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Multi-Modal Effects of the Repellent Deet Across Protostomia

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MULTI-MODAL EFFECTS OF THE REPELLENT DEET ACROSS PROTOSTOMIA

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DEET (*N, N*-diethyl-*m*-toluamide) is the most broadly effective and widely used personal repellent available, yet we do not understand what makes it so effective. Even in well-studied species like *Drosophila melanogaster* flies and *Aedes aegypti* mosquitoes, many mysteries remain as to how DEET can affect behavior in these species.

For example, *Ae. aegypti* mosquitoes are attracted to human arms. When an arm is covered in DEET, wild-type mosquitoes are not attracted to the arm, while mutant mosquitoes that lack the odorant receptor co-receptor (*orco*), approach the arm, but rarely bite. We investigated this *orco*-independent DEET repellency in *Ae. aegypti* and found that these mosquitoes can sense DEET with their tarsi as well as their proboscis. The tarsi are required for mosquitoes to be repelled after contact with a DEET-treated arm. The proboscis is required for the rejection of DEET-laced liquid food. These results suggest that DEET acts on multiple sensory modalities to repel insects.

Both this work and most prior literature has focused on studying how DEET affects Arthropods, yet one of the major open questions in the field is how DEET can be effective across so many different species. To identify genes and neurons required for DEET-sensitivity outside of Arthropoda, we turned to the nematode *C. elegans.* Here, we demonstrated that DEET affects chemotaxis to some odors but not others. We used this behavior as the basis for a forward genetic screen, and identified two genes as candidates required for complete DEET-sensitivity. We identified a natural isolate of *C. elegans* that was also resistant to DEET, and found that it contains a large deletion in one of the hits from our screen, the G protein-coupled receptor *str-217.* This gene is required for DEET-sensitivity in both wild-type and wild isolate strains. *str-217* is expressed in a single pair of chemosensory neurons called ADL, which are

required for complete DEET sensitivity, and respond to DEET as assayed by calcium imaging. Although we identified additional sensory neurons that respond to DEET, their behavioral contributions are unknown. Both ADL and *str-217* are required for a specific, DEET-induced behavior during chemotaxis and exploration: an increase in average pause duration. Through optogenetic stimulation of ADL, we demonstrated that ADL activity alone is sufficient to increase average pause duration.

Taken together, these experiments provide insights into the genetic and neural mechanisms underlying DEET-sensitivity in *C. elegans*, and allow for comparisons across Protostomes. We also establish *C. elegans* as a model non-Arthropod species for further investigation into the effects of DEET.

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INTRODUCTION

1.1 The discovery of DEET

> "From [the] beginning [humankind] has been prey to the lusts and appetites of hordes of insects. Very early in history [humans] devised methods of combatting these pests to which [they were] host. More often than not manual dexterity in the form of slapping and picking, as practiced in true anthropoid fashion, constituted, as it does in large measure to this day, the prime instrument of insect control… [Eventually, humans] learned that some substances applied to the body discouraged insect aggression. Thus originated the idea of repellents." (Dethier 1947)

1.1.1 Mosquito repellents

One of the first written mentions of insect repellents can be found in Natural History by Pliny the Elder, published between 77–79 AD. He took note of several practices at the time, from co-planting with leeks to deter caterpillars to the use of animal dung, ashes, decoction of wormwood, and burning galbanum plants to guard against garden pests and flies (Elder 1855).

Oral traditions tell us that humans have been using plants and plant-based extractions applied to the skin or smoked to protect against biting animals long before Pliny wrote down his local practices. The Lenape People of Manahatta, the land where this thesis work was completed, use Winkimakwsko (*Hierochloe odorata*), also known as sweetgrass, and Winakw oil (*Sassafras albidum*), also known as sassafrass, as insect repellents and have used these for generations (PaddlesUpstream 2017). Traditional knowledge has provided many of the plant oils used as insect repellents, and con-

tinues to provide new compounds for commercialization. For example, the Confederated Salish Peoples also use sweetgrass (*Hierochloe odorata*) as a repellent, and recently worked with a group of scientists to identify active compounds in this species. Phytol and coumarin were identified as the active compounds that deter biting of *Ae. aegypti* mosquitoes, and these plants were effective repellents in laboratory assays (Cantrell et al. 2016).

Smoking plants and pungent plant odors can be effective local repellents, but the United States (US) military has long been interested in the identification of long-lasting, low-odor, and smoke-free alternatives because smoke and odors can give away tactical positions. In 1900, the US military launched the Yellow Fever Commission to Cuba, which determined *Aedes* mosquitoes were responsible for the transmission of yellow fever in the Spanish-American War. This marks the beginning of the US military's active involvement in the development and use of insecticides and arthropod repellents (Kitchen 2009). In addition to effective but environmentally misguided efforts to curb insect-transmitted disease through the heavy use of the insecticide and environmental toxin DDT, the military funded several large scale chemical screens that produced today's most-used repellents.

1.1.2 A large chemical screen leads to DEET

Before World War II, Japan was the major exporter of both quinine — a compound isolated from cinchona bark and used as an anti-malarial drug — and pyrethrins — insecticidal compounds derived from pyrethrum or chrysanthemum flowers (Ware 1978; NIM 2003). After Japan joined World War II, the US military invested in identifying alternative synthetic repellents that could be produced in large quantities and funded large-scale efforts to identify such compounds. From 1942 – 1947, 6,241 chemicals were tested as mosquito repellents using *Ae. aegypti* mosquitoes. Many were also

tested for effectiveness against *Anopheles quadrimaculatus* in the laboratory, and in the field against *Aedes taeniorhynchus* (Fig. 1.1). The test consisted of “covering the fore-arm (wrist to elbow)… with a chemical or treated cloth and then exposing them in cages containing 2,000 to 4,000 hungry mosquitoes… Arms were exposed in the cages for 3 minutes at 30-minute intervals, or until the mosquitoes bit” (Morton 1947). These compounds were tested on 250 different subjects, and 9% were effective for at least three hours. All solid chemicals and many of the more effective liquid chemicals were tested for efficacy when applied to women’s mercerized cotton hose and tested the following day for 1-2 minutes in a cage of mosquitoes. This screen identified several putative repellents, including *N,N*-diethylbenzamide (Table 1.1, Item No. 2690). Many *N,N*-dialkylamides were identified as putative repellents in this study, and three were chosen for use by the United States military: *N,N*-diethylsuccinamate, *o*-chloro-*N,N*-diethylbenzamide, and *o*-ethoxy-*N,N*-diethylbenzamide and were patented for broad use from 1951-1953 (McCabe et al. 1954)

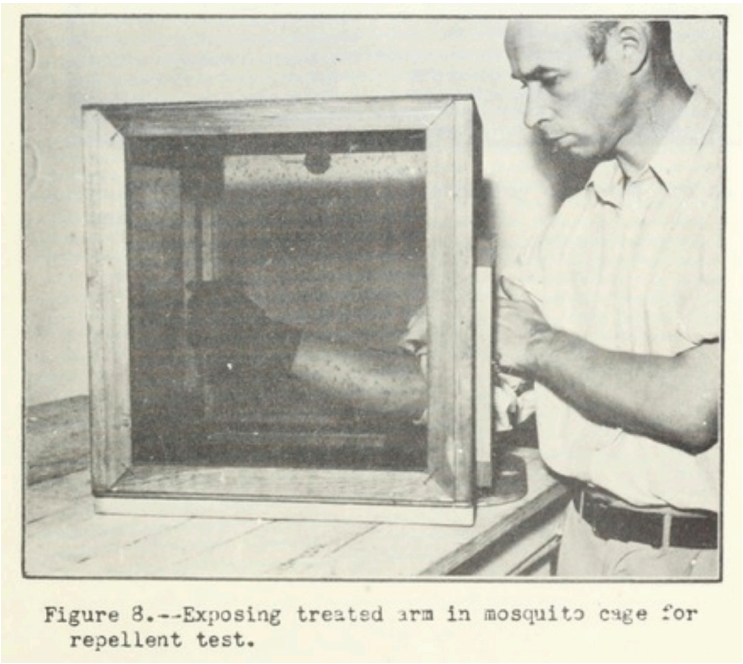


Figure 8.--Exposing treated arm in mosquito cage for repellent test.

Figure 1.1 Testing efficacy of chemicals applied to skin. An example of the assay used to test efficacy of 1 mL of individual compounds applied to skin. Reprinted from Morton et al 1947.

Table 1.1 Isolation of *N,N*-diethylbenzamide, a pre-cursor to DEET An excerpt from a table indicating the effectiveness of many chemicals as lousicides, miticides, and repellency to *Ae. aegypti* adult female mosquitoes when applied to skin (S) or cloth (C). All compounds were scored from 1 (un-effective) to 4 (very effective). *N,N*-diethylbenzamide is the last entry in this table excerpt, number 2690. Reprinted from Morton et al 1947.

Item No.	Orlando No. O-	Chemical	Lousicide Ins.	Lousicide Ov.	Lousicide K.D.	Miticide	Larvicide	Repellent S	Repellent C
2681	6198	N-4(1-Diethylamino pentyl) pyrolidene-1,3-dione	1	2	1	2	1		1
2682	6185	N-4(1-Diethylaminopentyl) urethane	3	3	2	2	1		4
2683	6475	2-(p-Diethylaminophenyl)-1,3-dioxolane	1	2	1	3	1		1
2684	6488	2-(p-Diethylaminophenyl)-4-methyl-1,3-dioxolane	1	1	1	3	1	1	
2685	13198	beta-Diethylaminopropionic acid, ethyl ester	1	1	1	1	1	1	
2686	13185	beta-Diethylaminopropionitrile	1	1	1	1	1	1	
2687	8973	Diethylaminosulfonic acid, trichloroethyl ester	3	3	2	3	1	2	
2688	9478	N,N-Diethylamylbenzenesulfonamide	1	1	1	2	1	1	
2689	2727	Diethylbarbituric acid	1	1	1	1	2		1
2690	1197	N,N-Diethylbenzamide	2	3	2	2	1	4	4

Although *N,N*-diethylbenzamide (CID: 15542) was effective, it often led to rashes when applied to skin (NCBI 2017). To identify additional compounds with better safety profiles, a follow-up study tested 33 ring-substitution derivatives of *N,N*-diethylbenzamide (McCabe et al. 1954). Each compound was applied to skin or cloth and the efficacy of repellency was rated as “excellent”, “very good”, “good”, “fair”, “little activity”, and “neg[ative]”. *N,N*-diethyl-*meta*-toluamide (which would later be re-named DEET) was highly effective on both skin and cloth in these assays (Table 1.2). What may look like a small change -- adding a methyl group --dramatically changed the safety properties while retaining efficacy.

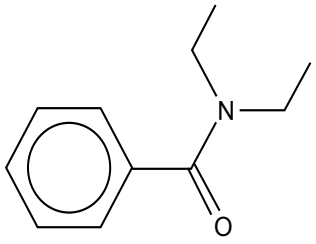
Table 1.2 DEET identified as an "excellent" repellent DEET is listed here as '*m*-Toluic' because this table refers to the base acid used (here *m*-Toluic acid) to make the *N,N*-diethylbenzamide ring substitutions. Table reprinted from McCabe et al 1954.

TABLE I

N,N-DIETHYLAMIDES RCON(C_2H_5)$_2$

DERIVED FROM (Acid)	YIELD,[b] %	n_D^{25}	B.P. °C./MM.	M.P. °C.	ANALYSES,[c] C		H		N		MOSQUITO REPELLENCY	
					Calc'd	Found	Calc'd	Found	Calc'd	Found	On Skin	On Cloth
Senecioic	82	1.4702	115/20[e]	—	—	—	—	—	—	—	—	Neg.
Sorbic	74	1.5244	145–147/16	—	71.81	70.53	10.25	10.03	8.38	8.78	—	++
o-Toluic	90	—	105/1.0[d]	49–50	75.35	75.65	8.96	8.58	7.32	7.52	++++	+++
m-Toluic	94	1.5206	111/1.0[e]	—	—	—	—	—	—	—	++++	++++
p-Toluic	94	—	110/1.0[f]	53.5–55.5	—	—	—	—	—	—	++++	++++

N,N-Diethylbenzamide



DEET
N,N-Diethyl-*meta*-toluamide

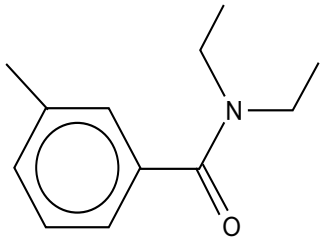


Figure 1.2 Chemical structures of *N,N*-diethylbenzamide and *N,N*-diethyl-*meta*-toluamide This figure demonstrates the structural difference between these two compounds is the toluene ring (a toluene is a benzene ring with a methyl group).

1.2 DEET across Protostomia

After its discovery, DEET was first used by the US military, then marketed for public use in 1957 ("Reregistration Eligibility Decision DEET" 1998). The name was shortened from *N,N*-diethyl-*meta*-toluamide to DEET in 1960 (Smith 1960). Although identified as a repellent for *Ae. aegypti* mosquitoes, DEET has become the gold standard repellent against many species.

1.2.1 The effectiveness of DEET beyond Arthropoda

Studies on the effectiveness of DEET have focused largely on blood-feeding arthropods. However, there are a few reports of other species responding to DEET.

Within Deuterostomia, humans have reported that DEET tastes bitter, though ingestion is not recommended and can lead to toxicity in high amounts (Ambrose 1959). In a study evaluating the safety properties of DEET in rats, DEET delivered in food pellets at greater than 400 mg/kg/day decreased food consumption, indicating taste aversion ("Review of the Toxicology Literature for the Topical Insect Repellent Diethyl-m-toluamide (DEET)" 2002).

Within Lophotrocozoa, a few studies have found DEET to be effective against several species of land leeches (Nath et al. 2002; Tawatsin et al. 2006).

Within Spiralia, DEET was effective in preventing infection of mice by the parasitic fluke *Schistosoma mansoni* in laboratory assays (Salafsky et al. 1998; Cooper et al. 2004) and in the field protected humans from infection.

It is difficult to make broad generalizations based on these data, but the effects of DEET appear to be far reaching across Animalia. It would be interesting to test additional species within Protostomia, including Onychophoran velvet worms or tardigrades. It may also be informative to branch out and test other lineages within Amoebozoa — like Dictyosetliidan slime molds — or plants — either with unicellular animals like *Chamydomonas* or multicellular plants like the Venus fly trap *Dionaea muscipula*.

1.2.2 The effectiveness of DEET in Arthropoda

The evidence for DEET-sensitivity is lacking across non-Dipteran arthropods and the existing evidence is largely concentrated on ticks and mites, both members of the taxon Acari. DEET is effective against ticks including the dog tick *Dermacantor*

variabalis, the lone star tick *Amblyomma americanum,* the South African bont tick *Amblyomma hebraeum*, and the deer tick *Ixodes scapularis* (Bissinger, Apperson, et al. 2009). The exact mode of repellency is unknown. One study proposed that in both the deer tick and lone star tick, DEET can act as a repellent at a distance when separated from DEET-treated skin by gauze but provided enhanced protection when contact was possible (Carroll et al. 2005). Another study demonstrated that deer ticks would spend less time in an air stream with DEET, providing further evidence of an olfactory effect of DEET (Romashchenko et al. 2012). It seems likely that ticks exhibit both contact and olfactory repellency.

Several mite species are DEET-sensitive. DEET disrupts attraction to host odor for the honey bee parasite *Verroa destructor* (Singh et al. 2014). DEET is effective against Trombiculidae chiggers both in laboratory assays (Hanifah et al. 2010) and in the field (Niebuhr et al. 2014) as well as *Sarcoptes scabiei* scabies infection (Fang et al. 2015). These data demonstrate that multiple Acari species are sensitive to DEET, but it would be interesting to test other Arthropod lineages such as millipedes and soil bugs.

1.2.3 The effectiveness of DEET in Diptera

The bulk of our understanding of how DEET works comes from Dipterans. Of blood-feeding Dipterans, DEET is effective against *Rhodnius prolixus* and *Triatoma infestans* kissing bug*s* (Buescher et al. 1985; Alzogaray et al. 2000)*;* several species of *Aedes*, *Anopheles*, and *Culex* mosquitoes (Syed et al. 2008; McIver 1981; Bernier et al. 2005); *Phlabatomous papatsi* sand flies (Klun et al. 2006), *Glossina morsitans* tsetse flies (Wirtz et al. 1985), and *Pediculus humanus* lice (Canyon et al. 2007). DEET even delayed the colonization of pig carcasses by blowflies (Shelomi et al. 2012).

There are far fewer studies of how DEET affects non-blood-feeding insects, with a few notable exceptions. *Apis mellifera* honey bees can taste DEET, as DEET

can be used as an unconditioned stimulus during proboscis extension-based learning (Abramson et al. 2010). The German cockroach, *Blatellica germanica*, avoids contact with DEET-treated filter paper (Sfara et al. 2016). Infestation of the flour beetle *Tribolium castaneum* and booklouse *Liposcelis bostrychophila* is similarly inhibited by DEET (Zhang et al. 2011). *Drosophila melanogaster* exhibits both olfactory (Ditzen et al. 2008) and anti-feedant (Lee et al. 2010) effects. The core of our understanding of the molecular mechanisms of DEET repellency come from one of these insects, *Drosophila melanogaster,* and are further explored later in this chapter.

1.3 Our understanding of DEET has advanced alongside our understanding of Dipteran chemosensation

Initial work studying DEET focused on its efficacy and safety for military use. Although its effectiveness against ticks was apparent by 1976 (Grothaus et al. 1976), the majority of studies focused on insects, especially *Ae. aegypti*. As new techniques for studying chemosensation and neuroscience emerged, new effects of DEET were discovered and hypotheses have progressed alongside these new findings.

1.3.1 Early DEET hypotheses

Initial work studying DEET focused on its efficacy and safety for military use. It was registered for public use in 1957 and re-named DEET in 1960 (Smith 1960). Although its effectiveness against ticks was apparent by 1976 (Grothaus et al. 1976), the majority of studies focused on insects, especially *Ae. aegypti*.

Lactic acid, a component of human sweat, was the first individual human-derived molecule identified as an attractant for *Ae. aegypti* (Acree et al. 1968). Edward Davis (Davis 1976a) identified lactic acid sensitive neurons, and examined how DEET affects these cells. In insects, peripheral olfactory sensory neurons reside in head appendag-

es—the antennae, maxillary palps, and occasionally the proboscis—and insert dendrites into small hairs called sensilla. These sensilla have pores that are permeable to odorants. It is possible to record extracellular spikes that originate from these sensory neurons by inserting an electrode into the sensillum. Davis identified two neurons in antennal grooved-peg sensilla that were sensitive to lactic acid: the spike frequency of one neuron was increased by lactic acid, and the other decreased. The lactic acid-inhibited cell was further inhibited by DEET, and the lactic acid excited cell was attenuated by DEET (Davis 1976a). Over subsequent years, several behaviorally active compounds were identified as host cues and oviposition cues. The neurons responding to these cues were also identified, and DEET proved to be effective inhibitor for many of these neurons that respond to behaviorally-active compounds (Davis 1976b; Davis 1985). This led to a hypothesis that DEET masks the attractive odors of humans by inhibiting the activation of sensory neurons that mediate attraction.

Based on these electrophysiological and behavioral data, several hypotheses arose to explain how repellents in general and DEET specifically may affect insects. Repellents may activate the response of sensory neurons required for behaviors like oviposition (Davis 1976b), repellents may inhibit the response of sensory neurons to normally attractive stimuli like lactic acid and make the host “invisible” (Davis 1976a), or repellents may activate a labeled line repellency that makes the animal avoid the host, or they may “jam” the sensory information system by activating several different receptor neuron types and change how the host is perceived (Davis 1985). Susan McIver proposed that DEET, as a highly lipophilic molecule, interacts with the lipid cell membranes and perturbs them in such a way that the normal responses to attractants are altered. This unique hypothesis allows for both labeled line repellency and the “jamming” of sensory information (McIver 1981). However, without genetic access to these organisms, and lacking the ability to identify receptors or manipulate neurons, it was not possible in

the 1980s to directly test these hypotheses.

1.3.2 Molecular revolution

The discovery of the insect Odorant Receptors (ORs) (Clyne et al. 1999; Vosshall et al. 1999) and subsequent finding that the functional OR is a complex of a ligand-selective OR and the odorant receptor co-receptor *orco* (Larsson et al. 2004) allowed for renewed investigation into the effects of DEET in insects.

DEET inhibits behavioral attraction of *D. melanogaster* flies to food odors. This preference requires intact antennae and *orco* (Ditzen et al. 2008), indicating that the odorant receptors are required for DEET to inhibit attraction to food odors. Additionally, sensilla recordings demonstrated that DEET can potentiate or inhibit odor-evoked activity and can inhibit odor-evoked suppression of activity (Pellegrino et al. 2011). Together, these data support the “jamming” or “confusant” hypothesis: many neurons are affected by DEET, and this confuses the aggregate signal that may form the percept for the animal.

In addition to confusing the odor code at the periphery, it is possible that DEET also acts as a true repellent in some species. DEET alone can increase the spiking activity in some neurons in *Culex quinquefasciatus* (Syed et al. 2008), and DEET generates an aggregate, electroantennographic response (Costantini et al. 2001; Leal et al. 1998) as measured by extracellular recordings of receptor potentials across a whole antenna. Behaviorally, *Cu. quinquefasciatus* animals show a preference for solvent over DEET in a two-choice assay, but animals injected with dsRNA against *CuOR136* lose this preference (Xu et al. 2014). In this assay, two heating elements are present, with a blood-soaked cotton ball placed on top, and a ring of DEET- or solvent-treated paper surrounds the element about three centimeters away. Animals are individually videotaped and the time spent on each side is scored. RNAi-mediated knockdown of

CuOR136 also decreased the electroantennographic response to DEET, and can confer DEET-sensitivity to oocytes. In *Cu. quinquefasciatus*, there may be an additional volatile repellent effect of DEET.

1.4 Investigating *orco*-independent DEET sensitivity in and beyond Arthropoda.

This thesis describes a quest toward understanding why DEET is so effective across so many different species, and how we can further probe that question using the tools available today. We begin in the mosquito *Ae. aegypti*, identifying and isolating multiple effects of DEET just within this one species (**Chapter 2**). We then describe a quantitative assay to test DEET avoidance in terrestrial animals, and demonstrate its effectiveness across Arthropoda (**Chapter 3**). We then establish the nematode *C. elegans* as an ideal organism for studying the effects of DEET, and describe the effects of DEET on chemotaxis in this species (**Chapter 4**). Next, we delve into the genetic (**Chapter 5**) and related neuronal (**Chapter 6**) requirements for this effect on chemotaxis behavior. Finally, we use single-animal tracking and optogenetics to detect and artificially reproduce specific contributions to the chemotaxis defect (**Chapter 7**), opening up this species for further investigation discussed in **Chapter 8**.

CHAPTER 2

CONTACT DEET REPELLENCY IN THE *AE. AEGYPTI* MOSQUITO IS INDEPENDENT OF BITTER TASTE AND REQUIRES THE TARSI.

Within seconds of inserting a human arm into a cage of female, *Ae. aegypti* mosquitoes, hundreds of animals cover the skin, and within minutes most have extracted enough blood to use the digested protein to produce and lay over one hundred eggs each. Female mosquitoes need blood to develop these large clutches of offspring, and therefore host-seeking and blood-feeding are important aspects of a female mosquito's life. If the arm is first covered in DEET, very few animals take flight, even though they are inches away from a perfectly good blood meal (DeGennaro et al. 2013). Are they "repelled" by the smell of DEET? Does DEET mask the scent of the arm? Or does the DEET alter the mosquito's perception of the scent of the arm by acting as a "confusant"? These are the prevailing hypotheses for how DEET acts in the volatile phase. If the cage is instead filled with *orco* mutant females, a buzz of activity ensues after the arm is inserted. The mosquitoes approach the arm, but shortly after contact the animals leave so that the majority of animals do not blood feed. In this example, volatile repellency occurs at a distance and only in the *orco* mutant mosquitoes do we see that DEET has an additional effect as a contact chemo-repellent.

With the development of *orco* mutant mosquitoes, we gained access to this mechanistically distinct, *orco*-independent DEET repellency. Our objective was to (1) observe this new phenomenon, (2) identify similarities and differences between this behavior and reported results in other species, and (3) investigate the appendages required for this repellency.

2.1 *Ae. aegypti orco* mutants land on DEET-treated arms and are insensitive to volatile DEET

Volatile DEET repellency occurs at a distance, preventing most wild-type mosquitoes from approaching a DEET-treated arm. In contrast, *orco* mutant mosquitoes remain attracted to DEET treated arms placed next to a cage (~2.5 cm away), but they do not blood-feed when the arm is inserted into the cage (DeGennaro et al. 2013). We knew that *orco* mutants were being repelled by DEET within 2-3 cm of the arm, but when observing arms inserted into cages, the animals moved too quickly for us to see by eye what was happening. Therefore, we needed a way to observe every interaction of each mosquito with DEET-treated skin, so we restricted the available skin area. Subjects wore a long latex glove with a small (2.5 cm) hole cut in it. We focused a digital video camera on this area of skin (Fig. 2.1) and then manually scored each video. From these videos, we were able to gather data on how often mosquitoes contacted the skin, how long they spent contacting the skin, and what appendages they used to contact to skin.

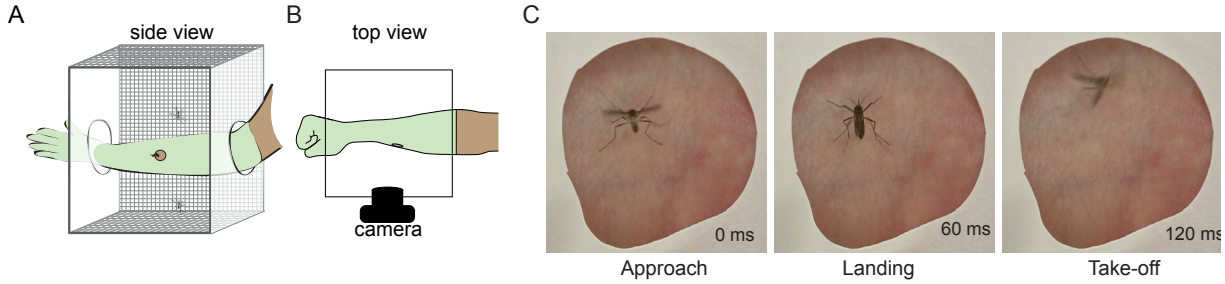


Figure 2.1: Schematic of video-based assay and example landing
(A) Side view of the assay set up, without the camera included. (B) Top view of the assay. (C) Cropped stills from an example video showing an example female *orco* mutant mosquito landing on a human arm.

We found that the *orco* mutants landed on DEET-treated arms as often as solvent treated arms (Fig. 2.2A), but rarely blood-fed or bit the arm (Fig. 2.2B). This both provided additional confirmation that *orco* mutants are attracted to DEET-treated arms but rarely bite them, and also provided evidence that DEET may act as a contact

repellent in the absence of volatile, *orco*-mediated avoidance.

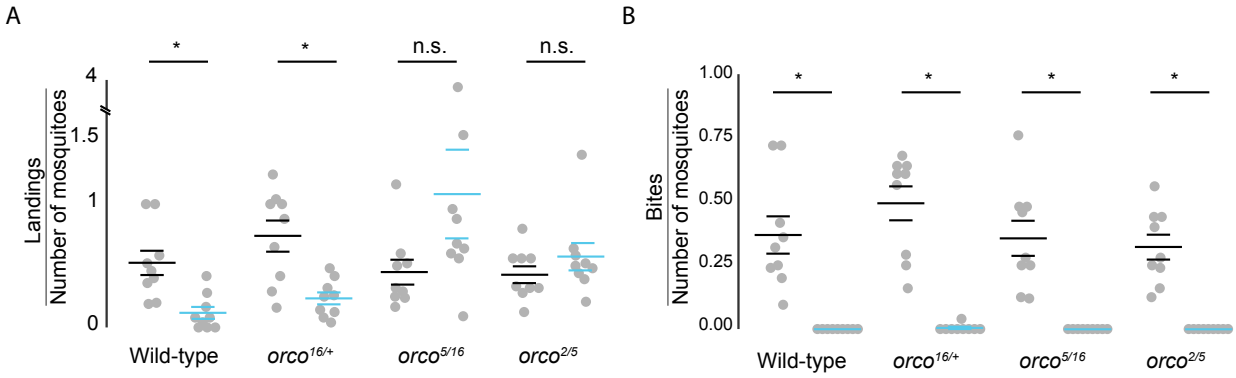


Figure 2.2: Female *orco* mutant *Ae. aegypti* mosquitoes are repelled by DEET on contact. (A) The average number of landings per mosquito in each 10 minute video landing on human skin treated with either ethanol solvent (black) or 10% DEET (blue). (B) The average number of biting events per mosquito in each video. (Bonferroni corrected *t*-test comparing solvent and DEET for each genotype. *p<0.0125; N=9 assays, n=23-25 female mosquitoes per assay)

Although male mosquitoes do not blood-feed and cannot bite humans (Lee 1974), we wanted to see if males were also repelled by DEET on contact. In the wild, male *Ae. aegypti* mosquitoes aggregate around human hosts. Attraction to humans is likely the primary way they find sexually mature mates (Hartberg 1971). Male attraction to human hosts was fortuitous for this study: we found that in this arm-in-cage assay, male mosquitoes would land on human skin, allowing us to ask if males also exhibit DEET-sensitivity in this assay (Fig. 2.3A). Similar to female mosquitoes, DEET is an effective volatile repellent for wild-type and heterozygous genetic controls, but *orco* mutant males landed on both solvent- and DEET-treated arms (Fig. 2.3B). Because males cannot blood-feed, we could not compare how many bites occurred in these videos. Instead, we noted the amount of time they spent on the arm and found that male mosquitoes spent significantly less time on DEET-treated arms, regardless of genotype (Fig.

