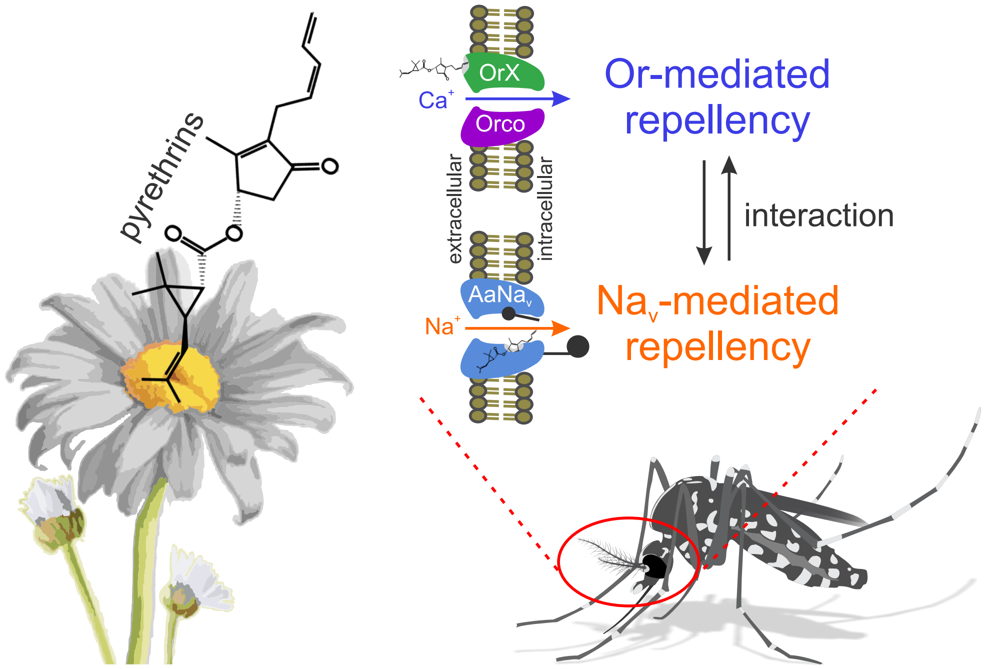
Chapter 1

A dual-target molecular mechanism of pyrethrum repellency against mosquitoes



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Abstract

For centuries, pyrethrum extracts from flower heads of *Chrysanthemum* (*Tanacetum*) spp. have been used worldwide as a potent natural insect repellent. A previous study had shown that the action of a minor component of pyrethrum extract [i.e., (*E*)-*β*-farnesene (EBF)] on the *Aedes aegypti* odorant receptor (Or) AaOr31 only explain the repellency partially. Therefore, a comprehensive full understanding of the physiological and molecular basis of pyrethrum repellency remains to be described. In this study, we found that to elicit spatial repellency, the principal components of pyrethrum, pyrethrin I (P-I) and pyrethrin II (P-II), activate both a olfactory receptor neuron located in a short sharp-tipped sensilla (i.e., sst-1), and the voltage-gated sodium channels of *Ae. aegypti* mosquitoes. Interestingly, we also found that pyrethrin-induced activation of voltage-gated sodium channels significantly synergizes the Or-mediated repellency by EBF. Thus, pyrethrum exerts spatial repellency through a novel, dual-target mechanism. Elucidation of this two-target mechanism for a popular natural insect repellent has significant implications in the design of a new generation of synthetic repellent mixtures against major mosquito vectors of infectious human diseases, including, malaria, dengue and Zika.

Keywords: Olfaction; Sodium channel; Pyrethrins; Repellency; Synergism.

1.1 Introduction

Mosquito-transmitted human diseases, such as malaria and dengue fever, represent significant burdens to global human health and cause considerable human suffering. One of the most effective measures to reduce disease transmission involves the use of insect repellents to prevent human contacts with mosquitoes. Pyrethrum extract from the dried and crushed flower heads of *Chrysanthemum* (*Tanacetum cinerariifolium*) began to be used as an insect repellent against biting arthropods thousands of years ago (Casida and Quistad, 1995; Moore and Debboun, 2007). Today, *T. cinerariifolium* plants are grown commercially in many parts of the world, particularly in East Africa and Australia, for extraction of pyrethrum (Crombie, 1995; Greenhill, 2007) and pyrethrum and its synthetic analogs, pyrethroids, are key active ingredients in a variety of commercial insect-repelling products, such as mosquito coils, emanators, vaporizer mats, textile finishes, hessian strips/ribbons (Achee et al., 2012; Bibbs and Kaufman, 2017; Chatha et al., 2019; Hill et al., 2014; Kawasaki et al., 2017; Mmbando et al., 2018, 2019; Ogoma et al., 2014, 2017). In addition, pyrethrum-producing *Chrysanthemum* spp. are recommended as companion plants to repel pest insects (Riotte, 1998).

Pyrethrins and pyrethroids exert potent insecticidal activities by hyper-activating insect voltage-gated sodium channels, thereby causing rapid paralysis, known as knockdown, and eventual lethality (Bloomquist, 1996; Narahashi, 2000; Soderlund, 2005). While the molecular mechanism of insecticidal lethal action of these compounds is well established, the molecular mechanism(s) underlying the repellency elicited by pyrethrum and pyrethroids, at sublethal levels, remains a mystery. Various possibilities have been proposed, including contact-based repellency, spatial repellency, neuronal irritation, olfactory responses (Bohbot et al., 2011; Chareonviriyaphap et al., 2013; Hadis et al., 2003; Hu et al., 2018; Jiang et al., 2019; Nentwig et al., 2017; Ogoma et al., 2014; Wagman et al., 2015; Yang et al., 2012, 2020), but none had been confirmed/understood at molecular level.

Pyrethrum is an extract containing a mixture of six insecticidal esters (i.e., collectively known as pyrethrins) as major components, and several sesquiterpenes, at much lower concentrations, that provides the flowering fragrance of the extract (Cai et al., 2013; Henrick, 1994; Maciver, 1995). In a still unpublished study, *Liu F.*, *Wang Q.* and *Xu P.*, associates in research at our laboratory, determined that pyrethrum elicits olfactory-like, odorant-receptor mediated repellency by the activation of a specific odorant receptor (Or) (i.e., AaOr31) expressed in the A neuron of a short blunt-tipped sensillum (named sbt-1) in *Aedes aegypti*, the primary vector of viruses that cause dengue fever, Zika, yellow fever and chikungunya. In brief, *Liu et al* discovered that (*E*)-β-farnesene (EBF), one of the minor components of pyrethrum extract, was the only component that could activate the sensillum called sbt-1. After using several molecular and electrophysiology approaches to identify the AaOr31 as the Or been expressed in the A neuron of sbt-1 sensilla and using the mosquito strain Rockefeller as background, it was generated a new mosquito strain with the *AaOr31* gene partially removed by CRISPR-Cas9. The deletion rendered a strongly impaired and functionally inactive Or protein. The activation by EBF in the sbt-1A neurons of the *AaOr31*$^{-/-}$ mosquitos did not happen, so did the repellency by EBF alone (Supplementary Fig. 1a and b). This new mutant strain also showed reduced repellency to pyrethrum (Supplementary Fig. 1c), confirming the EBF mediate activation of AaOr31 as an important component of pyrethrum repellency.

The discovery of this strictly Or mediated repellency pathway is a breakthrough toward the understanding of pyrethrum repellency in this species. However, the percentage of EBF in commercial pyrethrum extracts is generally very low (ranging from 1.25% to 1.97% based on our analysis of the pyrethrum extracts used in this study, and in *Liu et al*), which alone would not be sufficient to evoke any repellency (Supplementary Fig. 2a). Furthermore, we noted that more than 40% of pyrethrum repellency remained in *AaOr31*$^{-/-}$ mosquitoes (Supplementary Fig. 1c), suggesting additional mechanism(s) underlying the pyrethrum repellency, acting either alone or in interaction with EBF mediated repellency.

Considering the major constituents of pyrethrum extract are pyrethrin I (P-I) and pyrethrin II (P-II), which are well known to act on insect voltage-gated sodium channels (Bloomquist, 1996; Narahashi, 2000; Soderlund, 2005), we hypothesised that pyrethrins may also be involved in pyrethrum repellency, possible by acting on sodium-channel or interacting with EBF mediated repellency. Therefore, we took a combination of electrophysiological and behavioral approaches to gain further insights into pyrethrum repellency in *Ae. aegypti* mosquitoes. We aimed to address the following key questions: i) Does pyrethrins elicit repellency in *Ae. aegypti* mosquitoes? ii) If yes, is the repellency mediated by action on Ors or by activation of *Ae. aegypti* voltage-gated sodium channels (AaNa$_v$)? iii) Does a mixture of pyrethrins and EBF elicit stronger repellency, such as is observed for pyrethrum? And iv) If yes, does the interaction happens prior or post target site level? Our study resolved all these questions and together the results of *Liu et al* (to be published together as a single work), revealed a novel, dual-target mechanism for insect repellency, involving dual activation of olfactory repellency pathways and voltage-gated sodium channels. The discovery has significant basic and practical implications in understanding the mechanisms and development of insect repellents against mosquitoes and other human disease vectors.

1.2 Results

1.2.1 Pyrethrum elicits Na$_v$-dependent spatial repellency

Because pyrethrum major components are pyrethrins, which are known to hyperactivate voltage-gated sodium channels that are critical for neural electrical signaling (Chen et al., 2018a; Narahashi, 1971), first, we examined a possible involvement of activation of sodium channels in pyrethrum repellency. For this purpose, we used the KDR:ROCK mutant strain, which is near isogenic to the wild-type Rockefeller strain and resistant to pyrethrum due to two defined mutations in the *AaNa$_v$* gene (Smith et al., 2018) (Supplementary Fig. 3a). To evaluate the repellency toward pyrethrum in both these mosquito strains, it was used the hand-in-cage assay. In this behavioral assay, mosquitoes are attracted to a human hand in a modified glove (Fig. 1a) that has a screened window on its back. Mosquitoes would land on a piece of mesh secured on the top of the window. Between the top mesh and the hand is a second mesh, which was above the hand and treated with a test compound or solvent, but mosquitoes cannot make direct contact with the second mesh (Fig. 1a). The landing frequency of female Rockefeller mosquitoes (wild-type *Ae. aegypti*) onto the top mesh was significantly reduced when the second mesh was treated with pyrethrum, but pyrethrum repellency decreased in KDR:ROCK mosquitoes (Fig. 1b).

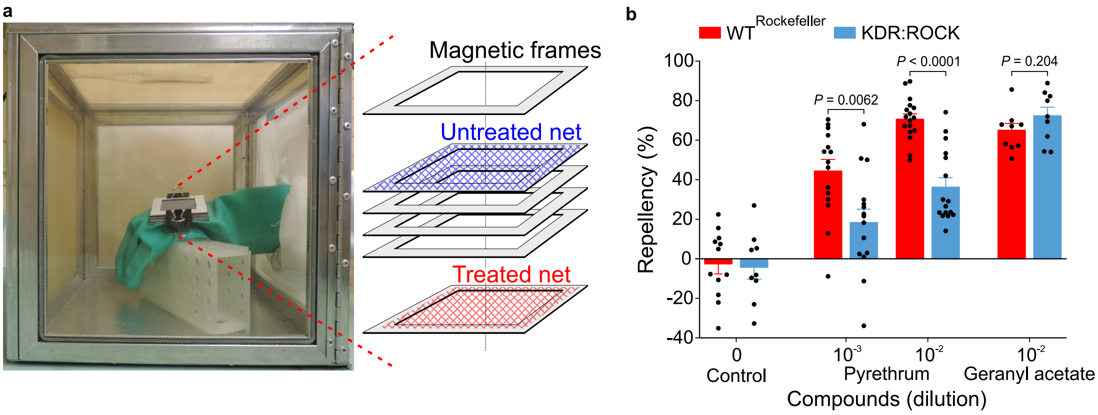

Figure 1: Pyrethrum elicits Na$_v$-dependent spatial repellency. **a**, A schematic depiction of the setup for the hand-in-cage assay as previously described (Boyle et al., 2016). **b**, Repellency by pyrethrum and geranyl acetate in KDR:ROCK mosquitoes compared to that in Rockefeller mosquitoes (n = 10 cages for control in Rockefeller and n = 9 cages for control in KDR:ROCK from 2 batches of mosquitoes; pyrethrum at the 10^{-3} dilution: $t = 2.96$, $df = 28$, n = 15 cages for Rockefeller and n = 15 cages for KDR:ROCK from 4 batches of mosquitoes; pyrethrum at the 10^{-2} dilution: $t = 6.56$, $df = 31$, n = 17 cages for Rockefeller and n = 16 cages for KDR:ROCK from 4 batches of mosquitoes; geranyl acetate: $t = 1.32$, $df = 16$, n = 9 cages for each strain from 2 batches of mosquitoes). Data are plotted as mean ± s.e.m. and dots denote the value of each repeat. Two-tailed unpaired student's t-test was used to compare each of two sets of data. Exact P-value are indicated for each comparison in the figure. The control for each mosquito strain represents the baseline activity in response to the solvent.

In insects, in addition to individual Ors that are responsible for recognizing specific odorants, an obligate co-receptor (Orco) is required for detection of diverse odorants (Joseph and Carlson, 2015; Ray, 2015; Vosshall and Stocker, 2007) and can be used to infer whether insect attraction or avoidance response is Or-mediated. Geranyl acetate is an essential oil component used as mosquito repellent (Kwon et al., 2014; Michaelakis et al., 2014), and when tested for repellency in a mosquito strain that is olfactory impaired because of deletion of the *orco* gene (Degennaro et al., 2013), we concluded geranyl acetate repellency is Orx:Orco dependent (Supplementary Fig. 3a). Therefore, we used geranyl acetate, an olfaction dependent odorant, as a positive control in our Rockefeller *vs.* KDR:ROCK strains comparison repellency assay. As expected, while geranyl acetate repellency was reduced in *orco*$^{-/-}$ mosquitoes in comparison with its wild-type strain, Orlando (Supplementary Fig. 3b), its repellency in the KDR:ROCK was not impacted (Fig. 1b). The results of our positive control further support that a reduction in repellency by an already proved AaNa$_v$ ligand compound (pyrethrum) (Chen et al., 2018a) in the KDR:ROCK strain, when compared to its near isogenic Rockefeller strain, is a valid assay to conclude the involvement of AaNa$_v$ in pyrethrum/pyrethroid repellency.

1.2.2 Pyrethrins repellency is not only AaNa$_v$-mediated, but also Or-mediated

In order to confirm that pyrethrins are the components eliciting the AaNa$_v$-mediated repellency in pyrethrum, we evaluated if isolated and purified P-I and P-II (Supplementary Fig. 4) was able to elicit repellency in both the wild-type strains used in this study, and if the repellency in KDR:ROCK and *orco*$^{-/-}$ was impacted. Indeed, both P-I and P-II could elicit repellency in wild-type mosquitoes (Fig. 2a and b). As expected, the repellency by both pyrethrins was reduced in KDR:ROCK mosquitos (Fig. 2a). Surprisingly, the repellency by both compounds was also reduced in the *orco*$^{-/-}$ strain (Fig. 2b). These results indicate that in addition to act on AaNa$_v$ to elicit repellency, pyrethrins are also able to target Or(s), and that its action in a putative Orx does contribute to repellency.

1.2.3 Pyrethrins activates a specific olfactory receptor neuron

Insect Ors are mainly expressed in olfactory receptor neurons (ORNs) in antenna (Carey and Carlson, 2011; Leal, 2013; Potter, 2014; Vosshall and Stocker, 2007). Three major morphologically distinct types of antennal trichodae sensilla are recognized in *Ae. aegypti* antennae: short sharp-tipped (sst), long sharp-tipped (lst), and short blunt-tipped (sbt) (Ghaninia et al., 2007; Chen et al., 2018b). Each *Ae. aegypti* sensil-

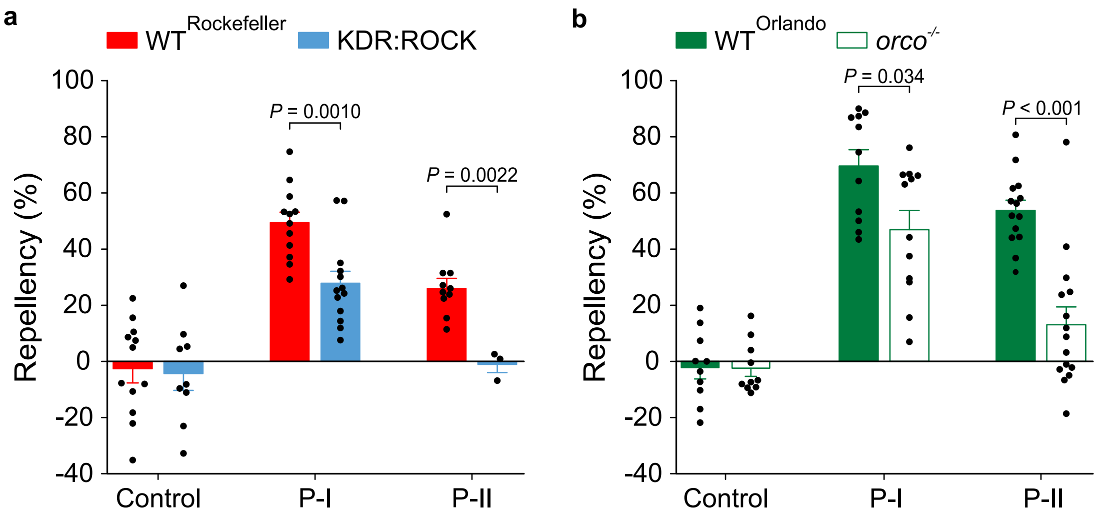

Figure 2: Pyrethrins repellency is not only AaNa$_v$-mediated, but also Or-mediated. **a**, Reduced pyrethrin (10^{-3} v/v) repellency in KDR:ROCK mosquitoes compared to that in Rockefeller (wild-type) mosquitoes (n = 10 cages for control in Rockefeller and n = 9 cages for control in KDR:ROCK from 2 batches of mosquitoes; P-I: t = 3.78, df = 23, n = 12 cages for Rockefeller and n = 13 cages for KDR:ROCK from 3 batches of mosquitoes; P-II: t = 3.97, df = 11, n = 10 cages for Rockefeller and n = 3 cages for KDR:ROCK from 2 batches of mosquitoes). **b**, Repellency elicited by P-I and P-II (10^{-3} v/v) in Orlando (wild-type) and *orco*$^{-/-}$ mosquitoes (n = 10 cages for Orlando and *orco*$^{-/-}$ controls from 2 batches of mosquitoes; P-I: U = 31, n = 11 cages for Orlando and n = 12 cages for *orco*$^{-/-}$ from 3 batches of mosquitoes; P-II: U = 15, n = 14 cages for Orlando and n = 15 cages for *orco*$^{-/-}$ from 4 batches of mosquitoes). Data are plotted as mean ± s.e.m. and dots denote the value of each repeat. Two-tailed unpaired student's t-test or two-tailed Mann-Whitney Rank Sum test was used to compare each of two sets of data. Exact P-value are indicated for each comparison in the figure.

lum houses two neurons: The neuron that generates larger spikes (i.e., action potentials) is called the A neuron and the neuron that produces smaller spikes is called the B neuron. To identify which mosquito ORN(s) responds to pyrethrum, *Liu et al* performed single sensillum recordings (SSR) of antennal olfactory sensilla of Rockefeller mosquitoes in response to a panel of odorants including pyrethrum, (±)-citronellal, geranyl acetate and (-)-borneol, most of which are plant-derived mosquito repellents. In their work *Liu* and *Wang* identified three responsive sst sensilla, sst-1 to sst-3, and six responsive sbt sensilla, sbt-1 to sbt-6, based on their response profiles to the odorants in the panel (Supplementary Fig. 5). The lst sensilla did not respond to any odorants in their panel.

Out of the nine different sensilla functionally mapped in their work and in addition to the sbt-1, a second sensilla (i.e., sst-1) present a weaker but still significant activation by pyrethrum (Supplementary Fig. 6). To test if pyrethrins were responsible for the activation of sst-1 sensilla by pyrethrum, we first applied the same panel of odorants in order to verify the sensilla identity, and then tested pyrethrum and pure pyrethrins. Indeed, we confirmed pyrethrum activation of sst-1, and found that P-I and P-II, both activate sst-1A neurons in Rockefeller mosquitoes (Fig. 3). A similar response of sst-1A neurons to P-I and the entire odorant panel was also observed in Orlando and *AaOr31*$^{-/-}$ mosquitoes (Supplementary Fig. 7).

Combined, these results demonstrate pyrethrins contribute to pyrethrum repellency via two mechanisms: (i) activation of sst-1A-associated olfactory pathway and (ii) hyper-activation of voltage-gated sodium channels.

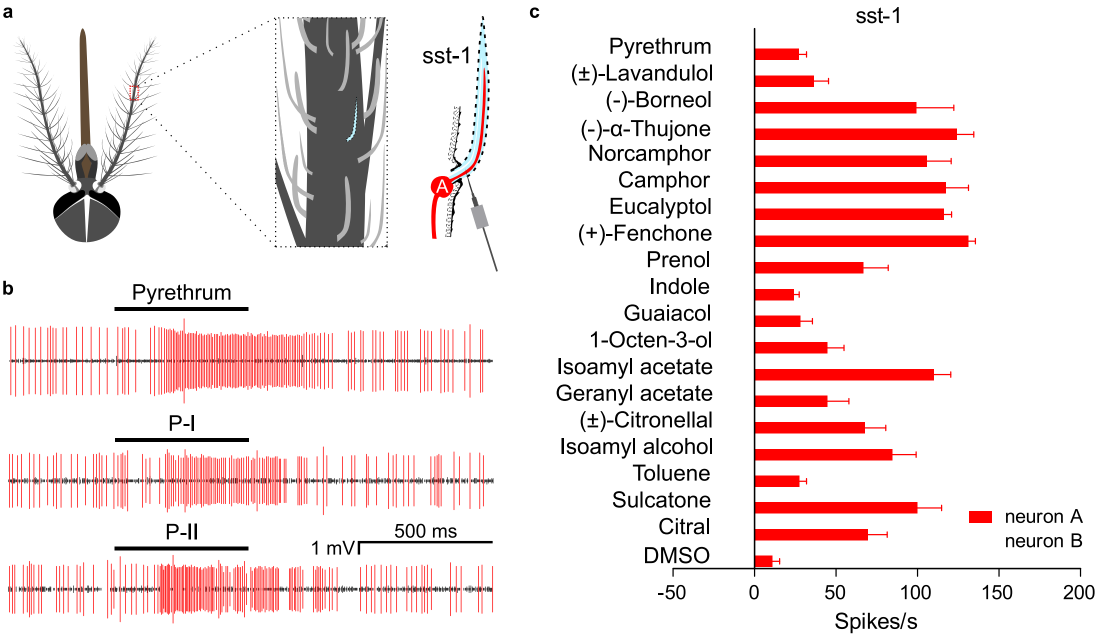

Figure 3: Pyrethrins activates a specific olfactory receptor neuron. a, A schematic drawing illustrating SSR from *Ae. aegypti* mosquito antennae. **b,** Representative SSR traces of sst-1 sensilla responding to Pyrethrum (n = 7 sensilla), pyrethrin I (P-I) (n = 8 sensilla) and pyrethrin II (P-II) (n = 6 sensilla) at the 10^{-1} dilution from Rockefeller mosquitoes. **c,** The response profiles of sst-1 sensilla identified in the single sensillum recording in Rockefeller mosquitoes (n = 7 sensilla).

1.2.4 Synergism between AaOr31-mediated repellency and sodium channel-activated repellency

Identification of sst-1A neuron and voltage-gated sodium channels as targets of a single component, as well as the fact that activation of AaOr31 by EBF is involved in pyrethrum repellency, even though EBF alone at such low concentrations it occurs inside commercial pyrethrum extract cannot elicit repellency (Supplementary Fig. 2) raised a fundamental question with respect to the relationship between activation of sodium channels and olfactory pathways in pyrethrum-mediated mosquito repulsion. To address this question, we examined mosquito repellency in response to pyrethrins I/II and EBF alone or in combination. Remarkably, EBF enhanced pyrethrin I and pyrethrin II repellency at a concentration of as low as 4x 10^{-6} (Fig. 4a), whereas EBF alone at these concentrations did not evoke any repellency (Supplementary Fig. 2). Similarly, the synergism between pyrethrins and EBF could be observed even when the concentration of pyrethrin I was reduced to 10^{-5} and 10^{-6} which alone did not elicit repellency (Fig. 4b). Importantly, the enhancement was specific to pyrethrins, as no enhancement in repellency was observed between EBF and another plant-derived repellent, (-)-borneol (Fig. 4c). Although, like pyrethrins, (-)-borneol activates sst-1A neurons (Fig. 3b, and Supplementary Fig. 7) and elicits Orco-dependent repellency (Supplementary Fig. 8a), it cannot activate sodium channels (Supplementary Fig. 8b), suggesting that EBF enhancement may be specific to sodium channel-activating chemicals, such as pyrethrins, and is not a result from interactions between activation of two different ORN neurons. Furthermore, the enhancement was abolished in *AaOr31*$^{-/-}$ mosquitoes (Fig. 4d), indicating that pyrethrins enhance AaOr31-dependent repellency evoked by EBF through a mechanism downstream from the Or-activation. Finally the synergism between P-I and EBF disappeared in KDR:ROCK strain (Fig. 4e), confirming the synergism is a result of pyrethrins action on the AaNa$_v$ rather than an artefact of the mixture, prior vapors reach the insect.

