Generic Insect Repellent Detector from the Fruit Fly

*Drosophila melanogaster*

Zainulabeuddin Syed, Julien Pelletier, Eric Flounders, Rodrigo F. Chitolina, Walter S. Leal

Department of Entomology, University of California Davis, Davis, California, United States of America

Abstract

**Background:** Insect repellents are prophylactic tools against a number of vector-borne diseases. There is growing demand for repellents outperforming DEET in cost and safety, but with the current technologies R&D of a new product takes almost 10 years, with a prohibitive cost of $30 million dollar in part due to the demand for large-scale synthesis of thousands of test compounds of which only 1 may reach the market. R&D could be expedited and cost dramatically reduced with a molecular/physiological target to streamline putative repellents for final efficacy and toxicological tests.

**Methodology:** Using olfactory-based choice assay we show here that the fruit fly is repelled by not only DEET, but also IR3535 and picaridin thus suggesting they might have “generic repellent detector(s),” which may be of practical applications in new repellent screenings. We performed single unit recordings from all olfactory sensilla in the antennae and maxillary palps. Although the ab3A neuron in the wild type flies responded to picaridin, it was unresponsive to DEET and IR3535. By contrast, a neuron housed in the palp basiconic sensilla pb1 responded to DEET, IR3535, and picaridin, with apparent sensitivity higher than that of the DEET detectors in the mosquitoes *Culex quinquefasciatus* and *Aedes aegypti*. DmOr42a was transplanted from pb1 to the “empty neuron” and showed to be sensitive to the three insect repellents.

**Conclusions:** For the first time we have demonstrated that the fruit fly avoids not only DEET but also IR3535 and picaridin, and identified an olfactory receptor neuron (ORN), which is sensitive to these three major insect repellents. We have also identified the insect repellent-sensitive receptor, DmOr42a. This generic detector fulfills the requirements for a simplified bioassay for early screening of test insect repellents.

Introduction

Arthropod-borne diseases cause considerable human suffering and death. Mosquitoes, in particular, are notorious for their deleterious transmission of pathogens and parasites while feeding on human blood. *Anopheles* mosquitoes, particularly *An. gambiae* and *An. funestus*, are implicated in the deaths of about one million humans, particularly women and children, every year [1]. While feeding on their victim's blood, they unwittingly transmit the malaria-causing parasite that threatens half of the world's population. Globally, the number of people who get malaria each year is greater than the population of the United States [2]. The yellow fever mosquito, *Aedes aegypti*, is the primary vector of dengue throughout the tropical and subtropical world, thus accounting every year for several million cases globally [3]. *Culex* mosquitoes are major vectors of pathogens including the human filarial nematode, *Wuchereria bancrofti*, and arboviruses such as St. Louis encephalitis, Japanese encephalitis, Venezuela equine encephalitis, Western equine encephalitis and West Nile virus [4]. Newborn babies and immunocompromised patients from endemic areas, as well as military personnel and travelers moving into these areas, are at particularly higher risk given that typically they do not have immunity to pathogens locally transmitted by mosquitoes. Insect repellents are prophylactic tools against all these maladies. They may be used in conjunction with bednets and other integrated vector management (IVM) tools to reduce mosquito bites [5,6,7,8], but typically they are applied to the skin of uncovered parts of the body.

Despite its safety record [9], there is growing concern regarding topical applications of DEET (IUPAC name: N,N-diethyl-3-methylbenzamide) at high concentrations because deeper skin penetration can cause potential toxicity [10]. Additionally, DEET does not fulfill the ideal properties of insect repellents [9]. For example, DEET is a plasticizer that reacts with synthetic rubber and certain plastics and has several cosmetic concerns, including unpleasant odor. More importantly, most DEET formulations have a short duration of action (limited to seven hours) [10], which is a serious hindrance for military use as well as for civilians residing in areas with high temperatures. However, since its discovery more than five decades ago [11], DEET remains the most effective repellent in use today [12], and only a handful of new products have reached the market in the United States.
Insect Repellents Reception in the Fruit Fly

