UNIVERSITY OF CALIFORNIA
RIVERSIDE

Conservation of Avoidance Behavior in *Drosophila* Species Exposed to Volatile Repellents

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

by

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December 2016

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ACKNOWLEDGEMENTS

Professional:

1. Portions of the research presented in chapters II and III was published: Krause Pham C, Ray A (2015) Conservation of olfactory avoidance in *Drosophila* species and identification of repellents for *Drosophila suzukii*. Sci Rep 5:11527.
2. *Drosophila* Species Stock Center at the Unviersity of California, San Diego provided non- melanogaster species and newly caught *D. melanogaster*. Dr. Richard Stouthamer donated *D. suzukii*.
3. Sana Tharadra conducted single-sensillia electrophysiological recordings for responses to carbon dioxide in different species.
4. CRISPR design and molecular cloning were done by Dr. Maria Irigoyen, Ph.D.
5. Compounds tested were hypothesized to be repellents in a computational analysis done by Dr. Sean Michael Boyle, Ph.D.
6. Dr. Wynand Van der Goes van Naters provided comments about *D. suzukii* electrophysiological responses to food sources.
7. To my committee, Dr. Anand Ray, Dr. Anupama Dahanukar, Dr. Michael Adams and Dr. Linda Walling for their never ending support during this process and critical reading of this manuscript.
8. Funding from Dr. Anand Ray's grants and UC Riverside Graduate Division for financial support.

Personal:

In scientific disciplines, it truly takes a village to transform a first year graduate student into a PhD holder who can go on to make a mark on the world. Thank you to all of the people, former and current members of the Ray and Dahanukar Labs. Your efforts to keep our lab running smoothly and conversations to push our science further were critical. My colleagues in the Adams and Yamanka labs broadened my horizons and understanding of neuroendocrinology in insects. Faculty and students in the Interdepartmental Neuroscience Program and in particular my class cohort, Kevin, Tom, Kelly and Zev created a rich learning environment for me to grow. The Entomology Department helped me to expand my knowledge of insects. Finally, my family and friends for your support, I would not have made it without your encouragement.

ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Neuroscience
University of California, Riverside, December 2016
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Insects are a highly successful class of arthropods consisting of diverse species adapted to live in many environments across the globe. Insects can increase their survivability by avoiding harmful compounds in their environment. Here, we investigate innate avoidance pathways from five species of *Drosophila* at different evolutionary distances to determine the degree to which different species have adapted to avoid odorants in the environment. In examining *D. melanogaster, D. yakuba, D. suzukii, D. pseudoobscura,* and *D. virilis,* we have determined that avoidance to repellents such as carbon dioxide, ethyl-3-hydroxybutyrate, and citronellal vary greatly across these species. For example, *D. melanogaster* robustly avoids carbon dioxide, while *D.suzukii* has olfactory neurons that can respond to carbon dioxide but behaviorally does not avoid carbon dioxide. On the other hand, DEET, a synthetic chemical, is observed to be highly repellent across all species tested behaviorally. In this analysis, the role of olfactory neurons in DEET avoidance is investigated. In addition, the relationship between compound vapor pressure and avoidance is tested.

Results presented here imply that the chemosensory mechanism these *Drosophila* species use to avoid DEET is dependent on multiple factors and complex. Nevertheless, we have identified several related chemicals that appear to be highly repellent for *D. melanogaster*.

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Chapter 1: Introduction

Sensory systems are critical to an organism's survival. Like higher organisms, insects have developed sensory systems to detect chemicals, sound and light in their environment. Chemosensory systems play a prominent role in an insect's ability to be successful by helping the animal to forage for food and to reproduce. Olfactory systems allow for insects to interact with other insects by sensing and responding to pheromones from conspecifics and kairomones from species where they have a predator-prey relationship (Ebrahim et al., 2015). Many insects are attracted to plants volatiles. Plants produce defensive chemicals to protect themselves from insect feeding in the form of repellents (both benign and toxic to the insect) (Versace et al., 2016). Other insects such as female mosquitoes and bed bugs need to locate hosts to blood-feed for reproduction or their survival. Processes such as attraction are well studied. Less is known about how insects use chemosensation in repellency. Understanding aversive mechanisms in insects is of high importance to humans because avoidance cues may deter blood-feeding insects that transmit disease as well as protect food crops that insects feed on.

Historically, *Drosophila melanogaster* has been the model insect organism. Scientists have studied the vinegar fly for over 100 years in the laboratory. Naturally occurring random mutations and later mutations generated by exposing flies to chemicals that cause mutations (such as EMS) or x-rays

allowed scientists to isolate flies (in laborious screens) with specific gene deficiencies. Later it was found that flies have transposable elements that can be harnessed to delete and/or insert specific genes into their genome. Many genes have been “knocked out” or removed from the fly genome to allow for studies of flies to determine the function of individual genes. Also, transposable elements can insert in multiple locations of a genome causing extra copies of a gene to be transcribed and translated in a cell. This allows for studies to determine the effect of over-expression of a gene of interest. These tools, as well as a newer technology, CRISPR-Cas9 genome editing (that will be discussed in chapter four) have allowed creation of fly lines that have non-functional or completely absent proteins that would ordinarily form receptors on the olfactory receptor neurons. In chapter four, we use *Drosophila melanogaster* mutant in olfactory receptor genes to better understand ways DEET activates chemosensory circuits.

In *D. melanogaster* olfactory sensory neurons are housed in sensilla located on two pairs of olfactory organs: a pair of antenna and a pair of maxillary palps (Laissue and Vosshall, 2008). Sensilla of different morphological types are located in a stereotypical pattern on the antenna and palps. Neurons send their dendrites into the sensilla and are surrounded by lymph. Responses to odorants can be measured using electrophysiology by exposing the fly to odorants and measuring the responses in the sensilla. Each Olfactory Receptor (OR) gene has been shown by *in situ* hybridization analysis to be expressed in a

subpopulation of olfactory receptor neurons (ORN) in these two olfactory organs (de Bruyne et al., 1999). Generally, each ORN expresses one OR gene (Couto et al., 2005). ORNs are housed in sensilla of four morphological types: small basiconic, large basiconic, trichoid, and coeloconic sensilla. Olfactory neurons are named based on a four-part code:

1) the sensory organ (a for antenna, p for palp)
2) the sensilla (basiconic (b), tricoid (t) and coeloconic (c)) in which it is located
3) a number designating the different type of neuron defined by tuning to odorants
4) letters A-D for the 1-4 neurons per sensilla

For example, the Or56a receptor is expressed in the ab4B neuron, which places it on the antenna (a) in the fourth basiconic sensilla (b4) and is the neuron with the second largest action potential spike amplitude (B) in this sensillum. Active ORs require expression of two proteins. One protein determines the unique ligand-binding site for the receptor and the other protein is a co-receptor referred to as Orco (formerly Or83b), which forms a ligand-gated ion channel (Sato et al., 2008),(Larsson et al., 2004).

Olfactory sensory neurons expressing one receptor type send axons to a distinct target glomerulus in the antennal lobe of the brain (Fishilevich and Vosshall, 2005). Glomeruli are named according to their three dimensional spatial location in the antennal lobe. Olfactory receptor neurons are cholinergic (Kazama and Wilson, 2009). Sensory processing occurs in the antennal lobe,

where ORN are pre-synaptically inhibited by GABAergic local interneurons (Olsen and Wilson, 2008). In addition, excitatory interneurons synapse onto inhibitory local interneurons and the projection neurons that target locations in the lateral horn of the protocerebrum (Yaksi and Wilson, 2010) for innate responses. Additional processing in the antennal lobe occurs with excitatory gap-junction neurons (Huang et al., 2010). Third order projections go to the lateral horn of the protocerebrum (LH) and/or Kenyon cells in the mushroom body (MB).

Multiple types of neurons may be involved to accommodate changes in state. For example, it was shown that two classes of projection neurons activated by the olfactory neurons expressing the Ir64a receptor form connections outside of the antenna lobe (Gao et al., 2015). One type of projection neuron targets the lateral horn of the protocerebrum as expected; however, a second structurally different projection neuron has been identified that bifurcates and connects to both the lateral horn and the mushroom body (Lin et al., 2013).

In *D. melanogaster*, sensory neurons interpreting chemicals in the environment generally express one of three classes of identified chemoreceptors: (1) Olfactory Receptor, (OR), (2) Gustatory Receptor, (GR) and (3) Ionotropic Receptor, (IR). Initial identification of these receptor families occurred with bioinformatic approaches, which identified proteins with putative seven transmembrane domains, and selectively expressed in neurons of the

olfactory systems (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999; Abuin et al., 2011) and gustatory systems (Robertson et al., 2003) . The evolutionary conserved class of Ionotropic Receptors evolved from ionotropic glutamate receptors, which are thought to have lost the ability to bind glutamate and instead bind a variety of acids, amines and aldehydes (Croset et al., 2010; Silbering et al., 2011). Members of the OR family have one co-receptor called Orco, while IRs have at least two co-receptors, generally Ir8a and Ir25a, but sometimes Ir76b (Benton et al., 2009; Abuin et al., 2011). Olfactory avoidance circuits in *D. melanogaster* are constructed with sensory neurons containing receptors from each class.

The most robust olfactory avoidance pathway in *D. melanogaster* is activated by a complex formed from gustatory receptor proteins Gr63a and Gr21a that are housed in the ab1C neuron on the antenna (Suh et al., 2004). Carbon dioxide is a strong ligand for this receptor; however, the receptor can also be inhibited when exposed to some odorants from fermenting fruit (Turner and Ray, 2009).

Or85a is expressed on the ab2B neuron and is strongly activated by ethyl-3-hydroxybutyrate (Hallem et al.). In addition, activation of this receptor can lead to avoidance behavior in flies simultaneously exposed to otherwise attractive odorants (Semmelhack and Wang, 2009). Or56a located in ab4B was initially shown to be activated by geosmin, a compound produced by harmful microbes on yeast (Stensmyr et al., 2012). More recently, fenchone and α-

ionone have been shown to be additional ligands for this receptor (Münch and Galizia, 2016).

In *D. melanogaster*, citronellal activates two neurons classes, ab11A and ab12A (Kwon et al., 2010). In further mutant screening, activation of ab11A was shown to be independent of *Orco* and ab12A dependent on *Orco*. TrpA1 was the channel mediating the response in the ab11A neuron. The specific olfactory receptor activated by citronellal is not known.

Drosophila as a genus has species that have evolved to adapt to a wide range of conditions on the planet. *Drosophila melanogaster* was the first fruit fly to have its genome sequenced (Adams et al., 2000). Subsequently genome sequences were obtained for *D. pseudoobscura* (Richards et al., 2005) in 2005 and then ten additional species *D. sechellia, D. simulans, D. yakuba, D. erecta, D. ananassae, D. persimilis, D. willistoni, D. mojavensis, D. virilis and D. grimshawi* (Clark et al., 2007). More recently, in 2013, the sequence for *D. suzukii* became available (Chiu et al., 2013). Wild populations of *Drosophila* need carbohydrate sources that yeast can feed on to survive. Wild species don't breed and feed abundantly on domesticated food sources (Carson and Stalker, 1951). In Chapter 2 we investigated if these species would avoid compounds *D. melanogaster* found aversive. We found that the avoidance response to DEET (a man-made chemical) is the most conserved repellent across the species.