2.3C). These data confirm that DEET is an effective volatile repellent for both male and female *Ae. aegypti* mosquitoes and that this volatile repellency requires *orco*. This work also provides evidence that there is an additional, *orco*-independent, likely contact-mediated repellency in both males and females.

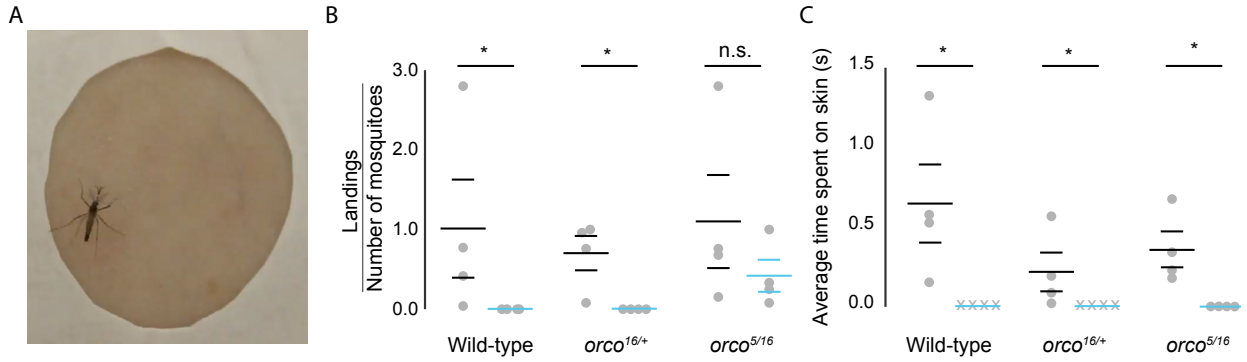


Figure 2.3 Male *orco* mutant *Ae. aegypti* mosquitoes are repelled by DEET on contact. (A) A male mosquito on an exposed portion of a solvent-treated human arm (B) The average number of landings per mosquito in each video landing on human skin treated with either ethanol solvent or 10% DEET. (B) The average time spent on the skin per landing in each video. (Bonferroni corrected, one-sided permutation test comparing solvent and DEET for each genotype. *p<0.0167; N=4 assays, n=23-25 male mosquitoes per assay)

2.2 *Orco*-independent DEET repellency is independent of bitter taste in *Ae. aegypti.*

Previous work in the honey bee *Apis mellifera* demonstrated that DEET can be used as an aversive, unconditioned stimulus when mixed with sucrose and delivered as a tastant (Abramson et al. 2010). In *Lymantria dispar* moth larvae, DEET applied to red oak leaves, a food source, is able to deter feeding, and neurons on the maxillary palps of these larvae are sensitive to DEET and bitters (Sanford et al. 2014). Another study in *D. melanogaster* also identified an *orco*-independent, anti-feedant effect of DEET that requires bitter-sensitive gustatory neurons and three bitter Gustatory Receptors (GRs):

Gr66a, Gr32a, and *Gr33a* (Lee et al. 2010).

In this experiment from the Montell group, 18-24 hour starved flies were given access to a 96-well plate with sucrose-laden agar food sources. Half of the available food contained 5 mM sucrose mixed with 0.2% DEET and food coloring, and the other half contained 1 mM sucrose with solvent and another color. Without DEET present, flies preferred the higher sucrose food. With the addition of DEET, animals, including *orco* mutants, strongly preferred the lower-sucrose food which lacks DEET. Bitter GR mutants ($Gr33a^{1}$, $Gr66a^{ex83}$, and $Gr32a^{\Delta}$) showed a significantly shifted preference toward the high-sucrose DEET food. However, these animals did not return to non-DEET levels of strong preference for higher sucrose, indicating that they may still be able to sense DEET. It would be interesting to repeat these experiments with the same concentration of sucrose in each solution, and determine if there are other receptors in flies that may be required for avoiding DEET. This group was also able to record from neurons that express these bitter gustatory receptors and found that these neurons are also DEET-sensitive, and their response requires expression of these receptors. These data from *D. melanogaster* present the bitter gustatory neurons as interesting candidates for *orco*-independent DEET-sensitivity in *Ae. aegypti.*

Bitter gustatory neurons are tantalizing candidates, especially when considering another study from the Dickens lab that identified bitter- and DEET-sensitive neurons in the labella of *Ae. aegypti* mosquito mouthparts (Sanford et al. 2013). The Dickens group recorded from medium length hairs on the tip of the proboscis of female *Ae. aegypti* mosquitoes. These hairs contain neurons that are sensitive to DEET and bitter compounds, like quinine. Although these neurons are intriguing based on their electrophysiological responses and their location at the tip of the proboscis, the behavioral function of these neurons is unknown.

To investigate if bitter taste can explain *orco*-independent contact DEET repellency, we wanted to first determine if *Ae. aegypti* mosquitoes avoid bitter compounds and DEET when presented in conjunction with an appetitive stimulus like sucrose. To do this, we used a mosquito-adapted version of the CAFE assay (Ja et al. 2007; Liesch et al. 2013), and gave starved female mosquitoes a choice between drinking 10% sucrose with solvent or 10% sucrose with 1% DEET or two intensely bitter compounds (1 mM lobeline or 5 mM quinine) added. *Ae. aegypti* mosquitoes avoided both bitters and DEET in this assay (Fig. 2.4).

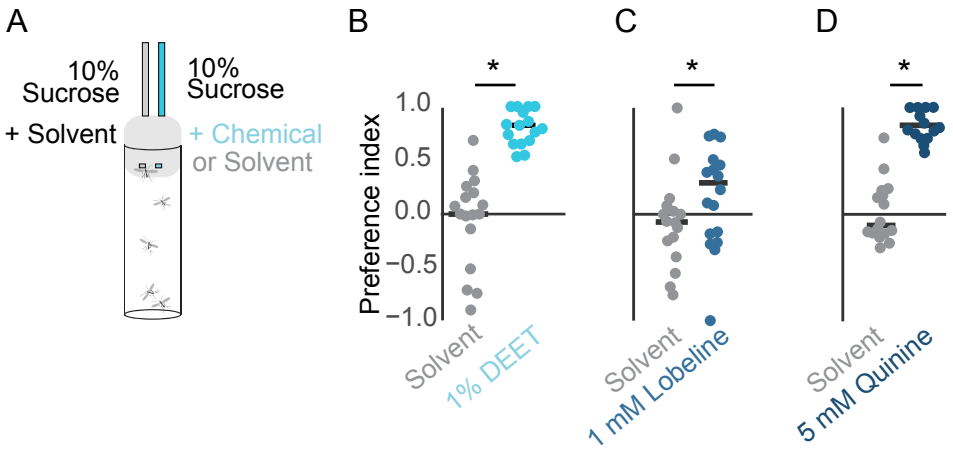


Figure 2.4 Both bitters and DEET are effective anti-feedants in a sucrose feeding assay. (A) Schematic of the mosquito CAFE assay. (B) Preference indices for the solvent-containing capillary in each vial of animals compared to the DEET-containing capillary [SOLVENT - COMPOUND/TOTAL]. (C) Preference indices for the solvent-containing capillary compared to the lobeline-containing capillary. (D) Preference indices for the solvent-containing capillary compared to the quinine-containing capillary (N=14-17 vials, n=5 animals per vial. Student's *t*-test, *p<0.05). These data were collected by Vineeta Reddy, a high school student.

These data demonstrate that in mosquitoes — like flies and bees — DEET and bitter tastants can induce avoidance of an otherwise attractive sucrose solution. Once we identified behaviorally-relevant bitter compounds, we wanted to know if these

compounds could mimic DEET as a repellent on skin. Towards this goal, we used the arm-in-cage assay (Fig. 2.5A) and found that even at 10-fold higher concentrations, bitters do not deter *orco* mutant mosquitoes from blood-feeding on bitter-treated arms (Fig 2.5). DEET is effective in both the CAFE and arm-in-cage assay at 1% concentration (52 mM).

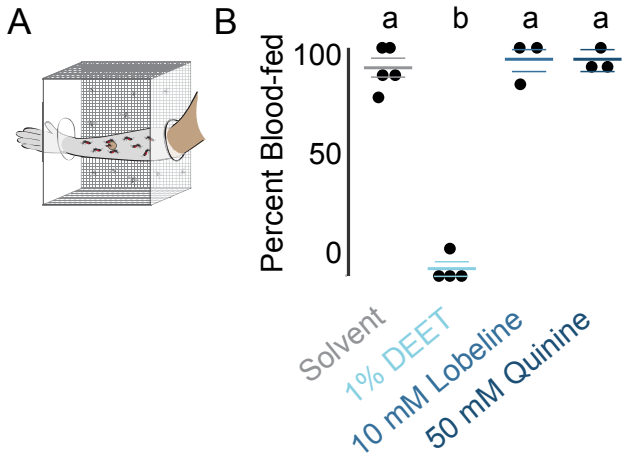


Figure 2.5 Lobeline and quinine are not effective contact repellents on human skin. (A) Schematic of the arm-in-cage assay. (B) Percent of animals blood-feeding on a human arm. Different letters indicate significant differences, *p<0.05, one-way permutation ANOVA with Tukey's post-hoc test. (N=3-5 assays, n=22-25 mosquitoes per assay).

When we tried to reconcile how bitters can be effective anti-feedants in sugar but not on skin, we developed two hypotheses. First, the labella may only be used to evaluate sources of sugar, but it may not be the primary source of information during host-seeking and blood-feeding. Second, it is possible that DEET is a stronger negative stimulus, and there may be positive taste cues on the skin that can override the bitter taste, but cannot override the potent taste of DEET. Although we attempted to avoid this by increasing the bitter concentration 10-fold between the CAFE assay and the blood-feeding assay while keeping the DEET concentration constant, it is possi-

ble that DEET is more than 10-fold more potent. If this is the case, using a taste-free blood-feeding system should allow bitter compounds to repel mosquitoes and decrease blood-feeding in the absence of any competition with attractive compounds on the skin.

To test this hypothesis, we used a Glytube feeding assay. The Glytube assay uses a piece of Parafilm as a skin-substitute, covering a small amount of warmed animal blood (Costa da Silva 2013). This allows us to deliver DEET and quinine specifically on the surface or in the blood (Fig. 2.6). In this assay, we saw that both DEET and quinine were effective anti-feedants when mixed into blood, and that only DEET was able to decrease feeding when applied to the surface (Fig. 2.6C). This agrees with recent results from *Cu. quinquefasciatus,* demonstrating that animals spent less time feeding on cotton balls soaked in blood with DEET and covered with Parafilm than cotton balls soaked in blood with solvent and covered in Parafilm (Lu 2017).

Our results disagree with previous work from Bar-Zeev and Schmidt (Bar-Zeev et al. 1959), which showed that *Ae. aegypti* rarely land on membranes containing DEET-laced blood. This study used animal intestine membranes to deliver P^{32} labeled blood, laced with DEET, and measured radiation levels in mosquito tarsi, and proboscis to determine if the animals landed and left, bit and rejected, or blood-fed. However, the intestinal membranes are semi-permeable, and could allow DEET to permeate the barrier and act as a volatile repellent when mixed with the blood and membrane odors, which would account for the discrepancies in landing events between our results.

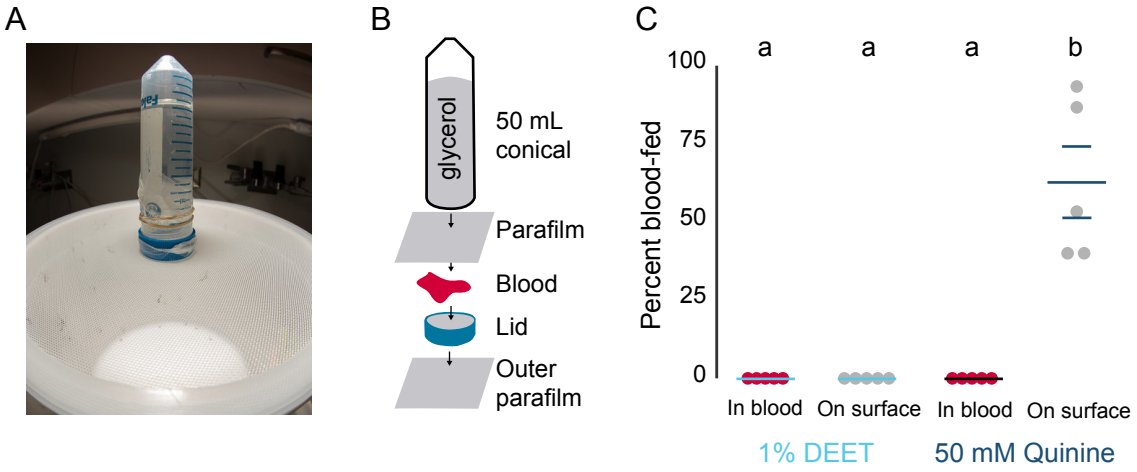


Figure 2.6 Quinine is an anti-feedant, but not an effective contact repellent, in a skin-free blood-feeding assay. (A) Example of a Glytube feeder. Image © Alex Wild, used with permission. (B) Schematic of Glytube assembly. DEET or quinine was added to either the blood ("In blood") or the outer Parafilm layer ("On surface"). (C) Percent of animals that blood-fed on DEET or quinine applied to the surface or delivered in the blood. Different letters indicate statistically significant differences between groups by two-way permutation ANOVA and Tukey's post-hoc test (N=5 assays, n=12-16 mosquitoes per assay, *p<0.05).

This work provides evidence against the idea that animals are ignoring bitter cues because they are in a blood-feeding state, as quinine can still act as an anti-feedant when mixed with blood (Fig. 2.6C). Because the surface is made of Parafilm and no skin cues are added, this also provides evidence against the hypothesis that positive tastants are overriding a bitter taste, though we cannot entirely rule out the possibility that some tastants from the blood are permeating the Parafilm barrier. These data instead support the hypothesis that contact DEET repellency is independent of bitter taste, and that an unknown, *orco*-independent mechanism is at work in *Ae. aegypti* mosquitoes.

In *D. melanogaster* feeding experiments, even bitter receptor mutant animals showed some residual bitter-independent DEET repellency (Lee et al. 2010). In the fly, it is difficult to parse these different effects as the flies are drinking and walking on the same substance (sucrose-laden agarose). We were able to clearly separate these effects in the mosquito because female mosquitoes land on the skin, while drinking blood below the skin, allowing us to experimentally separate the delivery of tastant cues (Fig. 2.7D-E).

Here we also provide evidence that there may be bitter- and DEET-sensitive neurons in the stylet of mosquitoes, in addition to the previously identified bitter- and DEET-sensitive neurons on the labellar lobes (Sanford et al. 2013). The stylet is a specialized feeding appendage that functions similar to a saw and needle, sawing through dermis and feeding from the blood underneath through the fascicle (Fig. 2.7A). The labellar lobes stay on the surface of the skin, and therefore cannot be the appendage detecting the DEET and quinine in the blood behind the parafilm barrier in the Glytube assay (Fig. 2.7G).

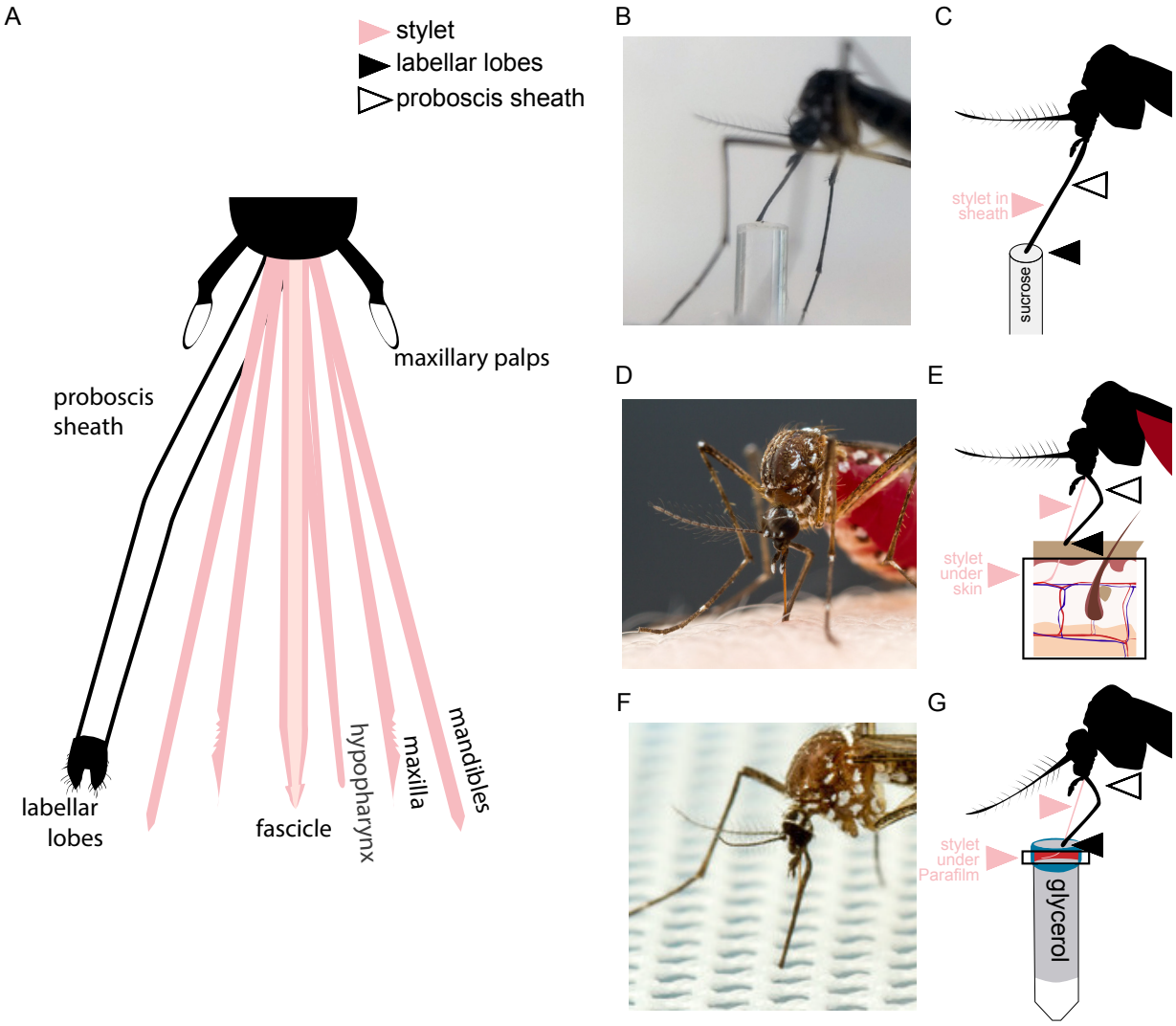


Figure 2.7 Bitter taste and DEET repellency may require different appendages (A) Diagram of the elements of an *Ae. aegypti* female proboscis. (B) Image of a female mosquito feeding from a 10% sucrose solution in a capillary. C) Schematic of the labellar lobes contacting the surface of the sucrose solution during feeding. (D) Image of a female mosquito feeding on a human arm. (E) Schematic of the stylet underneath the skin and the labellar lobes on the surface. (F) Image of a female mosquito feeding from a Glytube under a mesh. (G) Schematic of the stylet feeding from the blood-underneath the Parafilm surface. Photos in D and F © Alex Wild.

2.3 The proboscis is not sufficient for contact DEET repellency in *Ae. aegypti.*

Using *orco* mutant mosquitoes, we are now able to study *orco*- and bitter-independent DEET repellency. Although we have referred to this as “contact repellency”, it is also possible that this behavior does not require contact itself, but requires closeness to the skin only achieved during landing events. If contact is actually required, the proboscis or tarsi should be required for contact-mediated repellency, as they are the only appendages to contact the skin during landing (Fig. 2.8). Both the proboscis and the tarsi are covered in sensory hairs and propose intriguing candidate appendages for contact DEET repellency in *orco* mutant mosquitoes.

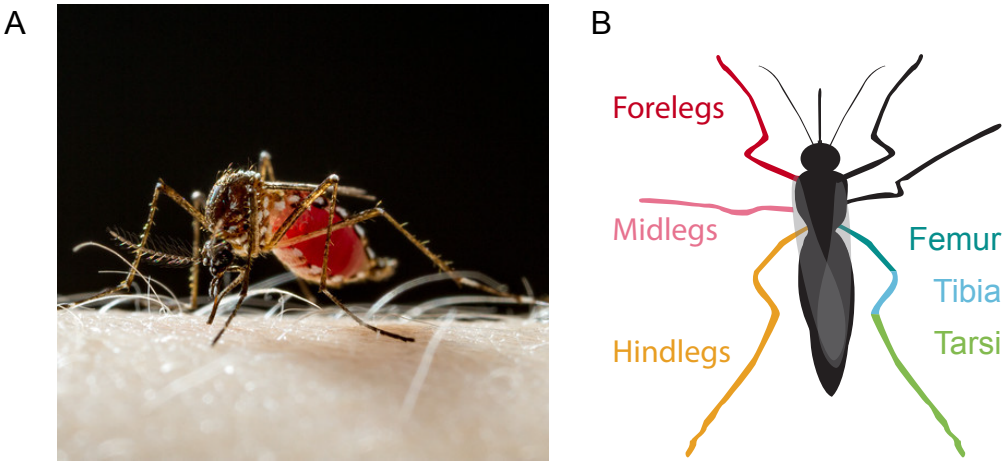


Figure 2.8 The proboscis and tarsi are the only appendages to contact the skin during blood-feeding (A) Image of a mosquito blood-feeding on a human arm. Image © Alex Wild, used with permission. (B) Illustration of the mosquito leg pair names (left) and segments (right).

To test if the proboscis is required for contact DEET repellency, we restricted the area of skin available for the mosquitoes to contact. The 1.5 mm diameter available skin is smaller than the distance between a mosquito’s forelegs, and therefore a mosquito cannot touch the skin with both her proboscis and her tarsi at the same time (Fig. 2.9D). In this assay, *orco* mutant mosquitoes blood-feed equally on solvent- and DEET-treated

arms suggesting that they are unable to sense DEET if only the proboscis touches the skin (Fig. 2.9E).

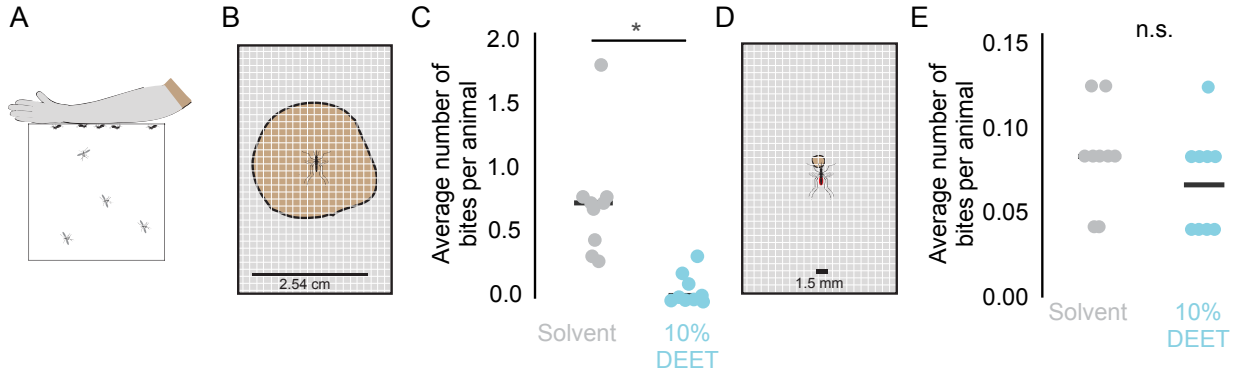


Figure 2.9 The proboscis is not sufficient for contact DEET repellency (A) Schematic of the arm-against-cage assay. (B) Illustration of a female mosquito feeding on a ~2.5 cm diameter area of human skin against the side of a cage. (C) The average number of biting events per animal in each video on DEET- or solvent-treated skin during the 10 minute assay. (D) Illustration of a female mosquito feeding on a 1.5 mm area of human skin against the side of a cage. (E) The average number of biting events per animal in each video on DEET- or solvent-treated skin. (N= 9 assays, n=23-25 female mosquitoes, Student's *t*-test, *p<0.05)

2.4 The tarsi are required for contact DEET repellency in *Ae. aegypti.*

These data provide evidence that the proboscis is not sufficient to confer DEET-sensitivity to *orco* mutant mosquitoes. (Fig. 2.9E). The tarsi are the only other appendages that contact the skin during blood-feeding, and we carried out experiments that asked whether some or all leg appendages mediate DEET contact chemorepellency. Attempts at removing all of the tarsi were not fruitful: the tarsi are important appendages that the mosquito requires to produce the necessary force and leverage to pierce the skin (Jones J. C. 1973). Therefore, we needed a way to disrupt tarsal chemosensation without removing the tarsi. Toward this goal, we covered the tarsi in UV-curing glues (Fig. 2.10), which have been used previously to occlude sensilla in taste organs (Olsen et al. 2008) and antennae (Wasserman et al. 2013) in *D. melanogaster*.

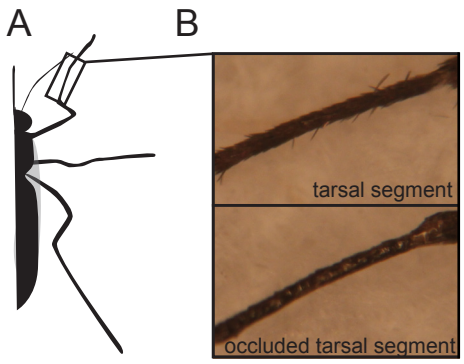


Figure 2.10 Gluing tarsal segments can effectively occlude the chemosensory sensilla on the legs. (A) Schematic of mosquito with focus on the fifth tarsal segment. (B) Example images of unglued (top) and glue-occluded (bottom) fifth tarsal segments.

When all tarsi were occluded, mosquitoes bit DEET-treated arms and solvent-treated arms at similar levels. Animals sham-treated or with their tibia glued were still repelled by DEET on contact (Fig. 2.11). Additionally, leaving any pair of tarsi un-glued was sufficient to significantly decrease biting events (Fig. 2.12).

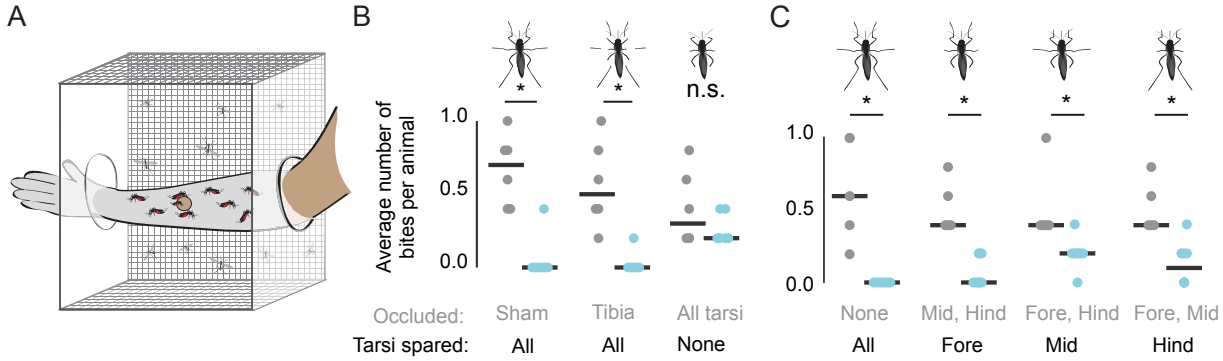


Figure 2.11 Tarsi are required for contact DEET repellency, and any pair of tarsi is sufficient to confer DEET-sensitivity. (A) Schematic of the gloved arm-in-cage assay. (B) Average number of bites per animal observed when all of the tarsi, tibia, or no appendages were glue-occluded. (C) Average number of bites per animal observed when a single pair of tarsi were left un-occluded (N=9 assays, n=4-5 female mosquitoes per assay, Bonferroni-corrected Student's *t*-test).