Collectively, these results strongly suggest that hyper-activation of sodium channels by pyrethrins, together with AaOr31-mediated repellency by EBF, produce a potent synergism that could explain the pyrethrum repellency against mosquitoes.

The potentiation of AaOr31-mediated repellency by pyrethrins raises the possibility of a broad effect of sodium channel hyper-activation on the mosquito olfactory system. We therefore examined a possible effect of pyrethrins on the activities of odorants other than EBF. We found that camphor and eucalyptol, which each activate multiple types of *Ae. aegypti* sensilla (Supplementary Fig. 5) elicit Or-dependent repellency (Supplementary Fig. 9a). Furthermore, P-I enhanced repellency by camphor and eucalyptol (Supplementary Fig. 9b), confirming a broad effect of pyrethrins on repellency by other mosquito repellents.

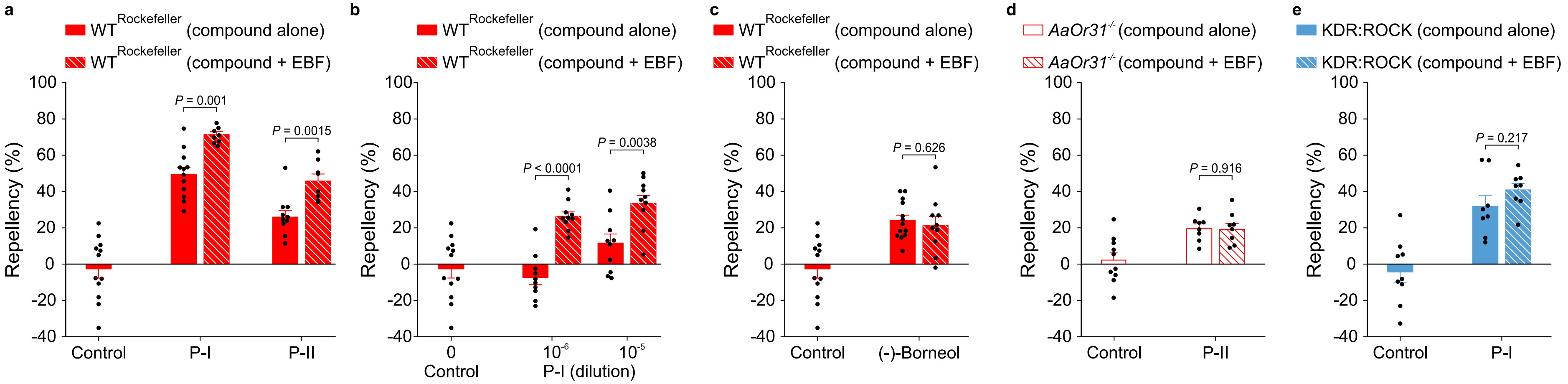

Figure 4: Synergism between AaOr31-mediated repellency and sodium channel-activated repellency. **a**, Effect of EBF (4x 10^{-6}) on P-I (10^{-3}) and P-II (10^{-3}) repellency in Rockefeller (n = 10 cages for control from 2 batches of mosquitoes; U = 6, n = 8 cages for P-I + EBF and n = 12 cages for P-I alone from 3 batches of mosquitoes; t = 3.82, df = 16, n = 10 cages for P-II alone and n = 8 cages for P-II + EBF from 3 batches of mosquitoes). **b**, Effect of EBF (10^{-5}) on repellency by P-I (10^{-6} and 10^{-5}) in Rockefeller (n = 10 cages for control; P-I at the 10^{-6}: t = 7.34, df = 18, n = 10 for P-I alone and n = 10 for P-I + EBF from 2 batches of mosquitoes; P-I at 10^{-5}: t = 3.32, df = 18, n = 10 cages for P-I alone and n = 10 cages for P-I + EBF from 2 batches of mosquitoes). **c**, Effect of EBF (4x 10^{-6}) on (-)-borneol (10^{-4}) repellency in Rockefeller (n = 10 cages for control from 2 batches of mosquitoes; t = 0.49, df = 21, n = 13 cages for (-)-borneol alone and n = 10 cages for (-)-borneol + EBF from 3 batches of mosquitoes). **d**, No effect of EBF (4x 10^{-6}) on P-II (10^{-3}) repellency in *AaOr31*$^{-/-}$ mosquitoes (n = 10 cages for control from 2 batches of mosquitoes; t = 0.11, df = 14, n = 8 cages for P-II alone and n = 8 for P-II + EBF from 2 batches of mosquitoes). **e**, Absence of EBF-P-I synergism in KDR:ROCK mosquitoes (t = 1.29, df = 14, n = 8 cages for P-I alone and n = 8 for P-I + EBF from 2 batches of mosquitoes). EBF alone at 4x 10^{-6} and 10^{-5} did not elicit repellency in Rockefeller or *AaOr31*$^{-/-}$ mosquitoes (Supplementary Fig. 2). Data are plotted as mean ± s.e.m. and dots denote the value of each repeat. Two-tailed unpaired student's t-test or two-tailed Mann-Whitney Rank Sum test was used to compare each of two sets of data. Exact P-value are indicated for each comparison in the figure.

Next, we investigated whether the pyrethrin/EBF synergism occur at the olfactory sensory level. Specifically, we evaluated the activity of sbt-1 neurons in response to EBF alone or co-application with P-I. As expected, EBF evoked increased firing of stb1A neurons, but co-application of P-I and EBF did not enhance the EBF-evoked activity (Fig. 5a, b). In addition, P-I did not alter the baseline firing of sbt-1 neurons in Rockefeller or *AaOr31*$^{-/-}$ mosquitoes (Fig. 5c), indicating that sodium channels in these ORNs are not sensitive to P-I. Collectively, these results suggest that pyrethrin potentiation of Or-mediated repellency does not occur at the olfactory sensory level, pointing to its effect at a subsequent step, possibly at the central neural processing level.

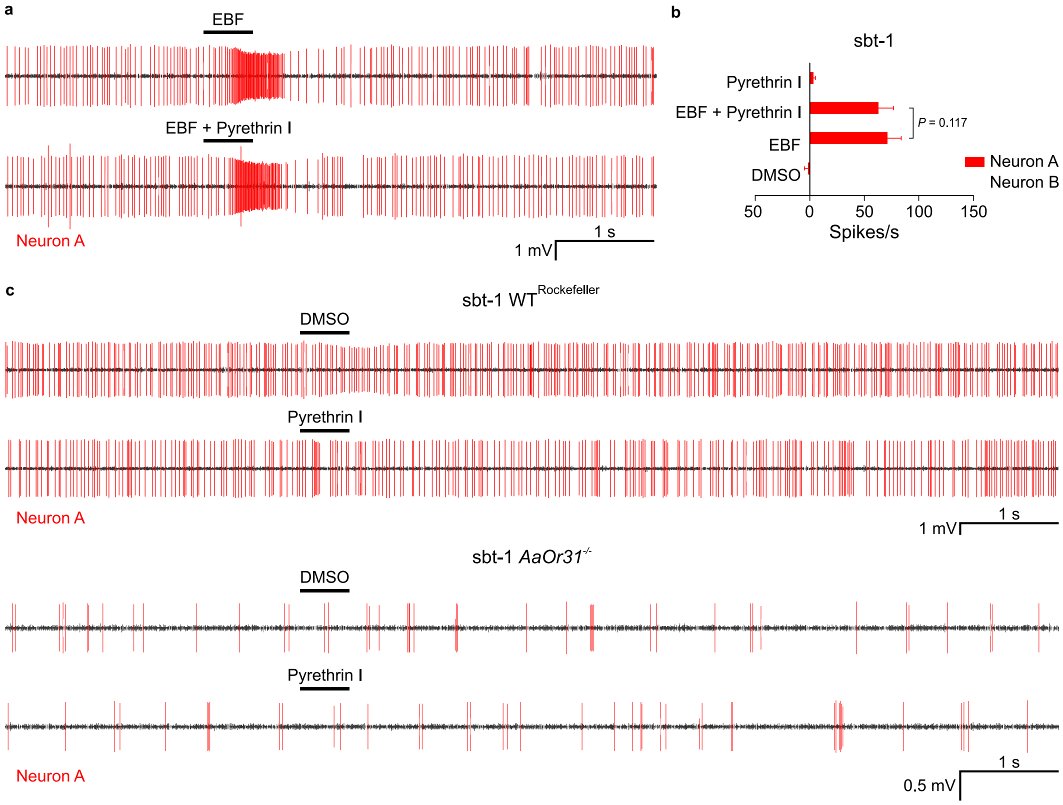

Figure 5: Pyrethrin does not impact the AaOr31 activation by EBF or AaNa$_v$ in sbt-1A neuron. a, Representative SSR traces (from n = 8 sensilla) evoked by EBF 10^{-5} with or without co-application of pyrethrin I 10^{-3} in sbt-1 sensilla in Rockefeller (wild-type) mosquitoes. **b**, Co-application of pyrethrin I (10^{-3} v/v) and EBF (10^{-5} v/v) did not affect the EBF-evoked activity (t = 1.79, df = 7, n = 8 sensilla). **c**, Pyrethrin I (10^{-1} v/v) did not affect the spontaneous firing of sbt-1 neurons from either Rockefeller or *AaOr31*$^{-/-}$ mosquitoes (from n = 7 sensilla for each mosquito strain). Two-tailed paired Student's t-test was used to compare the two sets of data. The exact P-value is indicated.

1.3 Discussion

In this study, we provide strong evidence that pyrethrum exerts spatial repellency through a novel, dual-target mechanism. While previous study in our lab (*Liu et al*) discovered that a minor component of pyrethrum, EBF, activates AaOr31, we found that the Or31-mediated repellency is significantly synergized by pyrethrins, which are activators of voltage-gated sodium channels and the principal components of pyrethrum. It is remarkable that, centuries ago, humans unknowingly exploited a hidden potent synergism between activation of sodium channels by pyrethrins and activation of AaOr31 by EBF in a natural plant extract against insect bites. Elucidation of this two-target mechanism for a popular natural insect repellent has significant implications in the design of a new generation of synthetic repellent mixtures against major mosquito vectors of infectious human diseases, including malaria, dengue and Zika.

For the first time we show that, besides acting on sodium channels, pyrethrins also directly activate Or(s), located in sst-1A neurons, although the identity of pyrethrin-activated Or(s) remains to be determined. We speculate that the EBF-pyrethrins synergism in pyrethrum repellency is likely the result of increased activity of EBF/AaOr31-mediated repellency by enhancing neuronal excitability via action of sodium channels by pyrethrins. How hyper-activation of sodium channels enhances Or-mediated repellency awaits future investigations. Previous research revealed extensive alternative splicing and RNA editing of the sodium channel transcript, which produces a wide range of functional diversity of insect sodium channels in *Drosophila* and cockroach, including differential sensitivities to pyrethroids (Dong et al., 2014). It is likely that non-lethal concentrations of pyrethrins present in pyrethrum activate pyrethrin–hypersensitive sodium channel variants expressed in certain neural circuits, including repellent circuits, which leads to depolarization of membrane potential and potentiates the sensitivity of neural circuits for firing in response to EBF and other Or-activating repellents. Our SSR in response to P-I alone or co-application with EBF showed that sodium channels in the ORNs were not the targets of pyrethrins, pointing to pyrethrin–hypersensitive sodium channel variants expressed at the central neural processing level. Functional identification and localization of such pyrethrin–hypersensitive sodium channel variants in the brain will be necessary to advance further mechanistic understanding of the pyrethrin-EBF synergism in repellency.

The discovery of a two-target mechanism in pyrethrum provides a framework for increasing the potency and durability of the repellency of commercial synthetic insect repellents. Specifically, our discovery of the intriguing synergism between activation of Ors and voltage-gated sodium channels could spur efforts to use natural pyrethrins, synthetic pyrethroids or other sodium channel activators as synergists to increase the potency of DEET and other Or-activating insect repellents. Synergized spatial repellency may be a promising new strategy that could be incorporated in the global fight against mosquito-borne human diseases.

1.4 Material & Methods

1.4.1 Mosquito strains.

Five *Ae. aegypti* mosquito strains were used: Rockefeller and Orlando are two wild-type strains and *orco*$^{-/-}$ (*orco*^{16}) is a mutant mosquito strain with the *orco* gene mutated (Degennaro et al., 2013) (BEI Resources, NIAID, NIH). KDR:ROCK is a pyrethroid-resistant strain (Smith et al., 2018). The *AaOr31*$^{-/-}$ mutant strain was generated in a previous study in our laboratory (*unpublished*) using the CRISPR-Cas9 technology and was backcrossed with parental Rockefeller mosquitoes for four generations to generate a near-isogenic strain for functional analysis.

1.4.2 Hand-in-cage assay.

Similar to the proposed by Boyle et al. (2016), the hand-in-cage assay includes a 30 × 30 × 30 cm mosquito cage (BioQuip, Rancho Dominguea, CA), with a mounted digital camera and a human hand in a modified glove with a screened window. The digital camera (e-con Systems Inc, San Jose, CA, model: e-CAM51A) for video recording is mounted on the cage top and connected to a laptop computer. A nitrile rubber glove (Ansell Protective Products, Coshoton, OH, part number: 37-155) was cut on the back side of the glove to create a window (6 x 5 cm) (Fig. 1a). A piece of magnetic frame (slightly larger than the dimension of the window) was glued onto the cut window which was used as a base for stacking more magnetic window frames (Fig. 1a; also further explained below). One piece of test compound-treated polyester netting (Shason Textile Inc., part number: WS-B532-111, Walmart # 567948282, white; 6.5 x 5.5 cm) was placed on this fixed magnetic frame, which was ∼3 mm above the glove. The second piece of the netting was untreated and placed ∼8 mm above the treated net using a stack of four magnetic frames. The stacked magnetic frames were further secured with a binder clip. The stacking creates sufficient space between the treated net and the untreated net so that mosquitoes that land on the open window were not able to contact the treated net or contact and pierce the skin of the hand in the glove. The hand makes no contact with the treated net.

The assay was run in a room with relative humidity around 50% and temperature between 27 °C to 30 °C. Twenty-four hours before an assay, four to nine days-old females (about 40, mated, non-blood fed) were transferred into a mosquito cage. The cage was kept in an incubator where mosquitoes were provided only with water in a cotton ball placed on the top of the cage. Immediately before the assay, one researcher (i.e., tester) treated a piece of netting with 500 µL test compound dissolved in acetone in a glass Petri dish in an adjacent room. Acetone served a control. After letting acetone evaporate (∼7 min), the researcher assembled and put on a modified glove. In the meantime, a lab assistant transferred the prepared cage from the incubator to a bench in the assay room. Both personnel avoided use of any hand lotions and cosmetic products and wore white lab coats and gloves. The hand in the modified glove was introduced into the cage to initiate the assay. Mosquitoes landing on the test window was recorded by the digital camera for five minutes. The number of mosquitoes landing during the second to fifth minutes was counted and recorded. For each cage, solvent (acetone) control was tested first and then followed with a treatment. The time interval of assays between control and treatment was at least 1.5 hours, allowing the mosquitoes to fully recover and residual vapors from experiments to be ventilated out of the room. Controls were from two trials of solvent 1.5 hours apart to make sure that mosquitoes continue landing in the second trial at the same rate. Data from any cage that gave a low landing number in a control trial were discarded. Percentage repellency was determined for each cage using the following equation: Percentage repellency $= \left[1 - \left(\frac{\text{number of mosquitoes that landed on treatment}}{\text{number of mosquitoes that landed on solvent}}\right)\right] \times 100$.

Between two assays, the cages were sprayed with ethanol (99%) followed with a thorough rinse using distilled water to remove any residual chemicals on the cages and then a second ethanol spray before the cages were left to air dry. The modified glove and its magnetic frames were soaked in ethanol (99%) in a container, then rinsed with distilled water and a second ethanol rinse, before being left to air dry.

1.4.3 Single sensillum recording.

Single sensillum recording was conducted as described in Liu et al. (2013). Female mosquitoes 4 days after eclosion were anesthetized (2-3 min on ice) and mounted on a microscope slide (76 × 26 mm)(Liu et al., 2013). An antenna was fixed using a double-sided tape to a cover slip resting on a small ball of dental wax to facilitate manipulation. The cover slip was placed at an appropriate angle to the mosquito head. Once mounted, the specimen was placed under a microscope (Nikon SMA645, Japan) and the antenna viewed at a high magnification (1000×). Two tungsten microelectrodes were sharpened in 10% KNO_2 at 2-10 V. The reference electrode, which was connected to ground, was inserted into the compound eye of the mosquito and the other was connected to the preamplifier (10×, Syntech, Kirchzarten, Germany) and inserted into the shaft of an olfactory sensillum (in 11^{th} flagellomere) to complete the electrical circuit to extracellularly record ORN potentials (Den Otter et al., 1980). Controlled manipulation of the electrodes was performed using a micromanipulator (Burleigh PCS-6000, CA). The preamplifier was connected to an analog-to-digital signal converter (IDAC-4, Syntech, Germany), which in turn was connected to a computer for signal recording and visualization. The activity of co-located ORNs in each sensillum was assessed based on the differences in spike amplitude. The ORN with the large spike amplitude was designated as cell A and cell B with the small spike amplitude

(Ghaninia et al., 2007). Signals were recorded for 10 s starting 1 s before stimulation, and the action potentials were counted off-line over a 500 ms period before and after stimulation. The spontaneous firing rates observed in the preceding 500 ms were subtracted from the total spike rates observed during the 500 ms stimulation, and counts were recorded in units of spikes s^{-1}.

Eighteen compounds were used for functional identification of the olfactory sensilla of interest. Each compound was diluted in dimethyl sulfoxide (DMSO) to a stock solution with a concentration of $100\,\mu g\,\mu L^{-1}$. Subsequently, a series of 10-fold dilutions were made from each of the stock solutions for each compound tested. For each dilution, a 10 µL portion was dispersed onto a filter paper strip (4 × 50 mm), which was then inserted into a Pasteur pipette to create the stimulus cartridge. A sample containing the solvent alone served as control. The airflow across the antennae was maintained constant at a $20\,mL\,s^{-1}$ throughout the experiment. Purified and humidified air was delivered to the preparation through a glass tube (10 mm inner diameter) perforated by a small hole 10 cm away from the end of the tube, into which the tip of the Pasteur pipette could be inserted. The stimulus was delivered to the sensilla by inserting the tip of the stimulus cartridge into this hole and diverting a portion of the air stream ($0.5\,L\,min^{-1}$) to flow through the stimulus cartridge for 500 ms using a stimulus controller (Syntech, Germany). The distance between the end of the glass tube and the antennae was ≤ 1 cm. The number of spikes s^{-1} was obtained by averaging the results for each sensillum/compound combination. Recording from each replicate/sensillum was done using different mosquito individuals.

1.4.4 Data analysis, statistics and experimental repeats.

All statistical analysis was done using SigmaPlot 12.5 (Systat Software). Data are presented as mean ± s.e.m. Unpaired Student's *t*-test was used to compare two sets of data. If the data did not meet the normality or equality of the variance assumptions needed for Student's *t*-test, the equivalent Mann-Whitney Rank Sum test was used instead. The significance for all the tests was set to a P-value < 0.05. Each hand-in-cage experiment were repeated by two or more researchers.

* * *

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1.6 Supplementary Information

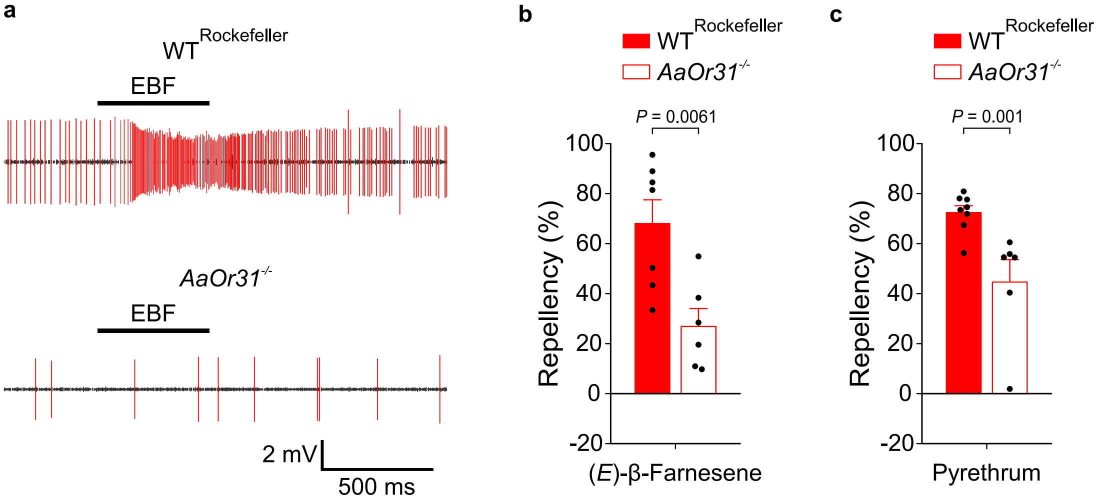
SI for Chapter 1 on page 26, partially redrafted from:

Liu F, Wang Q, Xu P, **Andreazza F**, Valbon WR, W Bandason E, Chen M, Yan R, Feng B, Smith L, Scott JG, Takamatsu G, Ihara M, Matsuda K, Klimavicz J, Coats J, Oliveira EE, Du Y, Dong K (2021) A dual-target molecular mechanism of pyrethrum repellency against mosquitoes. **Under review at: Nature Communications**.

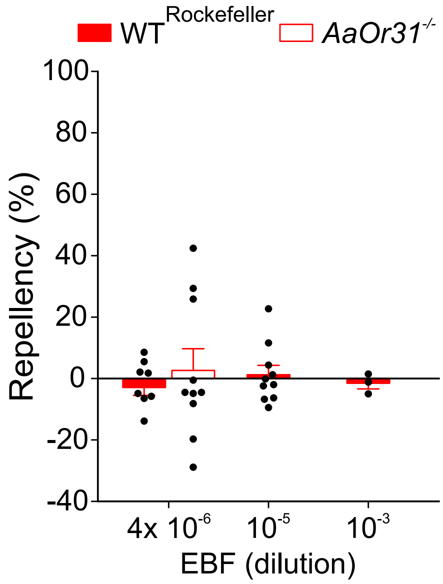
This SI section includes:

- Figures S1 to S9

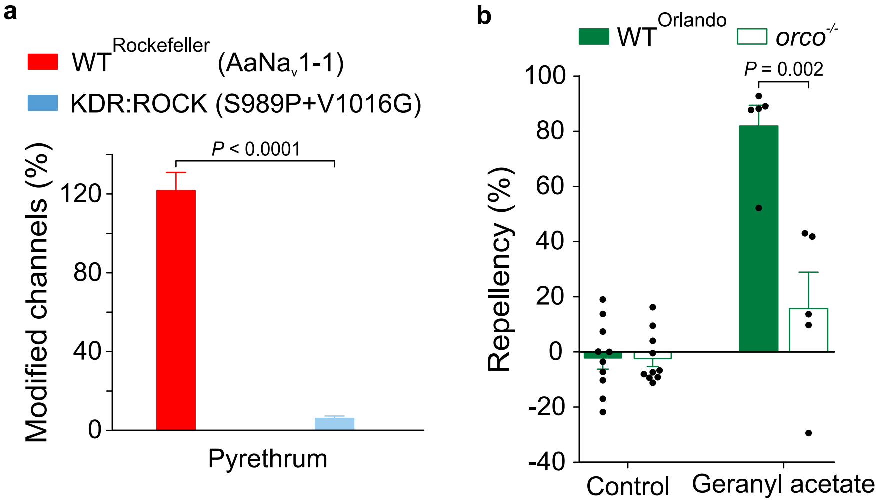
1.6.1 SI Figures



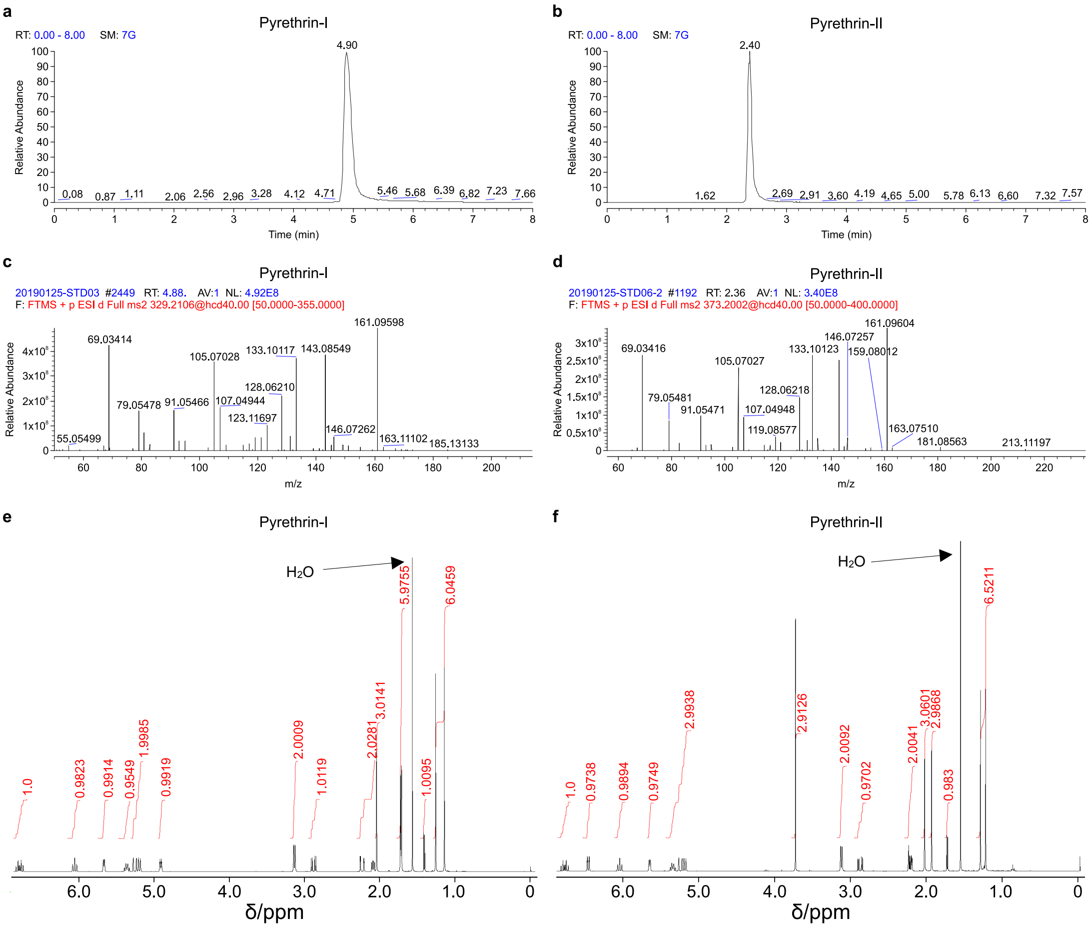
Supplementary Figure 1. **a**, Representative SSR traces from sbt-1 sensilla in Rockefeller (wild-type) and *AaOr31*$^{-/-}$ mosquitoes in response to EBF (n = 10 sensilla for Rockefeller and n = 7 sensilla for *AaOr31*$^{-/-}$) at 10^{-2} dilution. **b**, Repellency in *AaOr31*$^{-/-}$ compared with that of Rockefeller mosquitoes to EBF (t = 3.38, df = 11, n = 6 cages for *AaOr31*$^{-/-}$ and n = 7 cages for Rockefeller from 3 batches of mosquitoes) at 10^{-2} dilution. **c**, Repellency in *AaOr31*$^{-/-}$ compared with that of Rockefeller mosquitoes to pyrethrum (U = 1, n = 6 cages for *AaOr31*$^{-/-}$, n = 8 cages for Rockefeller from 3 batches of mosquitoes) at 10^{-2} dilution. Data are plotted as mean ± s.e.m. and dots denote the value of each repeat. Exact P-value are indicated for each comparison in the figure. **This data was provided by *Wang Q.*.**