Results and Discussion

Flies are repelled by DEET, IR3535, and picaridin

Using a previously described choice assay [14], we showed that the fruit fly is indeed repelled by DEET [14,16], with no difference between male and female responses (Fig. 1A). Flies placed in Petri dishes having food available only inside two food chambers (1.5 ml micro centrifuge, “Eppendorf like” tubes) crossed control filter paper strips (solvent only) placed at the entrance of these chambers, reached out to the food source, and remained trapped inside the feeding chambers (N = 180 flies, 100%). By contrast, in no occasion (N = 18 trials, 10 flies per trial) have we observed flies entering chambers treated with DEET (Figs. 1A). Under similar conditions, flies were also repelled by IR3535 and picaridin (Fig. 1B,C), and in each case only 1 out of 90 entered the treated chambers. The paradigm of the choice assay we used [14] suggests that the observed repellency to DEET, IR3535, and picaridin (Fig. 1) is mediated by the fly’s olfactory system. We then reasoned the olfactory system of the fruit fly houses ORN(s) sensitive to these insect repellents, which - as previously suggested [17] - might be detected through non-contact chemosensation.

Scanning the fruit fly antennae for generic repellent detector(s)

We scanned all olfactory sensilla in the antennae of the fruit fly by single sensillum recordings using DEET, IR3535, and picaridin as stimuli. During this mapping, at least three sensilla of each type (basiconic, coeloconic, and trichoid) were challenged with these insect repellents. Although we did not find a single ORN sensitive to DEET or IR3535, one neuron housed in ab3 sensilla responded to picaridin with high sensitivity (threshold 0.1 µg, source dose) (Fig. 2). Based on the large spike amplitude (Fig. 3), the picaridin-sensitive neuron was identified as ab3A, which is known to harbor DmOr22a/b [18]. Interestingly, signal termination of picaridin was very slow (Fig. 3). Normally spike frequencies decrease immediately at the end of the stimulus (see below) [19]. Considering this unusual signal termination and, more importantly, due to its insensitivity to two other insect repellents, ab3A neuron is not a good candidate for screening new insect repellents.

ORN in the maxillary palps is sensitive to DEET and other repellents

Next, we performed single unit recordings from all olfactory sensilla in the maxillary palps and found an ORN in the basiconic sensilla pbl1 that responded to DEET (Fig. 4) in a dose-dependent fashion (Fig. 5). Surprisingly, these sensilla showed apparent higher sensitivity (lower threshold) to DEET than sensilla previously identified in the Southern house [20] and the yellow fever [21] mosquitoes (Fig. 6). In contrast to mosquito ORNs, the DEET-detecting neuron in the fruit fly is a “generic repellent detector.”

Figure 1. Repellency assay indicating avoidance of D. melanogaster to three insect repellents: DEET, IR3535, and picaridin. (A) Male and female flies responded equally to DEET (N = 180 flies tested). Female flies avoid entering the food chambers treated with (B) IR3535 (N = 90 flies tested) and (C) picaridin (N = 90 flies tested). Data are from 9 independent trials for each test, with ten flies used in each trial. doi:10.1371/journal.pone.0017705.g001

Figure 2. Dose-dependent excitatory responses from a picaridin-sensitive ORN housed in an antennal basiconic sensillum ab3 on D. melanogaster antennae. Hexane (control) responses were subtracted. (N = 7). Error bars are standard error of the mean (SEM). doi:10.1371/journal.pone.0017705.g002
addition to DEET it responded dose-dependently to IR3535 and picaridin (Fig. 4,5). Interestingly, this repellent-detecting ORN discriminates enantiomers of PMD (IUPAC name: 2-(1-hydroxy-1-methylethyl)-5-methylcyclohexanol), a repellent derived from natural sources (Fig. 7). This is particularly interesting given that behavioral assays showed that a stereoisomer of another insect repellent, (1S,2S)-2-methylpiperidinyl-3-cyclohexene-1-carboxamide, is 2.5 times as effective as the racemic mixture against *Aedes aegypti* [12].