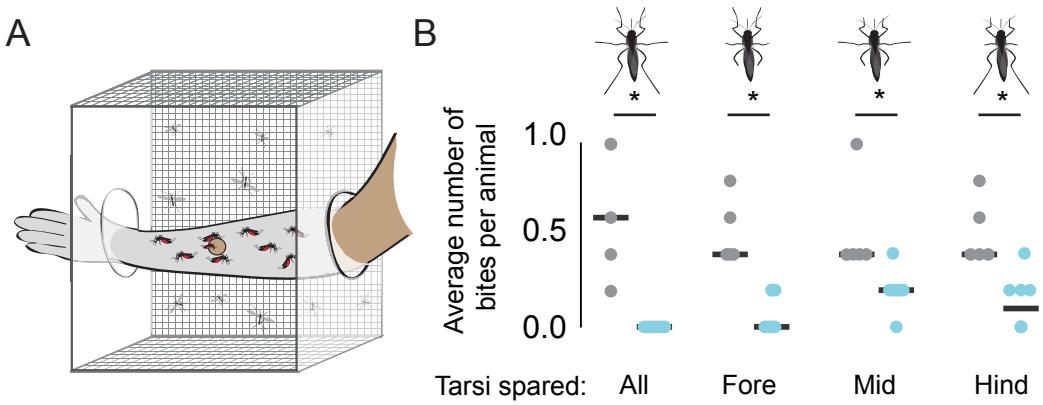


Figure 2.12 Animals with any pair of tarsi un-occluded remain sensitive to DEET. (A) Schematic of the gloved arm-in-cage assay. (B) Average number of bites per animal observed when specific pairs of tarsi were left un-occluded. (C) Average number of bites per animal observed when a single pair of tarsi were left un-occluded (N=8-9 assays, n=4-5 female mosquitoes per assay, *p<0.0125, Bonferroni-corrected Student's *t*-test).

Impressed with the effects of DEET, we wondered whether three recently identified compounds structurally related to DEET (Boyle et al. 2016), and another common repellent Picaridin, may have similar broad effects (Fig. 2.13). The three recently identified compounds were selected cheminformatically, based on the structures of DEET and another commonly used synthetic repellent, Picaridin, which is also branded as Icaridin in Europe. Picaridin has been shown to be effective against ticks, mosquitoes, and sand flies, but its mechanism of action has not been investigated. (Klun et al. 2006; Bissinger, Zhu, et al. 2009). We first asked how Picaridin performed in our arm-in-cage assay, and whether like DEET it had both volatile and non-volatile mechanisms of action. We were surprised to see that this compound is a primarily contact-based repellent: wild-type animals landed on both Picaridin- and solvent-treated arms.

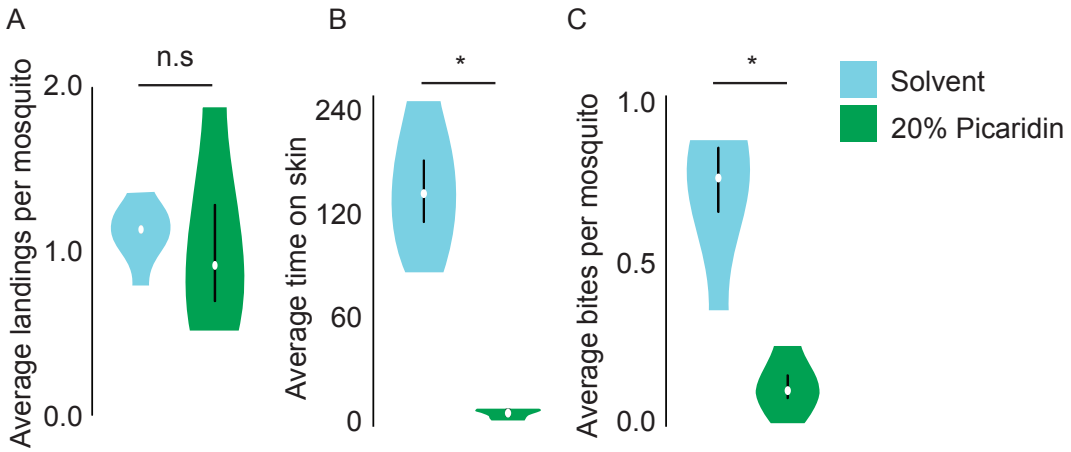


Figure 2.13 Picaridin is a primarily contact-mediated repellent in *Ae. aegypti*. (A) The average number of landings per mosquito in each video of female mosquitoes landing on either a solvent (blue) or Picaridin (green) treated arm. (B) The average length (seconds) of the landing events in each video. (C) The average number of bites per mosquito for each video. (N=8-9 assays, n=23-25 female mosquitoes per assay, *p<0.0167, Bonferroni-corrected Student's *t*-test).

We then evaluated the effectiveness, *orco*-dependence, and olfactory contribution to repellency for the three cheminformatically identified compounds (Fig. 2.14). Although these data are preliminary, we identified several trends from these data. Each of these compounds was at least partially effective, as they all decreased the number of bites received from wild-type mosquitoes (filled bars, Fig. 2.14C). By this standard, EA was the most effective, and appears to act as a contact repellent, as wild-type and *orco* mutant mosquitoes both land on the skin, but rarely blood feed (Fig. 2.14B and C). DA exhibited intermediate effects: both wild-type and *orco* mutant mosquitoes landed on DA-treated arms, and both genotypes did occasionally blood feed, but not as often as controls. The BA results were particularly intriguing: BA appears to be an *orco*-mediated, contact-based repellent. Wild-type and *orco* mutant mosquitoes often landed on BA-treated arms. Wild-type mosquitoes rarely bit BA-treated arms, but *orco* mutants bit

BA-treated arms as often as solvent-treated arms. Unlike *D. melanogaster* flies, which do not seem to express any odorant receptors in their tarsi, a few odorant receptors are expressed in an RNAseq-generated transcription profiles of mosquito tarsi (Matthews 2016). These receptors could be mediating contact- or close-range avoidance of BA and would be interesting targets for future work. While these are all preliminary data, these compounds do highlight the uniqueness of DEET: even highly similar compounds can only mimic some of the effects of DEET, even within just one species.

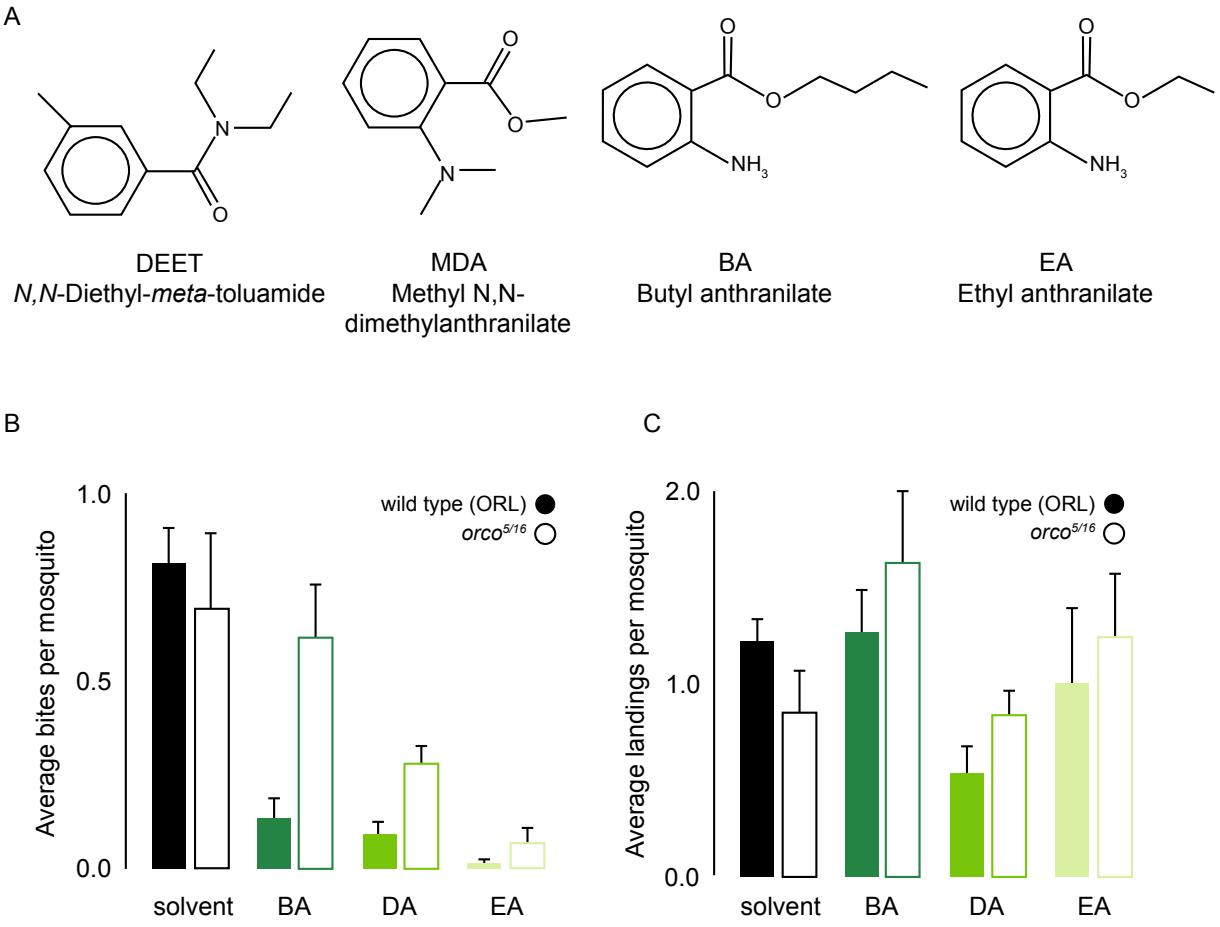


Figure 2.14 Cheminformatically identified repellents each mimic a subset of the effects of DEET. (A) Chemical structures of the repellents used in this assay. (B) The average number of landings per mosquito in each video of either wild-type (solid bars) or *orco*-mutant (open bars) mosquitoes landing on solvent- (black) or repellent- (green) treated arms. (C) The average number of bites per mosquito for each video. (N=3-4 assays, n=23-25 female mosquitoes per assay)

We then returned our focus on DEET and these behavioral data presented in this chapter. Looking to follow up on our results, we reasoned that there must exist DEET-sensitive neurons in the tarsi of *Ae. aegypti* male and female mosquitoes that can respond to DEET. We ruled out the primary candidate receptor neurons proposed in

the literature, the bitter gustatory receptor neurons. As we had no other obvious candidates, we turned to available RNAseq data set that profiled each pair of legs in female and male *Ae. aegypti* mosquitoes (Matthews 2016). Using these data, we hoped to find a small subset of genes expressed in both males and females, and in all pairs of tarsi. However, this resulted in thousands of candidates. We further narrowed down the candidates to include only genes with two or more predicted transmembrane domains not predicted to be in the endoplasmic reticulum or mitochondria, and over five hundred candidates remained, far too many for reverse genetic approaches. Given these difficulties, we did not attempt to identify candidate receptors mediating DEET contact chemorepellency, but our data do allow us to conclude that the tarsi are required for contact DEET repellency in *orco* mutant mosquitoes, any pair of tarsi are sufficient to confer contact DEET repellency, and that this repellency is independent of bitter taste. DEET is unique in its ability to affect all of these modes of behavior.

CHAPTER 3

A SCALABLE ASSAY FOR STUDYING CONTACT DEET REPELLENCY IN TERRESTRIAL INVERTEBRATES

The suggestion that DEET acts on a highly conserved family of receptors is an appealing hypothesis to explain its broad effectiveness across a multitude of species. Yet the experimental evidence for "effectiveness" differs wildly across species, making it difficult to compare experiments.

For many DEET-sensitive species, all of the published work focuses on DEET's effectiveness as a personal repellent. The World Health Organization defines the gold standard for evaluating mosquito repellents as requiring both laboratory assays and field studies (WHO 2009). They suggest that laboratory experiments should be used to determine effective doses providing 50% and 99.9% protection, and to estimate the length of time between application of the repellent and the first mosquito landing. 20% DEET should be used as a positive control. A minimum of two field tests are recommended, using human volunteers collecting mosquitoes landing on the skin. In practice, many species we consider 'DEET-sensitive' lack sufficient evidence by this definition. For example, in the tsetse fly — *Glossina morsitans,* the primary vector of the parasite that causes sleeping sickness — the evidence that DEET is an effective repellent rests largely on two studies. First, a laboratory assay using rabbits found DEET to be an effective biting deterrent even when compared to other repellents (Wirtz et al. 1985). Second, a field study where volunteers wore "repellents while riding in a vehicle that was driven slowly (4-6km/h), with the windows and rear door open, through fly-infested areas" (Sholdt et al. 1989). While these types of studies are useful to travelers, they do not tell us about *how* DEET is working: is DEET acting at a distance as a volatile repel-

lent, on the skin as a contact repellent, or in yet another way? Additionally, there are currently no guidelines for evaluating effects against non-blood-feeding animals, nor the contact effects of repellents. WHO guidelines are focused on time until landing in blood-feeding species only.

As demonstrated in **Chapter 2**, it is possible to separate volatile and contact effects experimentally during blood- and sugar- feeding. Together with a rotation student, Vikram Chandra, we set out to determine if we could study contact DEET repellency across species in a comparable and rigorous way.

3.1 Design and testing of the split Petri dish assay

We wanted to develop an assay that allowed us to control for volatile repellency and could be scaled up or down to accommodate different sized animals. We also wanted to test specifically for the avoidance of DEET itself, which is more likely to indicate active sensation, rather than a difference in attraction to food. This is in contrast to the anti-feedant effects seen in *D. melanogaster* flies (Lee et al. 2010), *A. mellifera* bees (Abramson et al. 2010), and presented here in *Ae. aegypti* mosquitoes (Fig. 2.4). Towards this goal, we developed a 3D-printed split Petri dish and removable mesh spacer layer (Fig. 3.1). We used standard Petri dish plate sizes and used the lids of commercially available Petri dishes, though a laser cut acrylic cover would also be effective if intermediate sizes were required.

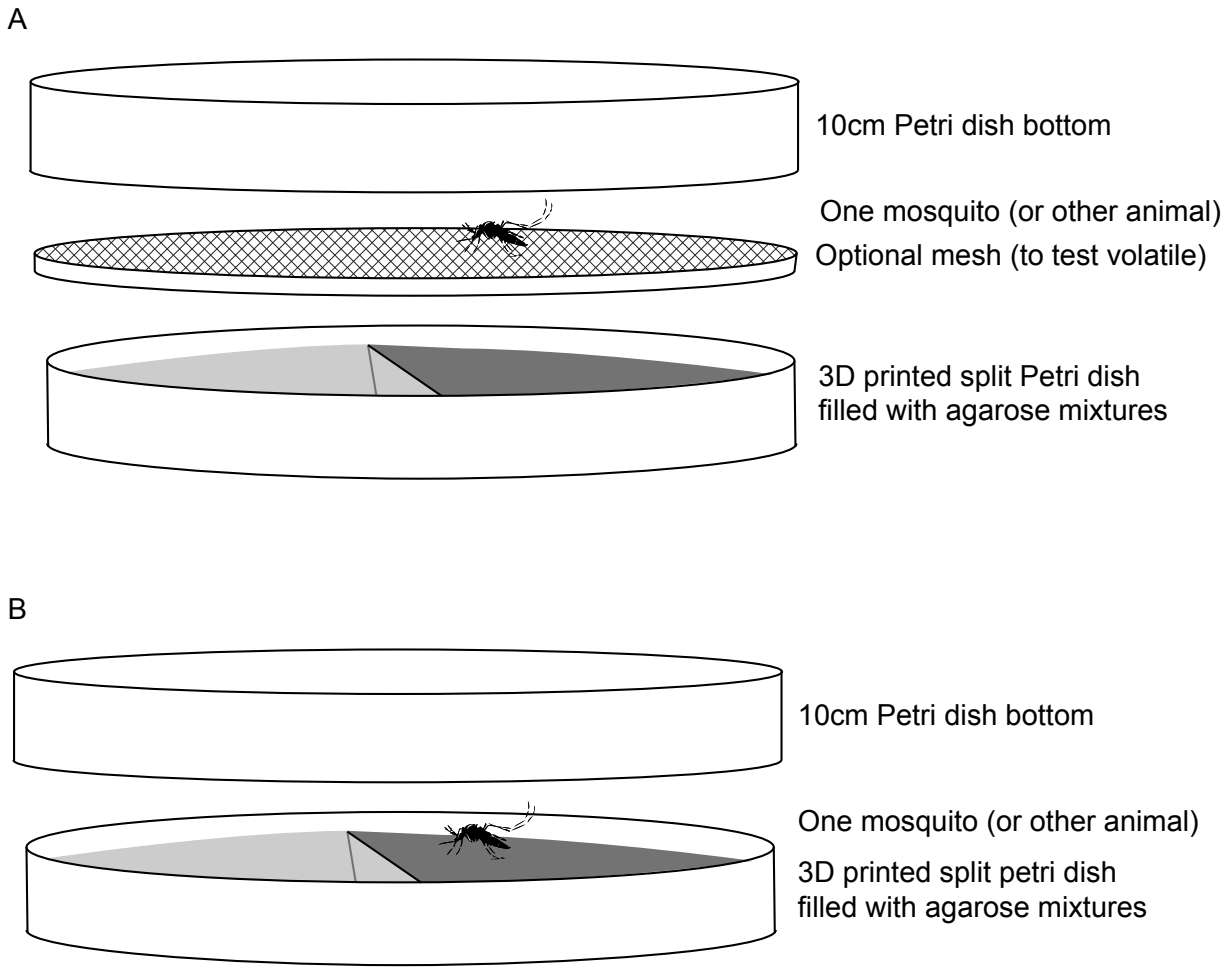


Figure 3.1 A scalable assay for studying contact DEET repellency (A) A schematic of a variation of the component pieces of the assay for testing the effects of volatile DEET. (B) A schematic of the component pieces of the contact DEET repellency assay.

To test this assay, we used wild-type (*w1118*) and *orco*^{2} mutant *D. melanogaster* flies and asked if they spend less time on 1% DEET in 2% agar mixture or ethanol solvent in 2% agar. Both wild-type and *orco* mutant flies were repelled by DEET in this assay (Fig. 3.2). Previous work from the Montell group showed that wild-type and *orco* mutant flies shifted their preference from high sucrose to low sucrose food when DEET was added to the high sucrose food (Lee et al. 2010). Our data demonstrate that *D. melanogaster* will avoid contact with DEET alone, and this repellency is not limited to

rejection of food or suppression of food intake, but extends to contact avoidance in general and is *orco*-independent.

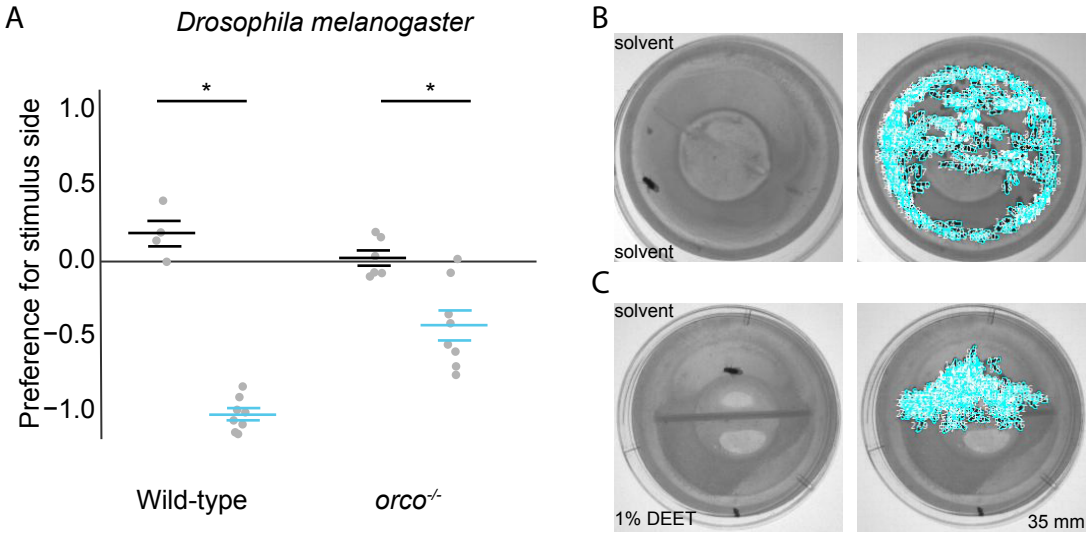


Figure 3.2 *D. melanogaster* are repelled by DEET on contact (A) Each data point represents the side preference of a single animal. (# frames on stimulus side - # frames on control side / total frames). (B-C) A single frame (left), and summary of automatic identification (right) of a video of a single fly either on a control (B) or DEET (C) split Petri dish. Blue ellipses are computer-identified body outlines from each frame. Data collected by Vikram Chandra.

3.2 Testing contact DEET repellency in terrestrial arthropods

After we confirmed that *orco*-independent contact DEET repellency can be tested with this assay, we then asked if DEET repellency can be tested in different terrestrial arthropods with different body sizes. We chose species were available for purchase, non-invasive, and represented different branches of Arthropoda. We chose at least one member of each extant class of Arthropoda to test the effectiveness of DEET across different species and the flexibility of this assay (Fig. 3.3). We were particularly intrigued by the possibility of studying pill bugs and sow bugs as they are crustaceans that have adapted to live on land independently from insects.

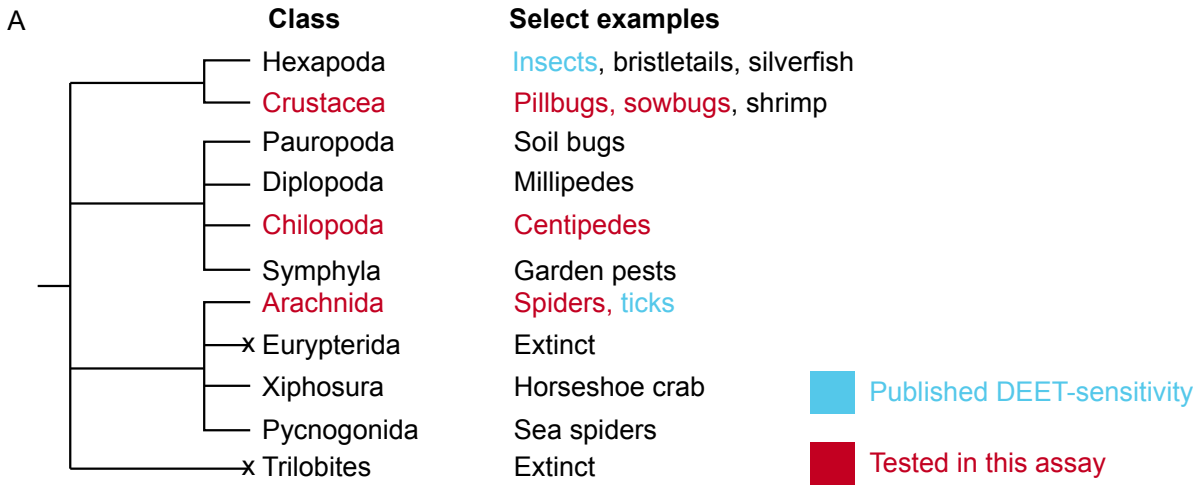


Figure 3.3 Evidence of DEET sensitivity in Arthropoda (A) Evolutionary tree highlighting species either with published DEET-sensitivity (blue) or tested in this chapter (red). (Regier et al. 2010; Letunic et al. 2016).

We scaled each assay such that the circumference of the assay was at least 8-fold longer than the animal. The purpose of this scaling was to allow for exploration of the arena and enough space for turning. Through these experiments we identified DEET-sensitivity in three of the four groups of animals tested for avoidance of 1% DEET in agar (Fig. 3.4). Notably, pill bugs appeared to be insensitive to these concentrations of DEET. It is possible that this species is completely DEET-insensitive. Because these animals are wild-caught and we lack many key details about them, therefore it is also possible that these animals are all one sex or one life stage and that particular attribute contributes to their DEET-sensitivity. Finally, it is also possible that at higher concentrations of DEET, we would see DEET-sensitivity in this species. All other classes of animal tested were sensitive to DEET.

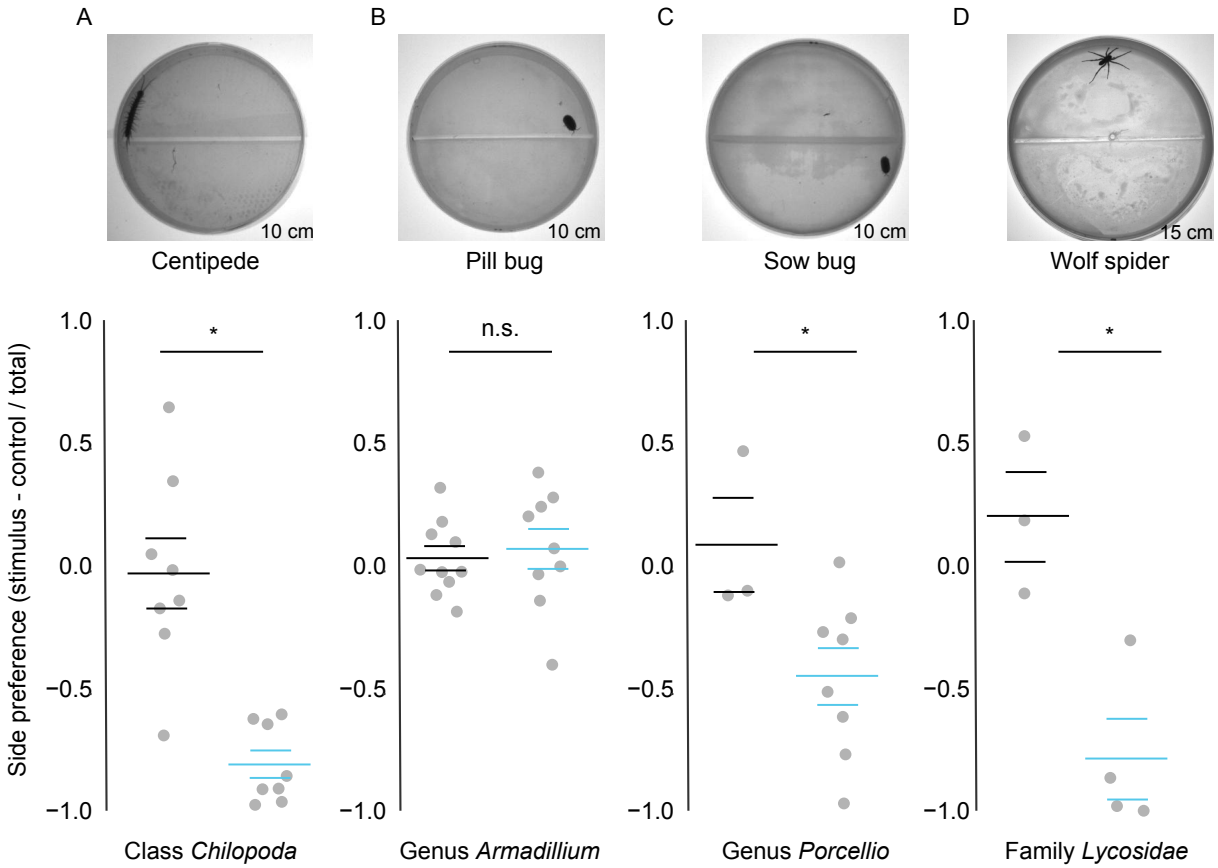


Figure 3.4 Contact DEET repellency in multiple Protostomes Preference indices for animals indicated above, with most accurate scientific name available below. Each data point represents the side preference of a single animal. (# frames on stimulus side - # frames on control side / total frames). (N=3-10 animals tested, *p<0.05, Student's *t*-test).

While these data are preliminary, they demonstrate that the split-Petri dish is a robust assay and opens up several avenues for future work. In *D. melanogaster*, it is now feasible to separate the anti-feedant effects of DEET and the avoidance of DEET-laced sucrose. It is conceivable that avoidance of DEET on contact in this split-Petri dish agarose assay requires the *Gr33a, Gr32a,* and *Gr66a* receptors, or that a distinct set of receptors is required for this DEET sensitivity. It is tempting to consider that the active avoidance of DEET may require a different set of receptors, and may

also explain the inability of bitter taste receptor mutants to return to wild-type levels of preference for high sucrose in the presence of DEET. It should be possible, using this assay or a similar one, to complete a screen for neurons or receptors required for DEET sensitivity in *D. melanogaster.* If this could be completed in a *Gr33a* genetic background, for example, this could be a powerful method for identifying new, additional DEET receptors.

More broadly, these data show that under comparable conditions in a highly similar assay, DEET affects animals across Athropoda. It would be interesting to expand these data to include non-Arthropod Ecdysozoans, like *Onychophora* velvet worms, non-Ecdysozoan Protostomes like *Helix aspersa* snails and to expand the characterization of each species with a dose-response curve. Using this simple, scalable assay, we open up the possibility of studying a specific aspect of DEET sensitivity with a consistent, comparable method across all terrestrial animals.