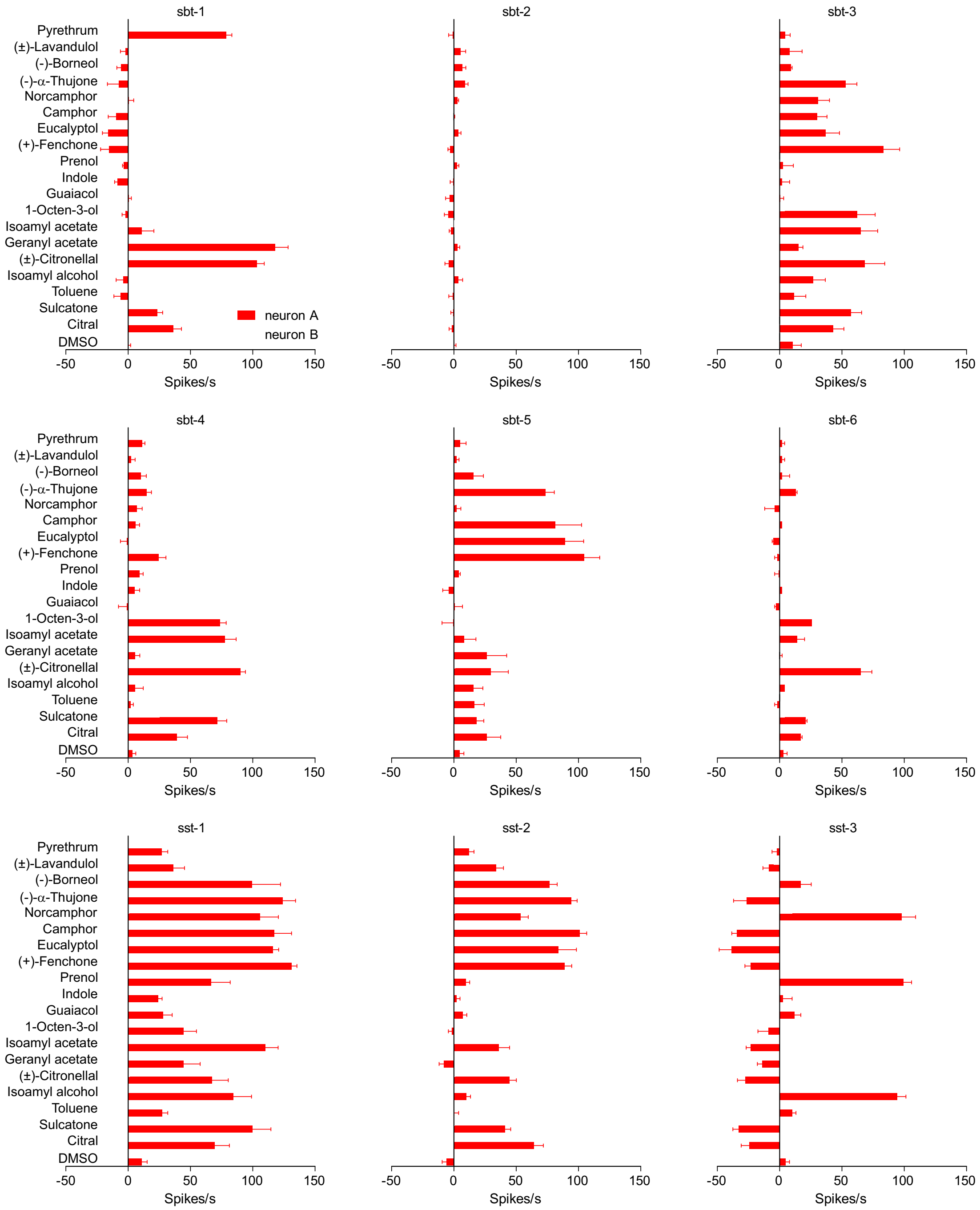
Supplementary Figure 2. EBF elicited no repellency at the 10^{-3} dilution (i.e., 11.4 µg cm^{-2}) or lower concentrations. Rockefeller: n = 8 cages for 4x 10^{-6}, n = 10 cages for 10^{-5}, n = 3 cages for 10^{-3}, from 2 batches of mosquitoes; *AaOr31*$^{-/-}$: n = 10 cages for 4x 10^{-6} from 2 batches of mosquitoes.



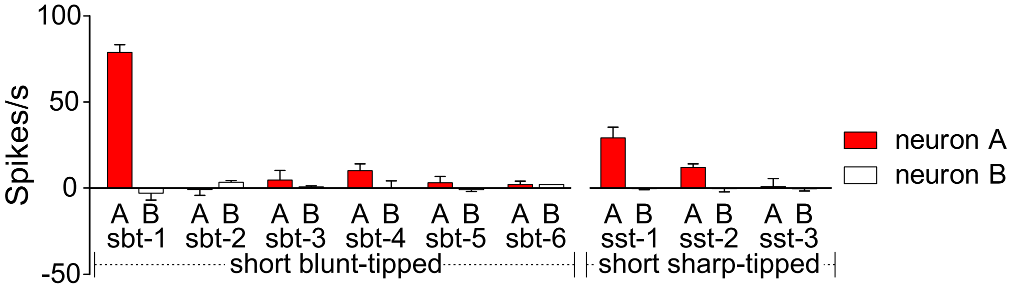
Supplementary Figure3. **a**, Two kdr mutations, S989P and V1016G, in the mosquito sodium channel (AaNa$_v$1-1) conferred AaNa$_v$1-1 channels resistance to pyrethrum (t = 9.8, df = 8, n = 5 for Rockefeller AaNa$_v$1-1 and n = 5 for KDR:ROCK AaNa$_v$1-1). AaNa$_v$1-1 wild type and the mutant channel carrying the double mutations were expressed in *Xenopus* oocytes and channel sensitivity to pyrethrum was examined using the two-electrode voltage clamp technique. The effect of pyrethrum was measured 10 min after its application. Pyrethrum-induced tail currents were recorded during a 100-pulse train of 5 ms step depolarizations from −120 to −10 mV with 5 ms inter-pulse intervals (Du et al., 2013). The percentage of pyrethrum-modified channels was calculated using the following equation: $M = \left[\frac{I_{tail}}{(E_h - E_{Na})}\right] / \left[\frac{I_{Na}}{(E_t - E_{Na})}\right] \times 100$; where I_{tail} is the maximal tail current amplitude, E_h is the potential to which the membrane is repolarized, E_{Na} is the reversal potential for sodium currents determined from the current-voltage curve, I_{Na} is the amplitude of the peak current during depolarization before pyrethrum exposure, and E_t is the potential of the step depolarization. **Data in panel "a" was provided by *Chen M.*.** **b**, Orco-dependency of geranyl acetate (n = 10 cages for each strain for control; geranyl acetate: t = 4.35, df = 8, P = 0.002, n = 8 cages for each strain). Data are plotted as mean ± s.e.m. and dots denote the value of each repeat. The exact P-value is indicated in the figure.



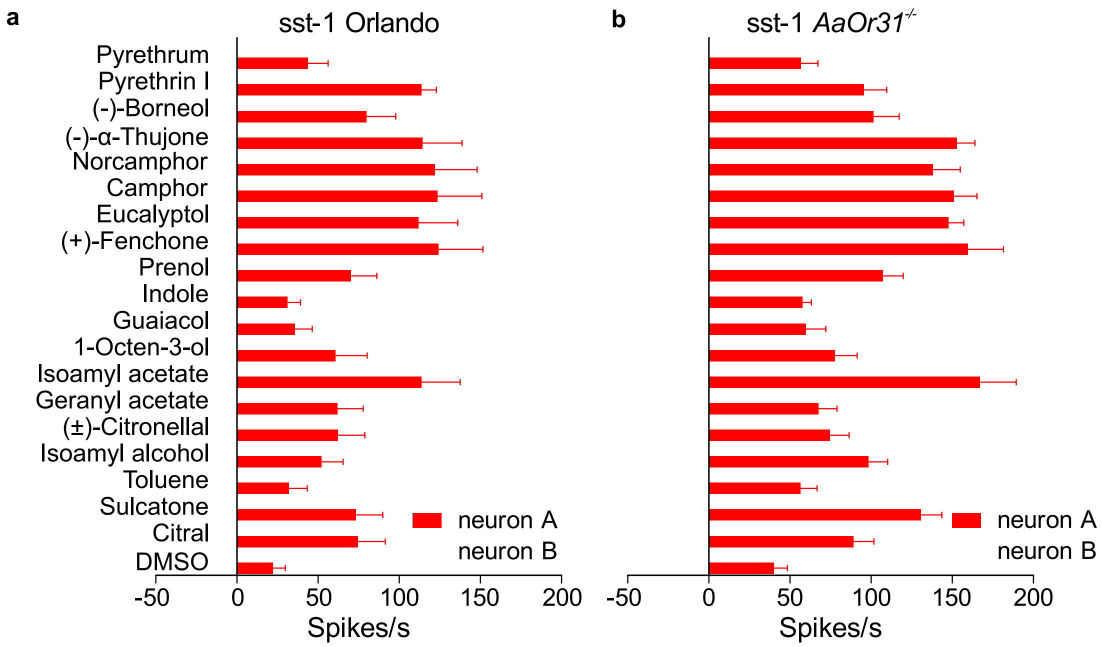
Supplementary Figure 4. Purities of pyrethrins I and II used in this study were validated by high resolution liquid chromatography tandem mass spectrometry (LC-MS) and nuclear magnetic resonance spectroscopy (NMR). **a** and **b**, LC chromatograms of pyrethrin I (**a**) and pyrethrin II (**b**). **c** and **d**, MS spectra of pyrethrin I (**c**) and pyrethrin II (**d**). For liquid chromatography, chromatographic column: Acquity UPLC BEH C18 (2.1 mm × 150 mm, 1.7 μm, Waters, USA), mobile phase: 0.1% formic acid-aqueous solution (mobile phase A) and 0.1% formic acid-acetonitrile solution (mobile phase B), run time: 10 min, flow rate: 0.3 mL min^{-1}, column temperature: 35 °C, isocratic elution mode: 80%B. For Mass spectrometry, Ion source: HESI+, Spray voltage: 3.8 kV, Sheath gas flow rate: 40, Aux gas flow rate: 10, Capillary temperature: 320 °C, S-lens RF level: 55, Aux gas heater temperature: 350 °C, Data acquisition mode: full MS /dd-ms^2(top N), full MS mode: Resolution: 70000, AGC target: 1e6, maximum IT: 200 Ms, Scan range: 50 to 750 m/z, spectrum data type: profile; dd MS^2 mode: Resolution: 17500, AGC target: 1e5, maximum IT: 50 Ms, Top N: 5, Isolation window: 1.6 m/z, (N)ce/stepped (N) CE: 20, 40, 60, spectrum data type: centroid; dd settings with Minimum AGC target: 8.00e3, Dynamic exclusion: 10 s. **e** and **f**, ^{1}H-NMR spectra of pyrethrin I (**e**) and pyrethrin II (**f**). ^{1}H-NMR spectra were recorded in CDCl$_3$ using an AV400M spectrometer (Bruker). **This data was provided by *Takamatsu G., Ihara M., Matsuda K.*.**



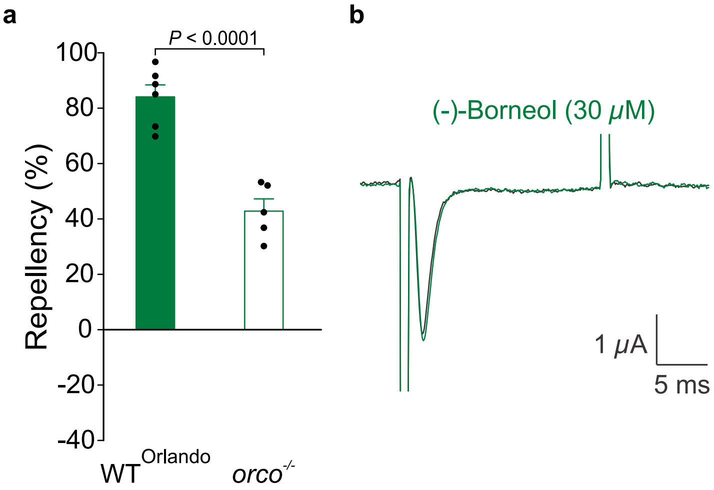
Supplementary Figure 5. The response profiles of three sst and six sbt sensilla identified in the single sensillum recording in Rockefeller mosquitoes (n = 2 for sbt-5 and sbt-6; n = 3 for sbt-3; n = 4 for sbt-4; n = 5 for sst-1 and sst-2; n = 7 for sbt-2; n = 8 for sbt-1 and sst-3). **This data was provided by *Liu F.* and *Wang Q.*.**



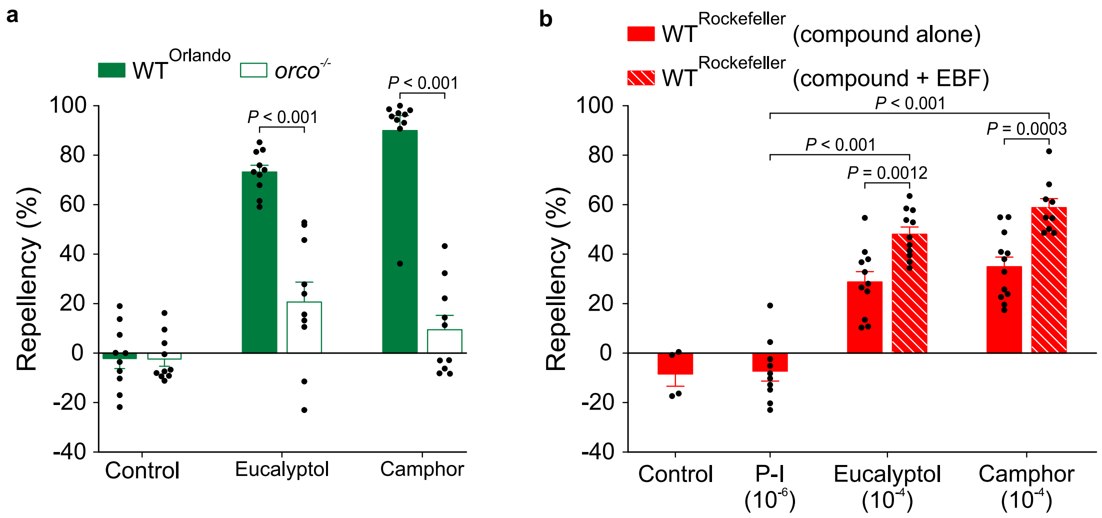
Supplementary Figure 6. SSR responses of sst and sbt sensilla to pyrethrum (10^{-2} v/v) (*n* = 8 sensilla for sbt-1; *n* = 7 for sbt-2; *n* = 3 for sbt-3 and sbt-4; *n* = 2 for sbt-5 and sbt-6; *n*= 5 for sst-1 and sst-2; *n* = 8 for sst-3). **This data was provided by *Liu F.* and *Wang Q.*.**



Supplementary Figure 7. The response profiles of sst-1 sensilla from Orlando (n = 7 sensilla) and *AaOr31*$^{-/-}$ (n = 7 sensilla) mosquitoes, including P-I.



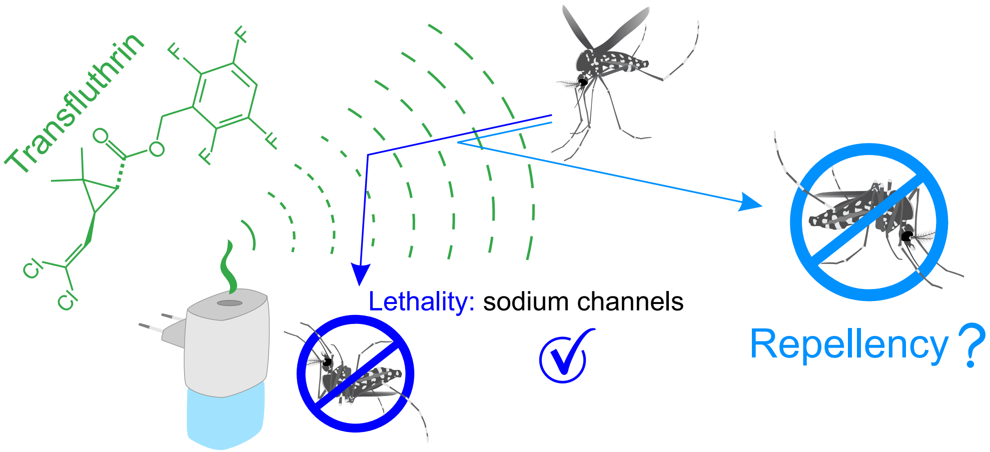
Supplementary Figure 8. **a**, Repellency by (-)-borneol (10^{-2} v/v) is Orco-mediated (t = 6.64, df = 9, n = 6 cages for Orlando (wild-type) and n = 5 cages for *orco*$^{-/-}$ from 3 batches of mosquitoes). **b**, Sodium current recording traces from AaNa$_v$1-1 channels expressed in *Xenopus* oocytes in response to 20 ms step-depolarization before and after exposed (-)-borneol (from n = 5 oocytes from two separated batches). (-)-borneol had no effects on peak current and channel gating. Data are plotted as mean ± s.e.m. and dots denote the value of each repeat. The exact P-value is indicated in the figure.



Supplementary Figure 9. **a**, Repellency by eucalyptol and camphor (10^{-2} v/v) is Orco-mediated (eucalyptol: $U = 0$; camphor: $U = 1.0$; $n = 10$ cages for each mosquito strain/compound, from two batches of mosquitoes). **b**, Pyrethrin I enhanced Orco-dependent repellency by eucalyptol and camphor in Rockefeller (wild-type) mosquitoes. (Two-tailed unpaired student's t-test: P-I *vs.* P-I + eucalyptol: $t = 11.46$, $df = 19$; P-I *vs.* P-I + camphor: $t = 12.33$, $df = 17$; eucalyptol *vs.* P-I + eucalyptol: $t = 3.78$, $df = 20$; camphor *vs.* P-I + camphor: $t = 4.37$, $df = 19$; $n = 4$ cages for control, $n = 9$ cages for camphor + P-I, $n = 10$ cages for P-I, $n = 11$ cages for eucalyptol and eucalyptol + P-I, and $n = 12$ cages for camphor, from 2 batches of mosquitoes). Data are plotted as mean ± s.e.m. and dots denote the value of each repeat. Exact P-values are indicated for each comparison. **Data for panel "b" was provided by *Valbon W.R.*.**

Chapter 2

Sodium channel activation underlies transfluthrin repellency and potentiation of odorant receptor-dependent aversion in *Aedes aegypti*



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Abstract

Volatile pyrethroid insecticides, such as transfluthrin, have received great attention as an insect repellent in recent years for controlling human disease vectors. Although it has been long understood that pyrethroids kill insects by prolonged activation of insect voltage-gated sodium channels, the mechanism of pyrethroid repellency remains poorly understood and controversial. Here, we show that transfluthrin is a potent spatial repellent and its vapor could repel *Aedes aegypti* in a hand-in-cage assay at nonlethal concentrations as low as 1 ppm. Contrary to previous reports, transfluthrin does not activate olfactory receptor neurons or affect mosquito's olfactory receptor neurons used to detect host odorants and other mosquito repellents. Remarkably, transfluthrin enhanced repellency by DEET and several plant-derived repellents that are known to activate odorant receptors (Ors). The *1S-cis* isomer of transfluthrin, which does not activate sodium channels, neither elicited nor enhanced Or-mediated repellency. Mutations in the voltage-gated sodium channel gene that reduce the potency of transfluthrin on sodium channels decreased transfluthrin repellency but did not affect repellency by DEET. These results provide a surprising first example that sodium channel activation alone, not activation of olfactory receptors, is sufficient for repelling mosquitoes. Our finding of sodium channel activation as the principal mechanism of transfluthrin repellency has broad implications in future development of a new generation of dual-target repellent formulations to more effectively repel a variety of human disease vectors.

Keywords: Sodium channel; Mosquito; Pyrethroid; Repellency; Synergism; Olfaction.

* * *

Significance

Vector-transmitted human diseases, such as dengue fever, represent serious global health burdens. Pyrethroids, including transfluthrin, are widely used as insecticides and mosquito repellents due to their relatively benign environmental impact. Pyrethroids target voltage-gated sodium channels for their insecticidal action. However, the mechanism of pyrethroid repellency remains unclear and often controversial. Insect repellency is traditionally thought to be mediated by olfactory receptors. We made two important discoveries in this study, showing that transfluthrin repellency is via activation of sodium channels and transfluthrin activation of sodium channel potentiates odorant receptor-mediated repellents including DEET. Discovery of sodium channel activation as a major mechanism of pyrethroid repellency has broad significance in insect olfaction study, repellents development, and control of human disease vectors.

2.1 Introduction

Pyrethroid insecticides are synthetic analogues of natural pyrethrins, which are major insecticidal components of the pyrethrum extract from the flowers of *Chrysanthemum* (*Tanacetum*) species (Elliott, 1977). Pyrethroids have been used extensively in the control of vectors of human diseases, including malaria, dengue, Zika, Lyme disease and leishmaniasis. The insecticidal activity of pyrethroids is through their action on voltage-gated sodium channels, which are critical for electrical signaling in the nervous system. Pyrethroids prolong activation of sodium channels, resulting in repetitive firing and/or membrane depolarization and eventual nerve conduction block and paralysis (i.e., knockdown) (Narahashi, 2000; Soderlund et al., 2002). Mutations in the sodium channel confer a major type of pyrethroid resistance, known as knockdown resistance (kdr) (Dong et al., 2014; Rinkevich et al., 2013; Soderlund et al., 2002).

Volatile pyrethroids are also used as popular repellents globally, in the form of vaporizers, emanators, mats, and coils, against mosquitoes (Achee et al., 2012; Bibbs and Kaufman, 2017; Govella et al., 2015; Hill et al., 2014; Kawada et al., 2005; Mmbando et al., 2019; Ogoma et al., 2017; Revay et al., 2013; Xue et al., 2012). For example, as one of the most widely used volatile pyrethroids, transfluthrin has been incorporated into a variety of new mosquito control products and programs. When mosquito coils treated with 0.03% transfluthrin either alone or in combination with long-lasting insecticide bed nets (LLINs), malaria parasite prevalence was reduced by up to 94% (Hill et al., 2014). Application of transfluthrin to hessian or sisal decorations/products, eave ribbons, eave-baffles and window screens offers a promising method of mosquito contact/bit prevention in both indoor or outdoor settings (Andrés et al., 2015; Govella et al., 2015; Killeen et al., 2017; Masalu et al., 2020; Mmbando et al., 2018, 2019; Ogoma et al., 2012, 2017). For example, transfluthrin-treated decorative baskets and wall decorations reduced bites of *Anopheles arabiensis* and *Culex* spp. mosquitoes by more than 80 and 60%, respectively (Masalu et al., 2017, 2018). Similarly, eave ribbons treated with transfluthrin were reported to significantly prevent outdoor-biting and indoor-biting by malaria vectors (Mmbando et al., 2018, 2019).