The two ORNs housed in the pb1 sensilla of the fruit fly were clearly distinguished (Fig. 4) by their odorant response profiles and the amplitude of their spikes [22,23]. The neurons with larger and smaller spike amplitudes are named ORN-A and ORN-B, respectively [22,23] (Fig. 4). In agreement with previous studies [22,23], ORN-A responded to ethyl acetate, ethyl propionate, isoamyl acetate, (E)-2-hexenal, and heptan-2-one, but not to 4-methylphenol. By contrast, ORN-B was stimulated by 4-methylphenol, but was silent to the other odorants. Therefore, we were able to unambiguously conclude that DEET, IR3535, and picaridin stimulated ORN-A, but not ORN-B. It is technically challenging to correlate the previously discovered DEET-sensitive ORNs from the Southern house mosquito [20] with the ORs in the *Culex* genome [24,25]; same is true for *Ae. aegypti*. However, the wealth of information on the mapping of *Drosophila* ORs vis-à-vis ORNs [18,23,26,27] allows us to identify the putative insect repellent receptor in the fruit fly. It has been previously demonstrated [23], and later corroborated [26], that ORN-A of the pb1 sensilla expresses the odorant receptor DmOr42a (= Or42a). This prompted us to test Or42a expressed in the “empty neuron system” [18].

Response of Or42a in the “empty neuron” to insect repellents

We performed single unit recordings from the “empty neuron” system of Or42a-expressing fruit fly (Fig. 8). Recordings from the
ab3 sensilla of the Δhalo background flies showed complete absence of large amplitude spikes (spike A) when challenged with DEET, IR3535 or picaridin (Fig. 8, top trace). The transgenic flies (w; Δhalo: UAS-Or42a/Or22a-GAL4) showed spontaneous activity of ORN-A and B (large and small spikes), as expected when heterologous expression is achieved [18]. We noticed, however, that in our hands the maximal response of ab3A neuron to one of the best ligands, ethyl butyrate, was much lower (38.1 ± 8.7 spikes/s; source dose, 10 μg) than that recorded from wild type flies (Fig. 9), as well as reported in the literature [28]. To make certain that the observed low responses were not generated by a weak driver, we performed a second crossing with newly received Or22a-Gal4 flies (a gift from Dr. J. R. Carlson). Again, Or42a expressed in the empty neuron gave 2.5x lower response to ethyl butyrate than previously observed [28]. When stimulated with DEET, IR3535, and picaridin Or42a responded, albeit with low sensitivity, in a dose-dependent fashion, except for picaridin at the highest dose tested (Fig. 10). Although the “empty neuron” has been demonstrated to be an invaluable system for testing/deorphanizing antennal ORs from the fruit fly [27] and other insects [29], it is not entirely surprising that a transplanted receptor does not perform well in the system [27, 29]. After all, odorant-binding proteins, odorant-degrading enzymes and other olfactory proteins are not transplanted along with test ORs. Low CO2 responses recorded from the “empty neuron” expressing the gustatory receptor Gr21a (co-expressed with Gr63a) [30, 31] have now been demonstrated to be due to the lack of the G-protein Gαq [32]. Likewise, the unavailability of other olfactory protein(s) may explain why the bombykol receptor from the silkworm moth, BmorOR1, is very sensitive to bombykol when expressed in T1 trichoid sensilla [19], but not in the “empty neuron” [33]. In the “empty neuron” the sensitivity was enhanced by co-expression of the silkworm pheromone-binding protein BmorPBPI [33]. It is conceivable that the absence of other olfactory protein(s) in the ab3 sensilla led to the lower responses to DEET, IR3535, and picaridin recorded from the “empty neuron system” (Fig. 9) when compared to those obtained from the endogenous ORN (Fig 5). Additionally, limited expression of Or42a, as indicated by 2.5x lower responses to ethyl butyrate, may have contributed to the weaker responses to insect repellents elicited by Or42a expressed in the “empty neuron.” It remains an interesting question for future research to determine if other olfactory protein(s) account for the differences in Or42a responses to insect repellents in endogenous and exogenous systems.

**Conclusion**

Apparently, DEET has multiple modes of action. When tested at higher dosages it may act like an insecticide [34]. Recently, it has elegantly been demonstrated to be a feeding deterrent [17].