CHAPTER 4

THE EFFECTS OF DEET IN THE MODEL ORGANISM *CAENORHABDITIS ELEGANS*

DEET is an effective personal repellent for a diverse set of animals, and in **Chapter 3** we demonstrated the broad effectiveness of DEET across Arthropoda in a contact-based assay. This repellency could require a single, well-conserved DEET-sensitive receptor or receptor family that is necessary for all of these species to sense DEET, or DEET could interact with many different receptors with very few similarities. It is also possible that the answer lies somewhere in between: that many receptors can be affected by DEET, but they all have some homologous region or shared function. To tackle this question comprehensively, one would want to identify all of the DEET-sensitive receptors for each DEET-sensitive behavior in every species affected by DEET. It is possible that this analysis would reveal a single conserved receptor or receptor family, or perhaps DEET does not have a single mechanism of action, and instead the similarities across organisms require similar cell types, membranes, or neuronal connectivity.

While there is certainly more work to be done to gain a complete understanding of DEET-sensitivity in *D. melanogaster* and *Ae. aegypti*, we currently do not know of a single candidate chemoreceptor gene required for DEET-sensitivity outside of Arthropoda, making any inferences across taxa impossible. To learn more about how DEET may work outside of insects and Arthropods, one would want to study a non-Arthropod species with tools for forward genetics, reverse genetics, neuronal manipulation, and neuronal observation. In the following chapters we show that the nematode *C. elegans* fulfills all of these requirements, and we use this species to discover a receptor, a pair of neurons, and a partial mechanism for DEET's activity in a non-insect invertebrate.

The nematode *C. elegans* belongs to the phyla Ecdysozoa, which include the sister group to Arthropoda (Dunn et al. 2008). Their compact, well-studied genomes and ability to self-fertilize make forward genetic screens possible (Brenner 1974) and modern tools make reverse genetics feasible (Arribere et al. 2014). An adult hermaphrodite has 302 neurons deriving from a fixed, mapped cellular lineage and a "connectome" has been available for decades, identifying nearly all of the connections between individual neurons (Sulston 1988; White et al. 1986). Additionally, the ability to inject transgenes that can be transmitted to offspring allows for the identification of gene expression patterns, expression of calcium indicators for imaging, and access to subsets of cells for manipulation (Mello et al. 1991). Together, these characteristics make *C. elegans* the ideal species for this work, but only if these worms are indeed DEET-sensitive.

In 2013, we received an email from Dr. Phil Hartman that set us down the path that would ultimately become the primary focus of this thesis: Dr. Hartman and his students identified DEET as a potent modifier of *C. elegans* chemotaxis behavior and learned that a forward genetic screen for DEET-resistance could be effective, but he could not follow up on these preliminary results. Thanks to his email, we knew that there was something interesting to study here, and set out to find it.

4.1 Testing old hypotheses in a new species

To begin, we used standard chemotaxis assays (Bargmann et al. 1991; Troemel et al. 1997; Cho et al. 2016) (Fig. 4.1A) to explore how *C. elegans* nematodes respond to DEET. There are currently three competing hypotheses about the mechanism of DEET based on work in insects: "Smell-and-repel" —DEET is detected by olfactory pathways that trigger avoidance (Abramson et al. 2010; Syed et al. 2008; Xu et al. 2014; Syed et al. 2011), "masking" —DEET selectively blocks olfactory pathways that mediate attraction (Dogan et al. 1999; Ditzen et al. 2008; Syed et al. 2008), and "confusant" —

DEET modulates multiple olfactory sensory neurons to scramble the perception of an otherwise attractive stimulus (Pellegrino et al. 2011; DeGennaro et al. 2013). Inspired by these hypotheses, we tested how DEET may interfere with olfactory behaviors in nematodes to identify similarities and differences with work in insects.

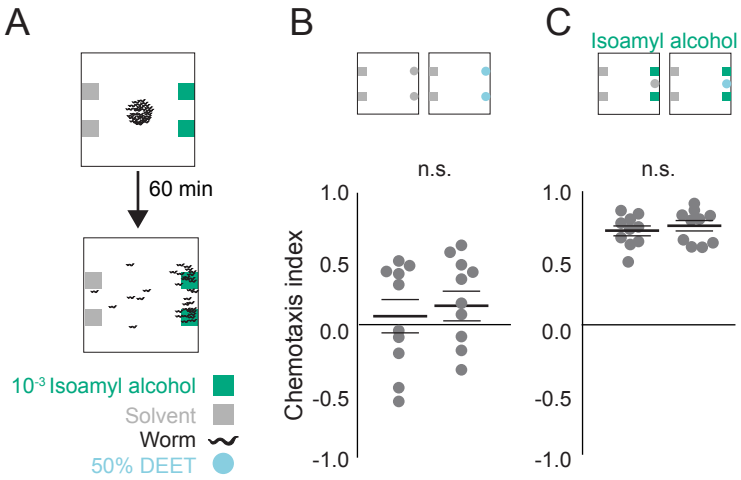


Figure 4.1 DEET is not a volatile repellent and does not mask attractive odor (A) Schematic of the chemotaxis assay. Chemotaxis of wild-type animals with point source stimuli of DEET alone (B) or DEET with isoamyl alcohol (C). We observed no chemotaxis to nor away from DEET alone, and no significant effect on isoamyl chemotaxis. Each dot represents a chemotaxis index of a single population assay [NEAR ODORANT - NEAR CONTROL / DISPERSED]. Horizontal lines indicate mean ± s.e.m. n.s. indicates no statistical significance. (N=10 experiments, n= 50-250 animals per experiment, *p>0.05, Student's *t*-test)

To test the smell-and-repel hypothesis, we presented DEET as a volatile point source. DEET was not repellent alone even at high concentrations of 50% (Fig. 4.1B), similar to previous results in *D. melanogaster* flies (Ditzen et al. 2008) and *Ae. aegypti* mosquitoes (DeGennaro et al. 2013). To address the possibility that DEET could be masking responses to attractive odorants (Dogan et al. 1999; Ditzen et al. 2008) or directly inhibiting their volatility (Syed et al. 2008), we presented DEET alongside the attractant isoamyl alcohol, both as point sources, and found that it had no effect on at-

traction (Fig. 4.1C).

In considering alternate ways to present DEET, and following personal communication from Dr. Philip Hartman, we mixed low doses of DEET uniformly into the chemotaxis agar and presented isoamyl alcohol as a point source (Fig. 4.2A). In this configuration, DEET-agar reduced chemotaxis to isoamyl alcohol in a dose-dependent manner (Fig. 4.2B).

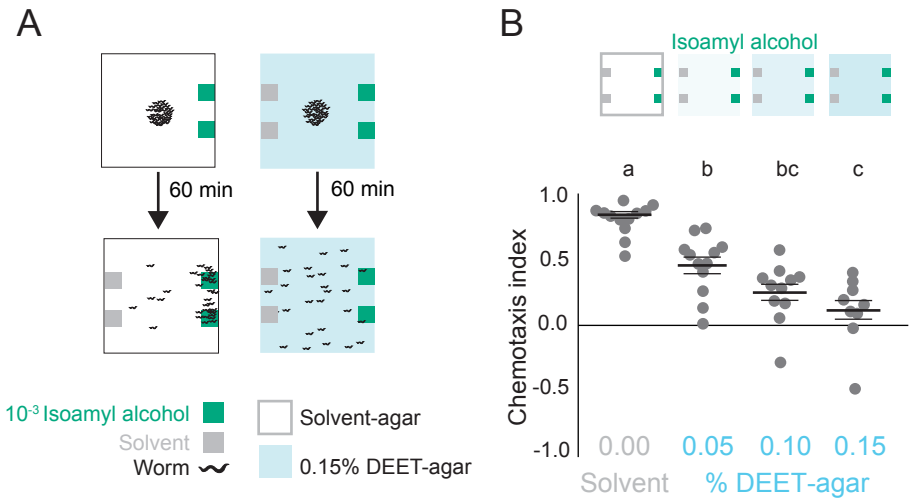


Figure 4.2 DEET interferes with *C. elegans* chemotaxis to isoamyl alcohol (A) Schematic of chemotaxis assay on solvent- and DEET-agar plates. (B) Wild-type chemotaxis to isoamyl alcohol on DEET-agar plates of the indicated concentrations. Each dot represents a chemotaxis index of a single population assay. Horizontal lines indicate mean ± s.e.m. Data labelled with different letters indicate significant differences (N=10-13 assays, n=50-250 animals per assay, *p<0.05, One-way ANOVA and Tukey's Post-hoc test).

4.2 DEET interferes with chemotaxis to some odors and not others

To ask if DEET has a general effect on chemotaxis, we tested two additional attractants, butanone and pyrazine, as well as the volatile repellent 2-nonanone. Behavioral responses to butanone requires overlapping primary sensory neurons as isoamyl

alcohol (AWC), while pyrazine and 2-nonanone require two different pairs of primary sensory neurons (AWA and AWB, respectively) (Troemel et al. 1997; Bargmann et al. 1993). DEET eliminated both attraction to butanone and avoidance of 2-nonanone, indicating that it can affect responses to both positive and negative chemosensory stimuli (Fig. 4.3A). In contrast, DEET-agar had no effect on chemotaxis toward the attractant pyrazine, an AWA odorant, but had an effect on diacetyl, another AWA odorant. The observation that pyrazine chemotaxis remains intact on a DEET-agar plate demonstrates that DEET is not having a general non-specific effect on their health or ability to move, but is instead selectively interfering with chemotaxis to some odors and not others.

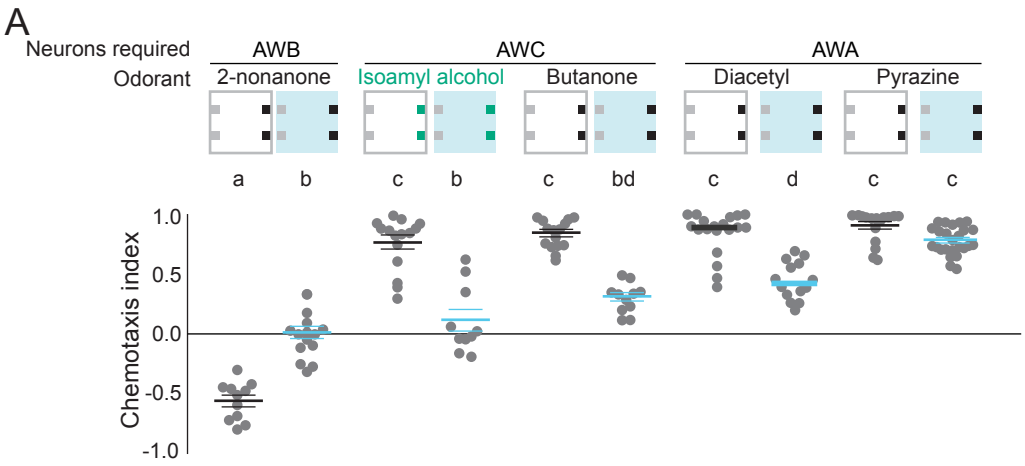


Figure 4.3 DEET interferes with *C. elegans* chemotaxis to several, but not all, odorants (A) Chemotaxis of wild-type animals on solvent-agar (grey) or DEET-agar (blue) in response to the indicated odorants. Each dot represents a chemotaxis index of a single population assay. Horizontal lines indicate mean ± s.e.m. Data labelled with different letters indicate significant differences (N=11-24 assays, n=50-250 animals per assay, *p<0.05, Two-way ANOVA and Tukey's Post-hoc test).

What makes pyrazine different from these other odors? One hypothesis is that AWA chemotaxis is less affected by DEET, either at the primary sensory level or down-

stream. However, diacetyl is affected by DEET, and diacetyl also requires AWA neurons for chemotaxis (Bargmann et al. 1993).

AWA sensory neurons respond to diacetyl as assayed by calcium imaging and are required for attraction to diacetyl (Bargmann et al. 1993; Larsch et al. 2015). Additional data suggest that AWB, ASK, and AWC neurons respond to the removal of 1:10,000 diacetyl as seen in calcium imaging experiments, and ablations of ASK and AWB decrease chemotaxis to diacetyl at 1:1000 and 1:10,000 concentrations respectively (Hale et al. 2017). Because attraction to diacetyl is already affected in these ablated animals, we did not further investigate their potential contribution to DEET-sensitivity. However, AWC neurons are not required for chemotaxis to these concentrations of diacetyl, but AWC neurons do respond to the removal of diacetyl as monitored by calcium imaging (Hale et al. 2017). To rule out a potential effect of AWC on diacetyl chemotaxis, we used *odr-1* mutants, which are defective in in chemotaxis to all AWC-sensed odorants but exhibit normal chemotaxis to attractive odors sensed by the AWA olfactory neurons (Bargmann et al. 1993) and found *odr-1* mutant animals are still affected by DEET (Fig. 4.4).

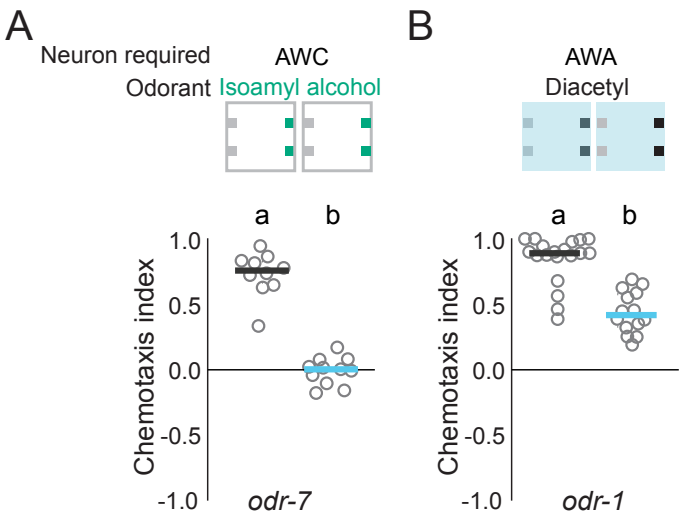


Figure 4.4 The effects of DEET on diacetyl chemotaxis are AWC-independent. (A) Chemotaxis of *odr-1* mutant animals on DEET-agar in response to diacetyl. Different letters indicate significant differences. (N=19 and 16 assays, n=50-250 animals per assay. $p<0.05$, Student's *t*-test).

It is possible that pyrazine is able to overcome the effects of DEET simply because it is a much more potent or attractive odorant at the concentrations tested. To address this, we decreased the concentration of pyrazine 10- and 100-fold, and increased the concentration of isoamyl alcohol 10- and 100-fold. At all concentrations, the same patterns held: attraction to isoamyl alcohol was affected by DEET at all concentrations and attraction to pyrazine on DEET- and solvent-agar was statistically indistinguishable at all three concentrations (Fig. 4.5B).

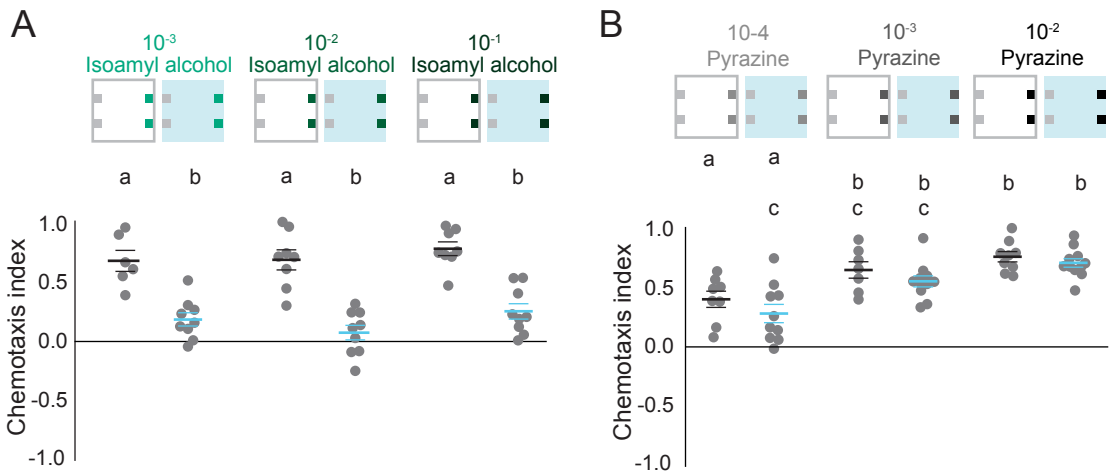


Figure 4.5 The differential effects of DEET on pyrazine and isoamyl alcohol chemotaxis are independent of odorant concentrations used over three orders of magnitude. (A) Chemotaxis of wild-type animals chemotaxing to increasing concentrations of isoamyl alcohol. (B) Chemotaxis of wild-type animals chemotaxing to decreasing concentrations of isoamyl alcohol. Each dot represents a chemotaxis index of a single population assay. Horizontal lines indicate mean ± s.e.m. Data labelled with different letters indicate significant differences. (n=50-250 animals, N=6-10 assays per condition, $p<0.05$, Two-way ANOVA and Tukey's Post-hoc test).

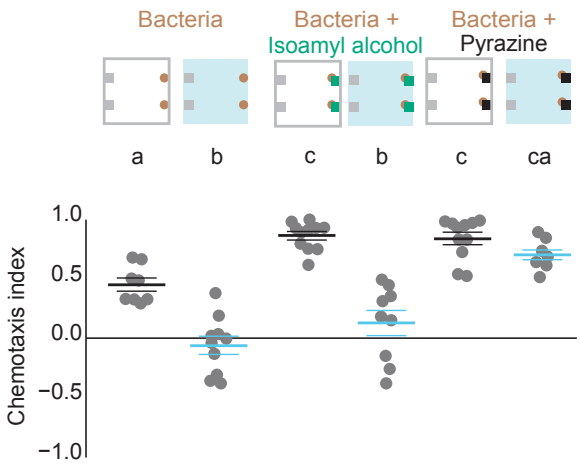


Figure 4.6 DEET acts as a behavioral confusant, and pyrazine is able to overcome the effects of DEET. Chemotaxis of wild-type animals chemotaxing to 20μL of bacteria, with either solvent, isoamyl alcohol, or pyrazine. Each dot represents a chemotaxis index of a single population assay (50-250 animals, N=7-11 assays per condition). Horizontal lines indicate mean ± s.e.m. Data labelled with different letters indicate significant differences ($p<0.05$, Two-way ANOVA and Tukey's Post-hoc test).

These results are reminiscent of the "confusant" hypothesis in insects, although the molecular and neuronal details by which DEET acts differ markedly between nematodes and insects. In insects, DEET alters responses of individual sensory neurons to attractive odorants (Pellegrino et al. 2011; Ditzen et al. 2008), thereby interfering with behavioral attraction. Our data in *C. elegans* are consistent with a mechanism where DEET can inhibit responses to some stimuli but not others by decreasing avoidance of 2-nonanone, decreasing attractiveness to multiple odorants, and leaving pyrazine behavioral responses intact. In both *D. melanogaster* flies and *Ae. aegypti* mosquitoes, the major behavioral effect of volatile DEET is inhibiting attraction to food odorants presented as mixtures, like food odor or human odor. To determine if DEET could similarly disrupt chemotaxis to a relevant mixed-odor stimulus, we used bacterial food as a chemo-

taxis stimulus for *C. elegans* (Fig. 4.6). These data demonstrate that DEET can interfere with chemotaxis to bacterial odor (Fig. 4.6A) and that pyrazine, but not isoamyl alcohol, is able to overcome this inhibition (Fig 4.6.B-C). We conclude that DEET chemosensory interference is odor-selective, can affect both attractive and repulsive stimuli, and is not a result of non-specific or toxic effects of DEET.

These data demonstrate that *C. elegans* is DEET-sensitive, and provide a robust assay to further explore the genes that underlie this behavior.

CHAPTER 5

GENETIC MECHANISMS OF DEET RESISTANCE AND SENSITIVITY IN *C. ELEGANS*

Many have been interested in identifying genes required for DEET-sensation. A forward genetic approach in *Drosophila melanogaster* flies yielded an X-linked DEET-insensitive mutant (Reeder et al. 2001) and a population genetics approach in mosquitoes identified a dominant genetic basis for DEET-insensitivity (Stanczyk et al. 2010), but neither study identified the genes underlying these behavioral phenotypes. Reverse genetic experiments in *D. melanogaster* flies and three mosquito species have identified the insect odorant receptors as a molecular target of DEET (Ditzen et al. 2008; Xu et al. 2014; Liu et al. 2010; Pellegrino et al. 2011; DeGennaro et al. 2013). However, this chemosensory gene family is not found outside of insects (Missbach et al. 2014; Robertson et al. 2003), raising the question of what pathways are required for DEET-sensitivity in non-insect invertebrates.

5.1 A forward genetic screen for DEET-resistant *C. elegans*

To gain insights into the mechanisms of DEET repellency in *C. elegans*, we carried out a forward genetic screen for mutants capable of chemotaxing toward isoamyl alcohol on DEET-agar plates (Fig. 5.1A). Following ethyl methanesulfonate (EMS) mutagenesis, we obtained five DEET-resistant animals, three of which produced offspring that consistently chemotaxed toward isoamyl alcohol on DEET-agar plates (Fig. 5.1B) even after four generations of outcrossing to the wild-type strain (Fig. 5.1C).

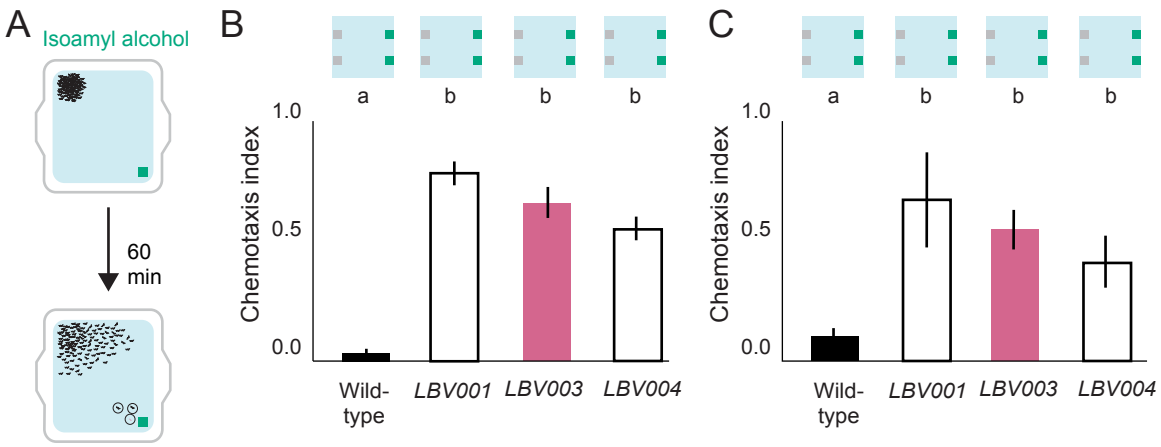


Figure 5.1 A genetic screen for DEET-resistance. (A) Schematic of forward genetic screen with hypothetical DEET-resistant mutants circled. (B-C) Chemotaxis of wild-type (black), *LBV003* mutant (pink), and two additional isolated strains (open bars) before (B) and after 4 generations of outcrossing (C). For all plots, the height of the bar indicates the mean and vertical bars the s.e.m. Data labelled with different letters indicate significant differences (N=3-8 population assays of 30-180 animals per assay, $p<0.05$, One-way ANOVA and Tukey's Post-hoc test).

We next used whole-genome sequencing to identify candidate mutations in these strains (Sarin et al. 2010) using the methods outlined in Figure 5.2. We failed to identify mutations in *LBV001* that were homozygous in all chemotaxing offspring. It is possible that there is a single, large-effect mutation that was missed in the original sequencing and analysis, that there are many mutations each with small effects that cannot be separated further using these methods, or that the mutation(s) are in regulatory regions and were excluded from our analyses.

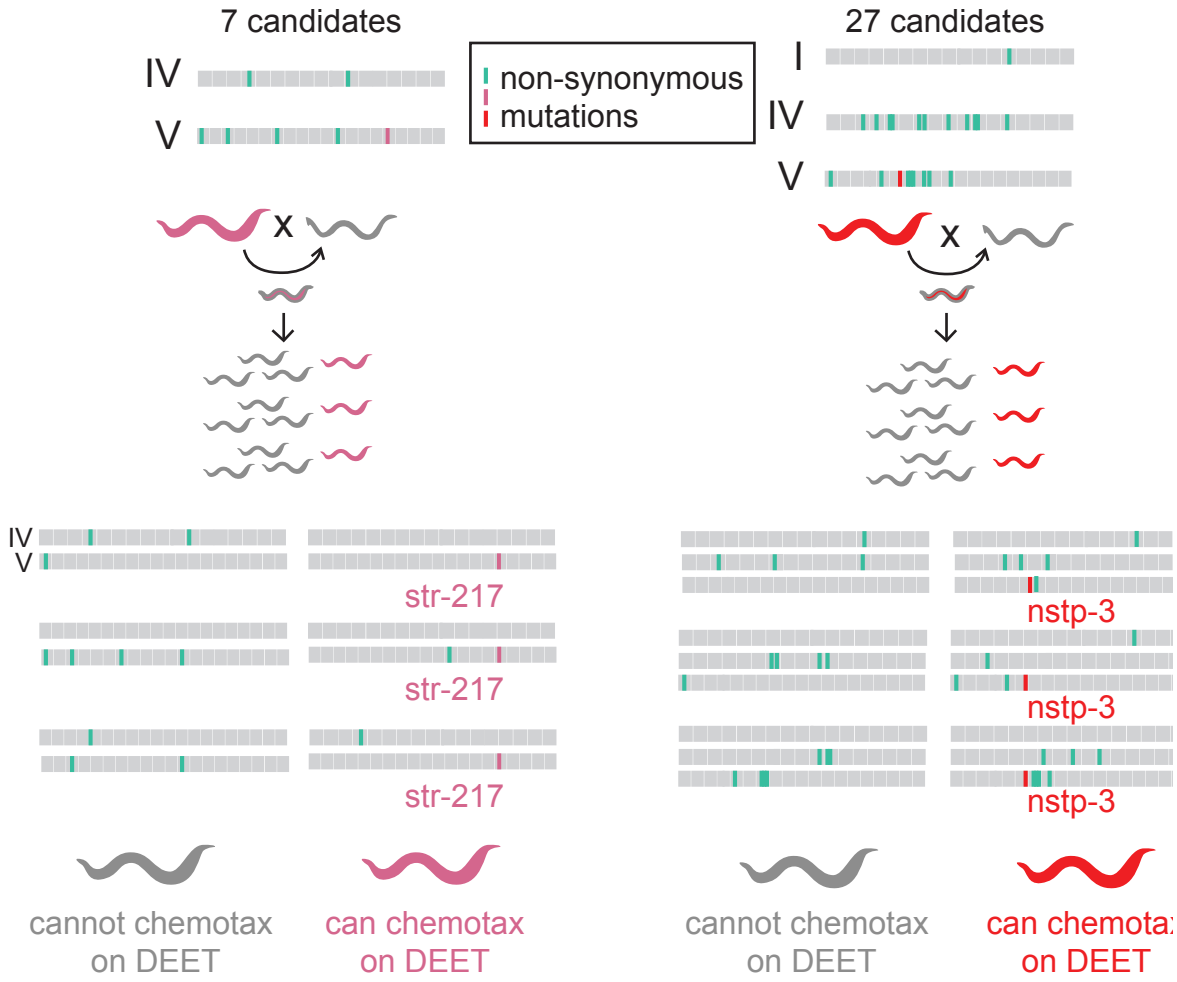


Figure 5.2 Schematic of the mapping strategy used to identify hits from the screen. (A) Whole genome sequencing after backcrossing identified 7 and 27 candidate non-synonymous mutations. (B) Diagram of the crosses producing animals whose offspring were tested and pooled to produce the example subset of data in (C).

We were able to narrow down our putative candidates to a single gene containing a non-synonymous mutation each in *LBV003* and *LBV004*. *LBV003* mapped to *str-217*, a G-coupled protein receptor (GPCR), and *LBV004* mapped to *nstp-3*, a predicted sugar:proton symporter with homology to the SLC transporter family ('Wormbase web site'). *str-217* was of immediate interest as it is a member of the *str* family of predicted

chemosensory GPCRs. The missense mutation isolated from the EMS mutagenesis screen is predicted to replace a proline in the C-terminal cytoplasmic tail segment of the protein with a serine. This was of particular interest because the mutation is predicted to change a Cys-Pro-Ser-Cys motif to Cys-Ser-Ser-Cys. The presence of these cysteines within 13-16 residues from the last transmembrane domain often indicates a site of thio-acylation of GPCRs (Escribá et al. 2007). In rhodopsins, thio-acylation of a similar sequence leads to incorporation of the palmitate into the plasma membrane, creating an additional intracellular loop. Although we do not have any data indicating the wild-type protein topology for *str-217*, we speculate that these cysteine residues are a candidate site for lipid interactions. Additionally, the Pro>Ser change was an exciting result as proline residues are known to be 'helix breakers' (Li et al. 1996) therefore it is possible that, without the wild-type proline residue, the mutated tail gains a helical conformation that could alter any binding properties or lipid interactions otherwise found in the wild-type protein.