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Chapter 2: Conservation of olfactory avoidance behavior in *Drosophila* species

Abstract

Attractive odorants have been studied extensively in *Drosophila* species, but little is understood about the role of avoidance pathways. Repellents are important because they cue insects to avoid chemicals in the environment that may be toxic. We investigated known innate avoidance pathways from five species at different evolutionary distances: *D. melanogaster, D. yakuba, D. suzukii, D. pseudoobscura* and *D. virilis*. First we examined carbon dioxide, a strong repellent and a robust activator of the ab1C neuron in *D. melanogaster* across multiple species using electrophysiological and behavioral analysis. Electrophysiology showed carbon dioxide activation of the ab1C-like receptor neuron to be well conserved in the above species; however aversive behavioral responses are not as conserved as one would expect. Next using a variety of established behavioral assays we investigated behavioral output of exposure to other known repellents. Surprisingly, only DEET showed strong repellency across all species, whereas CO_2, citronellal and ethyl 3-hydroxybutyrate showed only limited conservation. These findings highlighted a need for discovery of new repellents that activate a highly conserved pathway rather than approaching species-specific avoidance mechanisms.

Introduction

While attractive odor cues emitted from food play an important role in the differential selection process across species (Keesey et al., 2015; Revadi et al., 2015), here we investigated the less studied changes in behavior towards aversive cues. Previously published data have established that *Drosophila melanogaster* avoids odorants activating a number of types of receptors in olfactory organs: (1) Gr63a/Gr21a heterodimer receptor (i.e. ab1C neuron) (Suh et al., 2004), (2) the Or85a receptor (i.e. ab2B neuron)(Semmelhack and Wang, 2009), (3) Ir64a receptor (a neuron in the sacculus) (Ai et al., 2010), (4) Or56a receptors (i.e. ab4B neuron) (Stensmyr et al., 2012), (5) TrpA1 (ab11A) and (6) an unknown receptor in ab12A (Kwon et al., 2010). Another important insect repellent with a partially characterized avoidance circuit includes DEET (Ditzen et al., 2008; Lee et al., 2010; Pellegrino et al., 2011).

To determine the degree to which these avoidance pathways are conserved in related insects, we exposed a panel of other *Drosophila* species consisting of *D. yakuba, D. suzukii, D. pseudoobscura* and *D. virilis* to known *D. melanogaster* repellents in multiple behavioral assays. Using assays that presented CO_2 and ethyl-3-hydroxybutyrate, as well as two widely used insect repellents, DEET and citronellal; flies were given a choice to avoid these compounds in the presence and absence of attractive food odors. DEET was shown to be the most widely conserved aversive compound tested.

Material and Methods

Drosophila stocks

D. yakuba, *D. pseudoobscura, D. virilis* and *D. melanogaster* (wild-type lines) were obtained from the San Diego Stock Center. *D. suzukii* were a generous gift of R. Stouthammer. Unless otherwise indicated *D. melanogaster* were *white*^{1118} backcrossed 5X to Canton-S. *D. melanogaster* wild-type A1 was caught in La Jolla, California and A2 in Point Loma, California in July 2011. *D. melanogaster* species morphological identification was confirmed by sequencing the mitochondrial cytochrome oxidase gene (COI) gene and isogenized at the *Drosophila* Species Stock Center at the University of California, San Diego. These wild caught *D. melanogaster* were tested within five months of being captured. *D. melanogaster*, *D. yakuba* and *D. virilis* were raised on standard cornmeal in a humidified incubator at 25°C on a 12 hour light/12 hour dark cycle. *D. pseudoobscura* were raised in the dark at 18°C and *D. suzukii* were raised at room temperature on a modified cornmeal diet.

Single-sensillum electrophysiology

Recordings were obtained as described previously (Turner and Ray, 2009) and conducted by Sana K. Tharadra.

Short-term assays

Contact and non-contact short-term behavioral assays were conducted to determine responses to odorants.

The T-maze assay was ideally suited for highly volatile compounds such as CO_2. Avoidance to carbon dioxide and pyridine trials were conducted as before (Turner and Ray, 2009). Briefly, approximately 40 flies were released from an elevator into the horizontal intersection of a T-shaped apparatus. A test odorant was applied to one arm of the T-maze and a control odorant to the opposite arm. For trials with other odorants, paraffin oil was used as the solvent and in the control arm. Flies were given one minute to choose an arm before the elevator closed. Orientation of arms for test and control were switched between trials. Preference index was calculated as = (number of flies in test arm-number of flies in control arm)/(number of flies in test arm + number of flies in control arm).

The *Direct Airborne Repellent Test (DART)* was suited for testing volatile compounds that can also activate the taste system. Trials were performed using slight modifications to the previously reported assay (Kwon et al., 2010). Fifty flies were starved in vials with 2 Kimwipes moistened with 3 ml of water. A 6-mm diameter circle of Whatman #1 filter paper was placed in the bottom of a 10-cm length tube (VWR, #60818-661) to deliver the odor. A brass screen of

8/32-inch diameter was placed 5 mm from the bottom of the tube to gate off the filter paper. Approximately 100 flies were inserted into the control tube and joined to the tube with test odorant. After 30 minutes of exposure in the dark at 25°C the apparatus was photographed. Flies 5 cm from each screen were counted. Preference index was calculated as = (number of flies in test arm-number of flies in control arm)/(number of flies in test arm + number of flies in control arm).

The two-choice contact trap assay in a plate was used to test responses to less volatile odorants. Trials were performed as described (Reeder et al., 2001). Ten female flies were placed in a Petri dish containing two traps. Traps were made with 1.5-ml microcentrifuge tubes (USA Scientific) with an opening cut in the bottom of each tube. Both traps contained the fly's normal laboratory food at the base. The neck of one trap contained a filter paper with test odorant, the other trap had solvent. Five microliters of hexane (control) and five microliters of 10% DEET or test compound in hexane was applied to the stem part of filter paper inserted into the upper part of pipette tip near entrance of trap to allow flies to walk over the treated surface. Traps were placed in chemical hood for 5 minutes to allow hexane to volatilize before being placed in a Petri dish coated with 10ml 1% agarose to add humidity to the chamber.

The two-choice non-contact trap assay was performed to determine preference for an attractive food source in the context of a repellent odor. Briefly, ten male and ten female starved (4–7 day old) flies were placed in

a cylindrical chamber containing two traps: (1) with test odorant and lure, (2) with solvent and lure. Apple cider vinegar (10%) was the lure for all trials except for *D. virilis* where liquid malt (25%) was used. *D. virilis* was not attracted to apple cider vinegar (West, 1961). To create a well for separating lure from test odorant, a single-cap cut from a BioRad PCR 0.2-ml tube flat cap strip (#TCS0803) was inserted in a snap-top lid of a microcentrifuge tube. To run the assay, 35µl of test odorant was pipetted into the inner well and 90µl of lure into the outer ring. For all trials, the control trap had paraffin oil solvent in the inner well and lure in the outer ring. Flies were given six hours to enter traps. Preference index was calculated = (number of flies in test trap - number of flies in control trap)/(number of flies in test trap + number of flies in control trap).

Results

CO_2 avoidance behavior is not conserved in all *Drosophila* species

We first considered the robust repellent effect of CO_2 on our laboratory strain of *Drosophila melanogaster*. We cultured Canton S files in our laboratory for testing as wild-type *Drosophila melanogaster*. Because the preference index for these flies was so strongly negative, I wondered if this was the result of an adaptation in our wild-type flies, which were constantly anesthetized using CO_2 and kept for generations in the laboratory. I observed robust avoidance of CO_2 in the *D.melanogaster* laboratory strain and also in two recently caught wild-type strains tested in the T-maze assay, which suggested that repellency was not due to artificial selection in our laboratory strains (Figure 2.1).

These results pose an interesting question: How conserved is avoidance of CO_2 in other related *Drosophila* species? In order to answer this question we performed a series of behavioral and electrophysiological experiments with four additional *Drosophilid* species (Figure 2.2). Using the T-maze assay, we found that the closely related *D. yakuba* showed avoidance to carbon dioxide, albeit to a lower degree than *D. melanogaster* (Figure 2.2). However, *D. suzukii*, and the more distantly related *D. virilis* did not avoid carbon dioxide and *D. pseudobscura* was only mildly (but not significantly) repelled at the highest concentration of CO_2 tested (Figure 2.2). Relative to *D. melanogaster*, the Gr21a and Gr63a CO_2 receptor amino acid sequences were highly conserved across all tested species: *D. yakuba* (100% and 97%), *D. pseudoobscura* (97% and 93%) and *D .virilis* (88% and 85%). We found that a CO_2-sensitive ab1C-like neuron is present in each of these species from single sensillum recordings (Figure 2.3). These results suggested that detection of CO_2 is conserved; however, the behavioral changes could likely be due to other changes such as processing of CO_2-detection information in downstream circuitry in the brain.

Since the CO_2 pathway is the strongest known repellency pathway for some *Drosophila* species, we wondered whether other odorants that activate the CO_2 receptors would serve as practical repellents. In a previous study pyridine, an animal skin odorant, was identified to be one of the strongest activators of the CO_2 receptor (Turner et al., 2011). At a 10^{-2} concentration, pyridine elicited avoidance behavior in *D. melanogaster* and *D. yakuba* as

expected from my experimentally observed behavioral response to carbon dioxide, and also in *D. pseudoobscura,* which avoided CO_2 to a lesser degree (Figure 2.4). In *D. pseudoobscura,* it is conceivable that other olfactory receptors may also contribute to pyridine repellency such as an Ionotropic Receptor (Croset et al., 2010; Abuin et al., 2011). *D. suzukii* and *D. virilis* showed very little repellency to pyridine as we expected based on responses to CO_2 (Figure 2.2). A lower concentration of pyridine (at 10^{-4}) was also tested; however, none of the species showed behavioral response in the T-maze assay (data not shown).

The T-maze assay measures the instantaneous behavioral response of walking flies offered a choice between an odor and control (solvent). In order to test the behavioral response of flying *Drosophila* in the context of an attractive food, we utilized a two-choice non-contact trap assay. Ten male and ten female starved flies are placed in a cylindrical chamber containing two apple cider vinegar (ACV) traps, one trap containing the CO_2-neuron activator pyridine and the other solvent. Both *D. suzukii* and *D. melanogaster* showed no significant avoidance of the pyridine-treated trap (Figure 2.5) ($P = 0.86$). While behavioral responses of free flying *Drosophila* to CO_2 have not been tested, tethered *D. melanogaster* that can beat their wings do not demonstrate clear anti-tracking behavior to CO_2 (Wasserman et al., 2013). This taken together with our results suggest that CO_2-receptor activating odorants such as pyridine are unlikely to

act as broad-spectrum repellents for *Drosophila* species, particularly for the agriculturally important pest *D. suzukii.*

Conservation of other olfactory avoidance pathways

These findings prompted a systematic investigation of known repellent olfactory pathways in *D. melanogaster* and analysis of their conservation in related species. A second avoidance pathway in *D. melanogaster* is mediated by activation of Or85a, a member of another receptor gene family (Semmelhack and Wang, 2009). The strongest known activator of this receptor (identified by electrophysiology) is the odorant ethyl 3-hydroxybutyrate (Stensmyr et al., 2003; Hallem et al., 2004; Hallem and Carlson, 2006). We tested two concentrations of ethyl 3-hydroxybutyrate (10^{-2} and 10^{-4}) in a T-maze assay. All species tested showed little preference at the lower concentration (data not shown). *D. melanogaster* had some avoidance of ethyl 3-hydroxybutyrate at the higher concentration (Figure 2.6). This response was conserved in *D. yakuba.* The *D. suzukii* showed a small repellency; however, the distantly related species *D. virilis* and *D. pseudoobscura* showed no behavioral response to ethyl 3-hydroxybutryate. These behaviors are consistent with the observation that *D. pseudoobscura* and *D. virilis* lack a functional copy of the *Or85a* gene (Guo and Kim, 2007; McBride et al., 2007; Robertson and Kent, 2009; de Bruyne et al., 2010).