![Figure 7](image7.png)  
**Figure 7. Repellent-sensitive ORN, pb1A, challenged with PMD stereoisomers.** (+)-PMD elicited higher response from the large spike neuron than (−)-PMD. Source dose, 100 μg. doi:10.1371/journal.pone.0017705.g007

![Figure 8](image8.png)  
**Figure 8. Action potentials recorded from ab3 sensilla.** Δhalo flies showed spontaneous activity of neuron B, but not A, thus showing the ab3A is indeed “empty.” Lower traces were excitatory responses induced by DEET, IR3535, and picaridin and recorded from ab3 sensilla of Or42a-expressing flies (w; Δhalo; UAS-DmOr42a/Or22a-GAL4). doi:10.1371/journal.pone.0017705.g008

While gustatory receptors involved in this taste context [17] and an OR from larvae of the malaria mosquito have been previously identified [35], DEET odorant receptors from adult insects were hitherto terra incognita. The literature is even dichotomous regarding direct and indirect detection of DEET. One school favors a mode of action by “jamming” reception of other odorants [36,37], but antennal ORNs for direct detection of DEET have been identified from both the Southern House mosquito [20] and yellow fever mosquito [38]. Although it was not possible to unambiguously correlate ORN excitation vis-à-vis behavior as repellence was not impaired in flies with palps surgically removed (as well as those with antennae surgically excised), the discovery of an OR directly stimulated by DEET and other insect repellents and its ORN paves the way for practical applications in repellent R&D. There are a number of applications in reverse chemical ecology for which the “empty neuron system” is an invaluable surrogate. For example, flies carrying appropriate ORs from the

![Figure 9](image9.png)  
**Figure 9. Comparative responses of Or42a expressed in its native environment and in the “empty neuron.”** Ethyl butyrate (source dose, 10 μg) elicited higher responses from pb1 sensilla of wild type flies (top trace) than from the ab3 sensilla of Or42a-expressing flies (w; Δhalo; UAS-DmOr42a/Or22a-GAL4) (lower trace). SEM, standard error of the mean. doi:10.1371/journal.pone.0017705.g009
malaria mosquito, *An. gambiae* [29] can be used to prospect for novel attractants or repellents, with the benefits of (i) not having to deal with a quarantine issues related to maintaining a malaria vector in the lab, and (ii) performing single unit recordings on a more amenable insect. Here, the “empty neuron system” is a less desirable alternative. First, Or22a-expressing “empty neuron” does not match the sensitivity of the endogenous ORN sensitive to insect repellents. More importantly, the wild type flies are readily available to laboratories throughout the world, whereas the “empty neuron” still requires, albeit minimal, genetic manipulations. Therefore, we suggest that the ORN in the palpal sensilla “empty neuron” still requires, albeit minimal, genetic manipulation.

**Materials and Methods**

**Olfactory-based choice assay**

Tests were performed according to a previously described protocol [14] with slight modifications. In brief, traps (food chambers) were made of 1.5 ml “eppendorf-like” micro centrifuge pipette tips (USA Scientific, FL). The stem part of the filter paper was inserted through a slit on the upper part of pipette tip near the entrance of a food chamber so as to preclude flies from walking over the treated surface. Standard *Drosophila* corrmeal meal (UC Davis) was used as food bait. Traps were placed in OPTILUX™ Petri dishes (100 × 20 mm style; Becton-Dickinson, NJ) laid with 1% agarose.

**Expression of Or42a in the empty neuron system.** Test flies (w; Δhalo; UAS-DmOr42a/Or22a-GAL4) were obtained by crossing of transgenic lines (w; CyO/Δhalo; UAS-DmOr42a/TM3 and w; CyO/Δhalo; Or22a-GAL4) kindly provided by J. R. Carlson (Yale University).

**Acknowledgments**

We are grateful to Drs. John R Carlson (Yale University), Kamal Chauhan (USDA-ARS), and Artyom Kopp (UC Davis) for providing transgenic lines, samples of IR3535 and picaridin, and WT 89 flies, respectively. We thank also members of the lab, particularly Dr. Pingxi Xu, for comments on an earlier draft of the manuscript.