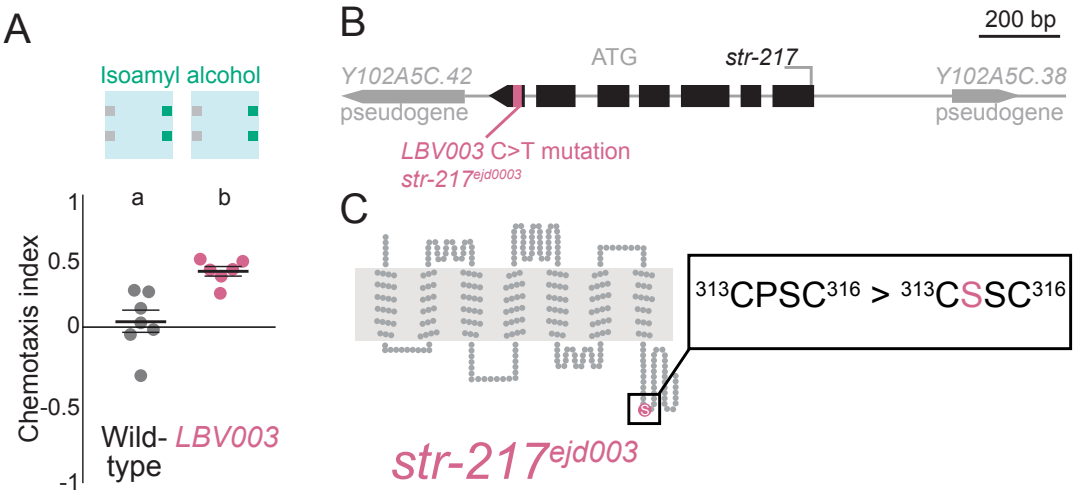


Figure 5.3 The candidate gene *str-217*. (A) Chemotaxis behavior of *LBV003* compared to wild-type animals. (B) The *str-217* locus and site of the EMS-induced mutation. (C) The predicted protein highlighting the non-synonymous mutation.

5.2 *str-217* is required for complete DEET sensitivity in a wild-isolate of *C. elegans*

In deciding how to follow up on these two genes, we discovered that a divergent strain of *C. elegans* isolated in Hawaii, CB4856 (Hawaiian), is naturally resistant to DEET (Fig. 5.4B-C). This Hawaiian strain contains a 138-base pair deletion in *str-217* (*str-217*^{HW}) that affects exons 2 and 3 and an intervening intron, leading to a mutant strain with a predicted frame shift insertion-deletion (indel) and early stop codon (Fig. 5.4A and 5.6C). Although we did not test them for their DEET-sensitivity, many other natural isolates contain deletions or predicted missense and early stop mutations in *str-217* (Fig. 5.5).

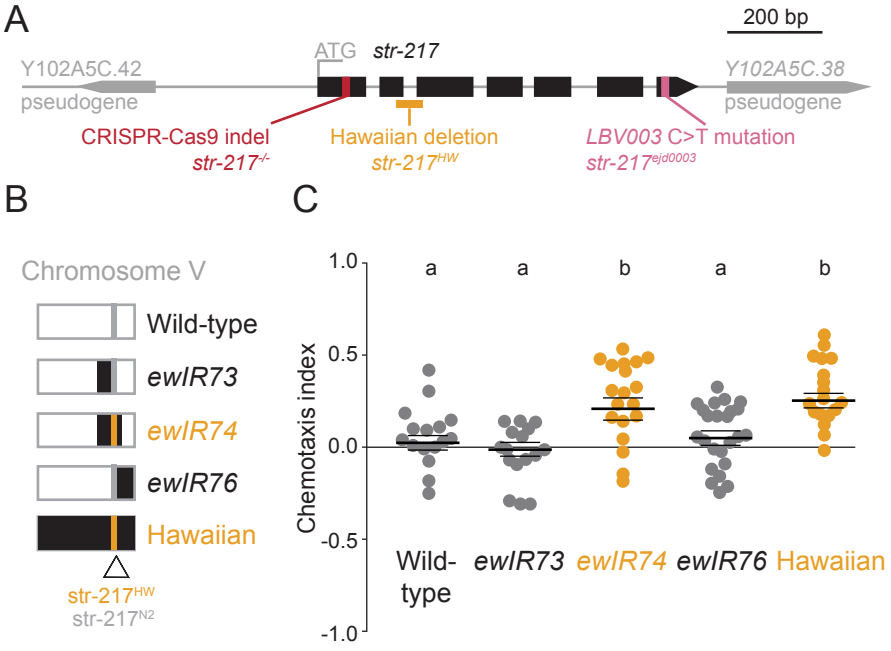


Figure 5.4 A wild isolate strain is DEET-resistant. (A) *str-217* genomic locus indicating the deletion in the Hawaiian strain in orange. (B) Schematic of chromosome V in each strain: wild-type (white), Hawaiian (black), *str-217*$^{+/+}$ (grey), *str-217*^{HW} (orange). (C) Chemotaxis of the indicated strains (N=16-24). Each dot represents a chemotaxis index of a single population assay (50-250 animals). Horizontal lines indicate mean ± s.e.m. Data labelled with different letters indicate significant differences ($p<0.05$ ANOVA and Tukey's Post-hoc test).

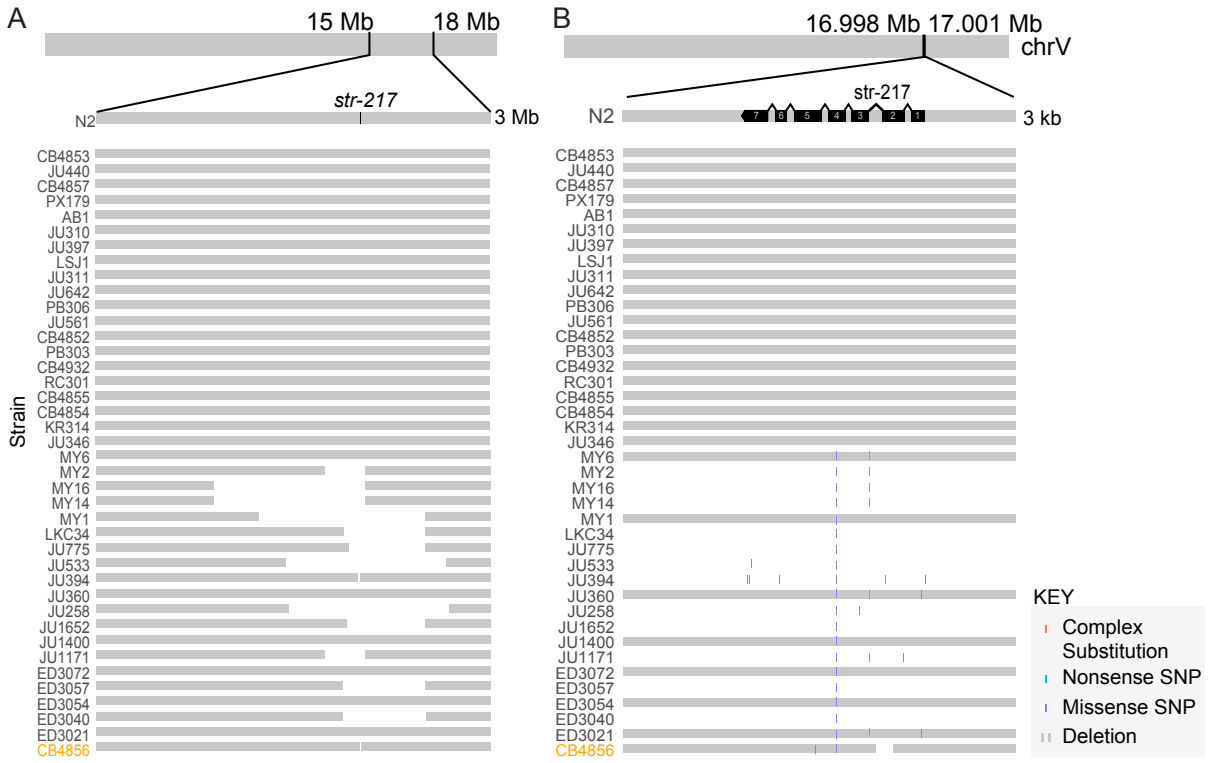


Figure 5.5 *str-217* varies in multiple wild-isolates of *C. elegans*. (A) Schematic depicting the large deletions overlapping the *str-217* locus across several natural isolates of *C. elegans.* (B) Schematic at a closer scale, depicting the predicted protein-disrupting changes identified in the wild-isolates. If there are large deletions not yet fixed in the population, any other mutations in the sequences are depicted on a white, instead of gray, background. Hawaiian strain (CB4856) is highlighted in orange. Data from CeNDR (Cook et al. 2017).

To confirm that the Hawaiian DEET resistance maps to *str-217*^{HW}, we tested three near-isogenic lines with a single, homozygous genomic segment of Hawaiian chromosome V introgressed into a wild-type (Bristol N2) background (Fig. 5.4B) (Doroszuk et al. 2009). Only the ewIR74 line contains *str-217*^{HW} and, like the parent Hawaiian strain, is DEET-resistant (Fig. 5.4C). To provide further confirmation that *str-217* is required for DEET sensitivity in these strains, we generated two additional genetic tools:

an engineered predicted null mutant produced by CRISPR-Cas9 genome-editing (*str-217*$^{-/-}$) (Fig. 5.6D), and a rescue/reporter plasmid that expresses both wild-type *str-217* and green fluorescent protein (GFP) under control of the predicted *str-217* promoter (Fig. 5.4A). The *LBV003* strain (Fig. 5.6B), Hawaiian introgressed strain *ewIR74* (Fig. 5.6C), and the str-217$^{-/-}$ engineered mutant strain (Fig. 5.6D) all showed chemotaxis on DEET-agar. Expression of a rescue/reporter construct in these three strains rendered all three DEET-resistant mutants fully sensitive to DEET, in that none chemotaxed to iso-amyl alcohol on DEET-agar (Fig. 5.6B-D).

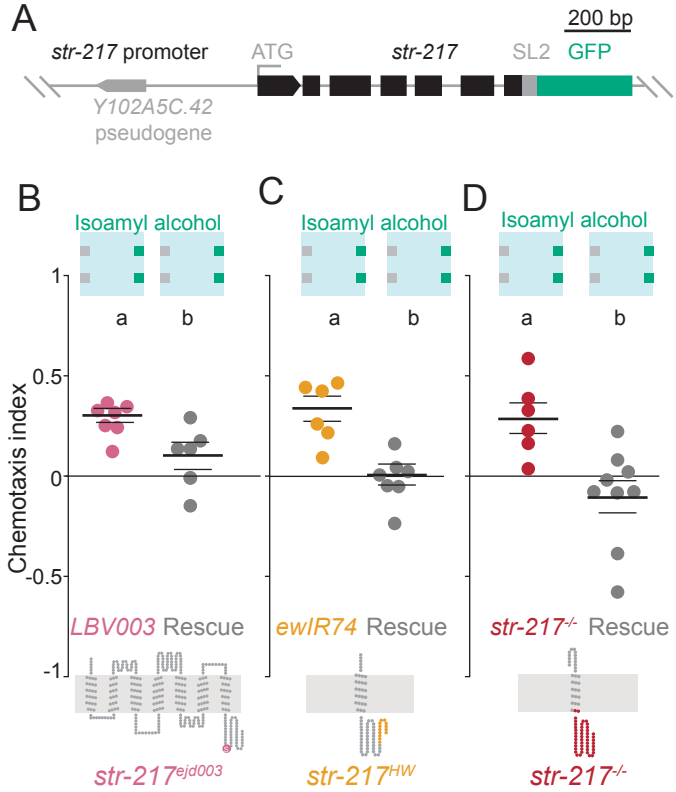


Figure 5.6 *str-217* is required for complete DEET-sensitivity (A) Schematic of *str-217* rescue construct. (B-D) Chemotaxis indices of the indicated strains. Predicted STR-217 protein topology of each mutant is indicated below each plot. Each dot represents a chemotaxis index of a single population assay. Horizontal lines indicate mean ± s.e.m. Data labelled with different letters indicate significant differences (N=6-9 assays, n=50-250 animals in each assay, $p<0.05$ two-sided Student's *t*-test).

These data identify two candidate genes required for complete DEET-sensitivity. One of these genes, *str-217*, is also required for DEET-sensitivity in an engineered *str-217* mutant and a wild isolate of *C. elegans.* This gene is part of a *C. elegans*-specific expansion of G-coupled protein receptors (GPCRs) and is not orthologous to any of the receptors required for DEET-sensitivity in insects (Robertson et al. 2006). We next wanted to determine where this gene is expressed, and to use it to learn more about how DEET is affecting chemotaxis behavior.

CHAPTER 6

CELLULAR MECHANISMS OF DEET RESISTANCE AND SENSITIVITY IN *C. ELEGANS*

We next turned to the neuronal mechanism by which DEET disrupts chemotaxis in *C. elegans*. In insects, DEET interacts directly with chemosensory neurons and this effect requires the odorant receptors that they express (Ditzen et al. 2008; Liu et al. 2010; Pellegrino et al. 2011; DeGennaro et al. 2013; Xu et al. 2014). To identify similarities and differences between insects and *C. elegans*, we wanted to evaluate DEET's effects on both the primary sensory neurons required for chemotaxis and also further investigate how *str-217* can disrupt the effects of DEET.

6.1 DEET affects AWC independent of *str-217*

In *C. elegans*, the primary sensory neuron for isoamyl alcohol is AWC (Bargmann et al. 1993). To ask if DEET modulates primary sensory detection of isoamyl alcohol, we used *in vivo* calcium imaging to monitor AWC activity in the presence and absence of DEET.

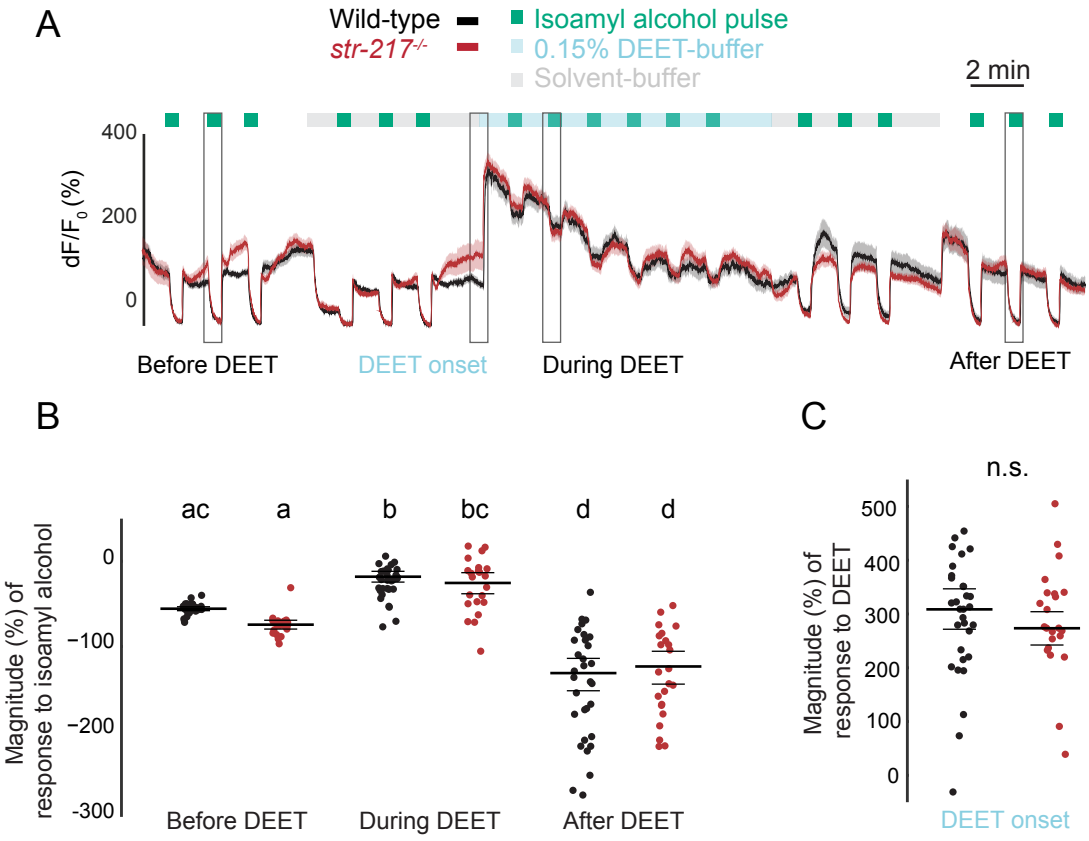


Figure 6.1 *str-217*-independent responses of chemosensory neuron AWC^{ON} to DEET. (A) Top: stimulus protocol. 30 second pulses of isoamyl alcohol (dark grey) were delivered in buffer, buffer with solvent (light grey), or buffer with 0.15% DEET (blue). Bottom: Average traces of GCaMP activity in AWC^{ON} in wild-type (black) and *str-217*$^{-/-}$ (red) animals over a 36 minute experiment, used for analysis in B and C. (B) Response magnitudes of the isoamyl alcohol response before, during, and after DEET. c, Response magnitude of the increase in calcium in AWC at DEET onset (N=23 *str-217*, N=31 wild-type animals in 3-4 experiments over 2-3 days). In b and c, each dot represents responses of single animals and the horizontal lines represent the mean and s.e.m. Data labelled with different letters indicate significant differences ($p<0.05$, two-way ANOVA and Tukey's Post-hoc test in B, and two-tailed Student's t-test in C). Data collected by May Dobosiewicz.

AWC responded to the addition of DEET with a rapid increase in calcium that decreased to baseline over the course of 11 minutes of chronic DEET stimulation (Fig. 6.1 A and C). In the presence of DEET, AWC responses to isoamyl alcohol decreased in magnitude, but there was no observed difference in AWC activity between wild-type and *str-217*$^{-/-}$ mutants in the presence or absence of DEET (Fig. 6.1A-B). This suggests that AWC sensory neurons are not the primary functional target of DEET.

6.2 *str-217* is required for ADL chemosensory neurons to respond to DEET

To identify the functionally relevant neurons, we determined where *str-217* is expressed by examining the *str-217* rescue/reporter strains, and found GFP expression in a single pair of chemosensory neurons, called ADL (Fig. 6.2A). As part of a large-scale effort characterize the expression patterns of the *C. elegans* chemoreceptors, the Hobert lab also found that *str-217* is expressed in ADL, and occasionally in an additional, non-chemosensory interneuron PVT (Vidal et al. 2018). ADL is not required for chemotaxis to isoamyl alcohol, suggesting an indirect role for ADL in DEET chemosensory interference (Zaslaver et al. 2015). To ask if ADL neuronal function is required for DEET-sensitivity, we used a strain expressing tetanus toxin light chain, which inhibits chemical synaptic transmission by cleaving the synaptic vesicle protein synaptobrevin, in ADL (Jang et al. 2012; Schiavo 1992). These animals showed the same level of DEET-resistance as *str-217* mutants (Fig. 6.2B). We note that neither *str-217* mutants nor ADL-deficient animals return fully to wild-type levels of chemotaxis (Fig. 6.2B), suggesting that additional genes and neurons contribute to DEET sensitivity in *C. elegans*.

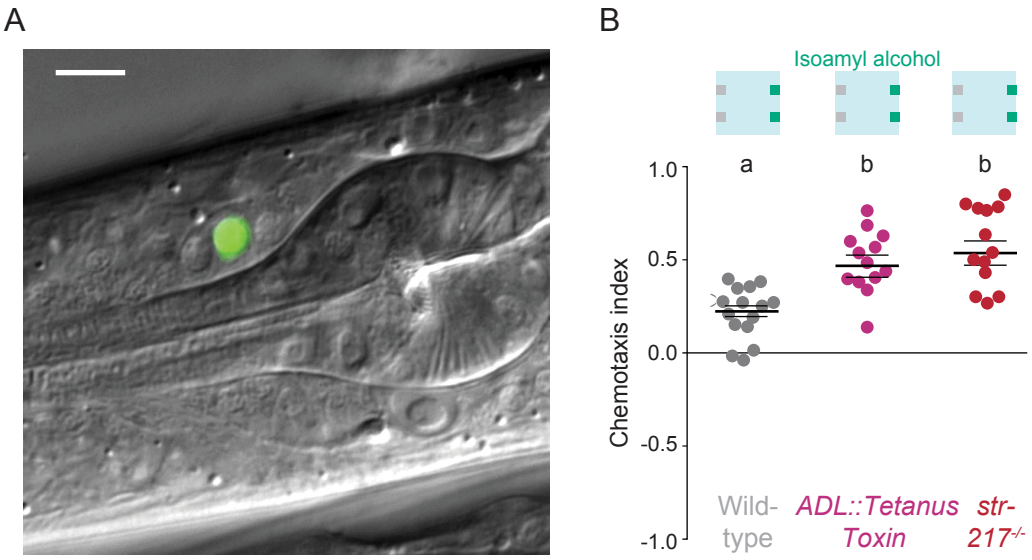


Figure 6.2 *str-217* is expressed in ADL and ADL is required for DEET-sensitivity (A) DEET-responsive chemosensory neuron required for DEET-sensitivity. a, GFP expression in a single ADL neuron from *str-217* rescue/reporter construct (scale bar: 10 μm). (B) Chemotaxis of the indicated strains. Image stack taken by Xin Jin.

Since both *str-217* and ADL function are required for DEET-sensitivity, we used calcium imaging to ask if ADL responds to DEET, and if this requires *str-217* (Fig. 6.3). Both wild-type and *str-217*$^{-/-}$ mutants carrying the rescue/reporter plasmid, but not *str-217*$^{-/-}$ mutants, showed calcium responses to DEET (Fig.6.3 B-C and F). In control experiments, we showed that the known ADL agonist, the pheromone C9 (Schiavo 1992), activated ADL in both wild-type and *str-217*$^{-/-}$ mutant animals (Fig. 6.3D-F) This suggests that the *str-217*$^{-/-}$ mutation has a selective effect on ADL response to DEET.

To exclude the possibility that DEET activates ADL indirectly by activating other sensory neurons that subsequently activate ADL, we carried out the same imaging experiments in genetic backgrounds that disrupt chemical synaptic transmission between neurons. We were able to see responses to DEET in ADL neurons in both *unc-13* and

unc-31 animals, which lack synaptic vesicle fusion (Richmond et al. 1999) and dense-core vesicle fusion (Jorgensen et al. 2002) respectively (Fig. 6.4). From these data, we conclude that DEET directly activates ADL. Further, we conclude that disrupting either ADL activity or *str-217* is sufficient to confer DEET-resistance in *C. elegans*, *str-217* is required for ADL neurons to respond to DEET, and DEET acts directly on ADL.

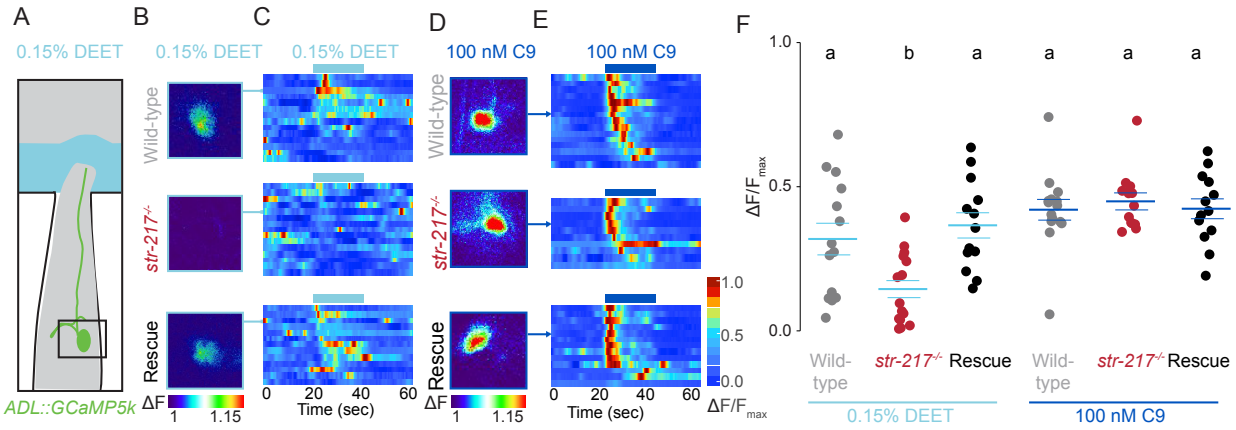


Figure 6.3 *str-217* is required for ADL to respond to DEET, but not the pheromone C9 (A) Schematic of microfluidic calcium imaging assay. (B) Pseudocolored images of ADL response to 0.15% DEET in animals of the indicated genotype (increase in mean fluorescence 20 seconds after the first DEET pulse minus mean of 20 seconds before the 0.15% DEET pulse). (C) Heat maps of calcium imaging data in response to 0.15% DEET. Each row represents ADL imaged in one animal, cropped to show only the first pulse. (D) Pseudocolored images of ADL response to 100 nM C9 pheromone in animals of the indicated genotype calculated as in B. (E) Heat maps of calcium imaging data in response to 100 nM C9 pheromone. Each row represents ADL imaged in one animal, cropped to show only the first pulse. (F) Mean normalized ADL calcium responses during the first DEET or C9 pulse in animals of the indicated genotype from data in C and E. In F, horizontal lines represent mean ± s.e.m. In F each dot represents a single neuron in a single animal. Data labelled with different letters indicate significant differences ($p<0.05$, Two-way ANOVA and Tukey's Post-hoc test).

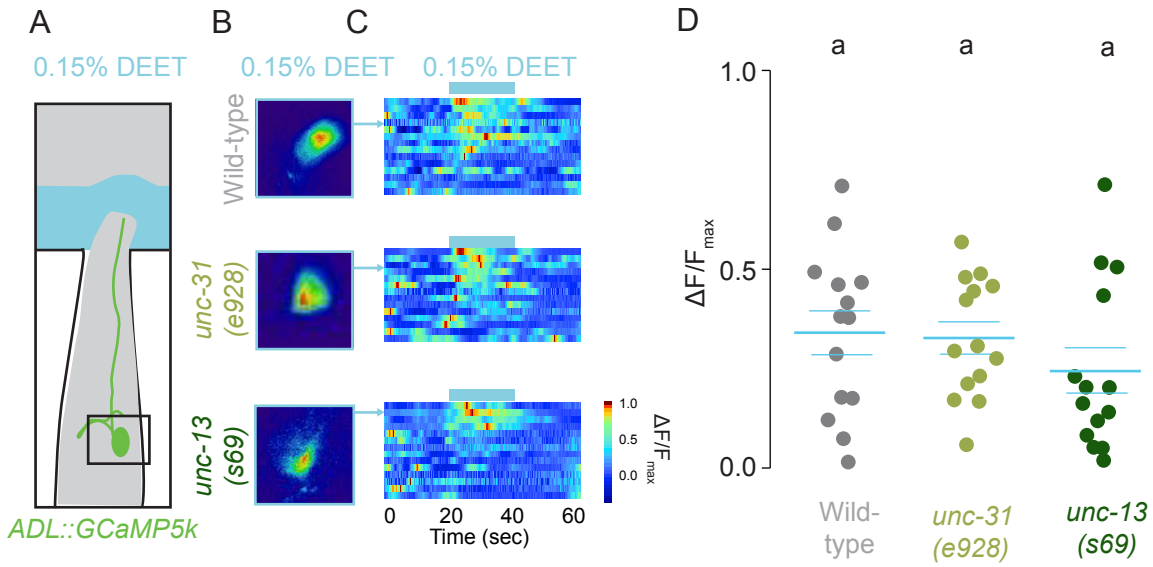


Figure 6.4 ADL responds directly to DEET. (A) Schematic of microfluidic calcium imaging assay. (B) Pseudocolored images of ADL response to 0.15% DEET in animals of the indicated genotype (increase in mean fluorescence 20 seconds after the first DEET pulse minus mean of 20 seconds before the 0.15% DEET pulse). (C) Heat maps of calcium imaging data in response to 0.15% DEET. Each row represents ADL imaged in one animal, cropped to show only the first pulse. (D) Mean normalized ADL calcium responses during the first DEET pulse in animals of the indicated genotype from data in C. In D each dot represents a single neuron in a single animal. Horizontal lines represent mean ± s.e.m. Data labelled with different letters indicate significant differences ($p<0.05$, Two-way ANOVA and Tukey's Post-hoc test).

6.3 *str-217* cannot confer DEET-sensitivity to HEK cells

While these data are consistent with the hypothesis that *str-217* is a DEET receptor, it is also possible that *str-217* is not the direct *in vivo* target of DEET, but is involved indirectly in signaling or modulation of DEET-specific responses in ADL. To ask if *str-217* is a direct molecular target of DEET, we expressed *str-217* in HEK293T cells and monitored activation by DEET using calcium imaging. Using this approach, we found that DEET did not activate HEK293T cells expressing *str-217* (Fig. 6.5).