In order to test whether ethyl 3-hydroxybutryate can reduce attraction towards an attractive odor source over time, we used a two-choice non-contact

trap assay. *D. melanogaster* avoids the apple cider vinegar trap with 1% ethyl 3-hydroxybutyrate (Figure 2.7). Participation in the two-choice trap assay for *D. suzukii* was very low and consequently, this data was not included in this analysis. The odorant was not avoided by three species tested. These results reinforce our view that for some odorants the avoidance is not conserved, because the receptor gene is not present in the genome.

A third known repellent is citronellal, a naturally occurring essential oil found in multiple plant species. Citronellal activates olfactory neurons in the antenna named ab11A and ab12A in *D. melanogaster.* A Trp channel (TRPA1) is believed to play a role in citronellal's activation of ab11A but not ab12A (Kwon et al., 2010). Other odorant receptors in these neurons are unknown. For odorants with low volatility, the one-minute duration of the T-maze assay was not adequate to elicit a response. Therefore, to test for conservation in repellency to citronellal, we used the previously described Direct Airborne Repellant Assay (DART) (Kwon et al., 2010) to allow for participation over a greater time scale. For this non-contact assay, odorant is placed on filter paper at the bottom of a standard 15-ml culture tube, with a mesh screen placed 0.5 ml from the bottom to prevent flies from contacting the filter paper with odorant. The open ends of two tubes are placed together to form a long tunnel in which ~100 flies are given 30 minutes to choose the odorant or solvent end of the tube. We found that *D. melanogaster, D. pseudoobscura* and *D. virilis* avoided 1% citronellal. Interestingly, *D.*

yakuba and *D. suzukii* gave mixed responses. A lower concentration (0.1%) of citronellal was slightly attractive and the higher concentration of 1% citronellal slightly repellent for *D. yakuba* and *D. suzukii* (Figure 2.8). These results suggest that citronellal is unlikely to be useful as a strong repellent for *D. suzukii* and *D. yakuba.*

Another compound shown to be a repellent for many insect species is DEET (Leal, 2014; Ray, 2015). The mechanisms used by *D. melanogaster* to avoid DEET remain controversial. Some report the contribution of an OR gene family receptor (Ditzen et al., 2008). Others observe DEET to be detected by bitter neurons in the gustatory system (Lee et al., 2010). In *D. melanogaster*, DEET response many be elicited by activation of both olfactory and taste receptors (DeGennaro, 2015). In *Culex quinquefasciatus* mosquitoes, DEET has been proposed to act via odorant receptor CquiOR136; however, none of the *Drosophila* species tested here have an ortholog of *CqiOR136* (Xu et al., 2014).

To examine the response to DEET in other *Drosophilids*, the two-choice contact trap assay in a plate is typically used (Syed et al., 2011). Briefly, 10 female flies are placed inside a Petri dish containing two food containing traps. In order to access the food, flies must crawl over a piece of filter paper impregnated with test compound. Four species strongly avoided DEET and *D. pseudoobscura* also avoided it, but to a lesser degree ($P = 0.023$) suggesting

that this pathway is highly conserved, unlike the other repellent pathways tested (Figure 2.9).

Geosmin is a recently identified repellant for *D. melanogaster* (Stensmyr et al., 2012). As in Stensmyr, I found *D. melanogaster* slightly avoided a 10^{-6} dilution of geosmin in the T-maze assay (Figure 2.10). *Drosophila melanogaster* showed a trend toward geosmin avoidance in the modified non-contact trap assay (Figure 2.11) over a number of concentrations. This brings up an interesting point that *Drosophila* typically avoided very high concentrations of odorants. This implies that receptor Or59a is narrowly tuned to the odorant. A large repertoire of receptors is not additionally activated by this odorant as observed for other natural compounds such as ACV. Therefore, geosmin is unlikely to cause avoidance by activating many receptors non-specifically.

Discussion

As expected *D.melanogaster* avoided all test odorants (Suh et al., 2004; Ditzen et al., 2008; Semmelhack and Wang, 2009; Ai et al., 2010; Kwon et al., 2010; Lee et al., 2010; Pellegrino et al., 2011; Stensmyr et al., 2012). For *D. yakuba*, *D. suzukii, D. pseudoobscura,* and *D. virilis*, some responses were conserved. Behavioral responses to CO_2 were not as expected. In single sensilla electrophysiological responses of *D. yakuba*, *D. suzukii*, *D. pseudoobscura,* and *D. virilis* to CO_2, ab1C-like neurons responded strongly to CO_2, but these species did not strongly avoid CO_2 in behavioral assays. In specific cases, reduced avoidance may partly be explained by starvation status.

Here, we starved flies before testing. Recently, it was reported that avoidance behavior towards aversive compounds is reduced in starved flies due to tachykinin (DTK) acting as a neuromodulator on glomerulus DM5 (Ko et al., 2015). Since Or85a neurons target this glomerulus, the effect of repellents such as ethyl-3-hydroxybutyrate may be suppressed during starvation. It would be interesting to re-test the response of unstarved flies to ethyl-3-hydroxybutyrate.

As additional genetic tools become available in non-model organism species, further investigation may determine the state of the CO_2 neuron circuit in *D. suzukii*. It would be beneficial to see if the ab1C neuron in *D. suzukii* targets the V glomerulus and then further map projection neurons leaving the antennal lobe. If projection neurons activated by the ab1C neuron in *D. suzukii* project to a different area in the lateral horn of the protocerebrum then the theory of a “repellency center” in *D. melanogaster* lateral horn would gain importance (Knaden et al., 2012). In addition, by further examining this pathway, we may be able to determine some new clues as to how the valance towards CO_2 changes in different insects. Whereas *D. melanogaster* robustly avoids CO_2, many insects, including mosquitoes (particularly those evolved to require human host blood for oogenesis) are attracted to CO_2.

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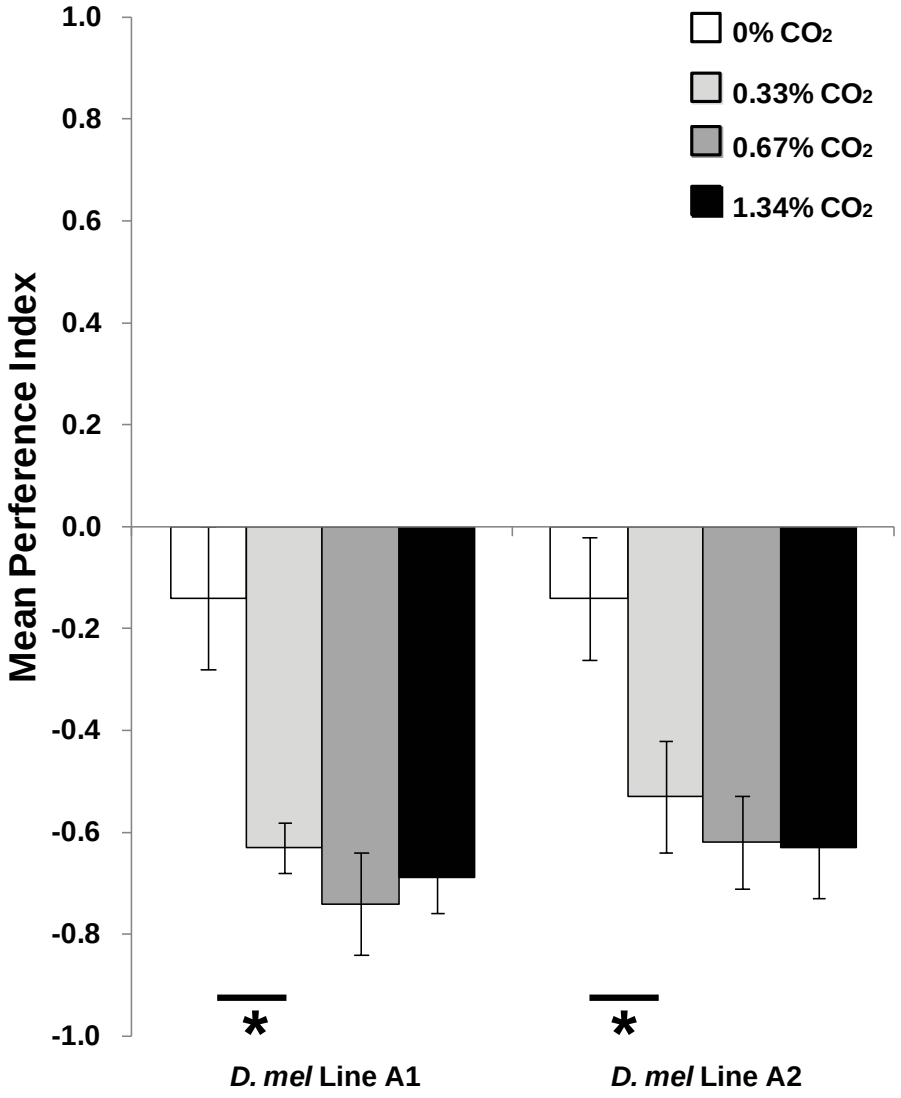

Figure 2.1: Wild-type *D. melanogaster* lines recently introduced into laboratory robustly avoid CO_2. Wild-caught *D. melanogaster* were tested in the T-maze assay within five months of being captured. Mean preference index to CO_2. N= 6-8 trials, ~40 flies/trial. Error Bars= S.E.M. Two-tailed t-test between 0 and 0.33% CO_2 (A1, p=0.01, A2, p=0.03). * $p < 0.05$.