**Author Contributions**

Conceived and designed the experiments: ZS JP EF RFC WSL. Performed the experiments: ZS JP EF RFC. Analyzed the data: ZS JP EF RFC WSL. Contributed reagents/materials/analysis tools: ZS JP EF RFC WSL. Wrote the paper: ZS WSL.

**References**

1. Anonymous (2004) The impact of malaria, a leading cause of death worldwide.

2. Leal WS (2010) Behavioural neurobiology: The treasure chest of a human. Nature 464: 37–38.

3. Gubler DJ (1997) Dengue and dengue hemorrhagic fever: its history and resurgence as a global health problem. In: Gubler DJ, Kuno G, eds. Dengue and dengue hemorrhagic fever. New York: CAB International. pp 1–22.

4. Nasci RS, Miller BR (1996) Culicine mosquitoes and the agents they transmit. In: Beaty BJ, Marquardt WC, eds. The biology of disease vectors. Niwot: University Press of Colorado. pp 85–97.

5. Faulde MK, Albeiz G, Nehring O (2010) Insecticidal, acaricidal and repellent effects of DEET- and IR3535-impregnated bed nets using a novel long-lasting polymer-coating technique. Parasitol Res 106: 957–965.

6. Hill N, Lenglet A, Arnez AM, Carneiro I (2007) Plant based insect repellent and insecticide treated bed nets to protect against malaria in areas of early evening biting vectors: double blind randomised placebo controlled clinical trial in the Bolivian Amazon. BMJ 335: 1023.

7. Moore SJ, Hill N, Ruiz C, Cameron MM (2007) Field evaluation of traditionally used plant-based insect repellents and fumigants against the malaria vector *Anopheles darlingi* in Riberaba, Bolivian Amazon. J Med Entomol 44: 624–630.

8. Pempeiter C, Costantini C, Corvel B, Lacciardi S, Dahire RK, et al. (2009) Synergy between repellents and organophosphates on bed nets: efficacy and behavioural response of natural free-flying An. gambiae mosquitoes. PLoS One 4: e7896.

9. Katz TM, Miller JH, Hebert AA (2008) Insect repellents: historical perspectives and new developments. J Am Acad Dermatol 58: 863–871.
10. Gupta RK, Bhattacharjee AK (2007) Discovery and design of new arthropod/insect repellents by computer-aided molecular modeling. In: Debboun M, Frances SF, Strickman D, eds. Insect Repellents: Principle, Methods, and Uses. New York: CRC Press. pp 195–228.
11. Moore SJ, Debboun M (2007) History of insect repellents. In: Debboun M, Frances SF, Strickman D, eds. Insect Repellents: Principle, Methods, and Uses. New York: CRC Press. pp 3–29.
12. Boeckh J, Beier H, Geier M, Hoever F-P, Kruger B-W, et al. (1996) Acylated 1,3-aminopropanols as repellents against bloodsucking arthropods. Pest Sci 48: 359–373.
13. Reeder NL, Ganz PJ, Carlson JR, Saunders CW (2001) Isolation of a deet-resistant mutant of Drosophila melanogaster (Diptera: Drosophilidae). J Econ Entomol 94: 1584–1588.
14. Syed Z, Ishida Y, Taylor K, Kimbrell DA, Leal WS (2006) Pheromone reception in fruit flies expressing a moth’s odorant receptor. Proc Natl Acad Sci U S A 103: 16530–16534.
15. Ditzen M, Pellegrino M, Vosshall LB (2008) Insect repellents: modulators of mosquito odorant receptor activity. PLoS One 3.
16. Stanczyk NM, Brookfield JF, Ignell R, Logan JG, Field LM (2010) Behavioral inactivity to DEET in Aedes aegypti is a genetically determined trait residing in changes in sensillum function. Proc Natl Acad Sci U S A 103: 16530–16534.
17. Stanczyk NM, Brookfield JF, Ignell R, Logan JG, Field LM (2010) Behavioral inactivity to DEET in Aedes aegypti is a genetically determined trait residing in changes in sensillum function. Proc Natl Acad Sci U S A 103: 16530–16534.
18. Ditzen M, Pellegrino M, Vosshall LB (2008) Insect odorant receptors are molecular targets of the insect repellent DEET. Science 319: 1838–1842.