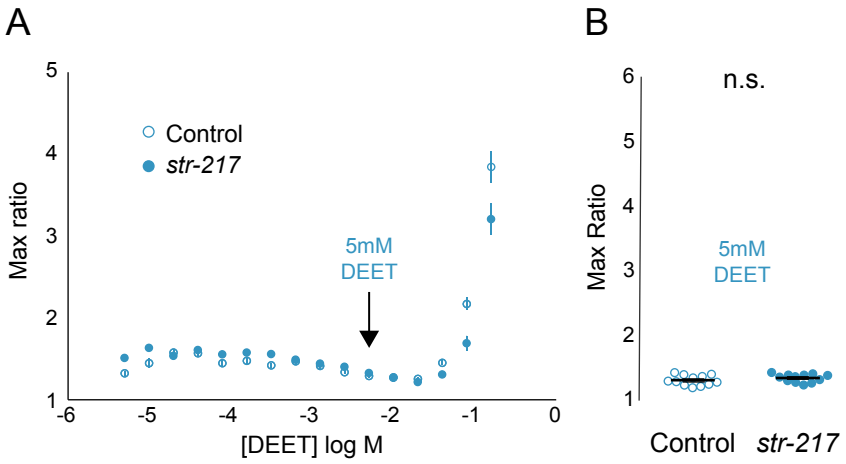


Figure 6.5 *str-217* does not respond to DEET when expressed in HEK-293T cells. (A) Max ratio (maximum fluorescence/baseline fluorescence) of calcium signal in HEK-293T cells transiently expressing *GCaMP6s* and $Gq_{\alpha}15$ without (control) or with *str-217* and stimulated by the indicated dose of DEET. (B) Summary of max ratio responses to 5 mM DEET. Data are plotted as mean ± s.e.m. (n=12, 3 replicates each in 4 separate plates; n.s., not significant, p>0.05, ANOVA and Tukey's Post-hoc test) with s.e.m. indicated by a vertical line in A and horizontal line in B. Data collected by Laura Duvall.

We cannot exclude the possibility that this nematode receptor is non-functional in mammalian tissue culture cells either because it is not trafficked to the cell membrane, or because essential signaling cofactors are not natively present in mammalian cells. It is also possible that *str-217* is required for ADL to be sensitive to DEET, but that this receptor acts downstream of a primary receptor for DEET.

6.4 Several *C. elegans* neurons respond to DEET

In *Drosophila*, many neurons show some sort of DEET response (Ditzen et al. 2008; Pellegrino et al. 2011) but it has not been possible to determine, which if any of these responses account for the behavioral effects of DEET on these flies because of the complexity of the olfactory system in these animals. In *C. elegans*, the relatively

small number of olfactory neurons and the genetic access to them allowed us to start to ask these questions. In addition to the increase in calcium seen in AWC (Fig. 6.1) and ASH (Fig. 6.6) we also saw increases in calcium in AWA (Fig 6.7G-H) and ASK (Fig 6.7A-B). Additionally, ASE responded with an increase in calcium after the removal of DEET (Fig 6.7). As ASH is often described as a repellency neuron, and DEET can evoke calcium responses in ASH, we wanted to determine if disrupting ASH could confer DEET-resistance. We used a strain expressing caspases in ASH, which should genetically ablate these neurons (Yoshida et al. 2012). We saw no effect on DEET-sensitivity in chemotaxis to isoamyl alcohol in this strain. Therefore although DEET activates ASH, this activation plays no role in the observed behavioral effect of DEET.

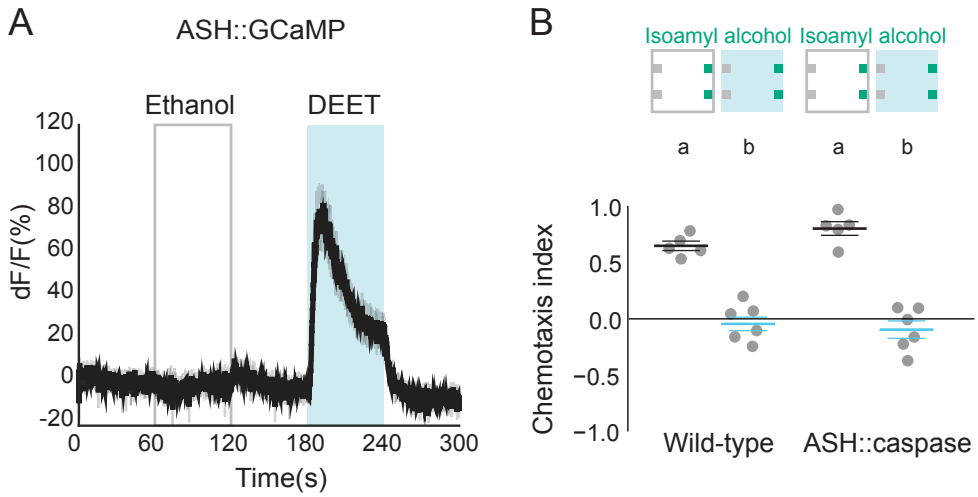


Figure 6.6 ASH neurons are also DEET-sensitive, but not required for DEET-sensitivity in chemotaxis to isoamyl alcohol. (A) Preliminary data showing average traces of GCaMP activity in ASH in wild-type (black) during exposure to ethanol (open gray box) and 0.15% DEET (blue). (B) Chemotaxis indices for wild-type animals and animals expressing caspase in ASH on solvent- and DEET-agar. Each dot represents responses of single animals and the horizontal lines represent the mean and s.e.m. Data labelled with different letters indicate significant differences (N=15 animals, over two experiments. $p<0.05$, two-way ANOVA and Tukey's Post-hoc test). Imaging data in A collected by May Dobosiewicz.

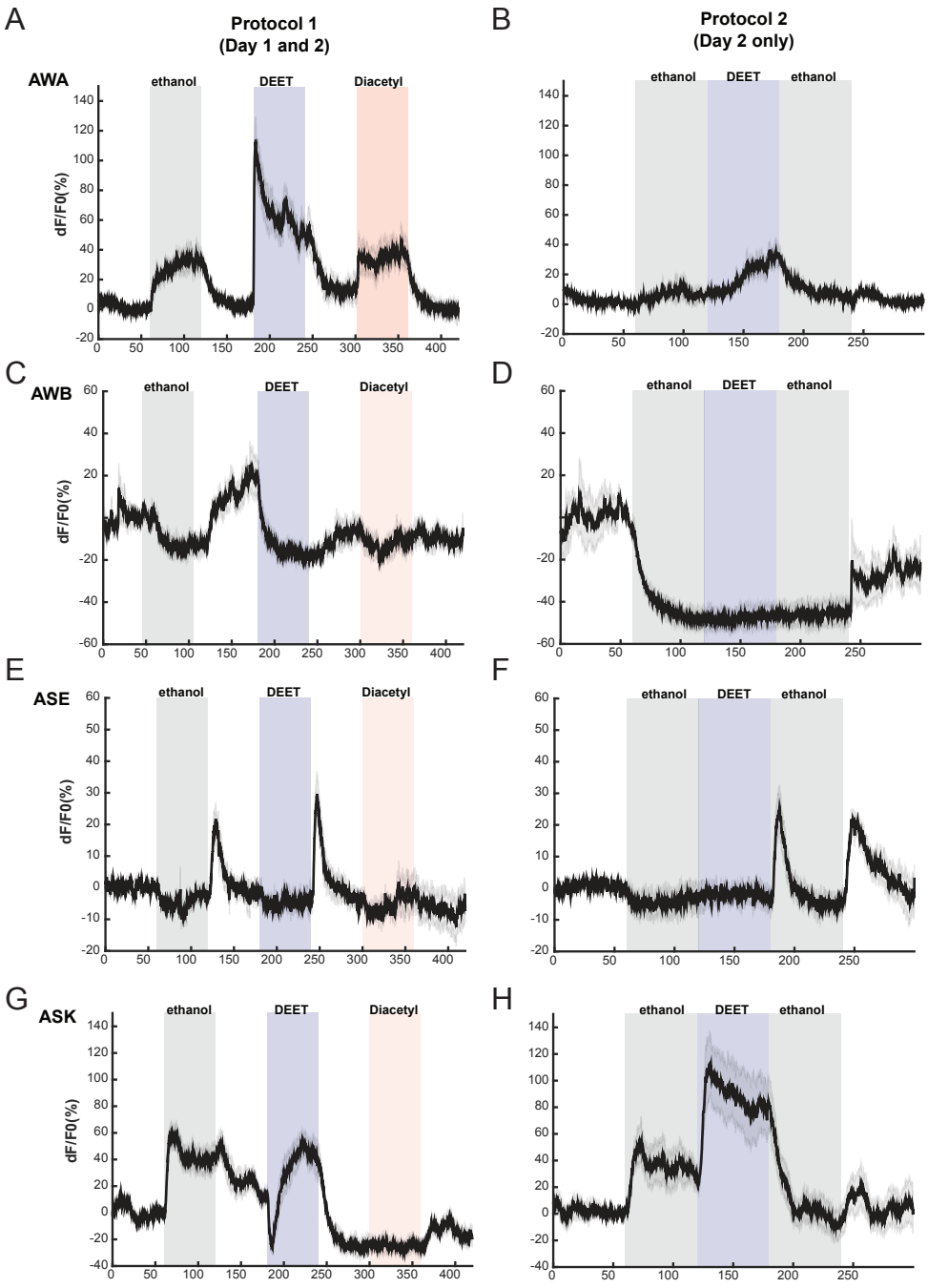


Figure 6.7 Many neurons respond to DEET. These preliminary data show varied responses to DEET and ethanol solvent in (A-B) AWA (C-D) AWB (E-F) ASE and (G-H) ASK neurons. These data show averaged traces from 8-11 animals on 1-2 days of experiments (black) and s.e.m. shown in gray. Data collected by May Dobosiewicz.

Although AWB responds to ethanol buffer, when in a constant stream of ethanol, there is no consistent response to DEET in AWB neurons (Fig. 6.7D). This makes expressing *str-217* in AWB an attractive direction for future study to determine if *str-217* can confer DEET sensitivity to another neuron.

Several neurons that do not express *str-217* are DEET-responsive, including ASH and AWC. In AWC, we were able to see these same responses to DEET in the *str-217* mutants and in wild-type animals. Together, these data indicate that cells that do not express *str-217* are able to respond to DEET. We do not understand the mechanism responsible for these responses. Because not all sensory neurons in *C. elegans* respond to DEET, and because responders aside from ADL do not express *str-217*, we hypothesize that additional genes confer selective responses to DEET in these neurons.

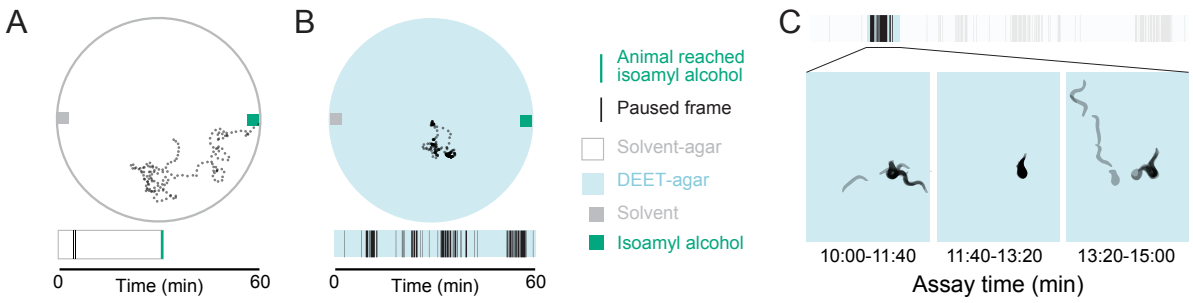
CHAPTER 7

DEET INCREASES AVERAGE PAUSE LENGTHS DURING EXPLORATION AND CHEMOTAXIS AND REQUIRES ADL

In **Chapter 4**, we described the effects of DEET on *C. elegans*, and in **Chapters 5 and 6** we identified genetic (*str-217*) and neuronal (ADL) requirements for this DEET-sensitivity. We showed that DEET has a specific effect on chemotaxis to some odors and not others, but we still do not know exactly how ADL activity may interfere with chemotaxis. Additionally, even in *str-217* mutants or *ADL::Tetanus* toxin animals, we never see complete return to solvent-agar levels of chemotaxis to isoamyl alcohol on DEET. To better understand how DEET is affecting chemotaxis and what role(s) ADL and *str-217* play, we recorded the behavior of animals during chemotaxis.

7.1 DEET increases average pause length during chemotaxis

Population chemotaxis assays report the location of the animal at the end of the experiment, and do not reveal the details of navigation strategy. To investigate which aspects of chemotaxis and exploration are affected by DEET, we tracked the position and posture of individual animals on DEET-agar or solvent-agar plates (Fig. 7.1). These data immediately revealed an obvious phenotype not visible in the population-based assays: DEET increases the frequency and duration of pausing in *C. elegans* during chemotaxis (Fig. 7.2)



7.1 Tracking animals' movement during chemotaxis reveals pausing phenotype. (A-B) Top: example trajectories of a single wild-type animal chemotaxing to isoamyl alcohol on solvent-agar (A) or a different animal on DEET-agar (B) plate. Each dot depicts the x, y position of a single animal once every 10 seconds. Bottom: raster plots indicating paused frames for each animal depicted above. C, Example pauses from the tracked animal in b. Images were extracted every 18 frames (6 seconds), cropped, and made into a silhouette. 16 silhouettes were overlaid to create each snapshot of activity.

To investigate if the DEET-induced increase in pause duration or frequency required *str-217* and ADL, we tracked single animals on solvent- and DEET-agar plates. Wild-type, but not *str-217*$^{-/-}$ mutant animals (Fig. 7.2), showed a dramatic increase in average pause length on DEET-agar.

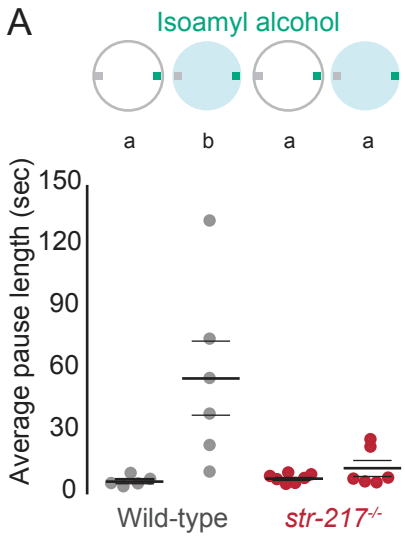


Figure 7.2 Increase in pause duration on DEET-agar during chemotaxis requires *str-217*. (A) Average pause length for each experiment on plates with the indicated stimuli and genotypes (N=6-7 plates, 4-15 animals per plate). Different letters indicate statistically significant differences ($p<0.05$ two-way ANOVA).

7.2 DEET increases average pause length during exploration

We then identified at least two hypotheses consistent with these results: DEET and isoamyl alcohol affect some number of neurons including ADL and AWC that lead to a 'pause state', or that DEET alone can lead to a 'pause state'. To determine if the increase in average pause length occurs only in the context of chemotaxis to isoamyl alcohol, or as a consequence of DEET alone, we tracked wild-type, *str-217*$^{-/-}$ mutant (Fig. 7.3A), and *ADL::Tetanus toxin* (Fig. 7.3B) animals on DEET-agar and solvent-agar plates with no additional odorants. Only wild-type animals had a higher average pause length on DEET-agar. Consistent with our prior observation that chemotaxis to pyrazine was unaffected by DEET, wild-type animals showed no increase in average pause length when chemotaxing to pyrazine on DEET-agar (Fig. 7.4). This suggests that pyra-

zine chemotaxis can overcome the effect of DEET on average pause length, and likely other DEET-affected aspects of exploration and chemotaxis.

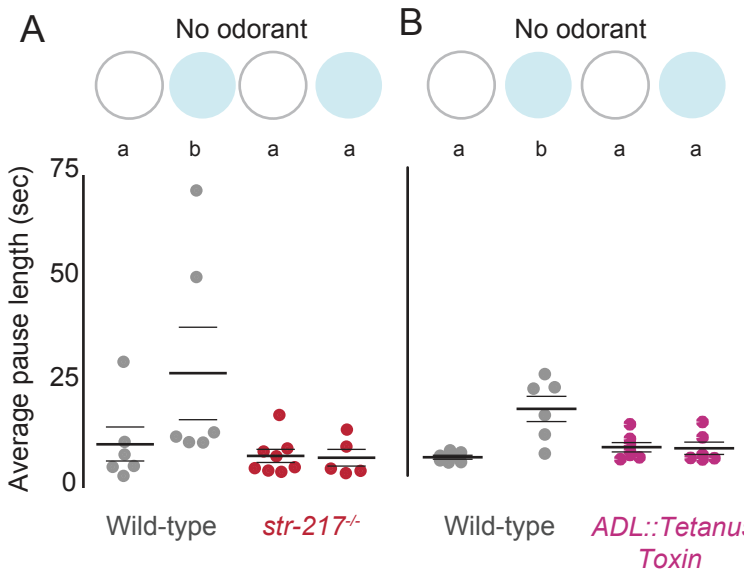


Figure 7.3 DEET increases average pause length and requires *str-217* and ADL. (A-B) Average pause length for each experiment on plates without any additional odor added (N=6-7 plates, 4-15 animals per plate). Different letters indicate statistically significant differences ($p<0.05$ two-way ANOVA).

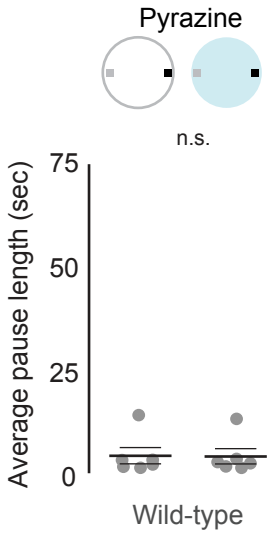


Figure 7.4 Wild-type animals chemotaxing to pyrazine do not show increased pause duration. Average pause length for each experiment (N=6 plates, 4-15 animals per plate). Different letters indicate statistically significant differences ($p<0.05$ Student's *t*-test).

Together, these data show that DEET has multiple effects on exploration and chemotaxis, including increasing the duration of pause lengths. ADL and *str-217* are required for this increase in average pause length, yet pyrazine is able to overcome these effects. It appears that the ADL response to DEET is necessary for an increase in pause duration, but with these data it is not possible to determine if ADL is sufficient to increase average pause duration. It could be that ADL activity increases pause duration, or that many neurons are affected by DEET, and the animal needs many DEET-affected neurons to act in concert to lead to the increase in average pause duration.

7.3 Artificial activation of ADL is sufficient to increase average pause length

To determine if ADL activity alone is sufficient to increase average pause length, we carried out an optogenetic experiment by expressing the light-sensitive ion channel ReaChR (Lin et al. 2013) in ADL neurons of wild-type animals, and tracking locomotor

behavior on chemotaxis plates. We observed an increase in average pause length when ADL was activated artificially (Fig. 7.6).

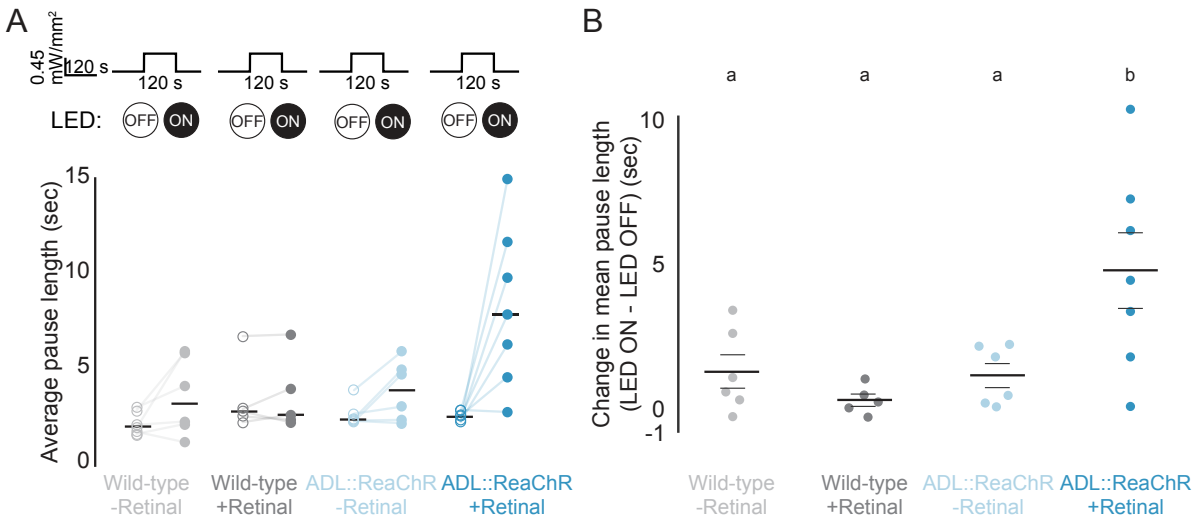


Figure 7.5 Artificial activation of ADL increases pause duration. (A) Average pause length of the indicated genotype with LED off (open circles) or on (closed circles), with lines connecting each experimental pair. (B) Difference in average pause length for each experiment in (A-B) (N=6 experiments, 4-15 animals per experiment). Horizontal lines indicate mean ± s.e.m. Data labelled with different letters indicate significant differences ($p<0.05$, two-way ANOVA and Tukey's Post-hoc test).

From these data, we conclude that ADL mediates the increase in average pause length seen on DEET-agar, and speculate that the increase in long pauses is one mechanism by which DEET interferes with chemotaxis.

7.4 The length and strength of ADL stimulation affects different aspects of exploration

Previous work on ADL demonstrated that the pheromone C9 increases the probability of reversals through ADL activity, yet we did not see an increase in reversals in our tracking data. This difference in behavioral response could be tied to the delivery of

the stimuli (acute C9 exposure compared to chronic DEET-exposure in the plates) or the strength and features of calcium response (Fig. 6.3). To further explore these differences, we used the *ADL::ReaChR* reagents and tested higher intensities of light as well as shorter stimulus regimes (Fig 7.7, Fig. 7.8).

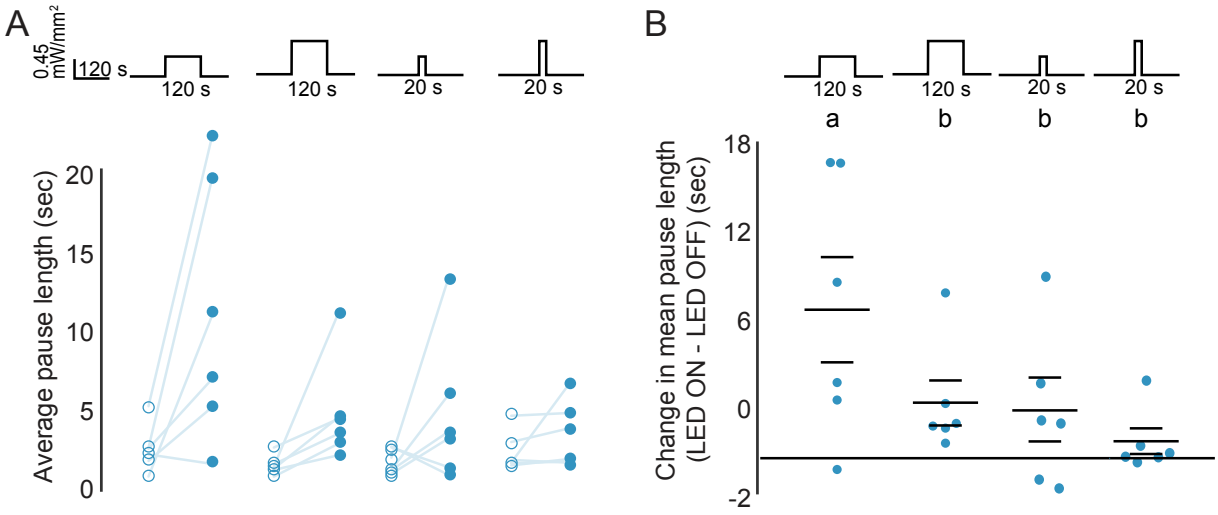


Figure 7.6 Increasing pause duration requires long stimulation. (A) Average pause length of the indicated stimulation scheme with LED off (right) or on (left), with lines connecting each experimental pair. (B) Difference in average pause length for each experiment in (A-B) (N=6 experiments, 4-15 animals per experiment). Horizontal lines indicate mean ± s.e.m. Data labelled with different letters indicate significant differences ($p<0.05$, two-way ANOVA and Tukey's Post-hoc test).

We found that the lower intensity, longer light pulses had the strongest effect on pause duration (Fig. 7.6) and that reversals were only seen at the shortest, highest light intensity (Fig. 7.7D). Previously we showed that the DEET-induced increase in pause duration requires ADL and *str-217* (Fig 7.3). Simiarly, here we show that artificial activation of ADL is not, under these conditions, able to increase pause frequency (Fig 7.7A and B), further supporting the hypothesis that ADL activity can lead to an increase in pause duration but not frequency.

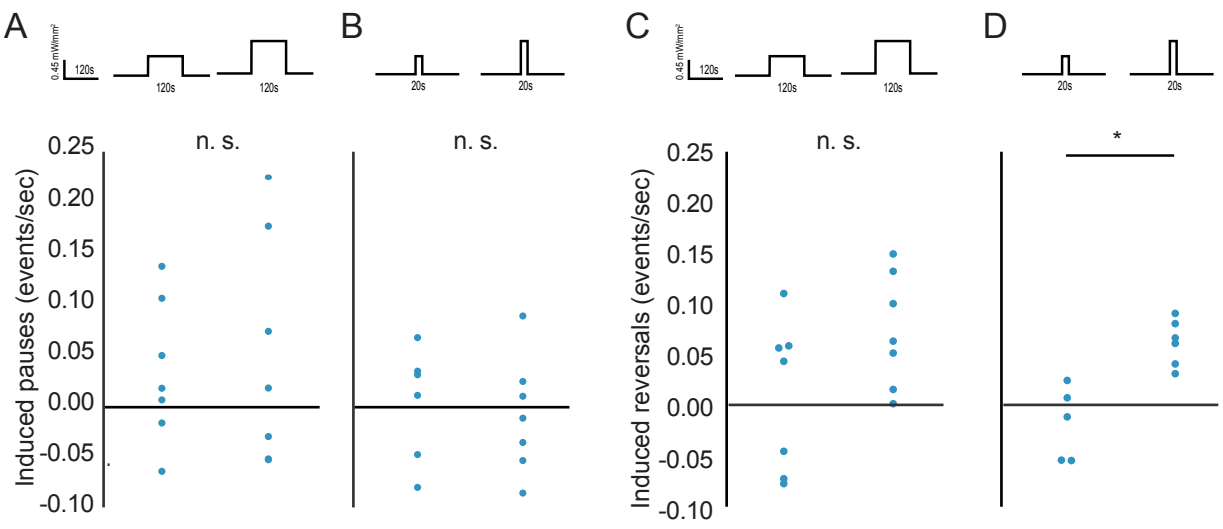


Figure 7.7 Increasing the intensity of the stimulation of ADL increases the number of reversals. (A) Increase in average pause frequency during two minutes of light stimulation compared to the two preceding minutes. (A) Increase in average pause frequency during twenty seconds of light stimulation compared to the twenty preceding seconds. (A) Increase in the frequency of long and short reversals during two minutes of light stimulation compared to the two preceding minutes. (A) Increase in the frequency of long and short reversals during twenty seconds of light stimulation compared to the twenty preceding seconds. (N=6 experiments, 4-15 animals per experiment). (*p<0.05, Student's *t*-test).

These data demonstrate that artificial activation of ADL is sufficient to increase average pause length, and the duration and intensity of ADL activation are both important variables in predicting the behavioral outcome of ADL activation. This concurs with both our results and the results from others. We have shown that delivery of the ADL-activating stimulus is important: mixing DEET into plates can increase average pause duration, at low concentrations but even high concentrations of DEET as a point source have no observable effect. This agrees with previous experiments from the Bargmann lab, where animals on agar containing 10 ng C9 explored less and spent more time dwelling on food (Greene et al. 2016) while acutely presented C9 can induce

reversals when presented at 100 nm and higher concentrations (Jang et al. 2012).

Together, these data identify a specific effect of DEET — an increase in average pause duration -- that requires both ADL and *str-217.* Artificial activation of ADL is sufficient to increase average pause length, demonstrating the sufficiency of ADL activation on pause duration. Finally, we provide evidence that the duration and intensity of stimulating ADL plays a role in the behavior elicited.

Chapter 8

DISCUSSION

In this thesis, we explored the basis of DEET-sensitivity across Protostomia, with a focus on *Ae. aegypti orco*-independent contact DEET repellency and the nematode *C. elegans.* We added *C. elegans* to the list of known DEET-sensitive animals, uncovered a neuronal mechanism for a DEET-induced behavior, and identified a molecular target required for complete DEET-sensitivity in an engineered mutant and in a wild-isolate of this species. Using these insights into non-Arthropod DEET sensitivity, we propose several areas for further study.