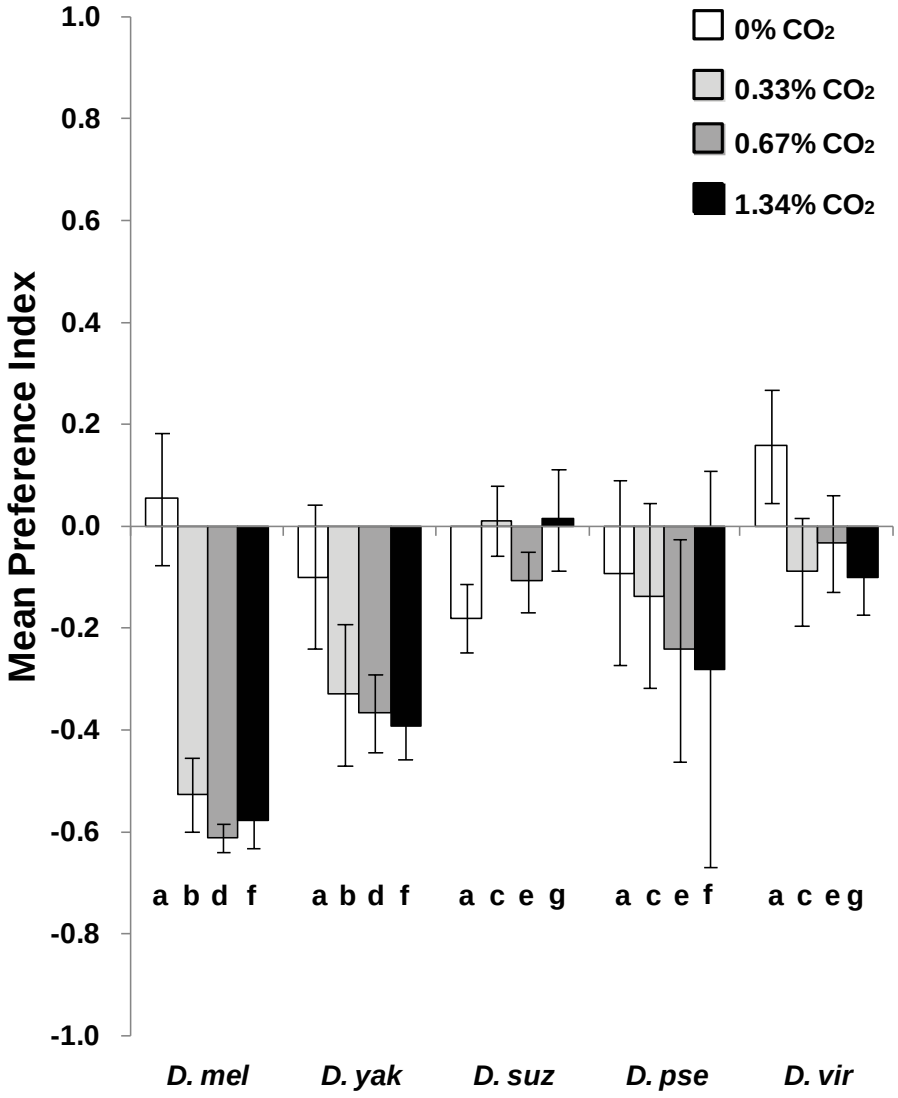

Figure 2.2: Mean preference index of the various *Drosophila* species to the indicated doses of carbon dioxide in a T-maze assay. N = 5–39 trials, 40 flies/trial, error bars = S.E.M. The Holm-Sidak method was used to conduct a pair-wise multiple comparison of species and CO_2 concentration. There was a significant difference between air and all three CO_2 concentrations in *D. melanogaster* ($P < 0.05$). *D. yakuba* avoided CO_2 at all concentrations, but not significantly.

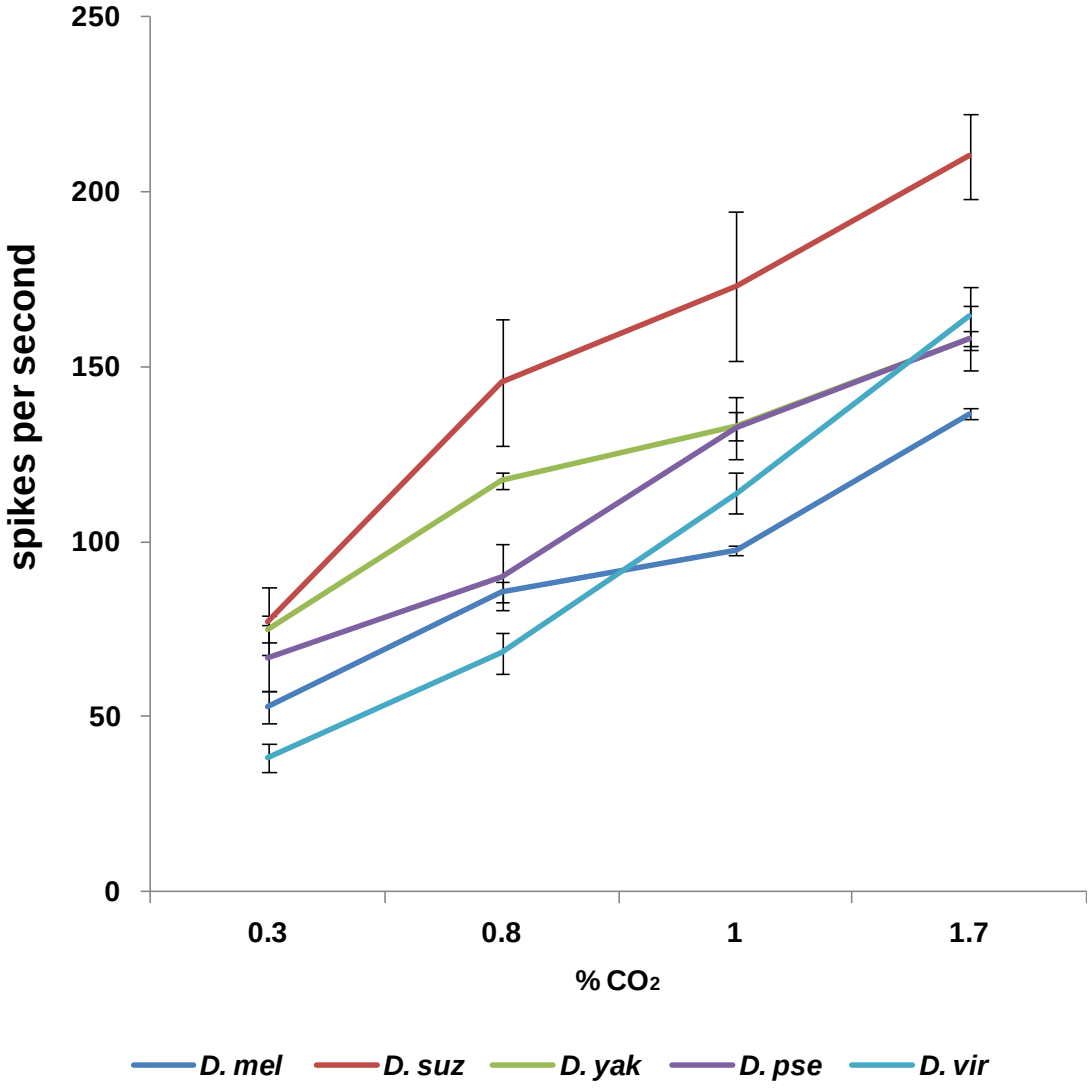

Figure 2.3: Mean electrophysiological responses of the antennal large basiconic CO_2-sensing neuron to different doses of carbon dioxide across the various species. N = 5–6 recordings/concentration. Electrophysiology recordings were conducted by Sana K. Tharadra.

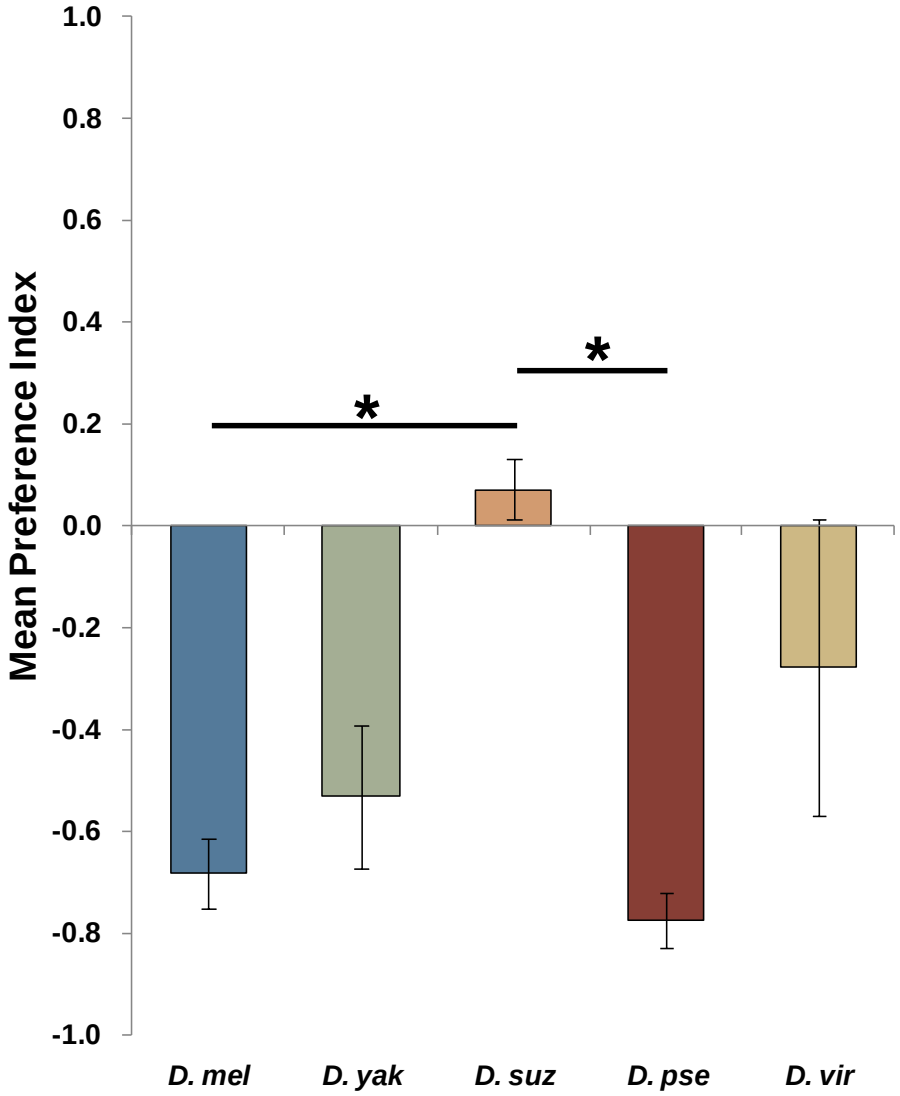

Figure 2.4: Mean preference index of *Drosophila* species to 1% pyridine in T-maze assay. Control arm contains paraffin oil. N = 5–10 trials, 40 flies/trial, error bars = S.E.M. For Kruskal-Wallis One Way Analysis of Variance on Ranks, there is a difference in the mean values ($P = 0.005$). Specifically, in a pair wise multiple comparison procedure (Dunn's Method), *D. suzukii* response differs from both *D. melanogaster* and *D. pseudoobscura* ($P < 0.05$).