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Despite the importance of pyrethroid-treated products in preventing mosquito biting and reducing disease transmission, the mechanistic basis of pyrethroid repellency remains not well understood and controversial. Several laboratory behavioral assays designed to evaluate spatial (i.e., non-contact) repellency showed that *Ae. aegypti* mosquitoes moved away from transfluthrin vapor, establishing transfluthrin elicits spatial repellency (Jiang et al., 2019; Nentwig et al., 2017; Wagman et al., 2015). In an earlier study, a pyrethroid (TL-I-73) was shown to inhibit the activities of odorant receptors (Ors) induced by volatiles indole and R-(-)-1-octen-3-ol in *Xenopus* oocytes, suggesting that the repellent activity of pyrethroids may be accounted for by their inhibitory effects on Ors (Bohbot et al., 2011). Transfluthrin repellency was reduced in pyrethroid-resistant *Ae. aegypti* mosquitoes carrying a kdr mutation (Wagman et al., 2015). It was proposed that neurotoxic irritation of mosquitoes by sublethal doses of transfluthrin as a mechanism of transfluthrin repellency (Wagman et al., 2015). A more recent study (Yang et al., 2020) also showed reduced repellency by transfluthrin and metofluthrin in a pyrethroid-resistant *Ae. aegypti* line, Puerto Rico, carrying multiple kdr mutations (Estep et al., 2017; Fan et al., 2020). However, surprisingly, these Puerto Rico mosquitoes also exhibited resistance to repellency by DEET, 2-undecanone and IR3535, which do not act on sodium channels. Furthermore, the same study (Yang et al., 2020) showed that transfluthrin and metofluthrin elicited electroantennogram (EAG) responses from adults of *Ae. aegypti* mosquitoes and the amplitudes of EAG signals elicited by pyrethroids and non-pyrethroid repellents were reduced in kdr mosquitoes. These authors (Yang et al., 2020) suggested that the reduced sensitivity of Puerto Rico mosquitoes to pyrethroids and non-pyrethroid repellents may represent a general fitness cost associated with the kdr mutations in Puerto Rico mosquitoes. Accordingly, the fundamental questions of (i) whether pyrethroids activate or inhibit Ors activities and (ii) whether activation of sodium channels per se mediates transfluthrin repellency remain unclear.

In this study we took a combination of molecular genetics, electrophysiological and behavioral approaches to determine (i) whether transfluthrin activates olfactory receptors; (ii) whether activation of sodium channels alone is sufficient to elicit spatial repellency; and (iii) whether activation of sodium channels by transfluthrin alters neural activities and spatial repellency by other plant derived mosquito repellents. Our results show that spatial repellency by transfluthrin solely relies on activation of sodium channels and does not depend on olfactory sensory inputs. Furthermore, we discovered that transfluthrin enhances repellency by DEET and plant-derived mosquito repellents via activation of sodium channels. Finally, we did not detect any effects by transfluthrin on the activities of olfactory receptor neurons (ORNs), suggesting that a sodium channel-mediated central neuronal mechanism underlies transfluthrin repellency and synergism. Our results provide the first example for sodium channel activation alone, not activation of olfactory receptors, as a principal mechanism of repelling insects. The demonstrated synergism between sodium channel activation and Or-mediated pathway have significant practical implications for the development of a new generation of synergistic multi-target repellent mixtures that would more effectively combat mosquitoes and other human disease vectors.

2.2 Results

2.2.1 Transfluthrin elicits spatial (i.e., non-contact) repellency in *Ae. aegypti*.

To evaluate spatial repellency by transfluthrin in the presence of an attraction, a human hand, we adopted a hand-in-cage assay developed by Boyle et al. (2016). This assay setup involves placement of a human hand with a modified glove in a mosquito cage (Fig. 1*A*). The modified glove has a window on its back, which is screened with two layers of mesh that are separated in space by a stack of magnetic window frames (Fig. 1*A*). When mosquitoes in the cage are attracted to the human hand, the top mesh serves as physical barrier preventing mosquitoes from direct contact with the bottom mesh (above the hand) and the hand. When the bottom mesh was treated with acetone as control, the landing of mosquitoes on the top mesh was not affected. However, when the bottom mesh was treated with transfluthrin, significant less frequent landing of mosquitoes was observed (Fig. 1*B*). This repellent effect caused by transfluthrin vapor was observed at concentrations as low as 1 ppm of transfluthrin (i.e., 20 ng/cm^2) in Rockefeller (wild-type) mosquitoes (Fig. 1*B*). Similarly, when the bottom mesh was treated with DEET (at 1 ppm), a gold-standard mosquito repellent, significant less frequent landing of mosquitoes was observed (Fig. 1*C*). Greater repellency was observed at higher concentrations of transfluthrin (Fig. 1*B*). However, at 100 ppm, about 10% of the mosquitoes exhibited uncoordinated locomotion, and at 1000 ppm, a small fraction of mosquitoes (8.42 ± 2.2%) were knocked down during the assay. No such locomotive modifications were observed at both 1 and 10 ppm. Therefore, in subsequent experiments in this study, we evaluated transfluthrin repellency only at the 1 ppm or below to avoid complications from the insecticidal and/or neurotoxic activity of transfluthrin at higher dosages.

2.2.2 Transfluthrin vapor does not activate olfactory receptor neurons (ORNs).

Yang and colleagues (Yang et al., 2020) recently reported that transfluthrin induces EAG responses in *Ae. aegypti*, including Orlando (wild-type) and Puerto Rico (pyrethroid-resistant) mosquitoes. During the early stage of this project, we also found that some transfluthrin samples, provided by a former collaborator, elicited EAG responses in *Ae. aegypti*. However, we subsequently found those transfluthrin samples contained impurities. When we repeated the experiment using transfluthrin from Sigma-Aldrich (99.2 - 99.9% purity), we could not detect any EAG signals in either Rockefeller, Orlando or Puerto Rico mosquitoes, even when undiluted transfluthrin was delivered in the EAG experiment (Fig. 1*D* and *SI Appendix*, Fig. S1*A*). Similarly, no EAG responses were detected by transfluthrin from Jiangsu Yangnong Chemical Co. Ltd. (Jiangsu, China; 98.5% purity) (*SI Appendix*, Fig. S1*B*). In contrast, DEET, 1-octen-3-ol and lactic acid all evoked EAG responses, as expected (Fig. 1*D* and *SI Appendix*, Fig. S1). Furthermore, consistent with our EAG results, we did not detect any response to transfluthrin from olfactory sensilla using single sensillum recording (SSR). In contrast, our SSR revealed that DEET activated a specific type of olfactory sensilla (*SI Appendix*, Fig. S2).

Next, we evaluated transfluthrin and DEET repellency in an *Ae. aegypti* mutant,

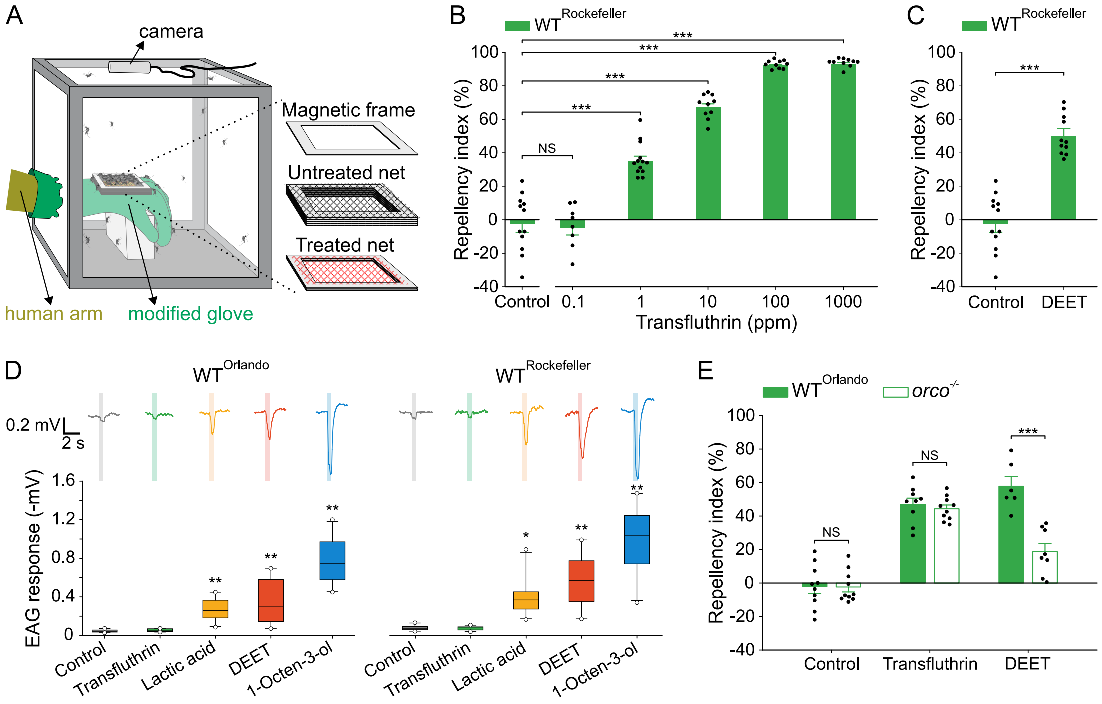

Figure 1: Transfluthrin elicits Or-independent spatial repellency in *Ae. aegypti*. (*A*) A schematic drawing of the hand-in-cage setup. (*B*) Dose-dependence of transfluthrin repellency; t = 0.25, df = 18, P = 0.802 for 0.1 ppm (v/v), U = 0, $P < 0.001$ for both 1, 10, 100 and 1000 ppm; NS = not significant, ***$P < 0.001$; n values for: control = 12, 0.1 ppm = 8, 1 ppm = 13, and the rest = 10. (*C*) Repellency by DEET (1 ppm); t = 8.45, df = 21, $P < 0.001$; ***$P < 0.001$; n values for: control = 12 and DEET = 11. (*D*) EAG performed with two wild-type (Orlando and Rockefeller) lines; 1 µL pure compound used; a representative trace is shown above each plot; the asterisks (*$P < 0.05$, **$P < 0.01$) indicate significant differences against control within each mosquito line, χ^2 = 46.49, df = 5, $P < 0.001$ for Orlando, and χ^2 = 43.83, df = 5, $P < 0.001$ for Rockefeller; n = 10 antennae. (*E*) Repellency by 1 ppm of both transfluthrin and DEET in Orlando and *orco*$^{-/-}$ mosquitoes; control: t = 0.05, df = 18, P = 0.963, transfluthrin: t = 0.64, df = 17, P = 0.530, DEET: t = 5.17, df = 12, $P < 0.001$, NS = not significant, ***$P < 0.001$; n values for: both controls and transfluthrin in *orco*$^{-/-}$ = 10, transfluthrin in Orlando = 9, DEET in Orlando = 6, and DEET in *orco*$^{-/-}$ = 8. The control represents the baseline activity in response to the solvent. Data are presented as mean ± SEM. Dots over the bars represent individual replicate values. Click for representative videos for panels (*B*), (*C*), and (*E*).

orco$^{-/-}$, in which the odorant receptor co-receptor gene (*orco*) was mutated resulting in impaired Or-mediated olfactory pathways (DeGennaro et al., 2013). DEET repellency was reduced in *orco*$^{-/-}$ mosquitoes (Fig. 1*E*) compared to the wild-type *Ae. aegypti* line Orlando, from which the *orco*$^{-/-}$ mutant was generated (DeGennaro et al., 2013), consistent with the observation that DEET non-contact repellency is Or/Orco-dependent (DeGennaro et al., 2013). However, transfluthrin repellency was not reduced in *orco*$^{-/-}$ mosquitoes (Fig. 1*E*), further indicating that transfluthrin repellency does not require activation of Or/Orco. Collectively, results from EAG, SSR and the *orco*$^{-/-}$ mutant contradict the notion that transfluthrin activates ORNs as part of its repellent mechanism. We speculate that impurities in our earlier transfluthrin samples were probably responsible for eliciting EAG signals in our initial experiments.

2.2.3 Transfluthrin vapor did not affect the ability of OSNs to detect human attractants.

To test whether transfluthrin repellency observed in the hand-in-cage assay is due to transfluthrin impairing the ability of ORNs to sense odorants emitted from the human hand, we examined the effect of transfluthrin on the EAG response to four attractants: 1-octen-3-ol, sulcatone, nonanal, and lactic acid (Mathew et al., 2013; McBride et al., 2014; Raji et al., 2019; Syed and Leal, 2009). Co-exposure of transfluthrin with any of the four attractants did not affect the amplitude or dynamics of EAG signals evoked by the attractants (Fig. 2). These results indicate that transfluthrin did not alter the ability of ORNs to sense these attractants emitted from the human hand and suggest that transfluthrin repellency is not a result of a general impairment of the olfactory sensory system.

2.2.4 Transfluthrin repellency depends on activation of voltage-gated sodium channels.

To determine the involvement of sodium channel activation in transfluthrin repellency, and minimize possible secondary effects due to background genomic differ-

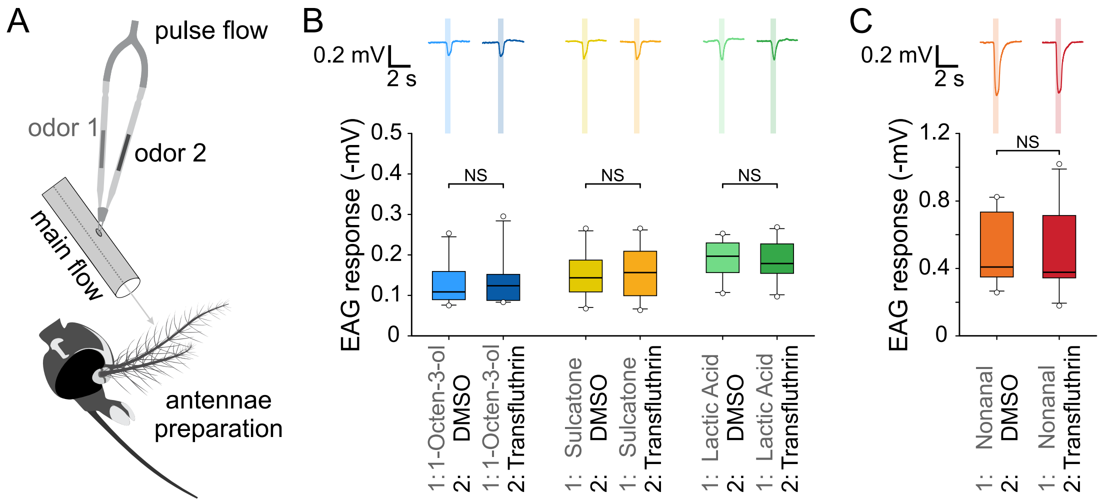

Figure 2: Transfluthrin does not impair the ability of *Ae. aegypti* to detect human odorants. (*A*) A schematic drawing of the EAG setup with a two-cartridge odor delivery system (Syed and Leal 2008). EAG responses by 1-octen-3-ol, sulcatone, and lactic acid (*B*) and nonanal (*C*) from Rockefeller mosquitoes; chemicals were diluted in DMSO to 10^{-2}; data is plotted as in Fig. 1*D*; for 1-octen-3-ol: $t = 1.1$, $df = 9$, $P = 0.298$, for sulcatone: $Z = 0.87$, $P = 0.432$, for lactic acid: $t = 0.93$, $df = 9$, $P = 0.378$, for nonanal: $Z = 1.68$, $P = 0.105$, NS = not significant; $n = 10$ antennae.

ences, we used two genetically related lines, Rockefeller (wild-type) and KDR:ROCK (pyrethroid -resistant, carrying the S989P+V1016G *kdr* allele (Smith et al., 2018)). KDR:ROCK mosquitoes were 30-fold more resistant to knockdown by transfluthrin than Rockefeller mosquitoes in a vapor toxicity bioassay (*SI Appendix*, Fig. S 3*A*). We also confirmed that AaNa$_v$1-1 channels carrying the two kdr mutations were resistant to transfluthrin compared to AaNa$_v$1-1 wild-type channels (*SI Appendix*, Fig. S 3*B*), similar to earlier studies reporting that the double mutation channel was resistant to permethrin and deltamethrin, two pyrethroids (Du et al., 2013; Hirata et al., 2014). As shown in Fig. 3*A*, transfluthrin repellency was reduced in KDR:ROCK compared to Rockefeller. Furthermore, we found that repellency by DEET was similar in Rockefeller and KDR:ROCK mosquitoes (Fig. 3*A*), indicating that S989P+V1016G mutations did not reduce DEET repellency in KDR:ROCK mosquitoes.

To further evaluate the role of sodium channels in transfluthrin repellency, we took advantage of stereospecific effects of pyrethroids on sodium channels and toxicity (Lund and Narahashi, 1983; Soderlund et al., 2002). Pyrethroids possess alterna-

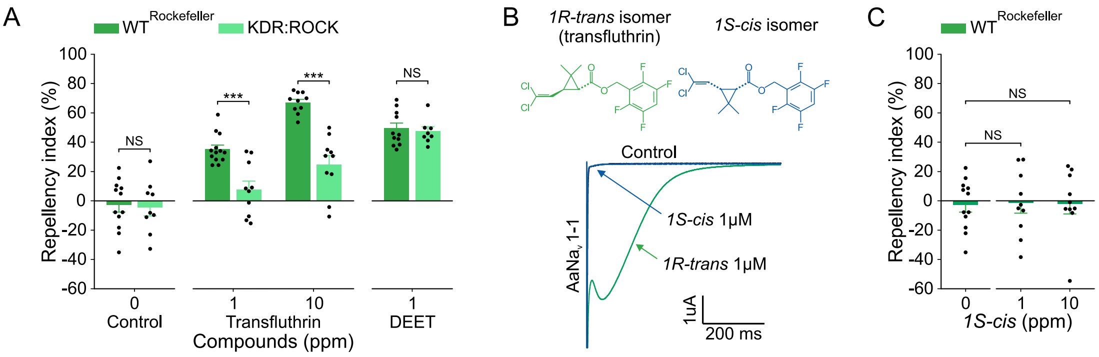

Figure 3: Transfluthrin repellency depends on its action on voltage-gated sodium channels. (*A*) Repellency by transfluthrin and DEET in Rockefeller and KDR:ROCK mosquitoes. For control: t = 0.20, df = 19, P = 0.840, for transfluthrin 1 ppm: t = 4.62, df = 21, $P < 0.001$, for transfluthrin 10 ppm: t = 6.39, df = 18, $P < 0.001$, for DEET: t = 0.39, df = 17, P = 0.701, NS = not significant, ***$P < 0.001$; n values for: KDR:ROCK for DEET = 8, KDR:ROCK for control = 9, Rockefeller for DEET = 11, Rockefeller for control = 12, Rockefeller for transfluthrin 1 ppm = 13, and the rest = 10. (*B*) Measurement of tail-current induced by *1R-trans* (transfluthrin) or *1S-cis* isomers following a 100-pulse train of 5-ms depolarization from -120 mV to 0 mV with 5-ms interval from AaNa$_v$1-1 channels in *Xenopus* oocytes (Du et al., 2013). (*C*) Repellency by *1S-cis* isomer in Rockefeller; For 0 *vs* 1 ppm: t = 0.1, df = 20, P = 0.922, for 0 *vs* 10 ppm: t = 0.16, df = 20, P = 0.871, NS = not significant; n values for: control = 12, and the rest =10. The control represents the baseline activity in response to the solvent. Data in panels *A* and *C* are presented as mean ± SEM. Dots over the bars represent individual replicate values. Click for representative videos for panels (*A*) and (*C*).

tive chiral configurations at C1 and C3 of the cyclopropane ring. *1R-cis* and *1R-trans* isomers are active, whereas *1S-cis*, and *1S-trans* isomers are inactive (Lund and Narahashi, 1983; Soderlund et al., 2002). Commercial transfluthrin is in *1R-trans* configuration (Fig. 3*B*). Upon repolarization under voltage-clamp conditions, transfluthrin induced tail-currents in the *Ae. aegypti* sodium channel, AaNa$_v$1-1, expressed in *Xenopus* oocytes (Fig. 3*B*), indicating the prolonged opening of sodium channels by transfluthrin. In contrast, *1S-cis* isomer did not induce any tail current (Fig. 3*B*), indicating that *1S-cis* isomer cannot act on sodium channels. As expected, *1S-cis* isomer did not induce knockdown in a vapor toxicity bioassay (*SI Appendix*, Fig. S3C). Importantly, *1S-cis* isomer did not elicit spatial repellency (Fig. 3*C*). These results clearly showed that activation of sodium channels is essential for transfluthrin to elicit repellency.

2.2.5 Transfluthrin enhances the potency of DEET and other mosquito repellents.

We were puzzled by the unexpected differences between our EAG results and those of Yang and colleagues (Yang et al., 2020). One possibility, we hypothesized, is that the effect of transfluthrin on olfactory responses, as observed in our preliminary experiments and in the study of Yang and colleagues (Yang et al., 2020), may be caused in part by some type of cross-interaction between transfluthrin and additional olfactory response-eliciting compounds that are fortuitously present in certain transfluthrin samples. To test this hypothesis, we examined whether transfluthrin affects DEET repellency in Rockefeller mosquitoes. Remarkably, transfluthrin at 0.1 ppm (i.e., $2\,\mathrm{ng/cm^2}$), which did not elicit repellency alone, enhanced DEET repellency in Rockefeller mosquitoes (Fig. 4*A*). This result raised the intriguing possibility that activation of sodium channels by transfluthrin can potentiate Or-dependent repellent activities.

The striking effect of transfluthrin on DEET repellency prompted us to test the hypothesis that hyper-activation of sodium channels by transfluthrin may be a general mechanism for synergizing the activities of a wide variety of other repellents. Indeed, similar to DEET, we found that transfluthrin also has mutually enhancing effects in other repellents. Geranyl acetate (GA), which has been reported to have repellent activity against mosquitoes (Kwon et al., 2014; Michaelakis et al., 2014), also elicited spatial repellency in our hand-in-cage assay (*SI Appendix*, Fig. S 4). Furthermore, GA repellency was reduced in *orco*$^{-/-}$ mosquitoes, indicating that GA repellency is Or-mediated (*SI Appendix*, Fig. S 4). We found that at 3000 ppm (i.e., $38\,\mathrm{\mu g\,cm^2}$) GA alone induced 41% of repellency, but when it was combined with transfluthrin at 0.1 ppm, repellency reached 69%, even though 0.1 ppm of transfluthrin by itself did not elicit repellency (Fig. 4*B*). Conversely, 35% repellency elicited by 1 ppm of transfluthrin was enhanced to 80% when mixed with 100 ppm of GA, which by itself had no repellency (Fig. 4*C*). Interestingly, combination of 0.1 ppm of transfluthrin and 100 ppm of GA, neither of which alone elicited repellency, evoked 33% repellency

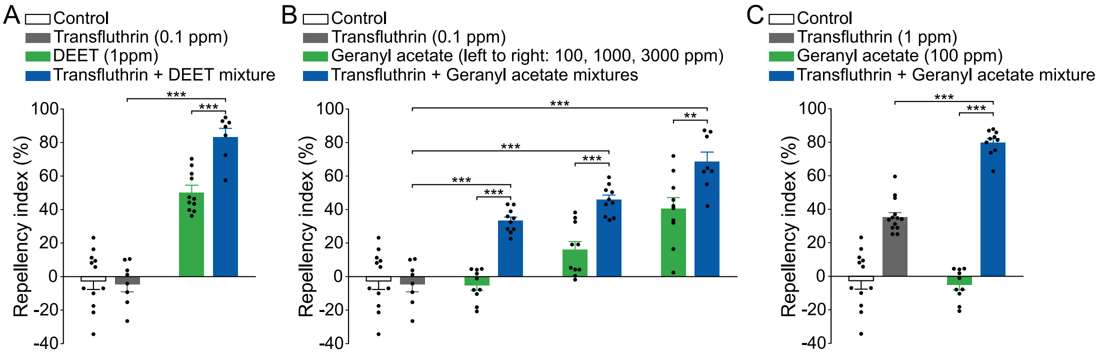

Figure 4: Transfluthrin potentiates the repellency by DEET (*A*) and geranyl acetate (*B* and *C*) in Rockefeller mosquitoes (*A*) DEET; for mixture *vs* transfluthrin: $t = 12.82$, $df = 13$, $P < 0.001$, for mixture *vs* DEET: $t = 5.57$, $df = 16$, $P < 0.001$, $^{***}P < 0.001$; n values for: mixture = 7, transfluthrin = 8, DEET = 11, and control = 12. (*B*) Geranyl acetate (GA); for GA (100 ppm) plus transfluthrin (0.1 ppm) *vs*: GA: $t = 10.39$, $df = 18$, $P < 0.001$ and transfluthrin: $t = 7.99$, $df = 16$, $P < 0.001$, for GA (1000 ppm) plus transfluthrin (0.1 ppm) *vs*: GA: $U = 5$, $P < 0.001$ and transfluthrin: $t = 9.86$, $df = 16$, $P < 0.001$; for GA (3000 ppm) plus transfluthrin (0.1 ppm) *vs*: GA: $t = 3.12$, $df = 16$, $P = 0.007$ and transfluthrin: $t = 9.97$, $df = 14$, $P < 0.001$; $^{**}P < 0.01$, $^{***}P < 0.001$; n values for: transfluthrin (0.1 ppm) and its mixture with GA (3000 ppm) = 8, control =12, and the rest = 10. (*C*) For GA (100 ppm) with transfluthrin (1 ppm) *vs*: GA: $t = 22.25$, $df = 18$, $P < 0.001$ and transfluthrin: $t = 11.72$, $df = 21$, $P < 0.001$, $^{***}P < 0.001$; n values for: transfluthrin (1 ppm) = 13, control = 12, and the rest = 10. The control represents the baseline activity in response to the solvent. Data are presented as mean ± SEM, and dots over the bars represent individual replicate values. Click for representative videos for panels (*A*) and (*B*).