This work opens up *C. elegans* as a system to test new repellents *in vivo* for both interference in chemotaxis and toxicity, and also for discovery of additional genes and neurons that respond to DEET. The molecular mechanism by which the *str-217* mutation renders ADL DEET-insensitive and worms DEET-resistant remains to be understood. *str-217* is a G protein-coupled receptor with no known ligand and that is evolutionarily unrelated to DEET-sensitive odorant receptor proteins and gustatory receptor proteins previously described in insects. Although we found no evidence that DEET can activate *str-217* in heterologous cells, it is conceivable that in the right milieu, *str-217* is indeed a DEET receptor. Alternatively*, str-217* could act indirectly in concert with an as-yet unknown DEET receptor in ADL. Interestingly, pyrazine chemotaxis is unaffected by DEET in any of our assays, consistent with our model that DEET is not a simple repellent, but a modulator of behavior to interfere with chemotaxis to some but not all odorants.

8.1 Why do *ADL::Tetanus toxin* animals and *str-217* mutants still have a chemotaxis defect?

We have shown that although *str-217*$^{-/-}$ mutants and animals expressing tetanus toxin in ADL are both DEET-resistant compared to wild-type animals, they are still DEET-sensitive. Several hypotheses could explain the residual DEET-sensitivity: DEET may act on some sensory neurons to blunt the normal response to odor as we saw in AWC imaging, DEET may interact with other sensory neurons to alter different aspects of locomotion which prevent the animals from chemotaxing efficiently, or DEET may interact with multiple neurons and affect the integration of sensory signals. None of these hypotheses are mutually exclusive, but we will propose experiments to start to untangle the different effects of DEET on chemotaxis and exploration, and to put these data in perspective of the broad efficacy of DEET.

8.1.1 DEET may interfere with the primary sensory neurons required for chemotaxis to specific odorants.

In **Chapter 6**, we showed that DEET alone can affect multiple sensory neurons (Fig. 6.7) and can decrease the magnitude of AWC calcium response to isoamyl alcohol (Fig. 6.1). We currently cannot predict the precise behavioral relevance of this change in magnitude, though it seems reasonable to speculate that a degraded calcium response may contribute to the lower chemotaxis indices.

The AWC calcium response to DEET does not require *str-217*, but it may still require other neurons. It would be possible to formally test this by imaging AWC in *unc-13* and *unc-31* animals, and identify if synaptic vesicle or dense core vesicle fusion is required. If AWC is directly affected by DEET, it is possible that continuous exposure to the DEET-agar plate is mimicking olfactory adaptation during this experiment. Odor history can influence chemotaxis and calcium responses in AWC: when animals were

incubated in the presence of high isoamyl alcohol, AWC showed a diminished response to isoamyl alcohol pulses and a decrease in chemotaxis indices after pre-exposure (Chalasani et al. 2010). However, animals mutant for the peptide *nlp-1* regained their chemotaxis to isoamyl alcohol. One could image from AWC neurons in both *nlp-1* and wild-type animals. If the AWC neurons showed normal responses to isoamyl alcohol in the *nlp-1* mutants, one could then test *nlp-1* mutants and wild-type animals in the population chemotaxis assays. If *nlp-1* animals were somewhat DEET-resistant, or if *str-217*$^{-/-}$*;nlp-1* double mutants showed additional DEET-resistance, it would support the hypothesis that DEET can affect chemotaxis by interfering with the AWC sensory neurons and their ability to respond to odorants.

We also showed that DEET can increase calcium in AWA and ASK neurons, and that ASE neurons showed a response to DEET removal. The AWA response is particularly interesting, because AWA is required for both pyrazine and diacetyl chemotaxis. In population chemotaxis assays, pyrazine chemotaxis is unaffected by DEET, while diacetyl chemotaxis remains affected (Fig. 4.3). To understand what makes these two odorants behave differently in these assays, it would be helpful to first know what the primary neural responses look like in the presence of DEET. One could image AWA responses to diacetyl and pyrazine in buffer, solvent-buffer, and DEET-buffer, similar to the experiment we performed with AWC and isoamyl alcohol (Fig. 6.1). If diacetyl responses were degraded in DEET-buffer, but pyrazine responses were intact, that would provide evidence that DEET may interfere with *odr-10*, the diacetyl receptor (Sengupta et al. 1996), but not the unidentified pyrazine receptor.

8.1.2 DEET may interact with sensory neurons to induce competing behaviors

We found that ADL is both necessary and sufficient for a DEET-induced increase

in pause duration. If animals spend more time paused, it will take them longer to chemotax toward an odor, even if all other aspects of chemotaxis are unaffected. This is an example of a competing behavior: an animal cannot be both moving and paused, and therefore increasing pause duration would decrease chemotaxis efficiency.

We also found that ADL is not required for DEET-induced pause frequency. This observation indicates that pause initiation and continuation require different subsets of neurons. Using existing strains expressing tetanus toxin or caspases in sensory neurons, it should be possible to identify neurons required for other DEET-induced behaviors, like pause frequency. If the sensory neurons required for the increase in, say, pause frequency were identified, it would be possible to determine if there are additional aspects of chemotaxis affected by DEET. For example, if neurons X were required for the increase in pause frequency, would animals expressing tetanus toxin in both neuron X and ADL completely regain DEET-sensitivity? If not, careful behavioral analysis of these animals may reveal additional effects of DEET.

8.1.3 The primary effects of DEET may be downstream of the sensory neurons

We observed that chemotaxis to pyrazine appears immune to the effects of DEET, both in population assays and in tracking experiments. This indicates that even if DEET is affecting the primary sensation of odorants and can induce competing behaviors, these effects can be overcome if the “right” signal is present. Intriguingly, diacetyl chemotaxis is affected by DEET, even though diacetyl and pyrazine both require the same AWA sensory neurons for chemotaxis (Bargmann et al. 1993). The diacetyl results provide evidence against the hypothesis that activation of AWA alone is able to overcome DEET-sensitivity, though we have not explicitly tested this assumption. If AWA responds similarly to diacetyl and pyrazine: what makes these two odorants lead to such

different outcomes?

One way of addressing this problem is to look at the information flowing through these circuits. It is now possible to perform large-scale neural imaging in freely moving animals (Nguyen et al. 2016), and it is becoming feasible to identify contributions from identifiable neurons (Nichols et al. 2017). Using these methods, it may be informative to image from freely moving *C. elegans* on DEET- and solvent-agar plates chemotaxing to diacetyl, and chemotaxing to pyrazine. Identifying differences between these patterns should produce testable hypotheses.

For example, we know that DEET increases pause duration in *C. elegans*. AVA and AVB are thought to be reverse and forward command interneurons, respectively. Each of these neurons runs the entire length of the ventral nerve cord, making synaptic connections to nearly all of the non-pharyngeal motor neurons in *C. elegans* (Chalfie et al. 1985). There is evidence that during a pause, both AVA and AVB are silent, or both AVA and AVB are active (Roberts et al. 2016). It could be that AWA response to pyrazine leads to AWA signaling differently to downstream neurons, or that pyrazine affects other sensory neurons. In either case, pyrazine could disrupt the balance of AVA and AVB excitation and inhibition, biasing the animal toward forward movement, while diacetyl does not. Using these large-scale imaging methods, we would be able to see the information flowing through these circuits, and ideally identify candidate neurons for further manipulation.

8.2 What other genes are required for DEET-sensitivity?

In this thesis, we identified *str-217*, a GPCR required for complete DEET-sensitivity in *C. elegans* However, *str-217*$^{-/-}$ mutants are not completely resistant to DEET as chemotaxis does not reach the levels seen on control plates lacking DEET, and multiple neurons that do not express *str-217* can respond to DEET by calcium imaging. Other genes required for DEET-sensitivity must therefore exist. Here we explore ways to iden-

tify more candidates required for DEET-sensitivity, and perhaps identify DEET-sensitive receptors

8.2.1 *nstp-3* is a candidate gene that may be required for DEET-sensitivity

Our genetic screen yielded three DEET-resistant strains of *C. elegans*, two of which we mapped to candidate genes. In addition to identifying *str-217*, we also identified *nstp-3*. *nstp-3* is annotated as a sugar-proton symporter in the SLC solute transporter family ('Wormbase web site'). Our early attempts at identifying the expression pattern of this gene with a small (0.7kb) promoter sequence were not fruitful, but using longer upstream putative regions could help identify the expression pattern of this gene and allow for rescue experiments to confirm this candidate.

8.2.2 A sensitized screen could reveal additional candidate genes

If we wanted to know which genes are required for the remaining DEET-sensitivity in *str-217*$^{-/-}$ mutants, we could repeat our forward genetic screen in a *str-217*$^{-/-}$ genetic background. This type of sensitized screen would bias us away from finding additional neurons required for ADL to sense DEET and increase average pause length.

8.3 How else might DEET affect behavior?

In **Chapter 2** we demonstrated that there are many, distinct behavioral effects of DEET in *Ae. aegypti*: olfactory repellency requiring *orco*, *orco*-independent contact DEET repellency that requires the tarsi, and rejection of DEET-laced liquid food. We propose that there are likely multiple unstudied effects of DEET not only in *Ae. aegypti*, but also in all of the other DEET-sensitive species. Here we propose several avenues to identify additional effects of DEET in nematodes and beyond.

8.3.1 Acute DEET sensitivity in *C. elegans*

Our experiments in *C. elegans* presented DEET uniformly at low concentrations, and did not explicitly test any acute effects of DEET. To model contact avoidance in nematodes, we attempted to convert the split Petri dish assay from **Chapter 3** for use with *C. elegans*. *C. elegans* crawl on the surface of the agar and are sensitive to surface variations, therefore this assay was not ideal for this species. To test for *C. elegans* sensitivity to acute DEET, we could instead perform a drop test (Jang et al. 2013) which places a small drop of liquid in front of a moving animal, and asks if the animal reverses. If DEET does have an acute effect on *C. elegans*, it would be interesting to screen for animals insensitive for this behavior as well. We could re-purpose the screen we performed in this thesis, using chemotaxis agar instead of DEET-agar, but adding a stripe of DEET down the middle of the assay. If animals crawl over the DEET to chemotax to the odor, they would be considered 'hits'. Candidates from this screen could then be tested for general osmotic and solvent sensitivity, and any mutants specifically DEET-sensitive could be further investigated. It would also be possible to do this on a smaller scale and screen for neurons required for acute DEET-sensitivity.

Together, these experiments in *C. elegans* can help us better understand how DEET is affecting this animal. Adding several more genes to the list of known genes required for DEET-sensitivity could help identify commonalities across these proteins, potentially leading to testable hypotheses that could be approached with protein engineering methods.

8.3.2 Can insects and nematodes smell DEET?

Several groups claim that DEET is an aversive odor to specific species of mosquitoes, but the behavioral evidence that DEET smells bad relies on behavioral assays that conflate contact and non-contact activity of DEET. Moreover, they do not test repellency of DEET per se, but the ability of DEET to inhibit attraction to other sensory

stimuli. These groups used an arm (Stanczyk et al. 2010), food odors (Syed et al. 2011), heat (Syed et al. 2008), or sucrose (Syed et al. 2008) to first attract mosquitoes, and many failed to control for contact repellency. If DEET itself is an aversive, unpleasant stimulus, presenting DEET alone should induce movement away from the DEET source, and a forced choice assay with no other stimuli should result in avoidance. In their home cages, most *Ae. aegypti* mosquitoes are on one of the four walls (Fig. 8.1). Adding a filter paper to each side of the cage, just outside, would allow for delivery of DEET by soaking the filter paper with syringes. If animals were repelled by DEET, more animals should leave the DEET side than the solvent side.

Figure 8.1 Mosquitoes spend time on the sides of cages. Image © Alex Wild, used with permission. Dark areas on the cages are mosquitoes.

It would also be informative to perform a forced-choice assay, similar to the olfactory two-choice assay developed in the Benton lab (Ramdya et al. 2015). In this assay, a laminar flow delivers either solvent (white) or odor (blue) in an air stream (Fig. 8.2). The body position of each animal would be tracked over time, and avoidance of the odor side could be compared. If DEET itself is an aversive, unpleasant stimulus, flies and mosquitoes should spend more time in the clean air stream in this experiment.

In *D. melanogaster*, geosmin would be an excellent control odor, as it is one of the few examples of labeled line repellency in *D. melanogaster*, though its effect on *Ae. aegypti* is unknown (Stensmyr et al. 2012).

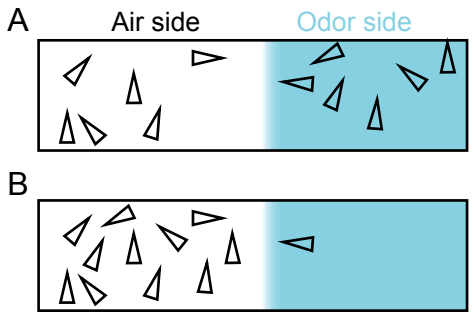


Fig. 8.2 Odor avoidance assay from Ramdya et al. 2014 (A) Schematic representing the assay, and example of triangles, which could symbolize either *Ae. aegypti* mosquitoes or *D. melanogaster* flies, with no side bias. (B) Example of animals avoiding the odor side.

Several studies have identified individual olfactory neurons that respond to DEET (Syed et al. 2008), or an aggregate, electroantennographic response in antennae (Costantini et al. 2001; Leal et al. 1998). If DEET is not a behavioral repellent on its own, it means either the responses are not sufficient to generate a perceptible odor, or DEET is a neutral stimulus. If DEET has a perceptible odor, it should be possible to train animals to avoid DEET or be attracted to DEET. Using classic learning assays in *A. mellifera* honeybees, DEET was mixed with sucrose and used as an effective unconditioned stimulus applied to the proboscis or tarsi during presentation of odorants (Abramson et al. 2010). Using these same assays, DEET could instead be used as the conditioned stimulus, and paired with either a shock or sucrose reward in an assay similar to the fly elevator described (Quinn et al. 1974; Tempel et al. 1983). If the animals can smell DEET, they should be able to learn using DEET as a conditioned volatile stimulus.

8.4 What does a perfect repellent look like?

Many investigators motivate their studies of DEET based on the assumption that the more information we have about how DEET works, the easier it will be to produce new, better repellents. DEET is already an amazingly effective compound and has a strong safety record, raising the question: what would improvements on DEET really look like? The ideal repellent would be applied infrequently, be completely non-toxic, and provide complete protection from all biting animals. A longer-lasting DEET formulation exists that lasts about 12 hours, but users must still apply at least once a day for protection (Salafsky et al. 1999). One idea that goes beyond existing products would be a single application of commensal skin bacteria producing repellents that could effectively protect someone for extended periods of time. While this may seem like science fiction, there are already methods to apply commensals producing natural products (Nakatsuji et al. 2017). However, DEET is not a natural product, and therefore is beyond the reach of this type of approach for the time being.

Several groups have explored the chemical space around DEET, looking for repellents (Katritzky et al. 2008). While they were able to identify compounds that could repel some species, nothing approaching the effectiveness of DEET has been identified. As we explored in **Chapter 2**, another group of compounds effective against mosquitoes show either *orco* dependency, contact-only repellency, or a lower effectiveness (Fig. 2.13). If the effectiveness of DEET lies in its multiple modes of action, these alternative repellents cannot mimic the effectiveness across modes. Even the original studies identifying DEET were able to identify related compounds with similar levels of repellency, but many of these compounds resulted in skin irritation, making them unsuitable for human use (McCabe et al. 1954). It could be that the uniqueness of DEET lies in its safety profile, coupled with its promiscuity and activity on multiple sensory systems. Together,

these observations demonstrate that we do not even know which aspects of DEET we should be trying to emulate.

8.5 What can we learn from other organisms?

To gain a broader understanding of how DEET works and what makes it so effective, we must synthesize information across all of the species DEET effects. This is currently difficult with the available information, but in this thesis, we demonstrate that performing carefully designed behavioral experiments can aid in dissecting the many effects of DEET within and across species. With this lesson in mind, we propose several avenues of future study to better understand the similarities and differences in the effects of DEET across invertebrates.

8.5.1 The effects of DEET on locomotion across species

This study identifies similar behavioral and peripheral sensory effects of DEET across both *D. melanogaster*, *Ae. aegypti,* and *C. elegans.* In studying DEET-like molecules, we noticed that some molecules can mimic the contact effects of DEET while others could mimic the *orco*-mediated effects. This implies that the many effects of DEET may be discrete elements which each contribute to its effectiveness, rather than one specific aspect of DEET required for all aspects of repellency.

If there are discrete properties of DEET, each could be explored independently, and a blend of odorants could be marketed as a DEET-replacement or used as a basis for designing repellent-producing bacteria. To mimic the effects of DEET, we must first understand and describe them.

We show that in *C. elegans*, DEET increases both the frequency and duration of pausing during exploration and chemotaxis (**Chapter 7**). In the German cockroach *Blattella germanica,* pre-exposure to DEET decreases locomotion in an exploration-based

assay, but repellency of contact DEET remains intact (Sfara et al. 2013). Anecdotally, in arm-in-cage assays in *Ae. aegypti* mosquitoes, we observed that animals do not move from the side of the cage when the DEET-treated arm is presented. This could indicate that DEET is inhibiting their activity. Inhibition of activity could be one common aspect of DEET-sensitivity. To test this, one could use an activity monitor, which measures gross activity of an animal by counting "beam breaks" — when the animals cross from one end of a tube to the other. If DEET is decreasing locomotion, DEET vapor should decrease the number of beam breaks in an assay of this type. This assay is scalable, and could be used to test many different terrestrial animals to determine if inhibition of locomotion is a consistent effect across species in similar assays.

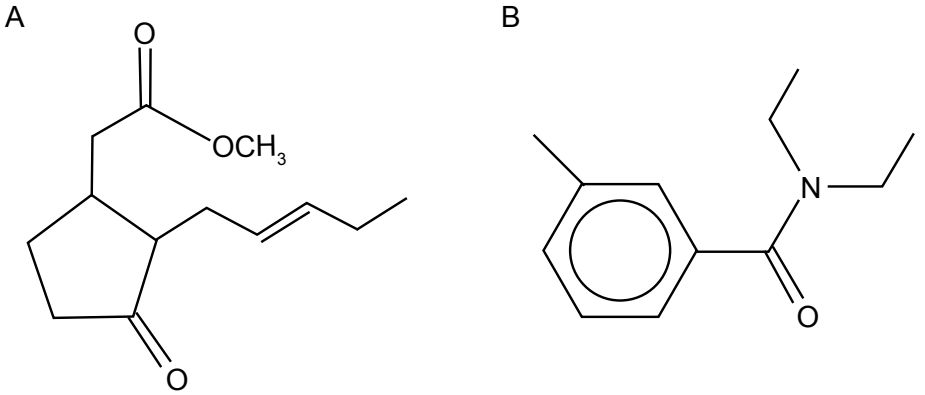
8.5.2 The effects of DEET on neural systems across species

DEET can affect primary sensory neurons and their responses to their cognate ligands: from blunting AWC responses to isoamyl alcohol in *C. elegans* to decreasing the spike frequency in bitter taste receptor neurons in *D. melanogaster*. DEET can also directly activate sensory neurons: from *CuOR136*-expressing neurons in *Cu. quinquefasciatus* to ADL neurons in the worm. Together, this broad and diverse activity appears to be a consistent effect of DEET across species at the neural level. The confusant hypothesis proposes that DEET changes the responses of many chemosensory neurons at the periphery, changing the percept of an otherwise attractive stimulus. In humans, the confusant hypothesis could be described this way: if one could perfectly re-create the smell of opening a box of pizza with vials of odorants presented at just the right concentration, the confusant hypothesis proposes that DEET removes a few of those odors, adds a few more odorants in, and changes the intensity of a few others. These changes should change the percept of the pizza box odor, and should no longer smell like pizza. In this way, the confusant hypothesis does focus on the peripheral sensory system, but is really a hypothesis about the integration of these signals, and so far, the

data from *C. elegans, D. melanogaster,* and *Ae. aegypti* all support this hypothesis. As it becomes more and more possible to study the neural responses to odor blends and begin dissecting how multiple odorants combine to form a percept or indicate an attractive source, studying how DEET affects these neural signatures will help test and refine the confusant hypothesis.

8.5.3 The effects of DEET on individual neurons and receptors

DEET is a synthetic molecule, but is broadly effective. One possible explanation for this effectiveness is that DEET is mimicking a natural compound. One of the current best candidates is methyl jasmonate, a plant defense compound that is important for plant wound healing, has a strong floral odor, and is derived from linoleic acid (Stintzi et al. 2000). Methyl jasmonate is an effective repellent against *Ixodes ricinus* ticks (Garboui et al. 2007) and *Cu. quinquefasciatus* mosquitoes (Xu et al. 2014). However, this compound does not eliminate blood feeding, and therefore seems unlikely to explain the effectiveness of DEET. Further chemoinformatic studies comparing intermediates and other similar compounds could be helpful in determining what makes DEET so useful.



Methyl jasmonate

DEET
N,N-Diethyl-*meta*-toluamide

Figure 8.3 Chemical structures of methyl jasmonate and DEET (A) Methyl jasmonate and (B) DEET

It is also possible that DEET does not mimic a single natural compound, but instead could act as a master key, unlocking responses of many receptors. With a list of DEET-sensitive receptors, and more detailed structures of these receptors, it may be possible to start identifying similarities across these DEET-sensitive proteins, and identify how it is so effective.

Another possibility is that DEET does not interact directly with receptors at all. Syed and Leal proposed that DEET can inhibit the release of odors applied to an arm (Syed et al. 2008), however this was not seen in subsequent experiments measuring odor used for sensillar recordings (Pellegrino et al. 2011). There was also a report that DEET can, *in vitro*, interact with an *Anopheles gambiae* Odorant Binding Protein of unknown functional significance (Tsitsanou et al. 2012). This could be just one more example of the promiscuity of DEET, or it could indicate that looking at the interactions of DEET with aspects of chemosensation that involve steps prior to receptor binding may be fruitful, such as chaperone competition, protein folding, or lipid membrane composition. Alternatively, this could be an artifact of the *in vitro* nature of this experiment, as there was poor binding affinity even at high concentrations of DEET mixed with this protein under specific buffered conditions which may not ever occur in sensilla.

None of these hypotheses are satisfying with our current evidence. Although we propose several methods to evaluate these claims, it is also conceivable that DEET has a much more general mechanism, and does not interact with receptors at all. The difficulty in pinning down the receptors and logic underlying the effectiveness of DEET is reminiscent of work studying anesthetics. Although reports of anesthetics date back to at least the twelfth century, in modern western medical tradition anesthesia was first publically successfully used in 1846 (Juvin et al. 2000). Although this first example used ether, several compounds were quickly identified that exhibit similar anesthetic proper-

ties, and the Meyer-Overton hypothesis claimed it what these compounds had in common was their lipid-solubility (Meyer 1899; Overton 1901). Although this hypothesis fell out of favor and many searched for receptors required for anesthesia for some time, the Meyer-Overton hypothesis has recently made a resurgence (Lugli et al. 2009). In 2017, researchers demonstrated that under anesthesia, the Venus fly trap plant loses autonomous and touch-induced movement, suggesting a very general mechanism that does not require neurons (Yokawa et al. 2017). This finding highlights the broad effectiveness of these compounds, and highlights the futility of focusing on the effects related to a loss of “consciousness”, unless one believes that Venus fly traps are conscious.

The search for the receptors required for DEET-sensitivity may be similarly fraught if DEET acts in a more general way. As early as 1981, Susan McIver proposed that DEET interacts with the lipid cell membranes and perturbs them in such a way that the normal responses to attractants are altered (McIver 1981). DEET is a small, lipophilic molecule, and could passively interact with the cell surface, disrupting some aspect of receptor-lipid interface or the cell surface itself. Plasma membranes are heterogeneous, and the somewhat controversial lipid raft hypothesis posits that lipids form groups or ‘rafts’ that appear to be important for transmembrane protein stability and activity in at least IgE-related signaling (Simons et al. 1997; Field et al. 1995). If DEET interacts with some lipid rafts and not others, it may explain the otherwise seemingly random effects: some odorant receptors may require lipid interactions with one type of raft that DEET can affect, while other odorant receptors may require a different raft for stability, and this may explain why some closely related receptors are affected differently by DEET. Without a multitude receptors to test, it is impossible to pursue this line of inquiry. In one study, several cation channels were found to confer DEET-sensitivity to HEK cells (Ditzen et al. 2008). Using detergents, it is possible to break up cell membranes and identify proteins in different detergent-soluble and insoluble fractions through antibody staining.

If all of the DEET-sensitive channels are found in the same membrane compartments, it would provide evidence that the lipid environment is important for DEET to be able to affect a receptor.

8.6 Mysteries remain

Here we have outlined many directions for further work to build on these data. The description of DEET-sensitive behaviors in *C. elegans* opens up exciting possibilities to better understand chemosensation and odor processing. How can two odorants requiring the same primary chemosensory neuron can lead to entirely different behaviors depending on context? How do differences in calcium imaging translate to behavioral changes? What makes one cell DEET-sensitive and another DEET-insensitive? As we identify additional genes required for DEET-sensitivity both in nematodes and beyond, we can also ask how neurons sensitive to DEET can require a wide variety of seemingly dissimilar membrane proteins, and what makes a protein, cell, or species DEET-sensitive. Studying DEET-sensitivity provides an exciting avenue to further explore these many facets of chemosensory behavior, from the chemical properties to the animal's percept. Even after six years, I am more fascinated by DEET today than I was in 2012, and am excited to see what new discoveries await the next batch of DEET researchers.

METHODS

Animals

Ae. aegypti

Ae. aegypti mosquitoes were reared and housed in mixed-sex cages in a heated and humidified room kept at 25-28C and 70-80% humidity on a 14:10 hour light:dark cycle with *ad libitum* access to 10% sucrose as previously described (DeGennaro et al. 2013). Only female mosquitoes 7-14 days old were used for behavior. Live mice or human volunteers were used as a blood source for egg production as needed, and all procedures with live hosts were approved by IACUC and IRB review. Humans gave their written informed consent to participate in these experiments. All animals used for behavior had never previously blood fed. The $orco^{2}$, $orco^{5}$, and $orco^{16}$ mutant lines were generated in the Orlando strain of *Ae. aegypti* as previously described (DeGennaro et al. 2013). Age-matched mosquitoes reared together and from the same cages were used in behavior experiments done on the same day.

D. melanogaster

Flies were maintained on conventional cornmeal-agar-molasses medium under a 12 h light:12 h dark cycle (lights on 9am) at 25°C and 60% relative humidity.

Select additional arthropods

Centipedes, (Class *Chilopoda*), sow bugs (Genus *Porcellio*), pill bugs (Genus *Armadillium*), and wolf spiders (Family *Lycosidae*) were obtained from Carolina Biological supply and were wild-caught. They were not sexed nor could we determine their age. We did not confirm their exact species. Wolf spiders and centipedes were fed wingless *D. melanogaster* once a day and housed individually. *D. melanogaster*, pill bugs and sow bugs were kept in the presence of food until 1 hour before the assay when they

were kept individually and allowed to acclimate to the room where the experiments were conducted. No anesthesia was used. Animals were given 1-2 minutes to acclimate to the chamber before recording began. Each video was 10 minutes long, and recorded at 1 Hz.

Nematode culture and strains.

C. elegans strains were maintained at room temperature (22-24°C) on nematode growth medium (NGM) plates (51.3 mM NaCl, 1.7% agar, 0.25% peptone, 1 mM $CaCl_2$, 12.9 µM cholesterol, 1 mM $MgSO_4$, 25 mM KPO_4, pH 6) seeded with *Escherichia coli* (OP50 strain) bacteria as a food source (Brenner 1974; Stiernagle 2006). Bristol N2 was used as the wild-type strain. The CB4856 (Hawaiian) strain, harboring WBVar02076179 (*str-217^{HW}*) (http://www.wormbase.org/db/get?name=WBVar02076179;class=variation) and Hawaiian recombinant inbred strains for chromosome V were previously generated (Doroszuk et al. 2009). Generation of extra-chromosomal array transgenes was carried out using standard procedures (Mello 1995), and included the transgene injected at 50 ng/mL, the fluorescent co-injection marker *Pelt-2::GFP* at 5 ng/ml (with the exception of LBV004 and LBV009, which did not include a co-injection marker), and an empty vector for a total DNA concentration of 100 ng/ml. CRISPR-Cas9-mediated mutagenesis of *str-217* was performed as described, using *rol-6* as a co-CRISPR marker (Arribere et al. 2014). The resulting *str-217* mutant strain [LBV004 *str-217(ejd001)*] results in a predicted frame-shift in the first exon [indel: insertion (AAAAAAA), deletion (CTGCTCCA), final sequence GCGTCGAAAAAAAATTTCAG; insertion is underlined]. The *str-217* rescue construct (*Pstr-217::str-217::SL2::GFP*) used a 1112 nucleotide length fragment 56 nucleotides upstream 5' of the translation start of *str-217*.

Human volunteers

All human volunteers gave informed written consent to participate in blood-feed-

ing and under Institutional Review Board protocol LV-0652.