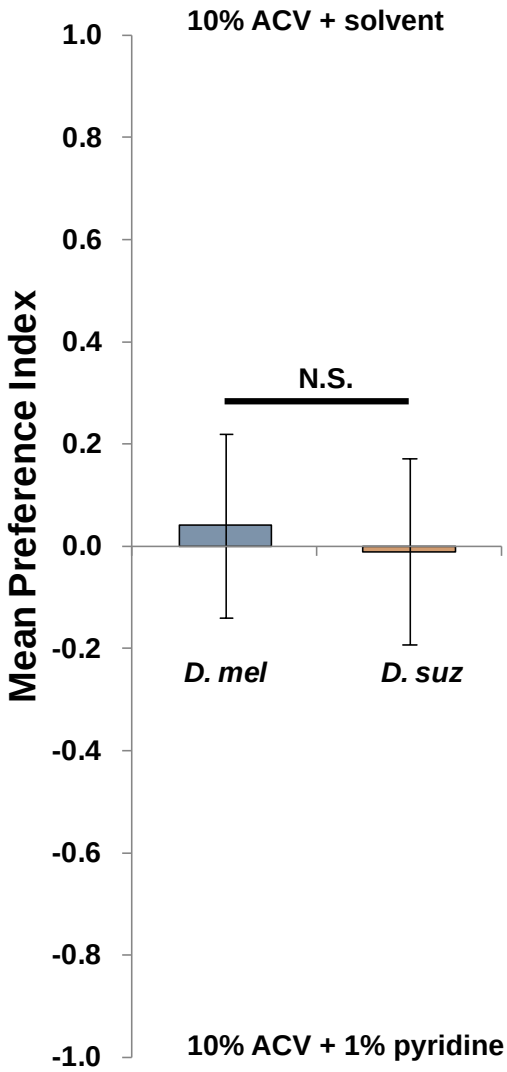

Figure 2.5: *D. melanogaster* and *D. suzukii* show no avoidance to an activator of the CO_2 receptor in the presence of apple cider vinegar (ACV). Two-choice non-contact trap assay with 1% pyridine dissolved in paraffin oil. Flies are given 6 hours to choose between the trap containing 10% ACV and pyridine or solvent (paraffin oil). N=5 trials, 20 flies (10 male + 10 female) / trial. Error bars = S.E.M., Two-tailed student's t-test is not significant (p=0.86).

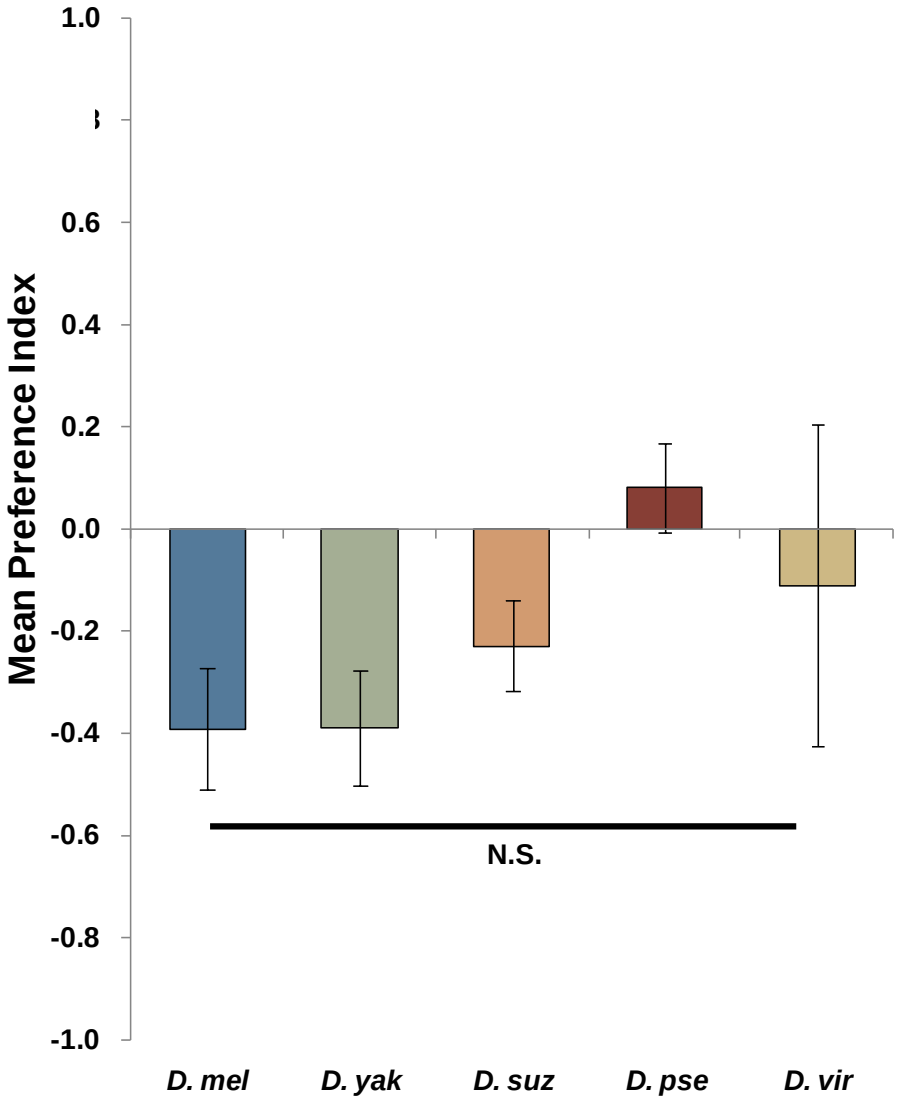

Figure 2.6: *D. melanogaster* and *D. yakuba* avoid ethyl 3-hydroxybutyrate in T-maze assay. Mean preference index of *Drosophila* species to 1% ethyl 3-hydroxybutyrate in T-maze assay. N = 5–6 trials, 40 flies/trial, error bars = S.E.M. For Kruskal-Wallis One Way Analysis of Variance on Ranks, there is no difference between the different species (P = 0.175).

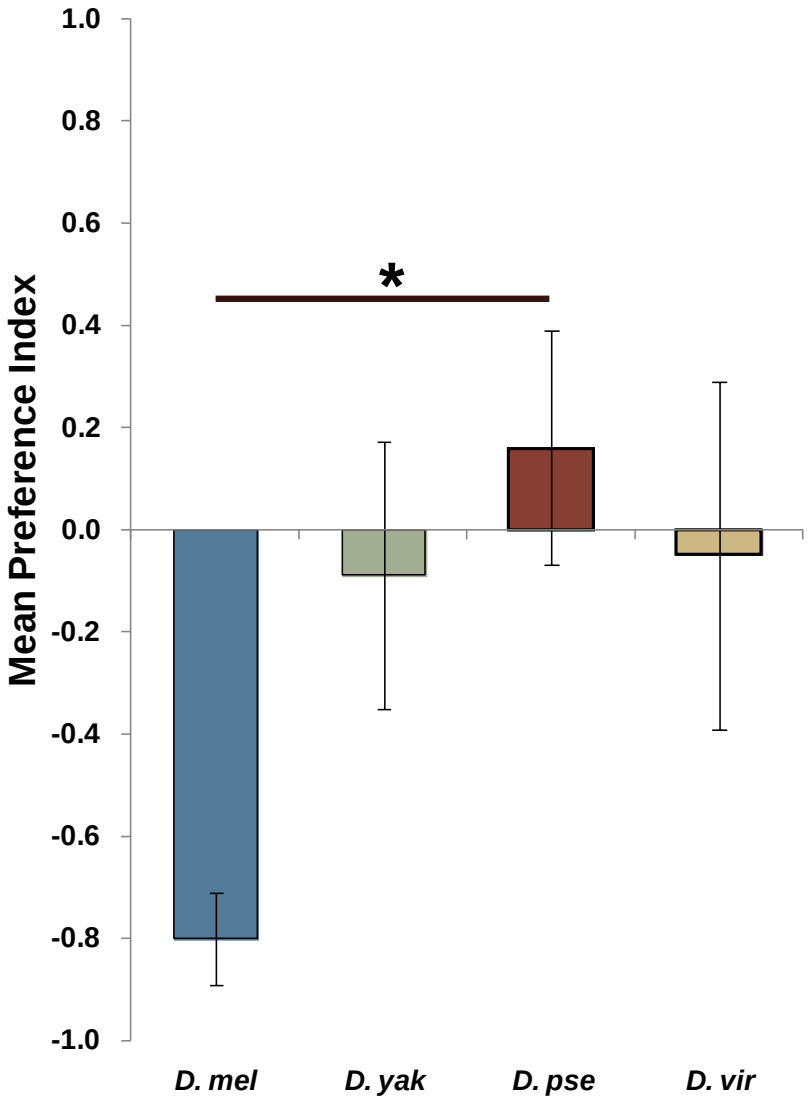

Figure 2.7: Only *D. melanogaster* avoid ethyl 3-hydroxybutyrate in trap assay. Mean preference index of *Drosophila* species to 1% ethyl 3-hydroxybutyrate in two-choice non-contact trap assay. N=6–7 trials, 20 flies/trial. Error bars = S.E.M. For Kruskal-Wallis One Way Analysis of Variance ($P = 0.043$), *$P < 0.05$ Dunn's Test.

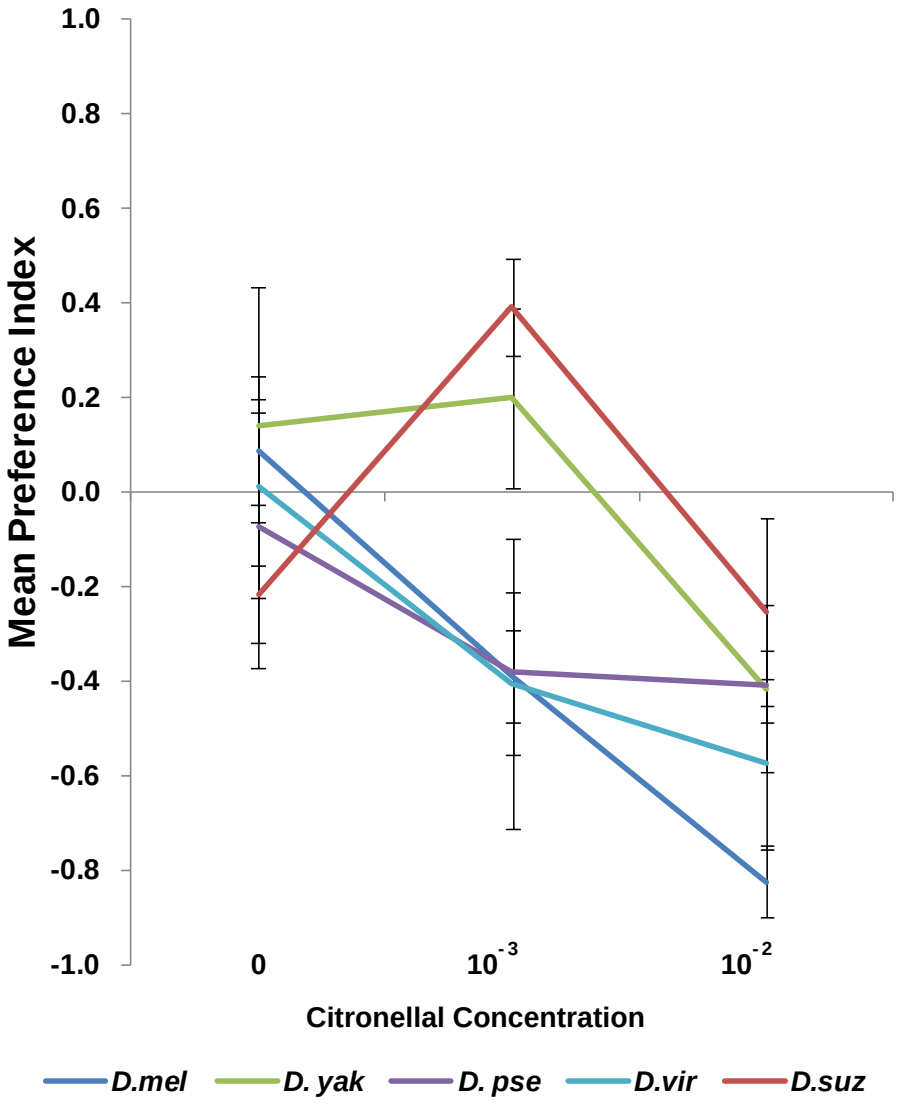

Figure 2.8: Other species show reduced avoidance to 1% citronellal in DART assay. Mean preference index of *Drosophila* species to citronellal in the DART assay. N = 4–8 trials, ~100 flies/trial. Error bars = S.E.M.