(Fig. 4*B*). Collectively, these results showed that there is mutual potentiation between GA and transfluthrin.

We then examined two additional plant-derived mosquito repellents, camphor and eucalyptol, for repellency and potential synergism with transfluthrin. Like GA, both compounds evoked Orco-dependent spatial repellency (*SI Appendix*, Fig. S4). When combined with 0.1 ppm of transfluthrin, repellency by camphor or eucalyptol was significantly enhanced (*SI Appendix*, Fig. S5*A* and *B*). Taken together, our results showed that activation of sodium channels by transfluthrin appears to have a general effect on enhancing the potency of potentially a variety of repellents that activate olfactory repellent pathways.

2.2.6 Transfluthrin potentiation of Or-mediated aversion is mediated by activation of sodium channels.

The interesting potentiation between transfluthrin and DEET, GA and other repellents propelled us to further examine the underlying mechanism. First, we examined whether the observed transfluthrin synergism with Or-mediated repellents requires involvement of transfluthrin's action on sodium channels. As previously mentioned, commercial transfluthrin is in *1R-trans* configuration, whereas *1S-cis* isomer is inactive on sodium channels (Fig. 3*B*). We found that transfluthrin-GA synergism was not detected when transfluthrin was replaced with *1S-cis* inactive isomer (Fig. 5*A* and *B*). These results showed that activation of sodium channels is essential for transfluthrin to function as an effective synergist of Or-based repellents. In further support of a critical role of sodium channel activation in synergism, the enhanced transfluthrin/GA combinational repellency was much less in transfluthrin-resistant KDR:ROCK mosquitoes compared to that in transfluthrin-sensitive Rockefeller mosquitoes (*SI Appendix*, Fig. S 6*A* and *B*). Collectively, these results showed that activation of sodium channels by transfluthrin is critical for potentiating the GA-activated repellency pathway.

2.2.7 Transfluthrin vapor does not affect the response of ORNs to volatiles that mediate Orco-dependent repellency.

Next, we evaluated whether transfluthrin affects the responses of antennal ORNs to these repellents by comparing EAG responses from each repellent alone or co-applied with transfluthrin. We observed that DEET, camphor, eucalyptol, and GA, all elicited EAG responses, as expected, and that transfluthrin did not alter the amplitude or dynamics of EAG signals evoked by any of these volatiles (*SI Appendix*, Fig. S 7).

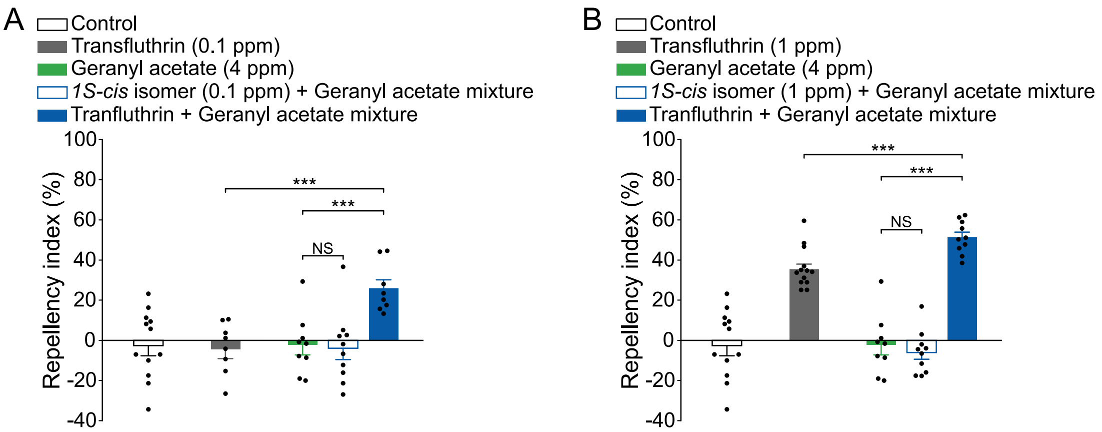

Figure 5: Transfluthrin potentiation of the Or-mediated repellency depends on its action on sodium channels. Rockefeller line. (*A*) For geranyl acetate (GA) plus transfluthrin (0.1 ppm) *vs*: GA: $t = 4.20$, $df = 15$, $P = 0.001$ and transfluthrin: $t = 4.83$, $df = 14$, $P < 0.001$; and for GA plus *1S-cis* isomer (0.1 ppm) *vs*: GA: $t = 0.22$, $df = 17$, $P = 0.830$. (*B*) For GA plus transfluthrin (1 ppm) *vs*: GA: $t = 9.87$, $df = 17$, $P < 0.001$ and transfluthrin: $t = 4.14$, $df = 21$, $P < 0.001$; for GA plus *1S-cis* isomer (1 ppm) *vs*: GA: $t = 0.64$, $df = 17$, $P = 0.530$. (*A* and *B*) NS = not significant, ***$P < 0.001$; n values for: transfluthrin (0.1ppm) and its mixture = 8, GA = 9, control 12, transfluthrin (1 ppm) = 13, and the rest = 10. The control represents the baseline activity in response to the solvent. Data are presented as mean ± SEM. Data from GA (4 ppm) are presented more than once to facilitate comparison. Dots over the bars represents individual replicate values. Click for representative videos for panels (*A*) and (*B*).

2.2.8 Commercial transfluthrin-based mosquito repellents possess both sodium channel-mediated and Or-mediated repellencies.

Our finding that transfluthrin can amplify Or-mediated repellency by other volatiles at extremely low concentrations led us to test whether repellency by transfluthrin in commercial products contains a component of Or-mediated repellency since these commercial products may contain impurities that could activate Ors. We collected three commercial transfluthrin liquid mosquito repellent products from Brazil, China, and France. The percentage of the active ingredient (i.e., transfluthrin) in these products ranged from 0.80 to 0.92%. We diluted each product to the concentration of 1 ppm of transfluthrin for direct comparison with the Sigma-Aldrich transfluthrin in the hand-in-cage assay. Remarkably, all three commercial transfluthrin products elicited repellency greater than that by Sigma-Aldrich's transfluthrin in Orlando mosquitoes. Furthermore, while the repellency by the Sigma-Aldrich transfluthrin in *orco*$^{-/-}$ mosquitoes remained intact (Fig. 1*E*), repellency by these products were significantly reduced in *orco*$^{-/-}$ mosquitoes (Fig. 6). Interestingly, the remaining percentage of repellency in *orco*$^{-/-}$ mosquitoes by the commercial products was comparable to that by the Sigma-Aldrich transfluthrin in wild-type Rockefeller and Orlando mosquitoes (Fig. 1*B* and *E*). Together, these results show all examined commercial transfluthrin products contain additional compounds that activate Ors, which contribute to repellency.

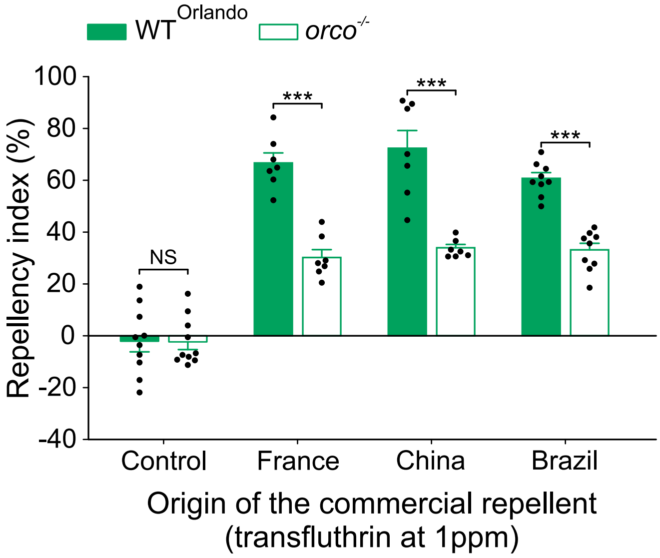

Figure 6: Repellency by commercial transfluthrin products consists of both Or-mediated and Or-independent repellency (mean ± SEM) in *Ae. aegypti*. Dilutions were made in order to adjust the concentration of transfluthrin to 1 ppm, accordingly to the original concentration indicated in the product label. For control: $t = 0.05$, $df = 18$, $P = 0.963$, for France: $t = 7.40$, $df = 12$, $P < 0.001$, for China: $U = 0.0$, $P < 0.001$, for Brazil: $t = 8.31$, $df = 16$, $P < 0.001$, NS = not significant, ***$P < 0.001$; $n = 9$ cages for Brazil in both lines, $n = 10$ cages for both controls, and $n = 7$ cages for the rest. The control represents the baseline activity in response to the solvent. Each black circle over the bars represents individual replicate values.

2.3 Discussion

Current understanding on how insect repellents, such as DEET, evoke spatial repellency can be summarized in three modes of action: activation of olfactory sensory receptors that mediate repellent pathways (DeGennaro et al., 2013; Lee et al., 2010; Ray, 2015; Stanczyk et al., 2010; Syed and Leal, 2008; Xu et al., 2014); inhibition of olfactory sensory processing of attraction cues from a host (Bohbot et al., 2011; Bohbot and Dickens, 2010; Ditzen et al., 2008; Pellegrino et al., 2011) and/or chemically masking attractants thereby reducing host attraction (Afify et al., 2019; Ray, 2015; Syed and Leal, 2008). In this study, we provide experimental evidence for a new mechanism of spatial repellency via activation of sodium channels alone, independently of activation or inhibition of ORNs, as a principal mechanism of transfluthrin repellency. Furthermore, our findings of synergism between sodium channel activating transfluthrin and mosquito repellents that elicit ORN responses could spur efforts to use natural pyrethrins, synthetic pyrethroids or other sodium channel activators as general synergists to increase the potency of DEET and other Or-activating insect repellents.

Repellency by insecticides that disrupt the function of the nervous system is often called irritancy, which occurs as a sublethal neurotoxic event and often at direct contact with treated surfaces (Manda et al., 2013; Mongkalangoon et al., 2009; Wagman et al., 2015). Prior to this study, a common belief of the mechanism of pyrethroid repellency is that pyrethroids causes a general neurotoxic effect, which consequently disables mosquitoes from finding hosts (Ray, 2015). This is an attractive hypothesis, especially when pyrethroid concentrations reach sublethal levels and mosquitoes are in direct contact and/or are very close to pyrethroid vapor-emitting devices. Transfluthrin at such sublethal concentrations would likely disrupt the normal function of electrical signaling by altering the gating of sodium channels, resulting certain behavioral impairments, as observed in our behavioral assay for 100 ppm or higher. In this study, however, we provide experimental evidence showing that transfluthrin-mediated spatial repellency can occur at nonlethal concentrations (e.g., at 1 ppm) at which no behavioral impairment was observed. We also found that transfluthrin did not impair the activity of ORNs evoked by mosquito attractants (Fig. 2), which is consistent with earlier observations in a push-pull approach that mosquitoes repelled by transfluthrin were still attracted to Biogents Sentinel™ (BGS) traps (Salazar et al., 2013). In a natural setting, mosquitoes would encounter such low concentrations of transfluthrin vapor first at a distance, before getting closer to the emanating source. Our results suggest that transfluthrin-mediated spatial repellency via activation of sodium channels already occurs in advancement of the hypothesized irritancy mechanism which may come into play when mosquitoes encounter higher transfluthrin concentrations.

Our findings of synergism between activation of sodium channels by transfluthrin (which does not activate any Ors) and Or-mediated repellency pathways by DEET, GA and other repellents provide strong evidence for potentiation of Or-mediated repellency by a sodium channel activator. It also provides new insight into the mechanism of transfluthrin repellency. In particular, we observed that transfluthrin could enhance the repellency by Or-activated repellents at extremely low concentrations. For example, at 0.1 ppm, transfluthrin by itself cannot elicit repellency, but it enhanced the repellency of other volatile repellents, including DEET. We speculate that the enhanced repellency is the result of enhanced activities of Or-mediated repellent pathways by transfluthrin because subthreshold depolarization of membrane potential by transfluthrin via hyper-activating sodium channels could potentiate neuronal excitability of Or-mediated repellent pathways. However, our results argue against the possibility that transfluthrin-mediated synergism occur at the peripheral sensory processing level (i.e., ORNs), as we did not observe alteration of ORN responses to other repellents by transfluthrin (*SI Appendix*, Fig. S7). Thus, it is likely that transfluthrin repels mosquitoes by directly affecting a post-ORN, central neural processing step(s). Of note, differential sensitivity of sodium channel variants (generated via alternative splicing or RNA editing) to pyrethroids has been well-documented in insect species (Dong et al., 2014). Functional identification and localization of post-OSN pyrethrin–hypersensitive sodium channel variants in the brain will be necessary to advance further mechanistic understanding of the transfluthrin synergism in insect olfaction. Future research should also use direct imaging of neural circuits in the mosquito brain to gain further insights into how transfluthrin-mediated sodium channel-activation impacts olfactory signal processing in the insect brain.

Results from this study likely have immediate and significant practical implications. In particular, the synergism between sodium channel-activating pyrethroids and Or-mediated repellents (e.g., DEET), as discovered in this study, could provide a basis for reciprocally augmenting the potency of two classes of insect repellents and lowering the concentration of each repellent used. This would be especially significant in terms of minimizing both, the costs and the toxic effects of transfluthrin and other repellent products, while maintaining adequate repellency and durability of commercial synthetic insect repellents. Fortuitously, current commercial transfluthrin repellent products may already exploited the hidden synergism in repellency by sodium channel-mediated transfluthrin and unknown Or-activating component(s) in these products. Future determination of these components that potentially synergize transfluthrin repellency may provide new lead compounds for the development of more potent insect repellent mixtures. Our findings could spur renewed interests in discovering new sodium channel activators with unique receptor sites on sodium channels that are distinct from the pyrethroid receptor sites (Catterall et al., 2005; Zhorov and Dong, 2017). Such new chemistries could be more effective in repel kdr mosquitoes. Overall, results from this study illustrate the first example of sodium channel activation-based repellent mechanism in insects, and provide a novel framework for future development of synergistic insect repellent mixtures to combat mosquitoes and other human disease vectors.

2.4 Material & Methods

2.4.1 Insects and chemicals.

It was used five mosquito lines: Two are wild-type *Ae. aegypti* lines [Rockefeller and Orlando (BEI Resources, NIAID, NIH)]; Two other are pyrethroid-resistant *Ae. aegypti* lines [KDR:ROCK (Smith et al., 2018), and Puerto Rico line (BEI Resources, NIAID, NIH)]; and the fifth one is the olfactory defective *orco*$^{-/-}$ line (orco^{16}) (BEI

Resources, NIAID, NIH) (DeGennaro et al., 2013). All the odorants used in this study and its sources are provided in *SI Appendix*, Table S 1.

2.4.2 Hand-in-cage assay.

The hand-in-cage behavioral assay followed similar procedures from Boyle et al. (2016). Briefly, a group of four to nine days-old females (about 40, mated, non-blood fed) inside a mosquito cage (30 x 30 x 30 cm) (BioQuip, Rancho Dominguea, CA) were exposed to a human hand wearing a modified glove with a window covered with two pieces of netting, as detailed in fig. 1*A*. The bottom netting (5.5 x 6.5 cm) was treated with 500 µL of either solvent (acetone), or test compounds. The top net was not treated and prevented mosquitoes from contacting the treated netting and the hand. A digital camera (e-con Systems Inc, San Jose, CA, model: e-CAM51A) on the top of the cages recoded mosquito landing on the top netting for 5 min. For each hand-in-cage experiment, ten cages were tested with acetone-treated net first, then, about 1.5h later, and in the same sequence, the ten cages were tested using test compound-treated netting (i.e., treatment). Similarly, the 2 trials for controls were also recorded 1.5 hours apart, and the landing numbers in the second trial presented to be at similar rate than in the first trial for each cage. Repellency index was calculated using the following equation: Percentage repellency $= \left[1 - \left(\frac{\text{number of mosquitoes that landed on treatment}}{\text{number of mosquitoes that landed on solvent}}\right)\right] \times 100$. The assay was run at 27-30 °C and relative humidity of 30-50%.

Note that for selected figures displaying behavioral data, representative videos for selected treatments are available following links either at the end of those respective figure legends, or at *SI Appendix*, Movie Legends section 2.7.4 on page 99. Those videos are deposited in the public data repository figshare (www.figshare.com). In *SI Appendix*, Methods section 2.7.1 on page 86, it is provided additional detail into the modified glove and assay set.

2.4.3 Electrophysiology recordings.

The EAG and SSR were performed as described elsewhere (Liu et al., 2013; Pelletier et al., 2010) using a recording system by Syntech company (Kirchzarten, Germany), which consisted of universal electrode holders, a preamplifier (10x), an analog-to-digital signal converter (IDAC-4) and a stimulus controller (CS-55). The software EAGPro and AutoSpike 3.1 (Syntech) were used to visualize, record and analyze EAG and SSR data, respectively. Insect antennae were bathed in a humidified air flow ($1.2\,\mathrm{L\,min^{-1}}$) under a microscope (100 - 1000x, Nikon Eclipse FN1, Japan), and odorants were delivered by air flow ($0.5\,\mathrm{mL\,min^{-1}}$) into a glass Pasteur pipet serving as an odorant cartridge. For most odorant delivery, odorants were diluted in DMSO, loaded (10 µL) onto filter paper stripes (4 x 50 mm) and placed inside the glass cartridge. However, transfluthrin and selected odorants were also applied without dilution at the inner wall of the glass cartridge, such as 1 µL of pure compound (equivalent to 10 µL of 10^{-1} solution) (Fig. 1*D*). For co-application of two compounds, the two-cartridge delivery system proposed by Syed and Leal (2008) was used (Fig. 2*A*).

To evaluate the activities of transfluthrin and its inactive *1S-cis* isomer on mosquito wild-type and mutant sodium channels, the wild-type AaNa$_v$1-1 and AaNa$_v$1-1 mutant carrying two kdr mutations (S989P and V1016G) were expressed in *Xenopus* oocytes system and functionally characterized using two-electrode voltage clamp as previously described (Tan et al., 2002; Tatebayashi and Narahashi, 1994). Any relevant detail is further denoted in the appropriate figure legend or text.

2.4.4 Data analysis and statistics.

SigmaPlot 12.5 (Systat Software) was used to perform the statistics analysis and plot the figures. Unpaired or paired (as appropriated) Student's *t*-tests were used to compare two sets of data. If data did not meet the normality or equality of the variance assumptions needed for Student's *t*-tests, the equivalent Mann-Whitney Rank Sum (for unpaired) or Wilcoxon Signed Rank (for paired) tests were used instead. For paired comparison of multiple treatments on a same individual against control either a One-way RM Anova or a Friedman RM Anova on Ranks was used, both with

Dunnett's multiple comparison against a control treatment.

2.5 Acknowledgments

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2.7 Supplementary Information Appendix

SI Appendix for Chapter 2 on page 58, redrafted from:

Andreazza F, Valbon WR, Wang Q, Liu F, Xu P, Bandason E, Chen M, Wua S, Smith LB, Scott JG, Jiang Y, Jiang D, Zhang A, Oliveira EE, Dong K (2021) Sodium channel activation underlies transfluthrin repellency and potentiation of odorant receptor-dependent aversion in *Aedes aegypti*. **Submitted to: Proceedings of the National Academy of Science of the United States of America**.

This SI Appendix section includes:

- SI Methods
- Figures S1 to S7
- Table S1
- Legends for Movies S1 to S9

Other supplementary materials for this Chapter include the following:

- Movies S1 to S9

SI movies can be accessed by following the links in selected main figure legends or at each respective movie legend on section 2.7.4 on page 99.

2.7.1 SI Methods

Hand-in-cage assay detailed set up.

The hand-in-cage assay set consisted of placing a human hand wearing a modified glove inside a 27 L cubic metal cage (part# 1450BSV - BioQuip, Rancho Dominguea, CA) containing about 40, mated, non-blood feed female mosquitoes. A left-hand glove (Ansell Protective Products, Coshoton, OH, part number: 37-155) was modified by cutting a 5 x 6 cm window on its back. Around the open hole, it was fixed a piece of magnetic frame 5 x 6 cm of inner and 7 x 8 cm of outer dimension, serving as a base to stack five other equally sized frames. Each frame was hand-cut from a magnetic card (Quotable^{TM}) with the aid of a single sided razor blade. In between the 1st (fixed to the glove) and 2nd frames, and in between the 5th and 6th frames it was fixed 2 pieces of polyester netting (Shason Textile Inc., part number: WS-B532-111, Walmart # 567948282, white). The top netting was never treated, and was slightly larger than the frame outer dimension, allowing easy placement at an outstretched manner. The first netting (closer to the glove/hand), which was treated with either the test-compound or with solvent control, was slightly smaller than the frame outer dimension. In this way, while still possible to keep it outstretched, that netting would be completely unaccessible to mosquitoes from the sides. The entire pack of magnetic frames containing the two nettings pieces were then firmly secured by two metal clamps (Fig. 1*A*). Using this design host cues from the hand could pass thought both nettings, attracting mosquitoes, but mosquitoes could only physically contact the untreated top netting. The treated netting was not physically accessible to mosquitoes and also did not make direct contact with the hand inside the glove.

For running the assay, the females was moved into the cage 24h in advance, and kept in an incubator (27 °C, relative humidity of 50% and photoperiod of 12h). A cotton ball soaked with distilled water was placed on the top of each cage. Just before each run of the hand-in-cage assay, a lab assistant transferred the prepared cage from the incubator to a bench in the assay room. In the meantime, in an adjacent room, one researcher (i.e., tester) treated a piece of netting with 500 µL acetone (control) or test compound (dissolved in acetone) on a glass Petri dish. After the acetone evaporated ($\sim$ 7 min), the researcher assembled and put on the customized glove set. To initiate the assay, the tested introduced the hand in the glove inside the mosquito cage allowing the mosquitoes to land on the top netting. The top of the modified glove was video recorded for five minutes by a digital camera (e-con Systems Inc, San Jose, CA, model: e-CAM51A) connected to a computer. Each of all cages were tested with the bottom netting treated only with acetone in a first time (first trial), then in the same sequence, each of the same cages were tested a second time (second trial), but using a test compound-treated netting (test-treatments) or acetone-treated netting (control-treatment). The second trials of each cage were always made about 1.5 hours after the first trial for that cage, interval time which rendered same numbers of mosquito landing in the control treatments. Any cage that had a low landing number (i.e.. $<$ than 50% of the average landing of the other cages in a same day) in the first run was not continued with the second run ($<$ 1% of all the replicates in our study).

From the recorded videos of each of the two trials in a cage, it was counted the number of mosquitoes landing on the window from the second to the fifth minutes (4 min total). Repellency index was calculated using the following equation: Percentage repellency $= \left[1 - \left(\frac{\text{number of mosquitoes that landed on treatment}}{\text{number of mosquitoes that landed on solvent}}\right)\right] \times 100$. After the second run, mosquitoes were visually assessed for signs of intoxication, such as uncoordinated movements, or knockdown, before disposal.

Before the cages be used in a new assay, to remove any residual chemicals left on the cages from previous assays, each cage was rinsed with: 1^{st}, 99% ethanol (spray); 2^{nd}, distilled water (thorough rinse); and 3^{rd}, 99% ethanol (spray). After that the cages were left to air dry. The modified glove set was soaked in ethanol (99%) in a container before being thoroughly rinsed with both distilled water and 99% ethanol, before being left to air dry.

Electroantennogram (EAG).

EAG was performed with 4-6 days old female mosquitoes. Both ground and recording electrodes were made from a glass capillary (Kimble Glass Inc. Kimax-51, Part. N° = 34502 99, size = 0.8 - 1.10 x 100 mm) pulled in a flaming/brown micropipette puller (Shutter Instrument Co. Model P-87, set to = Pull: 95, Vel: 150, Time: 155, Heat: ramp temperature + 7). Each electrode was filled with filtered standard EAG recording solution [0.1N KCl + 0.5% polyvinylpyrrolidone (PVP)]. The recording electrode consists of a glass electrode and a chlorinated silver wire (Beantown Chemical, Part. N° = 140740-5M; 0.25 mm of diameter) inside the glass electrode. Just prior the recording, the head of a female mosquito was excised with fine forceps and a small fraction of the right (looking from the top) antenna tip was cut off using a micro-dissecting precision blade. The ground electrode was introduced into the brain, from the soft membrane in the back portion of the head, and the whole set (i.e., electrode holder, electrode, head/antenna) was fixed to a hand micromanipulator (Märzhäuser Wetzlar Gmbh & Co. Model: MM33) to be visualized under a microscope. The tip of the antenna was placed inside the tip of the recording electrode with aid of a motorized micromanipulator (Burleigh PCS-6000, CA). The recording electrode were connected to the recoding set (as described in main text) and a 10 s signal was recorded for each odorant stimulation. The recordings were set to start 2 s before odor exposure, using EAG filter of 0.1 Hz, the smallest low-cutoff available. The purified and humidified air was delivered to the preparation through a glass tube (10 mm inner diameter) perforated by a small hole 2 cm away from the end of the tube, into which the tip of the odorant cartridge, could be inserted.