Mice

Animal care procedures were approved and monitored by The Rockefeller University Institutional Animal Care protocol 14756.

Strain list

A complete strain list can be found on the following page.

Text name	Strain name	Genotype	AddGene	References	Notes	Appears in:
Wild-type	N2 (Bristol)	Wild-type	N/A	Fatt 1963	N/A	Figs. 4.1-4.3, 4.5-4.6, 5.1, 5.3, 5.4, 5.6, 6.2, 7.2-7.8
LBV001	LBV001	Unknown	N/A	This thesis	EMS screen, backcrossed 4x	Fig. 5.1
LBV003	LBV002	*nstp-3(ejd002[F48V])*	N/A	This thesis	EMS screen, backcrossed 4x	Figs. 5.1, 5.3, 5.6
LBV004	LBV003	*str-217(ejd003[P314S])*	N/A	This thesis	EMS screen, backcrossed 4x	Fig. 5.1
LBV003 rescue	LBV004	*str-217(ejd003); ejdEx1[pLV001(Pstr-217::str-217::SL2::GFP)]*	pLV001	This thesis	N/A	Fig. 5.6
ewIR73	ewIR73	*str-217(N2)*	N/A	Doroszuk 2009	chrV:~14.0-17.4 Mb CB4856>N2	Fig. 5.4
ewIR74	ewIR74	*str-217(WBVar02076179)*	N/A	Doroszuk 2009	chrV:~14.0-18.6 Mb CB4856>N2	Figs. 5.4, 5.6
ewIR74 rescue	LBV009	*str-217(WBVar02076179); ejdEx1[pLV001(Pstr-217::str-217::SL2::GFP)]*	pLV001	Doroszuk 2009 and this thesis	N/A	Fig. 5.6
ewIR76	ewIR76	*str-217*(N2)	N/A	Doroszuk 2009	chrV:~17.4-21 Mb CB4856>N2	Fig. 5.4
Hawaiian	CB4856	Hawaiian strain	N/A	Hodgkin and Doniach 1997	N/A	Fig. 5.4
str-217-/-	LBV005	*str-217(ejd001)*	(pJA42, pDD162, pLV002)	This thesis	CRISPR-Cas9-induced lesion	Figs. 5.6, 6.2, 7.2 , 7.3, 7.5
str-217-/- rescue	LBV006	*str-217(ejd001); ejdEx2[pLV002(Psrh-220::str-217::mCherry)]*	pLV003	This thesis	N/A	Fig. 5.6
ADL::TeTX	CX12328	*kyEx3438[Psre-1(1kb)::TeTX::SL2:: mCherry + coel::DsRed]*	N/A	Schiavo 1992	N/A	Figs. 6.2, 7.3
ADL::ReaChR	LBV007	*ejdEx3[pES01(Psrh-220::ReaChR)]*	N/A	Lin 2013	Plasmid from the Bargmann lab	Figs. 7.7-7.8
ADL::GCaMP	CX16616	*mzmEx[Psre1::GCaMP5k opt + Psre-1::tagRFP]*	N/A	Jang 2017	N/A	Figs. 6.3-6.4

mutant ADL::GCaMP	LBV008	*217(ejd001);mzmEx[Psre 1:: GCaMP5kopt + Psre-1::tagRFP)]*	N/A	Jang 2012 and this thesis	N/A	Fig. 6.3
rescue ADL::GCaMP	LBV009	*str-217(ejd001); mzmEx[Psre1:: GCaMP5kopt + Psre-1::tagRFP))]; ejdEx2[Psrh-220::str-217::mCherry]*	pLV003	Jang 2012 and this thesis	N/A	Fig. 6.3
AWC::GCaMP	CX17256	*kyIs722[Pstr-2::GCaMP5a]*	N/A	this thesis	Plasmid from Sagi Levy (Bargmann Lab) integrated by UV, backcrossed 4x to N2	Fig. 6.1
str-217-/-; AWC::GCaMP		*str-217(ejd001);kyIs722[Pstr-2::GCaMP5a]*	N/A	this thesis	N/A	Fig. 6.1
odr-1	CX2054	*odr-1(n1936)*		L'Etoile and Bargmann 2000	N/A	Fig. 4.4
odr-7	CX4	*odr-7(ky4)*		Sengupta 1994	N/A	Fig. 4.4
ASK::GCaMP	CX10981	kyEx2866 [sra-9::GCaMP2.2b,unc-122::gfp]		Bargmann lab stock	N/A	Fig. 6.7
ASH::GCaMP	CX10979	*kyEx2865[Psra-6::GCaMP3.0 + Pofm::gfp]*		Hillard 2005	N/A	Fig. 6.6
ASH::mCasp	CX14597	*ls[sra-6p::mouseCaspase1]*		Yoshida 2012	N/A	Fig. 6.6
unc-31	CB928	*unc-31(e928)*		Jorgensen 2002	N/A	Fig. 6.4
unc-13	BC168	*unc-13(s69)*		Richmond 1999	N/A	Fig. 6.2
ASE::GCaMP	IV10	*ueEx7[gcy-7::GCaMP3, unc-122::gfp]*		Bargmann lab stock	N/A	Fig. 6.7
AWB::GCaMP	CX8446	*kyEx1423 [str-1::GCaMP 1.0, unc-122::gfp]*		Bargmann lab stock	N/A	Fig. 6.7
AWA:GCaMP	CX16152	*kyEx5511[gpa-6::NLS-GCaMP6S::SL2::NLS-mCherry, myo-3::mCherry]*		Bargmann lab stock	N/A	Fig. 6.7
Pptr10:: myrRFP	OS1907	nsIs108(*Pptr-10::myrRFP*)	N/A	Yoshimura 2008	N/A	Referenced in text

Glytube feeding (Fig. 2.8)

Groups of 12-15 female mosquitoes were mouth aspirated into cups and starved overnight 20-24 hours with access to water. These groups were then fed sheep blood using the Glytube membrane feeders as described (Costa da Silva 2013) with the variation that DEET in ethanol solvent or quinine (CID: 23424040, Sigma-Aldrich catalog #Q0132) in ethanol solvent were either added to the blood just before loading, or the assembled Glytube was dipped into a DEET or quinine solution just before placing the Glytubes on the top of the mesh covering each cup of mosquitoes. Animals were allowed to feed for 10 minutes. Next, the Glytube was removed and mosquitoes were moved to 4°C and fed females were scored by eye for engorgement and coloration of the abdomen.

Capillary feeder (CAFE) (Fig. 2.6)

Animals used for behavior were 7-14 day old female mosquitoes, sexed and sorted under cold anesthesia (4°C) and fasted 40-48 hours with access to water. This assay was adapted for the mosquito from similar assays for *Drosophila* (Ja et al. 2007) as described previously (Corfas et al. 2015). At the start of each trial five fasted female mosquitoes were transferred by mouth pipette to a polypropylene vial (#89092–742, VWR, Radnor, PA) with access to two 5 mL calibrated glass capillaries (#53432–706, VWR) embedded in cotton plugs (#49-101, Genesee Scientific, San Diego, CA) and barely protruding from the bottom of the plug surface. A small piece of red lab tape (VWR #89097-932) was also affixed to the bottom of the plug. One capillary served as the control, containing 10% (weight:volume) sucrose solution (Fisher Scientific 57-50-1) and 1% ethanol solvent (Millipore Sigma E7023) and the other had 10% sucrose with either 1% DEET and 1% ethanol (CID 24893319, Millipore Sigma catalog #D100951), 1mM lobeline (CID 101615, Millipore Sigma catalog #141879) and 1% ethanol, or 5mM

quinine and 1% ethanol. After four hours, the remaining liquid in all capillaries was manually measured by a blinded observer using a ruler and recorded to the nearest 0.1 millimeter. Control vials with capillaries but without mosquitoes were averaged to determine evaporation, and that value subtracted from measured values. Preference assays were measured by subtracting the amount consumed from the control capillary minus the amount consumed from the stimulus capillary and dividing by the total amount consumed in both capillaries. Time of day was randomized across conditions were blinded before manual scoring. Vials were excluded if any animals died during the assay.

Calculations were completed as follows. Mosquito-less control vials served as evaporation controls. An average evaporation amount for each batch of experiments was calculated (EVAP). For each vial of 5 mosquitoes, the drop in liquid level was manually measured with a ruler in millimeters (CONTROL and CHEMICAL). Then, the liquid consumed was calculated as follows for each vial: [(CHEMICAL – EVAP) – (CONTROL – EVAP)] / [(CHEMICAL – EVAP) + (CONTROL – EVAP)].

Mosquito biting assays

Landing assay (Fig. 2.2, 2.3, 2.13, and 2.14)

The landing assay was carried out similarly to the bitter feeding assays with the modification that a video camera was introduced into the cage perpendicular to the arm, the blood feeding status was not scored at the end of the assay but instead the resulting videos were scored manually for landing and biting events.

The number of landings per mosquito and biting events per mosquito were calculated by counting the number of events in the video, and dividing by the total number of mosquitoes in the cage (23-25). The average time spent on skin was calculated by adding together the lengths of each landing event in a single video, and dividing by the

total number of landing events in that video.

For analysis of non-DEET repellents, Picaridin (CID: 125098, a generous gift from Saltigo), MDA (methyl *N, N*-dimethylanthranilate, CID:82336 , Millipore Sigma catalog #PH011027), BA (butyl anthranilate, CID:24433, Millipore Sigma catalog #W218103), EA (ethyl anthranilate, CID: 6877, Millipore Sigma catalog #242977), ethanol, or the test compound was applied to skin prior to the subject putting on the glove.

Bitter compounds and blood-feeding (Fig. 2.7)

Standard arm-in-cage biting assays were carried out (Logan et al. 2010; Schreck 1977) with modifications as previously described (DeGennaro et al. 2013). Animals used for behavior were 7-14 day old mosquitoes, sexed and sorted under cold anesthesia (4°C) into groups of 25 females or 25 males, and fasted 18-24 hours with access to water. All assays were carried out ZT6-ZT10 in a heated and humidified room kept at 25-28°C and 70-80% humidity. A 25 mm diameter hole was cut into an elbow-length glove (Fisher Scientific #19-668-001). A group of 25 starved females were released and given five minutes to acclimate to the cage. During this time, 1 mL of either solvent or a test substance in solvent (lobeline, quinine, or DEET) was added to the skin of a human volunteer (27-year-old female). The glove was then stretched over the arm exposing only the treated, 25mm diameter area of the arm. After the five-minute acclimation, the treated arm was placed in the cage and held there for five minutes. After ten minutes, the arm was removed and cage moved to 4°C to anesthetize the animals. Animals were scored as blood-fed or non-blood-fed based on abdominal distention and coloring. No synthetic CO_2 was added to these cages but assays were carried out in close proximity to a breathing human subject.

At the end of the experiment, the cage was placed at 4°C to anesthetize the mosquitoes, and animals were sorted into fed and unfed based on color and abdominal

distention. Animals appearing unfed were squished in a clean tissue and counted as fed if any red blood was visible, and unfed if the excretions were white or yellow.

The percent blood-fed was calculated by counting the number of fed mosquitoes divided by the total number of mosquitoes, multiplied by 100.

Proboscis sufficiency assay (Fig. 2.9)

Standard arm-in-cage biting assays were carried out (Logan et al. 2010; Schreck 1977) with modifications as previously described as the mosquito landing assay (DeGennaro et al. 2013) with the additional modification that the hole cut in the glove was either 25 mm in diameter or 1.5 mm and the arms were placed on the side of the cage pressing against the mesh. This served to decrease the surface area that the mosquitoes would walk around on before finding the hole in the glove. Mosquitoes were able to bite through the mesh easily.

Biting index was calculated for each video by dividing the number of animals biting by the number of animals in the assay.

Occlusion assays (Fig. 2.10 and Fig. 2.11)

This assay was carried out similarly to the landing assay with several modifications. Standard arm-in-cage biting assays were carried out (Logan et al. 2010; Schreck 1977) with modifications as previously described as the mosquito landing assay (DeGennaro et al. 2013) with a 25 mm hole cut into the glove. Animals used for behavior were 7-14 day old female mosquitoes, were aspirated by mouth into cups and under cold anesthesia (in cups on wet ice) in groups of five 18-24 hours before the start of the experiment. Tarsi were inserted one at a time into a 1 mL pipette tip containing UV curing glue (Kemxert KOA 300-1) and cured with a 405 nm 5mW laser pointer (QQ-Tech) for 20 seconds. Tibia controls were treated similarly with the exception of glue

being slowly applied to the tibia through a 200 µL pipette tip until coated, then cured for 20 seconds. No-glue control animals had tarsi dipped into an empty 1 mL pipette tip and then exposed to the laser for 20 seconds. Animals were then grouped and housed 18-24 hours with access to water in groups of five females with the same glue treatment. If any animals died overnight, that group of females was discarded. If zero animals interacted with the skin, the video was excluded.

Biting index was calculated for each video by dividing the number of animals biting by the number of animals in the assay.

Split Petri-dish assay (Fig. 3.2 and 3.4)

Custom designed split-Petri dishes were 3D printed on a Project 3510 HD Plus 3D Printer using VisiJet Crysta plastic and rings were cut on a VLS 6.60 Laser Cutter. Mesh was attached to the surface of the laser cut acrylic using acetone.

Each chamber was filled with 2% agarose (Sigma Aldrich, 11685660001) containing either DEET or solvent. The agar mixtures were prepared by first heating a water and powdered agar mixture until dissolved. Once cooled, 50% DEET in ethanol or ethanol solvent was added in a 1:49 ratio of DEET or solvent to agar mixture for a final concentration of 1% DEET and 1% ethanol or 2% ethanol.

Chemotaxis assays

Chemotaxis was tested as described (Cho et al. 2016), on square plates containing 10 mL of chemotaxis agar (1.6% agar in chemotaxis buffer: 5 mM phosphate buffer pH 6.0, 1 mM $CaCl_2$, 1 mM $MgSO_4$) (Hart 2006). Additions of either ethanol (solvent-agar) or 50% DEET (CID: 4284, Sigma-Aldrich, catalog #D100951) in ethanol (DEET-agar) were added after agar cooled to <44°C and just before pouring. A total volume of 300 µL ethanol or DEET in ethanol was added to each 100 mL of agar mix-

ture for all experiments except Figure 4.1, 7.6 – 7.8, where nothing was added. Plates were poured on the day of each experiment, and dried with lids off for 4 hours prior to the start of the assay. 1 µl 1 M sodium azide was added to two spots on either side of the plate just before beginning the experiment to immobilize animals that reached the odorant or ethanol sources. Three days prior to all chemotaxis experiments, 4-6 L4 animals were transferred onto NGM plates seeded with *E. coli* (OP50 strain). The offspring of these 4-6 animals were then washed off of the plates and washed twice with S-Basal buffer (1 mM NaCl, 5.74 mM K_2HPO_4, 7.35 mM KH_2PO_4, 5 µg/mL cholesterol at pH 6-6.2) (Lin et al. 2013) to remove younger animals, and once with chemotaxis buffer. Immediately before the start of the experiment, two 1 µl drops of odorant diluted in ethanol, or ethanol solvent control, were spotted on each side of the plate on top of the sodium azide spots. 100-300 animals were then placed into the center of the plate in a small bubble of liquid. The excess liquid surrounding the animals was then removed using a Kimwipe. Odorants diluted in ethanol were used in this study: 1:1000 isoamyl alcohol (CID: 31260, Sigma-Aldrich, catalog #W205702), 1:1000 butanone (CID: 6569, Sigma-Aldrich, catalog #360473), 10 mg/µL pyrazine (CID: 9261, Sigma-Aldrich, catalog #W401501), 1:10 2-nonanone (CID: 13187, Sigma-Aldrich, catalog #W2787513). Assays were carried out for 60-90 minutes at room temperature (22-24°C) between 1pm – 8pm EST. Plates were scored as soon as possible, either immediately or, if a large number of plates was being scored on the same day, plates were moved to 4˚C to immobilize animals until they could be scored. The assay was quantified by counting animals that had left the origin in the center of the plate, moving to either side of the plate (#Odorant, #Control) or just above or below the origin (#Other), and calculating a chemotaxis index as [#Odorant - #Control] / [#Odorant + #Control + #Other]. A trial was discarded if fewer than 50 animals or more than 250 animals contributed to the chemotaxis index and participated in the assay.

Bacterial chemotaxis (Fig.4.6)

Plates were prepared as described for chemotaxis plates and as described previously (McMullan et al. 2012) with the following exceptions. Two hours into the four-hour drying time of the chemotaxis plates, two spots of 20 µL of LB media or two spots of *E. coli* (OP50 strain) bacteria grown in LB media overnight and diluted to OD 1.0 at 600 nm was applied to the chemotaxis plate. Two spots of 20 µL of media were applied to the other side of the plate and then left undisturbed for the remainder of the drying time.

Forward genetic *C. elegans* screen

About 100 wild-type (Bristol N2) L4 animals were mutagenized in M9 solution with 50 mM ethyl methanesulfonate (CID: 6113, Sigma-Aldrich, catalog #M0880) for 4 hours with rotation at room temperature. Mutagenized animals were picked to separate 9 cm NGM agar plates seeded with *E. coli* (OP50 strain) and cultivated at 20°C. ~5,000 F2 animals were screened for DEET resistance on 20.3 cm casserole dishes (ASIN B000LNS4NQ, model number 81932OBL11). Five animals across three assays were more than ~2 cm closer to the odor source than the rest of the animals on the plate and were defined as DEET-resistant. This phenotype was heritable in three strains, and each strain was backcrossed to OS1917 (Yoshimura et al. 2008) for 4 generations. Whole-genome sequencing was used to map the mutations to regions containing transversions presumably introduced by the EMS mutagenesis, parental alleles of the N2 strain used for mutagenesis, and missing alleles of the wild-type strain OS1917 used for backcrossing (Zuryn et al. 2010; Kutscher L. M. 2014). LBV003 mapped to a 5 Mb region on chromosome V, which was further mapped to *str-217*. LBV002 mapped to a 6.8 Mb region on chromosome V, which was further narrowed down to a likely candidate gene, *nstp-3(ejd002)*. In LBV002, *nstp-3(ejd002)* contains a T>G transversion of the 141st nucleotide in the coding sequence, which is predicted to produce a Phe48Val

substitution in this sugar:proton symporter. We were unable to map the DEET-resistant mutation(s) in LBV001.

Microscopy and image analysis

L2-adult stage hermaphrodites were mounted on 1% agarose pads with 10 mM sodium azide (CID 6331859, Sigma-Aldrich, catalog #S2002) in M9 solution (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85.6 mM NaCl, 1mM $MgSO_4$, pH 6). Images were acquired with an Axio Observer Z1 LSM 780 with Apotome a 63X objective (Zeiss), and were processed using ImageJ.

***str-217* heterologous expression.**

HEK-293T cells were maintained using standard protocols in a Thermo Scientific FORMA Series II water-jacketed CO_2 incubator. Cells were transiently transfected with 1 µg each of pME18s plasmid expressing *GCaMP6s*, $Gq_{\alpha}15$, and *str-217* using Lipofectamine 2000 (CID: 100984821, Invitrogen, catalog #1168019). Control cells excluded *str-217*, but were transfected with the other two plasmids. Transfected cells were seeded into 384 well plates at a density of 2 x 10^6 cells/ml, and incubated overnight in FluoroBrite DMEM media (ThermoFisher Scientific) supplemented with fetal bovine serum (Invitrogen, catalog #10082139) at 37°C and 5% CO_2. Cells were imaged in reading buffer [Hanks's Balanced Salt Solution (GIBCO) + 20 mM HEPES (Sigma-Aldrich)] using GFP-channel fluorescence of a Hamamatsu FDSS-6000 kinetic plate reader at The Rockefeller University High-Throughput Screening Resource Center. DEET was prepared at 3X final concentration in reading buffer in a 384-well plate (Greiner Bio-one) from a 46% (2 M) stock solution in DMSO (Sigma-Aldrich). Plates were imaged every 1 second for 5 minutes. 10 µl of DEET solution in reading buffer or vehicle (reading buffer + DMSO) was added to each well containing cells in 20 µl of media after 30 seconds of baseline fluorescence recording. The final concentration of vehicle DMSO was matched

to the DEET additions, with a maximum DMSO concentration of 7.8%. Fluorescence was normalized to baseline, and responses were calculated as max ratio (maximum fluorescence level/baseline fluorescence level).

Calcium imaging

ADL calcium imaging.

Calcium imaging and data analysis were performed as described (Larsch et al. 2015), using single young adult hermaphrodites immobilized in a custom-fabricated 3 x 3 x 3 mm polydimethylsiloxane (PDMS) imaging chip. *GCaMP5k* was expressed in ADL neurons under control of the *sre-1* promoter (Jang et al. 2012) and was crossed into *str-217*$^{-/-}$ and the *str-217*$^{-/-}$ rescue strain. Animals were acclimated to the imaging room overnight on *E.coli* (OP50 strain) seeded plates. All stimuli were prepared the day of each experiment, and were diluted in ethanol to 1000X the desired concentration before being further diluted 1:1000 in S-Basal buffer. Young adult animals were paralyzed briefly in (-)-tetramisole hydrochloride (CID: 27944, Sigma-Aldrich, catalog #L9756) at 1 mM for 2-5 minutes before transfer into the chip to paralyze body wall muscles to keep animals stationary during imaging. All animals were pre-exposed to light (470+/- 40 nm) for 100 seconds before recording to attenuate the light response of ADL (Jang et al. 2017). Experiments consisted of the following stimulation protocol: 20 seconds of S-Basal buffer, followed by 3 repetitions of 20 seconds of DEET (0.15% DEET and 0.15% ethanol in S-Basal) and then 20 seconds of S-basal buffer.

GCaMP signals were recorded with Metamorph Software (Molecular Devices) and an iXon3 DU-897 EMCCD camera (Andor) at 10 frames/second using a 40x objective on an upright Zeiss Axioskop 2 microscope. Custom ImageJ scripts (Cho et al. 2016) were used to track cells and quantify fluorescence. In Figures 6.3 and 6.4, all

frames in 20 seconds before the DEET pulse were averaged and subtracted from the average of the frames during the 20 seconds of DEET or C9 pulse to calculate ΔF. In Figures 6.3 and 6.4, traces were bleach corrected using a custom MATLAB script and then the 5% of frames with the lowest values were averaged to create $F_{0.}$ $\Delta F/F_0$ was calculated by $(F - F_0)/F_0$ and then divided by the maximum value to obtain $\Delta F/F_{max}$ (Larsch 2013). The heatmap traces in Figure 3e and g were also smoothed by 5 frames, such that each data point *n* is the running average of *n-2, n-1, n, n+1, and n+2*.

Arena Chip: AWC () and AWA, AWB, ASE, and ASK

Calcium imaging of freely moving worms and subsequent data analysis were performed as described (Larsch et al. 2013), using a 3 mm^2 microfluidic PDMS device with two arenas that enabled simultaneous imaging of two genotypes with approximately 10 animals each. We used an integrated line (CX17256) expressing GCaMP5a in AWC^{ON} neurons under control of the *str-2* promoter crossed into *str-217*$^{-/-}$ animals. Adult hermaphrodites were first paralyzed for 80-100 minutes in 1 mM (-)-tetramisole hydrochloride and then transferred to the arenas in S-Basal buffer. The stimulus protocol for AWC imaging was as follows: In S-Basal, three pulses of 60 seconds in buffer and 30 seconds isoamyl alcohol, followed by 120 seconds in buffer. Next, the animals were switched to S-Basal with 0.15% ethanol (solvent buffer) and three pulses of 60 seconds in buffer and 30 seconds in isoamyl alcohol in solvent buffer followed by 120 seconds in solvent buffer before a switch to S-Basal with 0.15% ethanol and 0.15% DEET (DEET buffer). In DEET buffer, animals were given 6 pulses of 60 seconds in DEET buffer and then 30 seconds in isoamyl alcohol in DEET buffer, followed by 120 seconds in DEET buffer before switching to solvent buffer. In solvent buffer, the animals received three pulses of 60 seconds in buffer and 30 seconds in isoamyl alcohol in solvent buffer followed by 120 seconds in solvent buffer before a switch to S-Basal. In S-Basal, the animals received three pulses of 60 seconds in buffer and 30 seconds isoamyl alcohol,

followed by 60 seconds in buffer.

Images were acquired at 10 frames/second at 5X magnification (Hamamatsu Orca Flash 4 sCMOS), with 10 msec pulsed illumination every 100 msec (Sola, Lumencor; 470/40 nm excitation). Fluorescence levels were analyzed using a custom ImageJ script that integrates and background-subtracts fluorescence levels of the AWC neuron cell body (6×6 pixel region of interest). Traces were normalized by subtracting and then dividing by the baseline fluorescence, defined as the average fluorescence of the last 2 seconds of the first three isoamyl alcohol pulses. The traces in Extended Data Figure 1 were also smoothed by 5 frames, such that each data point *n* is the running average of *n-2, n-1, n, n+1, and n+2*. The response magnitudes were calculated by taking the mean of the last 2 seconds of an isoamyl alcohol pulse, subtracting the mean of the 2 seconds before the isoamyl alcohol pulse (F0), and dividing by this F0. The response magnitudes were calculated for the 5^{th} (0.15% ethanol in S-Basal buffer), 8^{th} (0.15% DEET and 0.15% ethanol in S-Basal buffer), and 14^{th} (0.15% ethanol in S-Basal buffer) isoamyl alcohol pulses. We also quantified the response magnitude of the transition from S-Basal buffer with ethanol to S-Basal buffer with DEET. We took the mean of the first 2 seconds after switching to DEET buffer, subtracted the mean of the 2 seconds before switching (F0), and divided by this F0.

For AWA, AWB, ASE, and ASK experiments, the stimuli were instead delivered as indicated. For Protocol 1, animals were kept in S-Basal buffer, and exposed to one minute pulses as follows: one minute in S-Basal, one minute in 0.15% ethanol in S-Basal buffer, one minute in S-Basal buffer, one minute in 0.15% DEET and 0.15% ethanol in S-Basal buffer, one minute in S-Basal buffer, one minute in 10^{-5} diacetyl in S-Basal buffer, and one minute in S-Basal buffer. For Protocol 2, animals started in S-Basal buffer for one minute, exposed to 0.15% ethanol in S-Basal buffer for one minute, switched to 0.15% DEET and 0.15% ethanol in S-Basal buffer for one minute, switched back to

0.15% ethanol in S-Basal buffer for one minute, and then returned to S-Basal buffer for one minute.

Chemotaxis tracking and analysis.

8-20 adult hermaphrodites were first transferred to an empty NGM plate and then 4-15 were transferred to an assay plate to minimize bacterial transfer. Animals were then placed in the center on either a 0.15% DEET-agar or solvent-agar plate, and their movement was recorded for 60 minutes at 3 frames/second with 6.6 MP PL-B781F CMOS camera (PixeLINK) and Streampix software. Assays were carried out at room temperature, between 12pm-8pm, and lit from below. Worm trajectories were extracted by a custom Matlab (MathWorks) script (Cho et al. 2016), and discontinuous tracks were then manually linked. Tracks were discarded if the animal moved less than two body lengths from its origin over the course of the 60 minute trial. If an animal came within 1cm of the isoamyl alcohol stimulus, the track was truncated to remove information from animals immobilized at the odor source because of the addition of sodium azide.

ADL optogenetic stimulation.

L4 animals expressing an *Psrh-220::ReaChR* (Lin et al. 2013) array or array-negative animals from the same plate were raised overnight in the dark on an NGM plate freshly seeded with 100 µL of 10X concentrated *E. coli* (OP50 strain) with or without 50 µM all-*trans* retinal (CID: 720648, Sigma-Aldrich, catalog #R2500), which is required for ReaChR-induced activity. The next day, adult hermaphrodites were first transferred to an empty NGM plate and then 4-15 animals were transferred to the 10 cm circular assay plate to minimize bacterial transfer. Videos were recorded for 26 minutes at 3 frames/second with a 1.3 MP PL-A741 camera (PixeLINK) and Streampix software.

Blue light pulses were delivered with an LED (455 nm, 20 μW/mm^2, Mightex) controlled with a custom Matlab script (Gordus et al. 2015; Cho et al. 2016). Animals were exposed to normal light for 120 seconds, before exposure to 12 pulses of blue light (455 nm, 10 Hz strobing) for 120 seconds, followed by 120 seconds of recovery. This should activate ADL neurons only in retinal-fed animals expressing ReaChR. Worm trajectories were extracted by a custom Matlab script (Gordus et al. 2015). Pausing events were extracted, and all pauses ≥3 frames (1 second) were used for further analysis. Pauses were classified as “ON” if any frame included light illumination. A pause that began just before illumination began, but remained paused while the illumination occurred, was considered an ON pause, just as a pause that occurred in the middle of a light illumination time frame was considered ON. All other pauses were classified as “OFF” pauses. In the analysis in Figure 7.8A, we took an average pause length for all ON pauses and all OFF pauses for each animal, and pooled all of the animals on each plate. To control for any baseline differences between animals and experiment-to-experiment variation, we examined the increase in average pause length in Figure 7.8B.

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