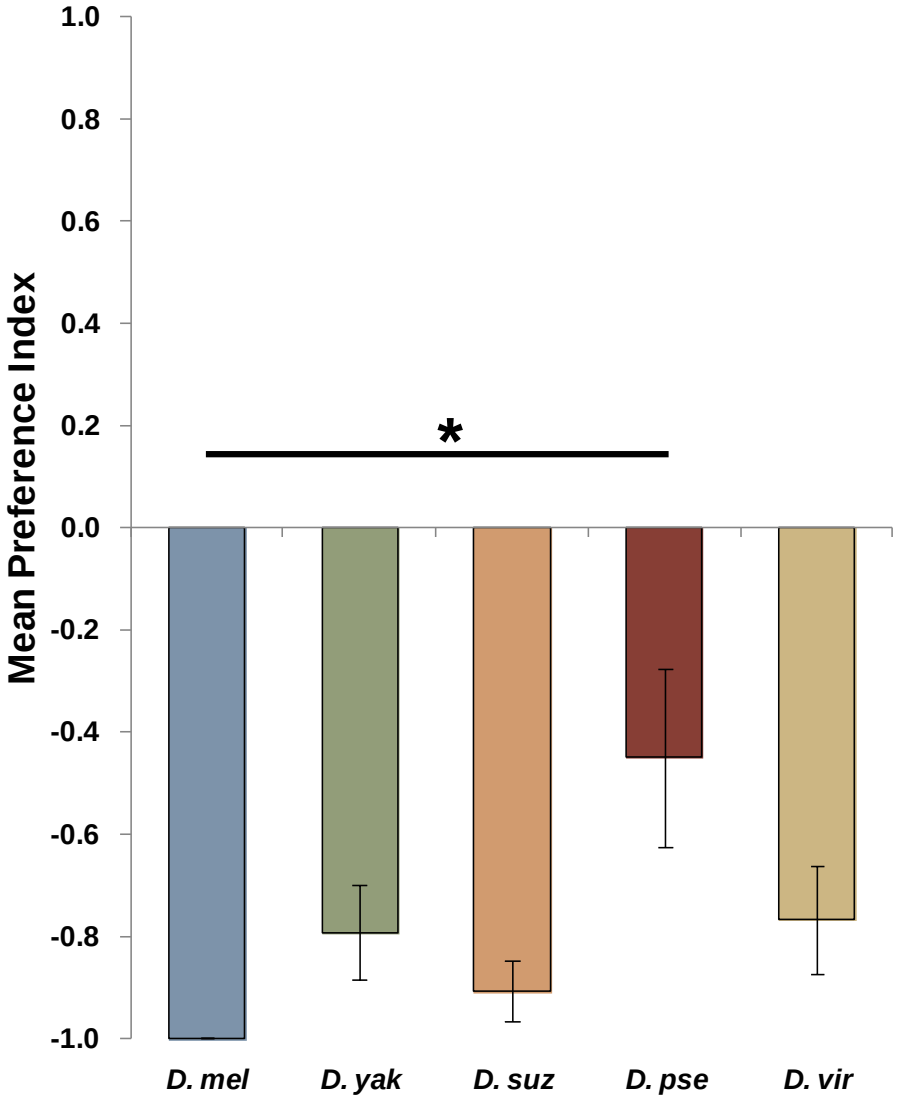

Figure 2.9: All species avoid DEET in two-choice contact trap assay. Mean preference index of *Drosophila* species to 10% DEET in two-choice contact trap assay in a plate. N = 8–10 trials, ~10 female flies/trial. Error bars = S.E.M. Kruskal-Wallis One Way Analysis of Variance (P = 0.023), specifically pairwise multiple comparison using Dunn's Method showed *D. melanogaster* and *D. pseudoobscura* response is significantly different (*P < 0.05).

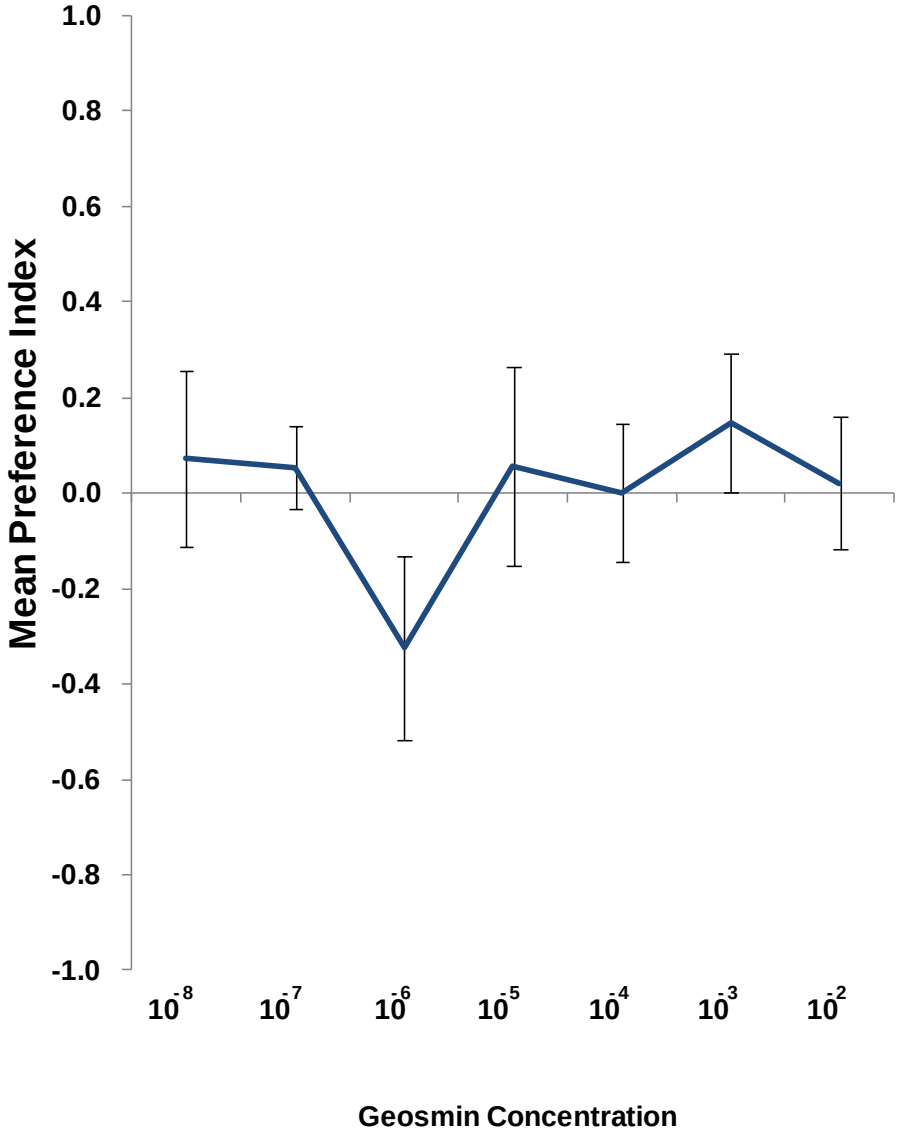

Figure 2.10: *D. melanogaster* avoids 10^{-6} geosmin in T-maze assay. Mean preference index of *Drosophila melanogaster* wildtype (wCs) to dose response of geosmin diluted in paraffin oil. T-maze assay with the following modifications (1) time was four minutes per trial, (2) 15 males/15 females per trial. N=4-8 trials, 40 flies per trial, error bars = S.E.M.

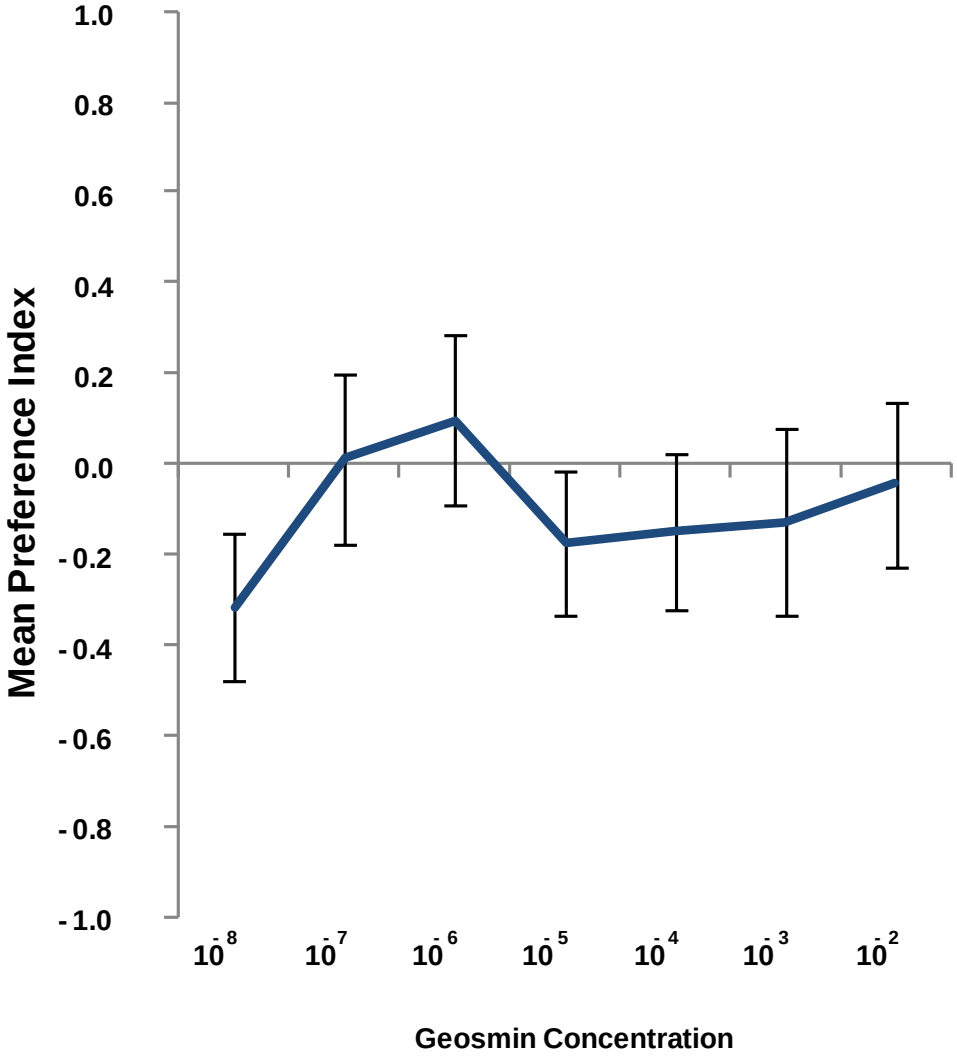

Figure 2.11: *D. melanogaster* shows minimal avoidance to geosmin in revised two-choice non-contact trap assay. Mean preference index of *Drosophila melanogaster* wild-type (wCs) to dose response of geosmin diluted in water. Flies are starved. This revised two-choice non-contact trap assay was conducted in the context of ACV. N=5-8 trials, 20 flies per trial (10 males and 10 females), error bars = S.E.M.