We applied 10 µL of odorant solution per pipet cartridge at the 10^{-1} or lower dilutions, which is commonly used in EAG recordings (Den Otter et al., 1980; Pelletier et al., 2010; Stanczyk et al., 2010; Xu et al., 2014). For some odorants such as (-)-borneol, which are solid crystals, we dissolved them in DSMO. However, dimethyl sulfoxide (DMSO), elicited small EAG signals. This effect was evident from single sensillum recording (SSR), when recording the A neuron of a short sharped tipped sensillum, hereafter called sst-1 (*SI Appendix*, Fig. S2). Therefore, a potential small signal from transfluthrin could be overlapped with this solvent induced signal. Thus, we tested transfluthrin without solvent (1 µL of pure compound corresponds to 10 µL of 10^{-1} solution when solvent-diluted) (Fig. 1*D* and *SI Appendix*, Fig. S1*B*). A blank empty pipet cartridge was used as control.

* * *

Single Sensillum Recording (SSR).

The insect preparation consisted of placing a female mosquito inside a 200 µL pipet tip thought its large end, gently pushing it toward the pipette tip. A small portion of the pipette tip was cut to allow the antennae and head of the mosquito to came out from the tip. That preparation was then mounted on a microscope slide (76 x 26 mm) with the aid of dental wax. The mosquito antenna was fixed using a double-sided tape to a cover slip resting on dental wax, to facilitate manipulation. The electrophysiological system for EAG recording described above was also used for SSR except for recording electrodes. Tungsten microelectrodes were sharpened in 10% KNO2 at 2-10 V. The ground electrode was inserted into the mosquito eye while the recording electrode was inserted into the shaft of an olfactory sensillum, to extracellularly record action potentials of olfactory receptor neuron (ORN). SSR alternated current (AC) signals was recorded for 10 s starting 2 second before odorant stimulation, at a sampling frequency of 10666.7 Hz. In our initial *Ae. aegypti* SSR records without filtering settings it was evident that AC frequencies for the extracellular action potentials signals range from about 300 to 2000 Hz, depending on the baseline activity of each neuron. Therefore, to reduce recording noise, it was used a low cut off filter of 100 Hz and a high cut off filter of 10kHz. The differences in action potential (spikes) amplitudes separated neurons A from B, with neuron A having the larger ones (Ghaninia et al., 2007). In addition to its morphological shape, our sst-1 sensilla was characterized by its functional responses to a panel of 19 compounds diluted in DMSO to 10^{-2} (*SI Appendix*, Fig. S 2*B*).

Toxicological assay.

The toxicities of transfluthrin and *1S-cis* isomer against female mosquitoes were evaluated using a vapor toxicity assay. We placed twenty 5-day old mosquitoes in a 200 mL plastic cup and then covered the cup with a thin Parafilm "M" (Bemis, Neenah, WI). A vapor cartridge was made from a glass Pasteur pipette containing a piece of filter paper (4 x 10 mm) impregnated with 10 µL of tested compound solution in DMSO. To inject a controlled amount of compound vapor into the cup, the cartridge was connected to a pulse flow stimulus controller system (CS-55, Syntech), and its tip inserted into the covered cup. The cartridge was quickly warmed with a lighter for 10 sec immediately followed by a 3 sec pulse at 0.5 L min. A vapor cartridge with DMSO was used as control. The cartridge warming-up step was used to generate more vapor which allowed us to observe knockdown of KDR:ROCK mosquitoes. One hour after vapor exposure, the mosquitoes in the cup were released into a mosquito cage (30 x 30 x 30 cm) (BioQuip) and the number of mosquitoes that were on their back were counted. Five replicates for each of 6 concentrations were carried out. Data was analyzed by the PROBIT procedure in the SAS statistical software package.

Data, figure and video processing

After being plotted, figures were further edited (for color, labelling, etc.) and assembled in CorelDRAW® Graphic Suit 2020 - version 22 (Corel Corporation, Ottawa, Canada). The representative supplemental videos from the hand-in-cage assay were trimmed to 4 minutes of evaluation and were cropped in its window dimensions to the landing region in the modified glove for better use of the view. The sound/noise were removed, and its speed was increase by 4x for quick visualization. All video assembly was done in iMovie application package (Apple, San Jose, CA).

2.7.2 SI Figures

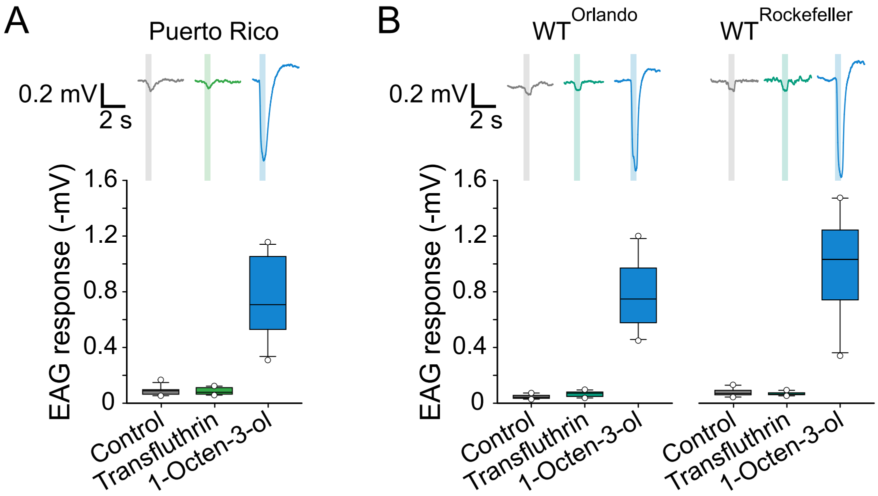


Figure S1. No EAG signals by transfluthrin were detected from wild-type (*A*) and pyrethroid-resistant Puerto Rico (*B*) mosquitoes. (*A*) Transfluthrin used was from Sigma (Lot: BCBT5175). n = 12 antennae. (*B*) Transfluthrin used was from Jiangsu Yangnong Chemical Co. Ltd. (Jiangsu, China; 98.5% purity); n = 10 antennae. 1-Octen-3-ol was applied as positive control following transfluthrin on the same antennae. In both panels representative traces are shown above each plot.

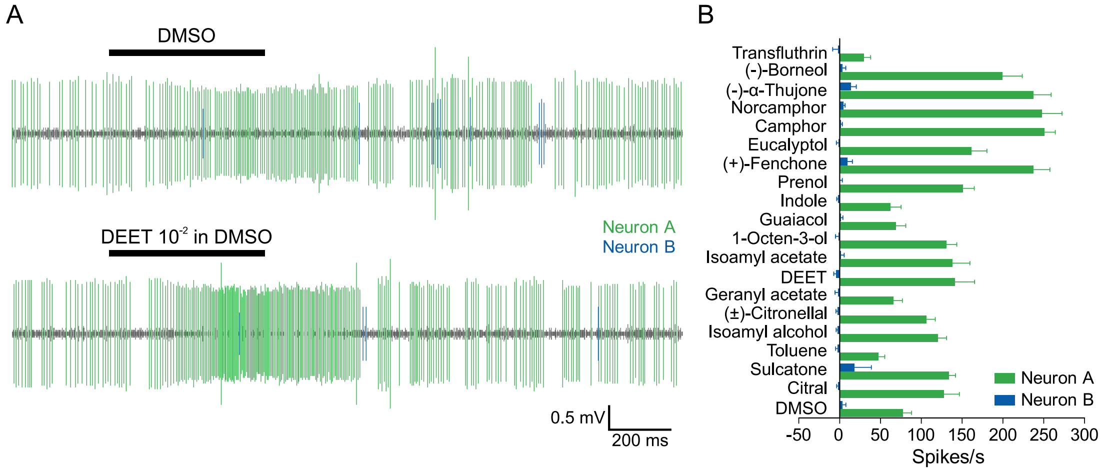


Figure S2. DEET activates a short sharped tipped (sst) sensilla of *Ae. aegypti*. (*A*) Representative traces of single sensillum recording (SSR) responses to DMSO and DEET from a sst (hereafter called sst-1) sensilla of *Ae. aegypti* Rockefeller mosquitoes (from n = 8 insects). (*B*) Functional response profiles of sst-1 sensilla using SSR (mean ± SEM); compounds diluted to 10^{-2} in DMSO (except transfluthrin which was diluted to 10^{-1} in DMSO), and applied with a 0.5 sec pulse flow; n = 6 insects for transfluthrin and n = 8 insects for the rest.

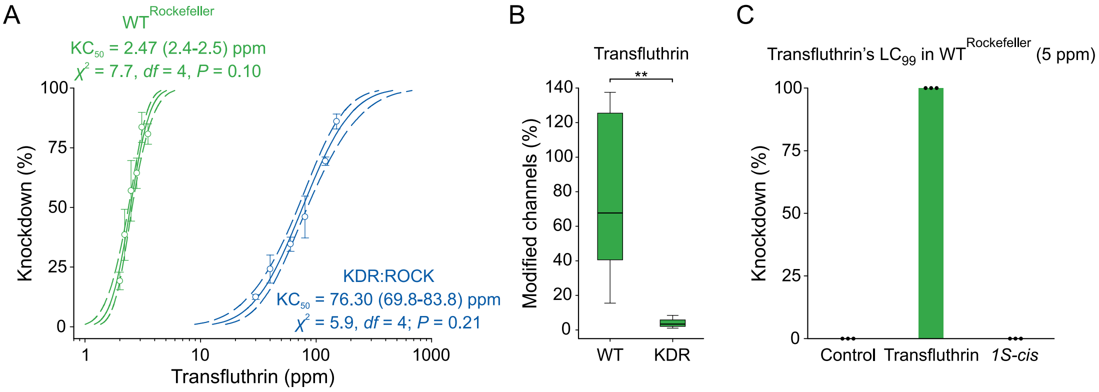


Figure S3. Transfluthrin resistance of KDR:ROCK mosquitoes and lack of toxicity of the *1S-cis* isomer against *Ae. aegypti* mosquitoes. (*A*) Concentration-dependent knockdown by transfluthrin vapor in Rockefeller and KDR:ROCK lines; values of parameters showed in the insets from the probit analysis; the solid line represents the curve estimate while the dashed lines represent both the lower and upper 95% confidence intervals; each circle represents the observed knockdown (mean ± SEM, from $n = 5$ replicates of 20 insects each). (*B*) Sensitivity of AaNa$_v$1-1 and mutant channels expressed in *Xenopus* oocytes to transfluthrin; $U = 0$, $P = 0.002$, $^{**}P < 0.01$, $n = 5$ oocytes for AaNa$_v$1-1, and $n = 8$ oocytes for mutant channels. (*C*) No knockdown effect of *1S-cis* isomer on Rockefeller mosquitoes at 5 ppm which is the KC$_{99}$ of transfluthrin for Rockefeller mosquitoes from panel *A*; $n = 3$ groups of 20 mosquitoes each.

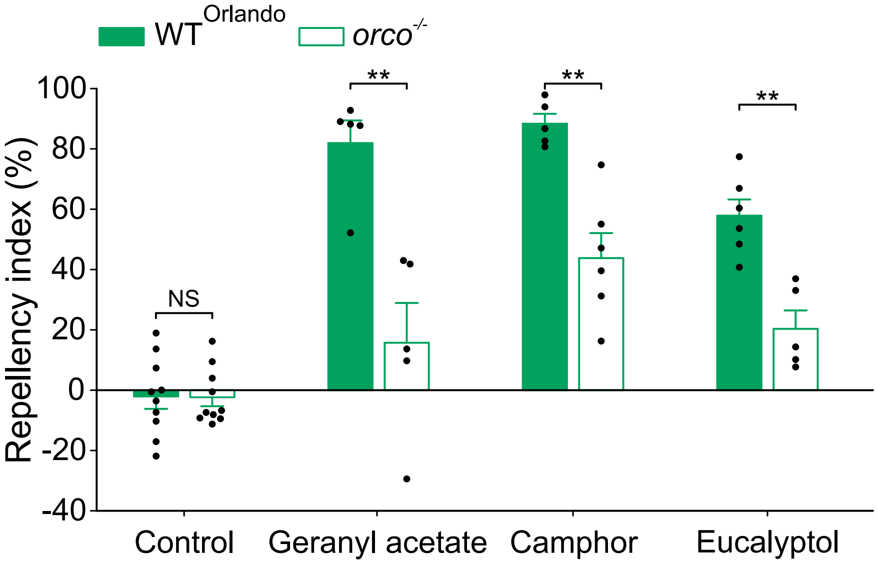


Figure S4. Orco-dependency of geranyl acetate, camphor and eucalyptol repellency. Concentration used was 10000 ppm. For control: t = 0.05, df = 18, P = 0.963, for geranyl acetate: t = 4.35, df = 8, P = 0.002; for camphor: t = 4.60, df = 9, P = 0.001, for eucalyptol: t = 4.63, df = 9, P = 0.001; NS = not significant, $^{**}P < 0.01$; n values for: both controls = 10, camphor in Orlando and eucalyptol in $orco^{-/-}$ = 5, and eucalyptol in Orlando and camphor in $orco^{-/-}$ = 6. The control represents the baseline activity in response to the solvent. All the data is plotted as mean ± SEM, and each black dot over the bars represents individual replicate values.

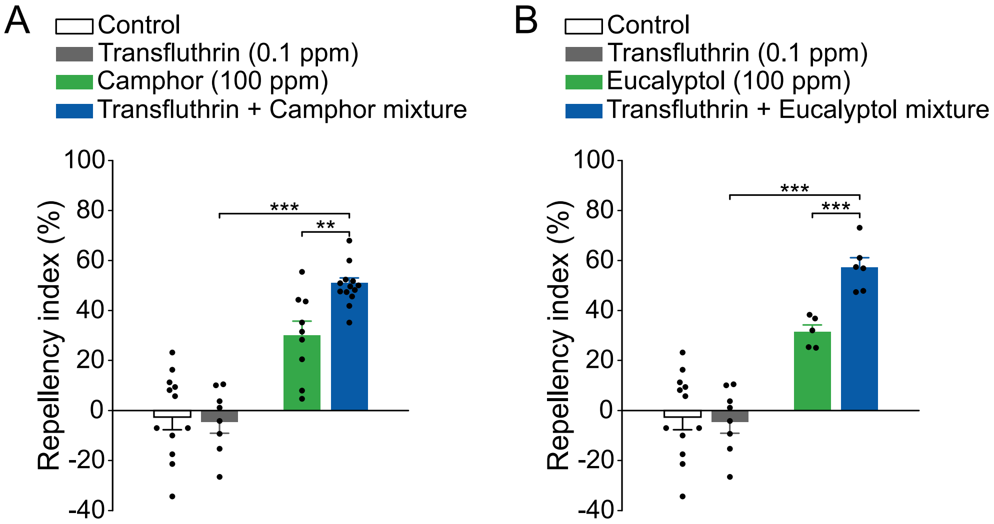


Figure S5. Transfluthrin potentiate the repellency by camphor (*A*) and eucalyptol (*B*) in *Ae. aegypti* wild-type Rockefeller mosquitoes. (*A*) For mixture *vs* transfluthrin: $t = 12.63$, $df = 18$, $P < 0.001$, for mixture *vs* camphor: $U = 12$, $P = 0.003$, $^{**}P < 0.01$, $^{***}P < 0.001$; n values for: transfluthrin = 8, control and mixture = 12, and camphor = 9. (*B*) For mixture *vs* transfluthrin: $t = 9.88$, $df = 12$, $P < 0.001$, for mixture *vs* eucalyptol: $t = 5.19$, $df = 9$, $P < 0.001$; $^{***}P < 0.001$; n values for: transfluthrin = 8, control = 10, eucalyptol = 5, and mixture = 6. The control represents the baseline activity in response to the solvent. All the data is plotted as mean ± SEM, and each black dot over the bars represents individual replicate values.

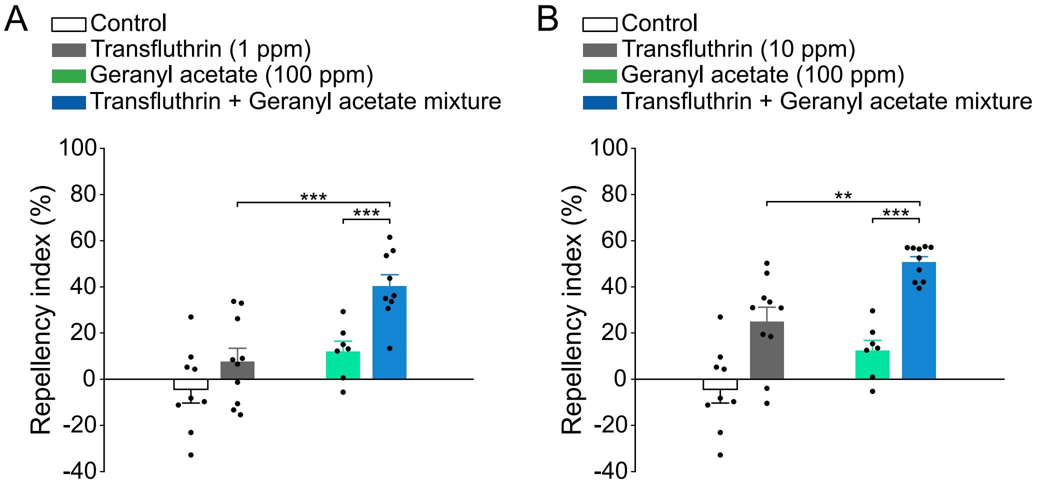


Figure S6. Transfluthrin potentiation of the Or-mediated repellency was reduced in KDR:ROCK mosquitoes. (*A*) For geranyl acetate (GA) plus transfluthrin (1 ppm) *vs*: GA: $t = 4.12$, $df = 14$, $P = 0.001$ and transfluthrin: $t = 4.22$, $df = 17$, $P = 0.001$; (*B*) for GA plus transfluthrin (10 ppm) *vs*: GA: $t = 8.36$, $df = 15$, $P < 0.001$ and transfluthrin: $t = 3.88$, $df = 18$, $P = 0.001$; (*A* and *B*) $^{**}P < 0.01$ $^{***}P < 0.001$; n values for: GA =7, control and for transfluthrin (1 ppm) plus GA = 9, and the rest = 10. The control represents the baseline activity in response to the solvent. Data are presented as mean ± SEM. Data from GA (100 ppm) are presented more than once to facilitate comparison. Each black dot over the bars represents individual replicate values.

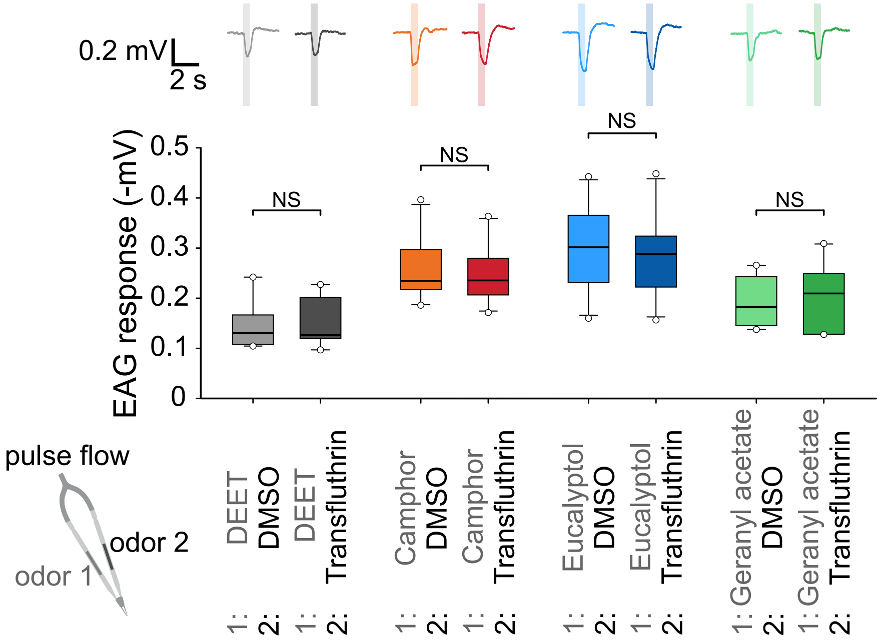


Figure S7. Transfluthrin vapor does not affect the olfactory response to Or-activating mosquito repellents. EAG responses by other mosquito repellents in the presence or absence of transfluthrin; data is plotted as in figure 1*D*; for DEET: $t = 1.13$, $df = 6$, $P = 0.301$, for camphor: $Z = 1.17$, $P = 0.275$, for eucalyptol: $Z = 1.78$, $P = 0.084$, for geranyl acetate: $t = 1.19$, $df = 9$, $P = 0.266$, NS = not significant; n values for: DEET = 7, and the rest = 10.

2.7.3 SI Tables

Table S1. Odorants products used in the current study.

Name	Brand/manufacturer	CAS/Prod. code	Lot or Batch	Purity (%)
Tranfluthrin Sample A	Sigma Aldrich	118712-89-3/46114	BCBV5909	99.20
Tranfluthrin Sample B	Jiangsu Yangnong Chem. Co.	118712-89-3/-	BCBV5909	98.50
Tranfluthrin Sample C	Sigma Aldrich	118712-89-3/46114	BCBT5175	99.90
1S-cis inactive isomer	Jiangsu Yangnong Chem. Co.	-/-	BCBV5909	97.75
DEET	Sigma Aldrich	134-62-3/36542	BCBS3631V	98.80
(-)-α-Thunjone	Aldrich	546-80-5/89231	1103512V	98.40
(-)-Borneol	Sigma Aldrich	464-45-9/15598	BCBR4601V	99.70
(+)-Fenchone	Sigma Aldrich	4695-62-9/46208	BCBT7523	99.70
(±)-Citronellal	Aldrich	106-23-0/27470	BCBD0932V	99.00
(*E*)-β-farnesene	Sigma Aldrich	18794-84-8/73492	BCBS0968V	98.50
Isoamyl alcohol	Aldrich	123-51-3/W205710	MKBQ8306V	99.50
1-octen-3-ol	Aldrich	3391-86-4/O5284	MKBT3320V	99.40
Acetone	Sigma Aldrich	67-64-1/270725	SHBK1371	99.98
Camphor	Aldrich	76-22-2/148075	STBG2753V	95.70
Citral	Aldrich	5392-40-5/W230316	MKBH9971V	95.70
Dimethyl sulfoxide	Sigma	67-68-5/D2650	RNBF1057	100.00
Eucalyptol	Aldrich	470-82-6/C80601	BCBR0344V	99.70
Geranyl acetate	Aldrich	105-87-3/173495	STBF9703V	98.30
Guaiacol	Aldrich	90-05-1/W253200	MKBR4220V	99.40
Indole	Aldrich	120-72-9/I3408	MKBL8993V	99.60
Isoamyl acetate	Sigma Aldrich	123-92-2/112674	06714HC	99.43
Nonanal	Aldrich	124-19-6/N30803	MKBX4673V	98.20
Norcamphor	Aldrich	497-38-1/N32601	MKBL6578V	99.10
Prenol	Aldrich	556-82-1/162353	10524KCV	99.60
Sulcatone	Aldrich	110-93-0/M48805	STBB8565V	99.20
Toluene	J.T. Baker	108-88-3/9460-01	T22B24	100.00
L-(+)-Lactic acid	Sigma	79-33-4/L1750	067K0748	98.00
Liquid Repellent - Brazil	SBP Reckitt Benckiser	-/-	ZOB2781O2T	0.80*
Liquid Repellent - China	Guard Sénce	-/-	-	0.90*
Liquid Repellent - France	Raid Johnson Johnson	-/-	-	0.90*

*Products composed of mixtures; value is for the active ingredient (i.e., transfluthrin).