Chapter 3: Identification of Repellents for *Drosophila suzukii*

Abstract

Flying insects navigate towards fruits in complex odor environments with remarkable accuracy. Some fruits change odor profiles substantially during ripening and related species can prefer different stages. In order to examine the role of the avoidance cue CO_2 emitted from fruit on the behavior of two species with different ripening stage preferences, we investigated the CO_2-detection pathway in *D. melanogaster* and in *D. suzukii*, a harmful pest of fruits. Using single sensillium electrophysiology and behavioral assays where flies are exposed to odorants with and without food sources, we compared preference between the two species. Avoidance to CO_2 is not conserved in *D. suzukii* suggesting a behavioral adaptation that could facilitate attraction to younger fruit with higher CO_2 emission levels. These findings guided us to test recently discovered safer repellents. We identified one that protects fruits from *D. suzukii*, thus providing a new behavioral strategy for controlling agricultural pests.

Introduction

The two related species, *Drosophila melanogaster* (vinegar fly) and *Drosophila suzukii* (spotted wing *Drosophila*), provide an excellent model to study how changes in olfactory behavior are associated with changes in food preference. Drastic changes occur in the composition of volatiles emitted during

fruit maturation, ripening and fermentation, potentially providing different cues for different species. *D. melanogaster* primarily feed and lay eggs on overripe and fermenting fruits fallen from plants, and they avoid fruit that is still ripening on the plant. During the ripening process, fruits undergo higher rates of respiration and emit a higher level of CO_2 (Faucher et al., 2006), which *D. melanogaster* strongly avoid (de Bruyne et al.; Suh et al., 2004; Suh et al., 2007; Robertson and Kent, 2009; Faucher et al., 2013). As the fruit over-ripens, levels of CO_2 emission decrease and a concomitant increase in yeast-derived volatiles occur. Volatiles from yeast can attract flies, and there are some volatiles (such as 1-hexanol and 2,3-butanedione) that can also inhibit the aversive CO_2 receptor (Turner and Ray, 2009) (Figure 3.1). Conversely, *D. suzukii* prefer to feed on ripening fruits and have evolved a specialized ovipositor that can tear through the skin of ripe berries to lay eggs (Kinjo et al., 2013; Atallah et al., 2014). Their larvae emerge inside causing hundreds of millions of dollars worth of agricultural damage worldwide (Goodhue et al., 2011; Lee et al., 2011).

D. suzukii oviposits in ripe berries, unlike other tested *Drosophilids* that prefer to oviposit in rotting, fermenting fruit. Benign repellents are needed to protect food crops such as ripe strawberries, blueberries, blackberries, cherries, and raspberries (Lee et al., 2011). Currently, the main line of defense is field application of pyrethoid, organophosphate, or spinosyn insecticides, which only provide control for 5-14 days (Bruck et al., 2011). Multiple applications of these compounds to each harvest may cause insects to

develop resistance. Based on preliminary data, *D. suzukii* is expected to have an olfactory or taste based mechanism for avoiding DEET and structurally similar molecules (Figure 2.9). By identifying benign odorants that activate multiple avoidance pathways, we can develop safe mechanisms to deter phytophagous insects such as *D. suzukii* from interacting with their hosts. Ideally, pest control may be achieved without adding broadly toxic synthetic substances to food crops.

Here we examine the olfactory determinants underlying these behavioral preferences. Our findings can serve not only as an important model to understand adaptations in behavior, but can also be employed to control insects that are harmful to crops.

Materials and Methods

Single-sensillum electrophysiology

Recordings were obtained as described previously (Turner and Ray, 2009) and were conducted by Sana K. Tharadra.

The T-maze assay

Carbon dioxide and pyridine avoidance trials were conducted based on prior experiments (Turner and Ray, 2009). Briefly, approximately 40 flies were released from an elevator into the horizontal intersection of a T-shaped apparatus. A test odorant was applied to one arm of the T-maze and a control odorant to the opposite arm. For trials with other odorants, paraffin oil was used as the solvent and in the control arm. Flies were given one minute to choose an

arm before the elevator closes. Orientation of arms for test and control were switched between trials. Preference index was calculated as = (number of flies in test arm - number of flies in control arm)/(number of flies in test arm + number of flies in control arm).

Two-choice oviposition assay

Test odorant or solvent was added to warm standard grape juice media and set to solidify in Petri dishes. A 100-ml beaker containing 40 ml of distilled water was placed in the center of a 10-gallon closed glass chamber to add moisture to the chamber. A grape plate with solvent was placed at one end of the chamber and a plate with test odorant at the other. The orientation of the grape plates were switched between trials. For each trial, 15 male and 25 female un-starved Canton-S flies were lightly anesthetized with carbon dioxide and released in the chamber. The assay was run for 24 hours at 25°C on a 12-hour light: 12-hour dark cycle. Preference Index was determined by counts of eggs = (number of eggs on test plate – number of eggs on control plate)/total number of eggs.

Two-choice blueberry assay in a glass chamber

Fresh blueberries were obtained from a local grocer and were soaked in distilled water for 30 minutes, then rinsed and dried. To prepare the chamber, 31 grams of blueberries were placed in each of two plastic bowls. Test compound (0.4 ml) is painted on blueberries in test bowl and solvent (0.4 ml) on

blueberries in control bowl. Bowls are placed at opposite ends of a 10-gallon closed glass chamber. A 100-ml beaker containing 40 ml of distilled water was place equidistant between fruit to add moisture to the chamber. For each trial, 15 male and 15 female un-starved flies were lightly anesthetized with carbon dioxide and released in the chamber. The assay was run for seven days at 25° Celsius on a 12-hour light: 12-hour dark cycle. After 7 days, each bowl was covered and set aside for an additional six days for eggs and larvae to develop after which the blueberries were dissected under the microscope and the number of eggs, larvae, pupae and newly emerging adults were recorded. Preference was determined by inferring egg-laying from a count of eggs, larvae and pupae emerging from each set of fruit.

Results

CO_2 avoidance

In order to determine whether the CO_2-avoidance pathway has been adapted to suit the *D. suzukii* food choice, we first investigated their ability to detect CO_2. The CO_2 receptor is comprised of two 7-transmembrane proteins Gr21a and Gr63a, which are housed in the ab1C neuron on the *D. melanogaster* antenna. We found that the amino acid sequences of both Gr21a and Gr63a are extremely well conserved in the *D. suzukii* genome (99% and 94%, respectively). In order to test whether the functional expression of the receptors occurs, we used single sensillum electrophysiology on the *D.*

suzukii antenna (N = 5–6). Our results indicated that an ab1C-like neuron was present in *D. suzukii* and is in fact more sensitive to CO_2 than *D. melanogaster* across different concentrations (Figure 3.2).

We next tested *D. suzukii* preference for CO_2 in a T-maze assay. Surprisingly, *D. suzukii* did not show avoidance to CO_2 at a level that elicits robust avoidance in *D. melanogaster* ($P < 0.001$) (Figure 3.3). These results suggested that while *D. suzukii* can detect CO_2, other changes in processing this sensory information have occurred. Since *D. suzukii* are attracted to ripening fruits, which emit CO_2, we also tested whether CO_2 can enhance attraction in the presence of food odors such as apple cider vinegar (ACV) as has been reported in *D. melanogaster* (Faucher et al., 2013). *D. suzukii* showed no significant increase in attraction to ACV in the presence of CO_2 ($P = 0.88$) (Figure 3.4). While we do not know the behavioral significance of CO_2 detection in this species yet, the ability to sense but not avoid CO_2 may offer a distinct evolutionary advantage since *D. suzukii* feed on ripening fruits that respire and emit CO_2.

Safer DEET substitutes repel an agricultural pest, the spotted wing *Drosophila*

Recently, a number of new naturally occurring repellents were discovered that can substitute for DEET and are strongly repellent to *D. melanogaster* and mosquitoes (Kain et al., 2013). Many of these repellent compounds are naturally present in fruits, have very mild and pleasant odors, and are commonly used flavor and fragrance components. They belong to a

category called generally recognized as safe (GRAS) by FEMA (Fragrance and Extracts Manufacturers Association) and are approved for human consumption through addition to food. We tested whether these compounds can be used to repel *D. suzukii.*

We measured behavioral responses of *D. suzukii* to three of these DEET-substitute compounds: (1) butyl anthranilate (BA), (2) methyl N,N-dimethylanthranilate (MDA) and (3) ethyl anthranilate (EA) in the previously used two-choice contact trap assay in a plate. *D. suzukii* avoided the traps containing 10% of all three compounds ($P = 0.926$); however, at 1%, ethyl anthranilate did not repel *D. suzukii* ($P < 0.05$) (Figure 3.5).

We then asked if DEET-like compounds would also act as oviposition deterrents by testing preference for the model organism, *D. melanogaster,* egg-laying using a two-choice oviposition assay (Figure 3.6). Briefly, 15 male and 25 female flies were released into a 10-gallon closed glass chamber with two (one with test odorant the other with solvent) Petri dishes containing standard grape juice media. After 24 hours, eggs laid on grape media containing test odorant and solvent were counted. *D. melanogaster* did not oviposit on grape media infused with 0.4% DEET (Figure 3.6). At the lower concentration of 0.2% DEET, there was no avoidance and even initially a preference for ovipositing on the DEET containing media. *D. melanogaster* avoided ovipositing on the higher, but not lower concentration of MDA. *D. melanogaster* did not oviposit on media containing BA and EA. Since BA and EA deterred attraction and oviposition of

D. melanogaster, we wondered if these substances would also deter *D. suzukii* from ovipositing on fruit.

In order to test whether BA can protect fruit from *D. suzukii,* we revised the previous assay by replacing the grape juice media with two bowls of fresh, ripe blueberries (a preferred fruit of *D. suzukii*) and extending the assay time to one week. One bowl of blueberries was coated with BA and the other solvent. This two-choice assay in a glass chamber (Figure 3.7A) allowed us to infer egg-laying from a count of eggs, larvae and pupae emerging from each bowl of fruit. As expected from the time elapsed between the end of the experiment and dissection of exposed blueberries, few eggs were observed, with the exception of one of the six trials of 10% BA where 43 unhatched eggs out of 159 total *D. suzukii* that were counted. Of the unhatched eggs, 95% were laid on the control blueberries. More importantly, we found a clear dose-dependent decrease in numbers of larvae and pupae emerging from the BA-treated blueberries (Figure 3.7B). Remarkably, decreases were observed from the week-long experiment after only a single treatment, with substantial decreases at 2.5% and nearly complete protection at the 10% concentration. This proof-of-principle experiment indicated that insect repellents with different safety profiles can indeed be useful to reduce fruit damage during ripening.

We then wanted to see if *D. suzukii* would avoid other BA-coated fruit equally as well. In an electrophysiological analysis of ab2A activation of fruit head space, it was reported that raspberry headspace strongly activates ab2A,

while blueberry headspace to a lesser extent (personal communication with W. van der Naters) in *D suzukii*. Next we coated raspberries with BA and analyzed different life stages associated with the highest concentration of this repellent. *D. suzukii* avoided the BA-coated raspberries. Interestingly, in some trials, *D. suzukii* laid eggs on the ripe raspberries, but the majority of eggs did not develop further. BA may make the raspberries a less hospitable site for egg development (Figure 3.8).