2.7.4 SI Movie Legends

- **Movie S1 (separate file).** Representative video for figure 1*B*. Each video pair (top and bottom) represents one bar from the figure panel, according to the labels provided within the video. Not all the bars within the panel are represented in the video.

  https://figshare.com/s/4782dc8a1d1dfec48993

- **Movie S2 (separate file).** Representative video for figure 1*C*. Each video pair (left and right) represents one bar from the figure panel, according to the labels provided within the video. Not all the bars within the panel are represented in the video.

  https://figshare.com/s/f42d63dbda0fc5cabdee

- **Movie S3 (separate file).** Representative video for figure 1*E*. Each video pair (top and bottom) represents one bar from the figure panel, according to the labels provided within the video. Not all the bars within the panel are represented in the video. Note: part one shows data for transfluthrin and part two shows data for DEET.

  https://figshare.com/s/1883b5024dacca3e9c11

- **Movie S4 (separate file).** Representative video for figure 3*A*. Each video pair (top and bottom) represents one bar from the figure panel, according to the labels provided within the video. Not all the bars within the panel are represented in the video. Note: the part one shows data for transfluthrin, and part two shows data for DEET.

  https://figshare.com/s/3ab01655918913e46102

- **Movie S5 (separate file).** Representative video for figure 3*C*. Each video pair (top and bottom) represents one bar from the figure panel, according to the labels provided within the video. Not all the bars within the panel are represented in the video.

  https://figshare.com/s/e247c0f58eaa08a02ead

- **Movie S6 (separate file).** Representative video for figure 4*A*. Each video pair (top and bottom) represents one bar from the figure panel, according to the labels provided within the video. Not all the bars within the panel are represented in the video.

  https://figshare.com/s/8055a83158010e670c9d

- **Movie S7 (separate file).** Representative video for figure 4*B*. Each video pair (top and bottom) represents one bar from the figure panel, according to the labels provided within the video. Not all the bars within the panel are represented in the video.

  https://figshare.com/s/c030c2997dcf6f7f121c

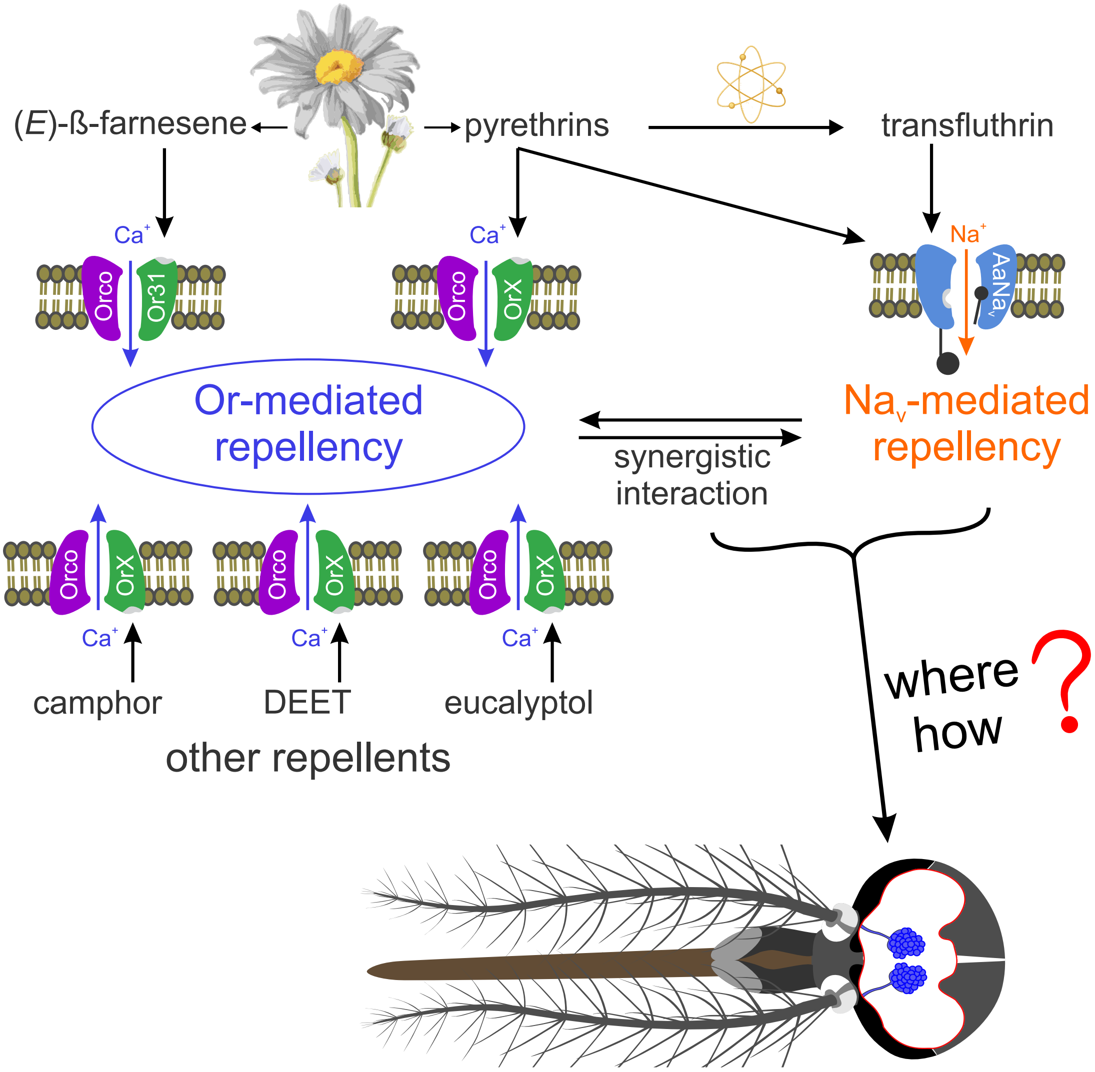
- **Movie S8 (separate file).** Representative video for figure 5*A*. Each video pair (top and bottom) represents one bar from the figure panel, according to the labels provided within the video. Not all the bars within the panel are represented in the video.

  https://figshare.com/s/a7fc05619cf4ecaf85fb

- **Movie S9 (separate file).** Representative video for figure 5*B*. Each video pair (top and bottom) represents one bar from the figure panel, according to the labels provided within the video. Not all the bars within the panel are represented in the video.

  https://figshare.com/s/904c8a24f81da56db6c3

Final considerations and future directions



* * *

The goal of this thesis project was to understand the molecular mechanism underlining the pyrethrum and pyrethroids spatial repellency in the medically important mosquito species, *Ae. aegypti*. The main findings are: 1^{st}, pyrethrins I and II from pyrethrum extract activates an Or in the sst-1 sensilla of *Ae. aegypti* antenna, and the repellency behavior observed is partially Or-dependent; 2^{nd}, activation of AaNa_v by either pyrethrin I, pyrethrin II or the pyrethroid transfluthrin is sufficient to elicit repellent behavior in *Ae. aegypti*; and 3^{rd}, even that at minute concentrations, both pyrethrins and transfluthrin action on the AaNa_v increases Or-mediated repellency by other mosquito repellents.

These advances in our understanding of pyrethrum and pyrethroid repellency also brought out new important questions which needs to be further addressed. The main questions are: 1) What is the identity of pyrethrin-activated Or(s) that mediates repellency; 2) What neural circuits are activated by pyrethrins and transfluthrin; and 3) What mechanism underlies pyrethrins/transfluthrin enhancement of Or-mediated repellency?

To address the first question in my future studies, I intend to use the Empty Neuron System technique (Carey et al., 2010; Dobritsa et al., 2003). Using this technique, each of the putative 131 *Ae. aegypti* Or genes (Bohbot et al., 2007) would need to be genetically introduced in a specific *D. melanogaster* line. Those mutant flies would then express the introduced Or gene in a specific known sensilla in the fly antennae, allowing SSR analysis with any desired compound. Building such a library for all *Ae. aegypti* Or genes is a safer, already tested approach, but still a very long term and resources consuming project. Alternatively, before taking this long term approach, I am working on development of a sampling technique able to extract the neuron body of a specific sensillum to supply Single Cell RT-PCR analysis which would then provide a short cut into the deorphanization of Ors in insects.

To identify which neural circuit is activated by transfluthrin (the second question), it can be used a mosquito line expressing a calcium sensor fluorescence protein (GCaMP6s, Chen et al. (2013)) in all of its neurons (Vinauger et al., 2019) to monitor neural activities in the brain in response to transfluthrin vapor. With this approach, known as calcium imaging, we will be able to visualize neurons (highlighted by fluorescence) when they are activated by transfluthrin vapor using specialty *in vivo* microscopy. To address the third question, on how or where Or-activated pathways interact with the transfluthrin-activated ones, we need to utilize tissue-specific expression of more than one calcium sensor to monitor activities from multiple circuits potentially in multiple regions of the brain This type of approach had just become available by the introduction of both GCaMP6s and CaMPARI2 (Moeyaert et al., 2018) calcium sensors together a recently developed binary expression system (QF2/QUAS, Riabinina et al. (2015)) in mosquitoes, allowing targeted expression of the sensors (linked to QUAS reporter) in cells expressing specific genes (linked to QF2 drivers) (Matthews et al., 2019; Shankar et al., 2020; Younger et al., 2020).

These future directions are challenging projects, but nonetheless, I hope that both my current finds and my future efforts will eventually lead to a better understand of repellency behavior in mosquitoes, assisting our society to cope not only with vector-borne diseases, but also other insect pests, in both urban and agricultural settings. While the path is challenging, it fortifies my scientific curiosity, and it is rewarding in most of the extends.

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Appendix A - Published/accepted side projects

Journal of Economic Entomology, 112(1), 2019, 425–433
doi: 10.1093/jee/toy321
Advance Access Publication Date: 1 November 2018
Research

Molecular Entomology

A Phylogeographic Approach to the *Drosophila suzukii* (Diptera: Drosophilidae) Invasion in Brazil

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Subject Editor: Raul Narciso Guedes

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Abstract

Biological invasions have reached large parts of the globe, due to human actions across the planet. *Drosophila suzukii* (Matsumura, 1931) is a globally invasive species, always associated with enormous and costly damage to agricultural crops. Native to Southeast Asia, *D. suzukii* recently (i.e., 2013) invaded and is dispersing through South America. Here, we used a phylogeographic approach based on the cytochrome c oxidase subunit I gene fragment to explore the invasion dynamics of *D. suzukii* populations in Brazil. We identified five haplotypes and moderate genetic diversity in Brazilian populations, which are undergoing demographic and spatial expansion. The analyses of molecular variance indicated a high genetic structure among the populations, which is partially explained by their morphoclimatic origin and invasion history. *Drosophila suzukii* expanded from southern to southeastern Brazil, aided by human-mediated transport of fruits from region to region. The sharing of haplotypes among Brazilian and other invaded regions of the world suggests a single invasion event of *D. suzukii* in Brazil, originating from previously invaded areas (e.g., North America and Europe). The rapid geographic dispersal and wide variety of fruits attacked by of *D. suzukii* require immediate implementation of control strategies (legal and phytosanitary) to manage this pest in Brazil.

Key words: spotted wing Drosophila, invasive pest, invasion origin, demography

Invasive species may alter the environment, representing a serious threat to native biodiversity as well as a potential cause of damage to agriculture and human health (Mack et al. 2000, Lee 2002, Paini et al. 2016). The impacts caused by invasive species on communities and ecosystems are highly complex (Simberloff 2013). Recent reports of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in South America and *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) in Africa exemplify the negative impacts of invasive insect pests in agriculture systems (Tay et al. 2013, Goergen et al. 2016, Day et al. 2017, Durigan et al. 2017).

Introduction events of invasive pests in agriculture have increased and are frequently related to the increase in the global world-trade market (Paini et al. 2016). Usually, the knowledge about the introduction routes of pests derives from historic and observational data, which are generally scarce, incomplete, or even misleading. In this context, population genetics has provided useful tools for reconstructing these routes, integrating population structure into genealogical and demographic models (Beheregaray 2008, Estoup and Guillemaud 2010). Intraspecific diversity studies of a species using molecular markers make it possible to evaluate the population structure, gene flow, and population expansion/retraction, which are valuable clues to understanding the invasion process of a species into a new habitat (Lombaert et al. 2014, Wei et al. 2015, Cao et al. 2017).

Among the different classes of molecular markers, mitochondrial DNA sequences (mtDNA) are historically used in phylogenetic and population studies of animals (Avise 2009). These markers have singular genetic traits, i.e., a high mutation rate, low rate of recombination, maternal inheritance, and a simple genetic organization, allowing one to identify genetic strains over time and space (Brown et al. 1982, Avise et al. 1987, Smith and Smith 2002). The mitochondrial gene cytochrome c oxidase subunit I (COI) is widely used for studies of insect population genetics (Yang et al. 2012, Oliveira et al. 2013, Corrêa et al. 2017, Soares et al. 2018), including for a recent and important invasive agricultural pest-species, *Drosophila suzukii* (Matsumura, 1931) (Carvajal 2010, Calabria et al. 2012, Choi et al.

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doi: 10.1093/jee/toz195
Advance Access Publication Date: 10 July 2019
Short Communication

Short Communication

Host Potential and Adaptive Responses of *Drosophila suzukii* (Diptera: Drosophilidae) to Barbados Cherries

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Abstract

Biological invasions are a global threat to agricultural crops worldwide. In the Neotropical region, the spotted-wing Drosophila [*Drosophila suzukii* (Matsumura)] has rapidly expanded its geographical range spreading throughout South America in recent years. Besides climatic factors, the remarkable success of its establishment and subsequent distribution in this region is closely dependent on the diversity and availability of host plants. We evaluated the host potential (e.g., as food and oviposition sources) of fruits of jabuticaba [*Plinia cauliflora* (Mart.) Kausel (Myrtales: Myrtaceae)], Barbados cherry (*Malpighia emarginata* DC) (Malpighiales: Malpighiaceae), bonnet pepper (*Capsicum chinense* Jacq.) (Solanales : Solanaceae), and coffee (*Coffea arabica* L.) (Gentianales : Rubiaceae) and their effects on the biological and physiological traits of *D. suzukii*. For the fruit types where fly emergence occurred, we assessed the biological and physiological performance of the flies and compared these parameters with those recorded for flies reared on strawberries (*Fragaria × ananassa* Duchesne) (Rosales : Rosaceae) and an artificial diet. Our results revealed that oviposition into fruits and completion of the life cycle occurred on Barbados cherries only. Furthermore, field surveys revealed a higher emergence rate of *D. suzukii* on undamaged ripe Barbados cherries than damaged ones. Moreover, flies developing on Barbados cherries and an artificial diet presented earlier emergence, shorter developmental time, lower number of adults per female, and a female-biased sex ratio compared to flies developing on strawberries. Overall, our findings demonstrated suitability of Barbados cherry as a host for *D. suzukii*, which renders management of *D. suzukii* in Neotropical region an even more challenging task.

Key words: spotted-wing drosophila, Barbados cherry, Neotropical host, oviposition

Drosophila suzukii (Matsumura), also called spotted-wing Drosophila (SWD), is a major pest of berries and cherries exhibiting a remarkable ability to invade a diverse range of environments (Asplen et al. 2015). Native to East and Southeast Asia (Ometto et al. 2013), *D. suzukii* has experienced rapid geographical expansion across the world (Rota-Stabelli et al. 2013, Asplen et al. 2015, Andreazza et al. 2017, Dos Santos et al. 2017).

Countries with vast territorial extensions, such as Brazil, present a wide array of climate types, biomes, and diversity of resources suitable for invasive insect pests like *D. suzukii*, which raises concerns about how and when to mitigate the losses caused by this species (Schlesener et al. 2015). In the Southern cone of Neotropical regions and since its initial report in Southern Brazil (Deprá et al. 2014), the potential for *D. suzukii* to attack and use native and exotic fruit species as a host has been recognized (Andreazza et al. 2017, Ferronato et al. 2018). With a total cultivated area exceeding 10,000 ha and a production of 33,000 tons, principally localized in the Brazilian Northeastern region (Delva and Schneider 2013, Marsaro Júnior et al. 2017), Barbados cherries (i.e., *Malpighia emarginata* DC) crop is a good example of potential exotic hosts of *D. suzukii*.

Using climatic variables, Benito (2016) and Dos Santos (2017) suggested that, in addition to regions where most of the known *D. suzukii* hosts are cultivated (i.e., Southern Brazil and parts of Uruguay, Argentina, and Chile), the potential spread of *D. suzukii* includes the Southeastern and at least part of Northeastern Brazil. These regions are at the edge of the optimal range of environmental conditions for *D. suzukii* due to high humidity and mild temperatures (Tochen et al. 2014, 2016; Benito et al. 2016). However, given

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Sex-dependent locomotion and physiological responses shape the insecticidal susceptibility of parasitoid wasps☆

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Diachasmimorpha longicaudata

ABSTRACT

The adaptive fitness of insect species can be shaped by how males and females respond, both physiologically and behaviorally, to environmental challenges, such as pesticide exposure. In parasitoid wasps, most toxicological investigations focus only on female responses (e.g., survival and especially parasitism abilities), leaving the male contributions to adaptive fitness (survival, locomotion, mate search) poorly investigated. Here, we evaluated the toxicity of the spinosyn insecticide spinosad against the South American fruit fly, *Anastrepha fraterculus*, and we used the parasitoid wasp *Diachasmimorpha longicaudata* (Ashmead) to evaluate whether sex-linked locomotory and physiological responses would influence the susceptibility of these organisms to spinosad. Our results revealed that *D. longicaudata* males were significantly more susceptible (median lethal time (LT_{50}) = 24 h) to spinosad than *D. longicaudata* females (LT_{50} = 120 h), which may reflect the differences in their locomotory and physiological (e.g., respiratory) responses to mitigate insecticide exposure. Compared to *D. longicaudata* females, male wasps were lighter ($P < 0.001$), walked for longer distances ($P < 0.001$) and periods ($P < 0.001$), and exhibited higher sensilla densities in their tarsi ($P = 0.008$), which may facilitate their intoxication with the insecticide. These findings indicate that male parasitoids should not be exempt from insecticide selectivity tests, as these organisms can be significantly more affected by such environmental challenges than their female conspecifics.

1. Introduction

Female insects are usually larger and play different adaptive roles than males (Field et al., 2015; Stillwell et al., 2010). Such sex-biased differences can also affect other life-history traits, such as behavioral patterns and physiological resource allocation, thereby adjusting selection pressure to characteristics that maximize fitness and result in higher chances for species adaptive success (Biro et al., 2014; Rennie et al., 2008; Stojković et al., 2015). Furthermore, according to the Bateman's principle (Bateman, 1948) and pace-of-life syndrome (POLS) hypothesis (Jones et al., 2008; Montiglio et al., 2018; Réale et al., 2010; Royauté et al., 2018; Wiersma et al., 2007; Wikelski et al., 2003), males and females often differ in their reproductive traits limiting fitness. These concepts also highlight that morphological, behavioral, and physiological differences among individuals are intimately linked to their energy balance and adaptive roles in a population.

Although lethal impacts of agricultural insecticides have been documented well before the 1960s, when Carson's book *Silent Spring* was released (Carson, 1962), sublethal exposure to environmental contaminants are often a neglected source for the variation of behavioral responses (Guedes et al., 2016, 2017; Montiglio and Royauté, 2014; Müller, 2018; Oliveira et al., 2007; Toledo et al., 2019; Ruiz et al., 2008). The lack of information regarding the different effects of sublethal exposures to pesticides contaminants on insect males and females becomes more intriguing when we consider that sex-biased morphological, behavioral and physiological differences can directly shape the

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☆ This paper has been recommended for acceptance by Dr. Da Chen.

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https://doi.org/10.1007/s10493-020-00501-6

Toxicities of acetogenin-based bioacaricides against two-spotted spider mite and selectivity to its phytoseiid predators

J. Miotto[1] · A. F. Duarte[1] · D. Bernardi[1] · L. P. Ribeiro[2] · F. Andreazza[3] · U. S. Cunha[1]

Received: 7 January 2020 / Accepted: 14 May 2020 / Published online: 18 May 2020

Abstract

Tetranychus urticae is the main pest of strawberry crops and can cause up to 80% of productivity losses under high infestations. Aiming to search *T. urticae* management alternatives compatible with eco-friendly or organic-based food production systems, this study evaluated the lethal and sublethal toxicities of formulated derivatives from Annonaceae (rich in acetogenins) against this pest species. In addition, it also evaluated the selectivity of the most promising formulation to the predatory mites *Neoseiulus californicus* and *Phytoseiulus macropilis*, which are largely applied in biological control in Brazil. Among the derivatives tested, the emulsion from the ethanolic seed extract of *Annona mucosa*—ESEAm (major component: acetogenin bis-tetrahydrofuran rolliniastatin-1) caused pronounced mortality of *T. urticae* after 120 h of exposure ($LC_{50} = 465.5$ mg L^{-1}), in a comparable or superior manner to an abamectin-based synthetic acaricide used as positive control ($LC_{50} = 1243.4$ mg L^{-1}). Moreover, ESEAm exposure resulted in a significant decrease in the number of eggs laid by females and caused the most pronounced ovicidal action for *T. urticae*, with only 5% embryonic viability. However, ESEAm also showed high toxicity to the predatory mites tested, causing 100% mortality for both species after 120 h exposure, similar to abamectin. The interaction between these bioacaricides and biological control agents should be tested under field conditions to further assess the potential ecological selectivity of these derivatives.

Keywords Botanical acaricides · Allelochemicals · Acetogenins · *Tetranychus urticae* · *Neoseiulus californicus* · *Phytoseiulus macropilis*

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10493-020-00501-6) contains supplementary material, which is available to authorized users.

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[2] Research Center for Family Agriculture, Agricultural Research and Rural Extension Company of Santa Catarina, Santa Catarina, Brazil

[3] Department of Entomology, Michigan State University, East Lansing, MI, USA

* * *

Behavioral and physiological responses of *Drosophila melanogaster* and *D. suzukii* to volatiles from plant essential oils

Running title: Repellency of essential oils against *Drosophila*

Qiang Wang[1, 2*], Peng Xu[1], Simon Sanchez[1], Phil Duran[1], Felipe Andreazza[1], Rufus Isaacs[1] and Ke Dong[1*]

[1]Department of Entomology, Michigan State University, East Lansing, MI, USA
[2]Department of Preventive Medicine and Public Health Laboratory Science, School of Medicine, Jiangsu University, Zhenjiang, China

[*] **Corresponding authors:** qiangwang@ujs.edu.cn; dongk@msu.edu

* * *

Appendix B - Undergoing/submitted side projects

- Wang Q*, Xu P*, **Andreazza F***, Hui Y*, Nomura Y, Duran P, Jiang L, Chen M, Takamatsu G, Ihara M, Matsuda K, Isaacs R, Oliveira EE, Du Y, Dong K (2021) Co-activation of multiple odorant receptors is essential for pyrethrum repellency in *Drosophila melanogaster*. *To be submitted to: Proceedings of the National Academy of Sciences of the USA*. ***Equal contributions**.

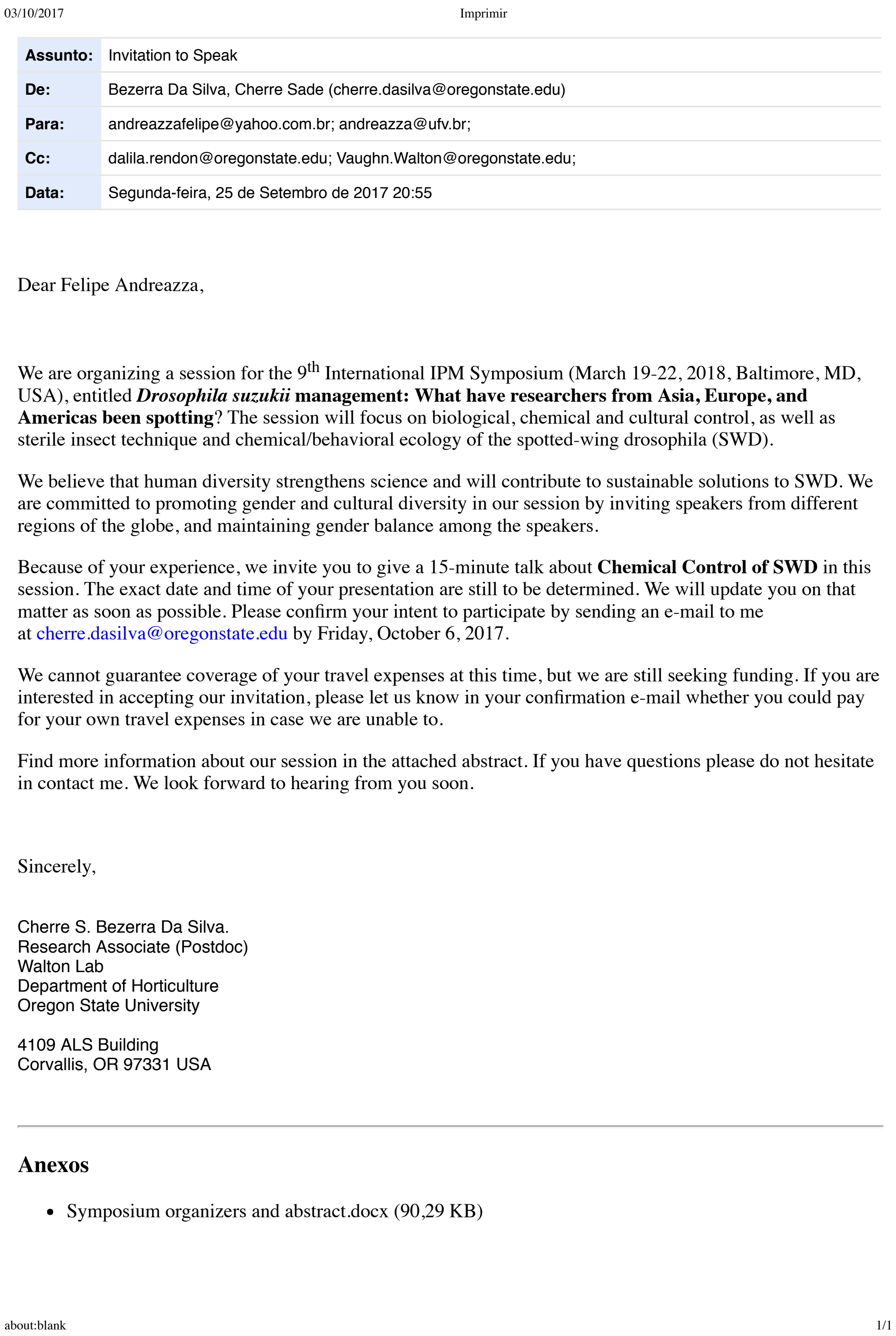
- Valbon WR*, **Andreazza F***, Oliveira EE, Hall M, Kilmavicz J, Coats J, Dong K (2021) Mechanisms of d-trans allethrin repellency in *Aedes aegypti*. *In preparation*. ***Equal contributions**.

- Valbon WR, **Andreazza F**, Machado FP, Rocha LM, Aguiar RWS, Dong K, Oliveira EE (2021). Characterization of potential targets for the *Siparuna guianensis* essential oil repellent actions on mosquitoes. *In preparation*.

- **Andreazza, F.**, Valbon, W.R., Nomura, Y., Chen, M., Du, Y., Oliveira, E.E., Dong, K. (2021). Novel sodium channel mutations confer pyrethroid resistance in the *Aedes aegypti* Puerto Rico pyrethroid-resistant strain. *In preparation*.

- Mantilla-Afanador JG, **Andreazza F**, Moura WS, Souza-Aguiar RW, Teixeira D, Alvarenga ES, Bernardi D, Oliveira EE (2021) Interspecific molecular differences in a lactone derivative target site mediates selectivity on a *Drosophila suzukii* parasitoid, *Trichopria anastrephae*. *In preparation*.

Appendix C - International invited talks



03/10/2017 Imprimir

Assunto:	Invitation to Speak
De:	Bezerra Da Silva, Cherre Sade (cherre.dasilva@oregonstate.edu)
Para:	andreazzafelipe@yahoo.com.br; andreazza@ufv.br;
Cc:	dalila.rendon@oregonstate.edu; Vaughn.Walton@oregonstate.edu;
Data:	Segunda-feira, 25 de Setembro de 2017 20:55

Dear Felipe Andreazza,

We are organizing a session for the 9th International IPM Symposium (March 19-22, 2018, Baltimore, MD, USA), entitled ***Drosophila suzukii* management: What have researchers from Asia, Europe, and Americas been spotting**? The session will focus on biological, chemical and cultural control, as well as sterile insect technique and chemical/behavioral ecology of the spotted-wing drosophila (SWD).

We believe that human diversity strengthens science and will contribute to sustainable solutions to SWD. We are committed to promoting gender and cultural diversity in our session by inviting speakers from different regions of the globe, and maintaining gender balance among the speakers.

Because of your experience, we invite you to give a 15-minute talk about **Chemical Control of SWD** in this session. The exact date and time of your presentation are still to be determined. We will update you on that matter as soon as possible. Please confirm your intent to participate by sending an e-mail to me at cherre.dasilva@oregonstate.edu by Friday, October 6, 2017.

We cannot guarantee coverage of your travel expenses at this time, but we are still seeking funding. If you are interested in accepting our invitation, please let us know in your confirmation e-mail whether you could pay for your own travel expenses in case we are unable to.

Find more information about our session in the attached abstract. If you have questions please do not hesitate in contact me. We look forward to hearing from you soon.

Sincerely,

Cherre S. Bezerra Da Silva.
Research Associate (Postdoc)
Walton Lab
Department of Horticulture
Oregon State University

4109 ALS Building
Corvallis, OR 97331 USA

Anexos

- Symposium organizers and abstract.docx (90,29 KB)

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for our environment • for our future

9th International IPM Symposium
Improving Health, Environment and Global Sustainability
March 19–22, 2018 | Baltimore, MD

March 22, 2018

To Whom It May Concern:

This is to certify that Felipe Andreazza attended the 9th International IPM Symposium held March 19-22, 2018 in Baltimore, Maryland, USA. Mr. Andreazza gave an oral presentation titled, "*Drosophila suzukii* chemical control options facing the current lack of regulated insecticides in Brazil" as part of the "*Drosophila suzukii* Management: What Have Researchers from Asia, Europe, and North America Been Spotting?" session.

More details about the event can be found on the website: ipmsymposium.org.

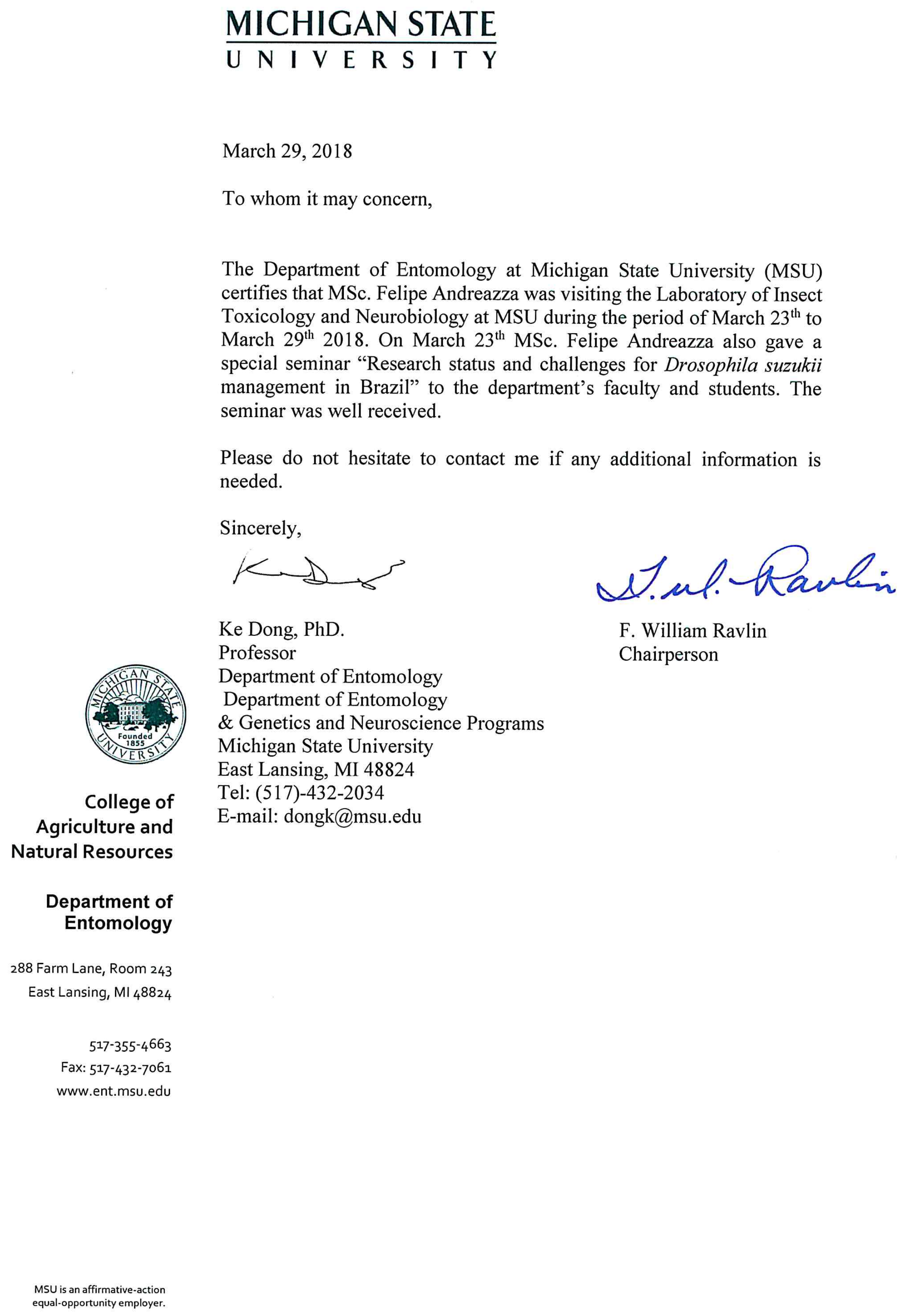
Please let me know if any additional information is needed.

Sincerely,

Michelle S. Marquart

Michelle Marquart
Lead Program Coordinator
mmarqua2@illinois.edu

telephone 217-333-1462 • *fax* 217-265-4114 • *url* citl.illinois.edu



MICHIGAN STATE
UNIVERSITY

March 29, 2018

To whom it may concern,

The Department of Entomology at Michigan State University (MSU) certifies that MSc. Felipe Andreazza was visiting the Laboratory of Insect Toxicology and Neurobiology at MSU during the period of March 23th to March 29th 2018. On March 23th MSc. Felipe Andreazza also gave a special seminar "Research status and challenges for *Drosophila suzukii* management in Brazil" to the department's faculty and students. The seminar was well received.

Please do not hesitate to contact me if any additional information is needed.

Sincerely,

Ke Dong, PhD.
Professor
Department of Entomology
Department of Entomology
& Genetics and Neuroscience Programs
Michigan State University
East Lansing, MI 48824
Tel: (517)-432-2034
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F. William Ravlin
Chairperson

College of Agriculture and Natural Resources

Department of Entomology

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Appendix D - Oral paper presentation

Advocate Entomology!

ENTOMOLOGY 2019

NOVEMBER 17-20 • ST. LOUIS, MO

PRESENTATION

1000: Roles of multiple sodium channel mutations in pyrethroid resistance in *Aedes aegypti*

Monday, November 18, 2019
10:20 AM - 10:30 AM
America's Center - Room 275

Pyrethroid insecticides are synthetic analogs of the botanical insecticide pyrethrum from *Chrysanthemum* species. Pyrethroids have a long history of use in the control of arthropod pests and human pathogen vectors because of their potent insecticidal activity and low mammalian toxicity. These compounds exert toxic effects by modifying the gating properties of voltage-gated sodium channels, which are critical for electrical signaling in excitable cells. However, due to extensive use of these compounds, many pest populations have developed resistance to pyrethroids. One of the major mechanisms of pyrethroid resistance is known as knockdown resistance (*kdr*) due to mutations in the sodium channel gene. In *Aedes aegypti*, multiple sodium channel mutations have already been reported to be associated with *kdr* in various populations around the world. Furthermore, new sodium channel mutations continue to emerge. In this study, we report the identification of six sodium channel mutations in a highly pyrethroid-resistant *Ae. aegypti* population. Three of the six mutations are new and have not been previously reported in any *Ae. aegypti* resistant population. We are currently evaluating the effects of these mutations, alone or in combinations, on the sensitivity to pyrethroids of mosquito sodium channels expressed in *Xenopus* oocytes. Findings from this study should provide valuable knowledge for better understanding of the mechanism of *kdr* and monitoring of *kdr* in a major human pathogen vector.

Authors

Felipe Andreazza
Universidade Federal de Viçosa

Michigan State University

Wilson Rodrigues Valbon
Michigan State University

Yoshiko Nomura
Michigan State University

Yuzhe Du
USDA - ARS

Mengli Chen
Zhejiang University

Advocate Entomology!

ENTOMOLOGY 2019
NOVEMBER 17-20 • ST. LOUIS, MO

Certificate of Participation

The Entomological Society of America (ESA)
certifies that

Felipe Andreazza

Presented an Oral Presentation

"Roles of multiple sodium channel mutations in pyrethroid resistance in *Aedes aegypti*"

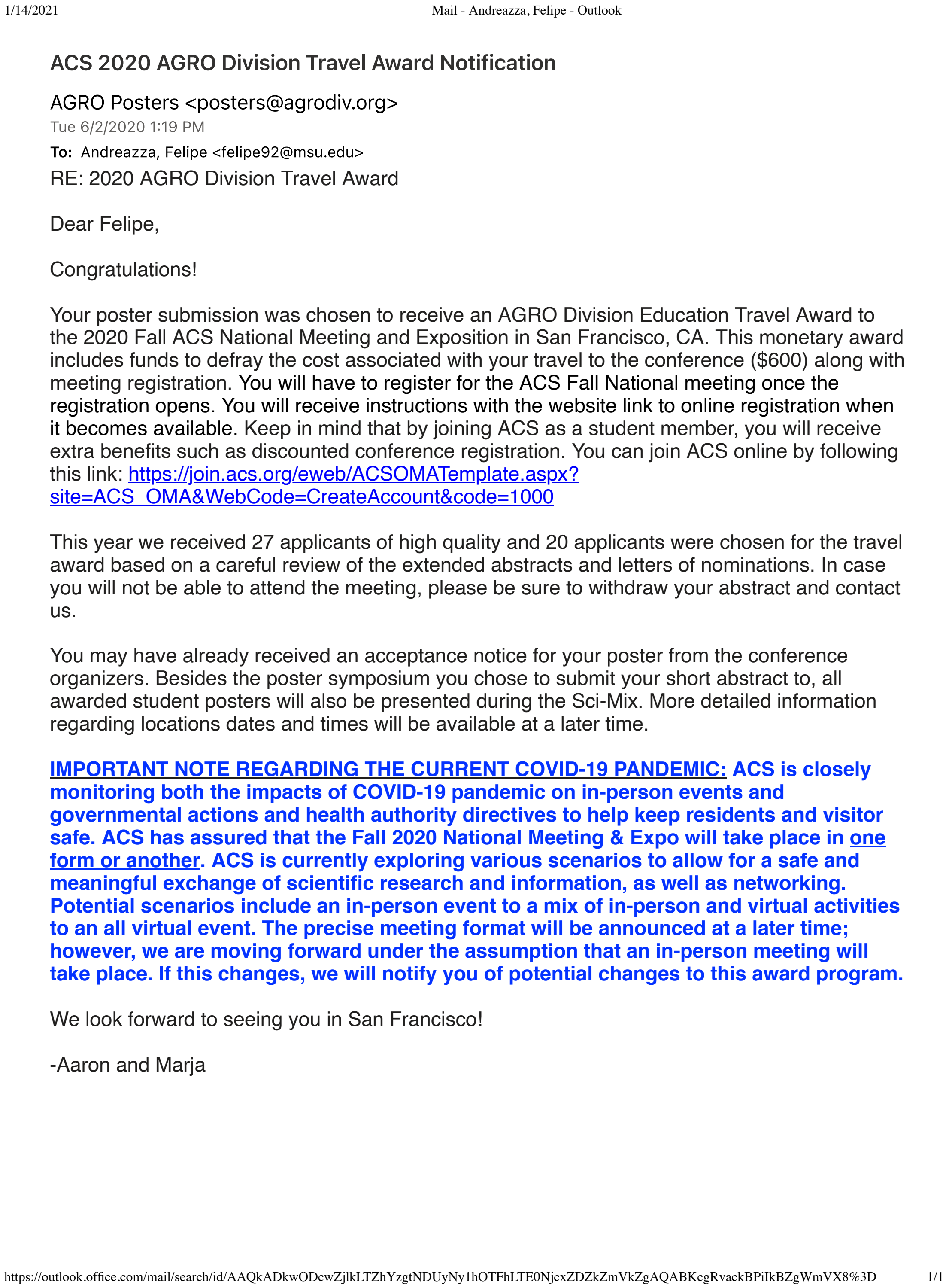
at Entomology 2019

November 17 - 20, 2019
St. Louis, Missouri, USA

C. David Gammel, CAE
ESA Executive Director

Appendix E - Awards

Award 1 - Student Travel Grant



1/14/2021 Mail - Andreazza, Felipe - Outlook

ACS 2020 AGRO Division Travel Award Notification

AGRO Posters <posters@agrodiv.org>
Tue 6/2/2020 1:19 PM
To: Andreazza, Felipe <felipe92@msu.edu>
RE: 2020 AGRO Division Travel Award

Dear Felipe,

Congratulations!

Your poster submission was chosen to receive an AGRO Division Education Travel Award to the 2020 Fall ACS National Meeting and Exposition in San Francisco, CA. This monetary award includes funds to defray the cost associated with your travel to the conference ($600) along with meeting registration. You will have to register for the ACS Fall National meeting once the registration opens. You will receive instructions with the website link to online registration when it becomes available. Keep in mind that by joining ACS as a student member, you will receive extra benefits such as discounted conference registration. You can join ACS online by following this link: https://join.acs.org/eweb/ACSOMATemplate.aspx?site=ACS_OMA&WebCode=CreateAccount&code=1000

This year we received 27 applicants of high quality and 20 applicants were chosen for the travel award based on a careful review of the extended abstracts and letters of nominations. In case you will not be able to attend the meeting, please be sure to withdraw your abstract and contact us.

You may have already received an acceptance notice for your poster from the conference organizers. Besides the poster symposium you chose to submit your short abstract to, all awarded student posters will also be presented during the Sci-Mix. More detailed information regarding locations dates and times will be available at a later time.

IMPORTANT NOTE REGARDING THE CURRENT COVID-19 PANDEMIC: ACS is closely monitoring both the impacts of COVID-19 pandemic on in-person events and governmental actions and health authority directives to help keep residents and visitor safe. ACS has assured that the Fall 2020 National Meeting & Expo will take place in one form or another. ACS is currently exploring various scenarios to allow for a safe and meaningful exchange of scientific research and information, as well as networking. Potential scenarios include an in-person event to a mix of in-person and virtual activities to an all virtual event. The precise meeting format will be announced at a later time; however, we are moving forward under the assumption that an in-person meeting will take place. If this changes, we will notify you of potential changes to this award program.

We look forward to seeing you in San Francisco!

-Aaron and Marja

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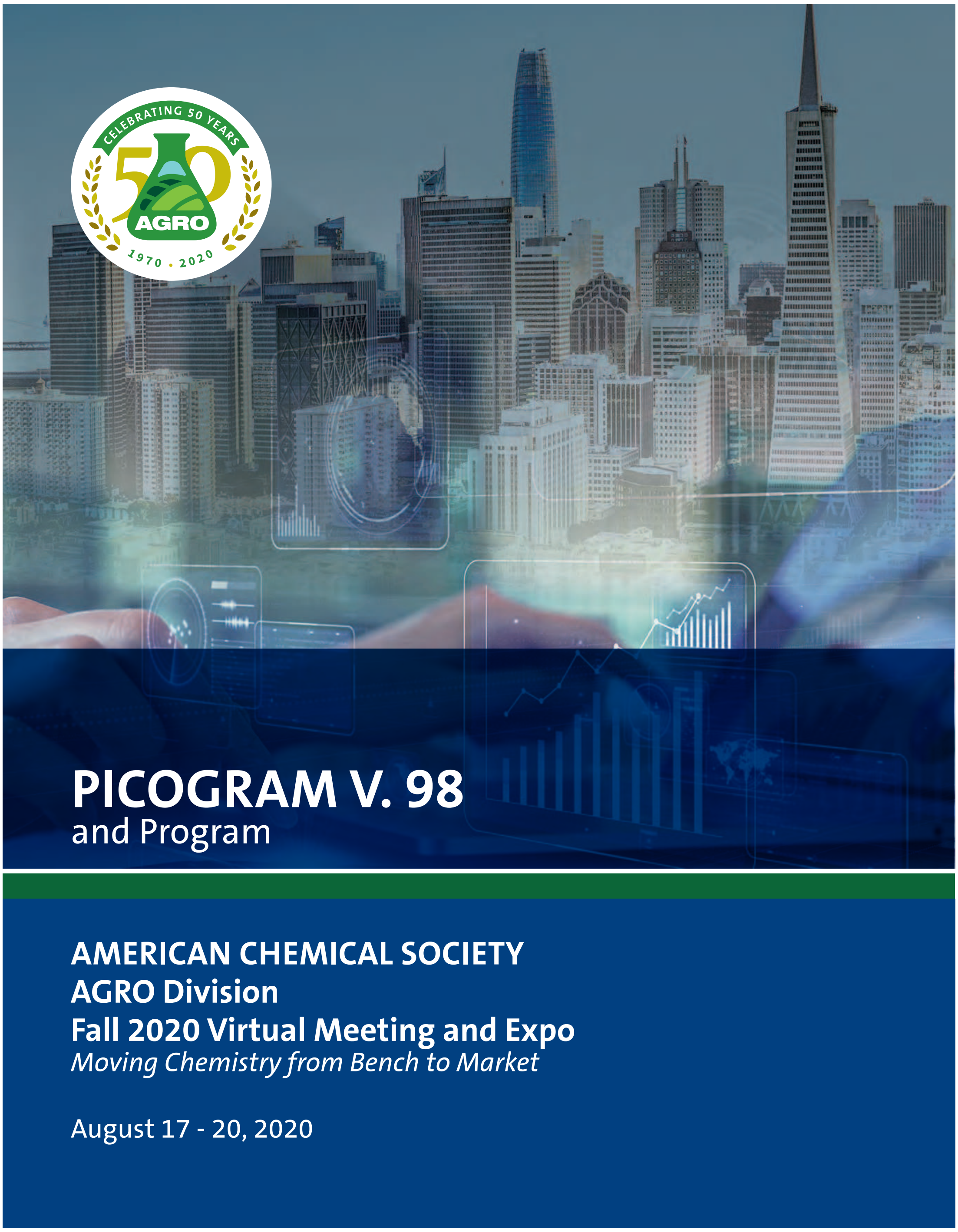


PICOGRAM V. 98
and Program

AMERICAN CHEMICAL SOCIETY
AGRO Division
Fall 2020 Virtual Meeting and Expo
Moving Chemistry from Bench to Market

August 17 - 20, 2020

2020 AGRO EDUCATION TRAVEL AWARDS

Sponsored by Bayer Cropscience

Congratulations to all our travel grant winners!

BROADCAST ORAL PRESENTATIONS

Maura J. Hall, Quantifying neonicotinoid insecticide residues in pollinator-attractive habitat adjacent to corn and soybean fields in Iowa. *Iowa State University, Joel Coats,* THURSDAY, 10:53 AM PDT

Jocelyn M. Macho, Novel mosquito-specific toxin from a marine strain of *streptomyces* for insecticide development, *UC Santa Cruz, John MacMillan*, THURSDAY, 2:18 PM PDT

Ryan Paul, Plants induce defense chemicals based on identity of parasitoid attacking an herbivore. *Colorado State University, Paul Ode*, MONDAY, 2:03 PM PDT

Zijiang Yang, Characterization of dispersion of particles from cotton gins and prediction of particle concentrations by AERMOD with dispersion correction factor. *University of Maryland at College Park, Alba Torrents***,** WEDNESDAY, 1:03 PM PDT

POSTER DISCUSSION SYMPOSIA, 9:00 – 10:00 AM PDT

MONDAY, POSTER DISCUSSION SESSION I
Analysis of Agriculturally-Important Chemicals

Jena Congilosi, Developing a multi-class LC-MS/MS method for the analysis of veterinary antimicrobials in water and manure matrices. *University of Buffalo – SUNY, Diana Aga*

Rebecca Dickman, Quantitative fluorine nuclear magnetic resonance ^{19}F-NMR method paired with liquid chromatography tandem mass spectrometry (LC/MS/MS) for a complete mass balance of per and poly-fluoroalkyl substances (PFAS) in biosolids. *University of Buffalo – SUNY, Diana Aga*

TUESDAY, POSTER DISCUSSION SESSION II
Discoveries in Crop Protection Chemistry

Kadie E. Britt, Evaluation of biological insecticides to aid arthropod pest management in hemp. *Virginia Polytechnic Institute and State University, Thomas Kuhar*

Courtney N. Huerter, Terpenoids from plant essential oils can upregulate detoxification genes in *Aedes aegypti*. *Iowa State University, Joel Coats*

Juliano Toniato, Bioactive compounds in food waste streams used as soil amendments to inactivate *Escherichia coli* during biosolarization. *University of California, Davis, Christopher Simmons*

Colin Wong, Nematode receptor ACR-16 as a target site for natural pesticides. *Iowa State University, Joel Coats*

WEDNESDAY, POSTER DISCUSSION SESSION III
Environmental Fate of Agrochemicals

Christopher J. Fellows, Synergistic and antagonistic effects of pesticides to the toxicity of organophosphate insecticides to *Apis mellifera. Louisiana State University, Daniel Swale*

Logan Running, Analysis of waste water using a parallel derivatization method for improved detection of 16 steroid hormones. *University of Buffalo – SUNY, Diana Aga*

THURSDAY, POSTER DISCUSSION SESSION IV
Assessing Health Risks of Agrochemicals

Felipe Andreazza, Mechanism of transfluthrin repellency in *Aedes aegypti. Michigan State University, Ke Dong*

Rui Chen, Enhancing the potency of GABAergic insecticides through chemical and genetic inhibition of K^+/Cl cotransporter. *Louisiana State University, Daniel Swale*

Caleb L. Corona, Biorational baits and their ability to control dipteran pests. *Iowa State University, Joel Coats*

Ellis Johnson, Spatial repellency, oviposition deterrence, and development inhibition of *Aedes aegypti* mosquitoes exposed to cajeput oil chemistries. *University of Nebraska-Lincoln, Troy Anderson*

Zhlilin Li, Inward rectifier potassium (Kir) channels are an integral component of mosquito vector competency. *Louisiana State University, Daniel Swale*

Sarah McComic, Reduced susceptibility and neural sensitivity to pyrethroids in the absence of the *kdr* genotype. *Louisiana State University, Daniel Swale*

Wilson Rodrigues Valbon, Dual-target mechanism of bioallethrin repellency in *Aedes aegypti* mosquitoes. *Michigan State University, Ke Dong*

Na Xie, Synergism of fipronil, lindane and dieldrin by the muscarinic acetylcholine receptor agonist pilocarpine in *Drosophila melanogaster*. *Virginia Polytechnic Institute and State University, Aaron Gross*

The AGRO Division is grateful for the sustained
support of the AGRO Education Travel Awards

Award 2 - Student Poster Award at the American Chemical Society Fall 2020 Virtual Meeting & Exposition

AGRO POSTER AWARD WINNERS

Congratulations!

Sponsored by BAYER

First Place

Na Xie

Virginia Tech

Second Place

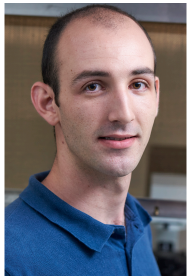
Sarah McComic

Louisiana State Univ.

Third Place (tie)

Rui Chen

Louisiana State Univ.



Felipe Andreazza

Michigan State Univ.

Thank you to the judges:
Drs. Edmund Norris, Sara Whiting, Nurhayat Tabanca, Daniel Swale and Troy Anderson