To test if other related compounds also deter *D. suzukii* from ovipositioning, we used 10% methyl N,N-dimethylanthranilate (MDA) and 10% methyl p-tert-butylphenylacetate (MBP) on blueberries. Trials with both compounds had too little eggs observed to draw any conclusions. The response may be compound specific.

Discussion

Each year *D. suzukii* damages hundreds of millions of dollars worth of fruits worldwide and there is a great need to find new ways to reduce this loss (Calabria et al., 2012; Poyet et al., 2015). Toxic insecticides are often risky to use directly on fruits, and a safe affordable repellent could provide protection and reduce use of toxic chemicals. Although DEET is repellent to *D. suzukii,* it is a synthetic chemical that is unlikely to be useful in protecting crops given the human health concerns regarding food supply contamination, as well as the high production cost for the large volumes that would be required in agriculture. Other insect repellents we test here provide an

opportunity to develop alternative effective strategies to reduce fruit damage. More generally, insects destroy a very large fraction of the global agricultural output and necessitate millions of tons of toxic insecticide use that is environmentally unfriendly and harmful to human populations. Further analysis of possibly environmentally safer and non-toxic repellents could decrease use of such insecticides. The analysis of conserved repellent pathways in the insect olfactory system offers an avenue to design behavioral control strategies of these dangerous pests and ultimately could form a foundation for novel and safe technologies that can improve both plant and human health.

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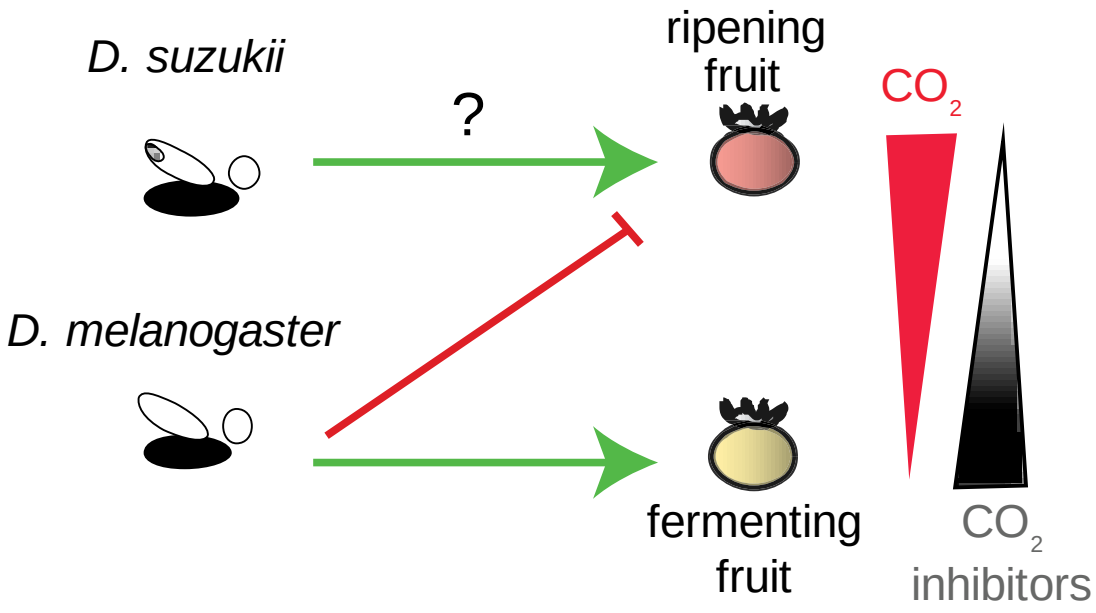

Figure 3.1: Schematic depicting proposed role of CO_2 and volatiles emitted from fruit contributing to attraction behavior of *D. melanogaster* to fermenting fruit and *D. suzukii* that are attracted to ripe fruit.

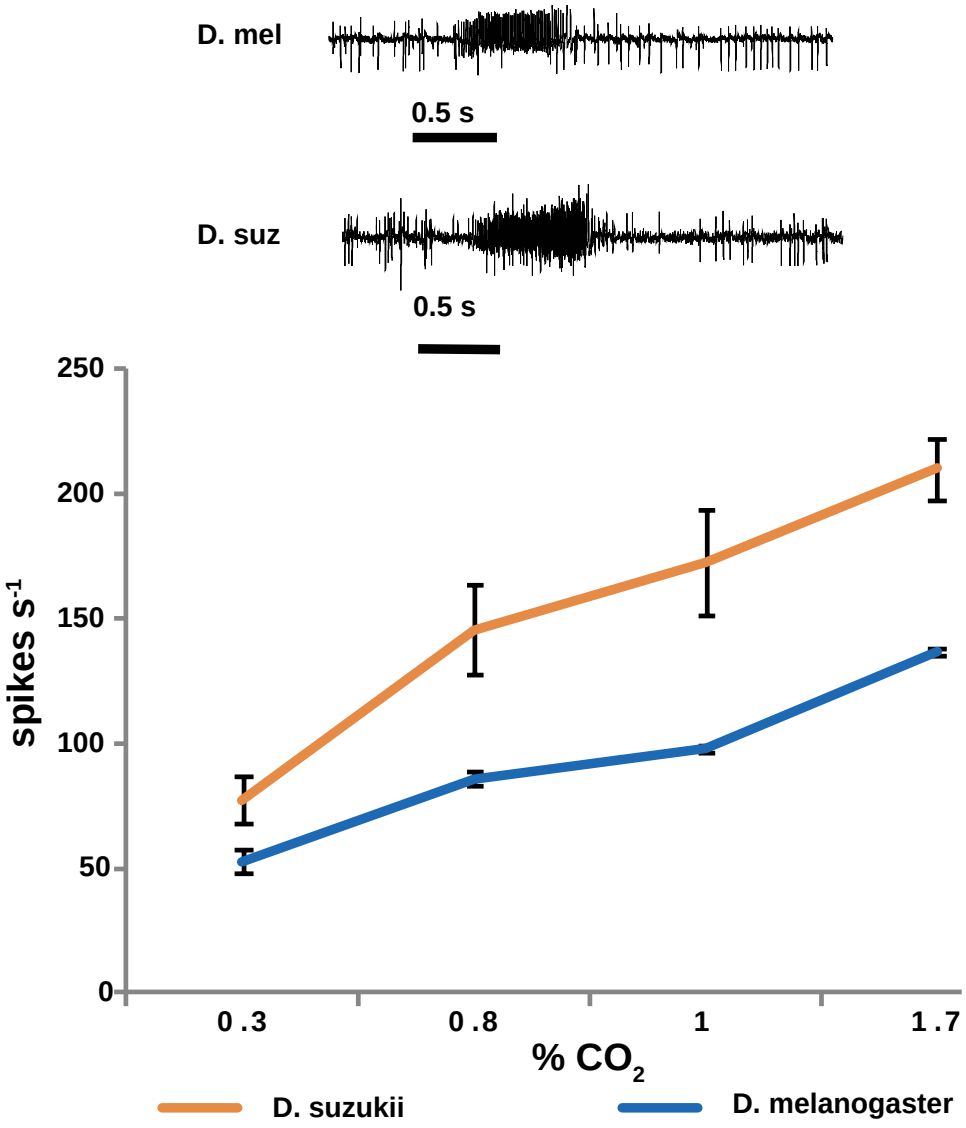

Figure 3.2: *D. suzukii* CO_2 neuron responds more strongly to CO_2 than *D. melanogaster* CO_2 neuron. Mean electrophysiological responses of the ab1C neuron to different doses of carbon dioxide. N = 5–6 recordings/concentration. Error bars= S.E.M.

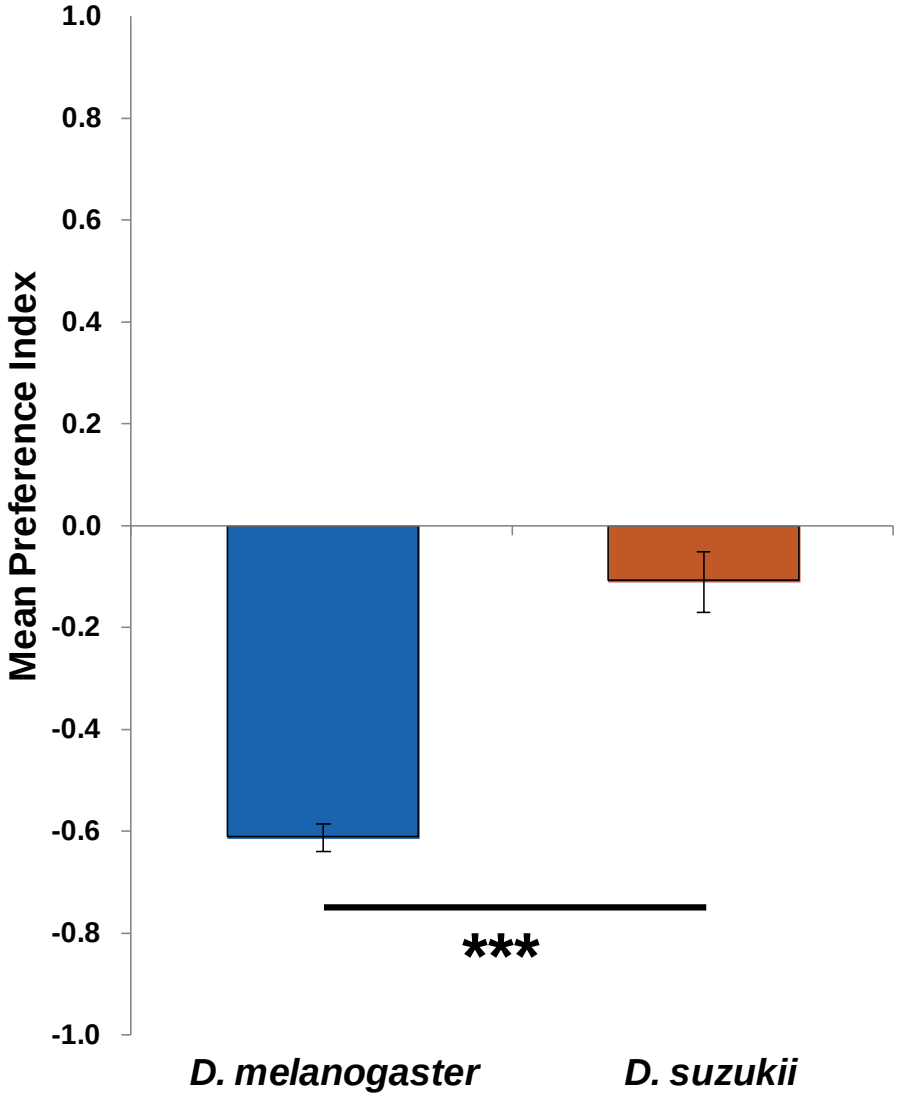

Figure 3.3: *D. suzukii* does not avoid CO_2. Mean preference index of *D. melanogaster* and *D. suzukii* to carbon dioxide (0.67%) in a T-maze assay. N = 11–39 trials, 40 flies/trial, T-test (***P < 0.001).

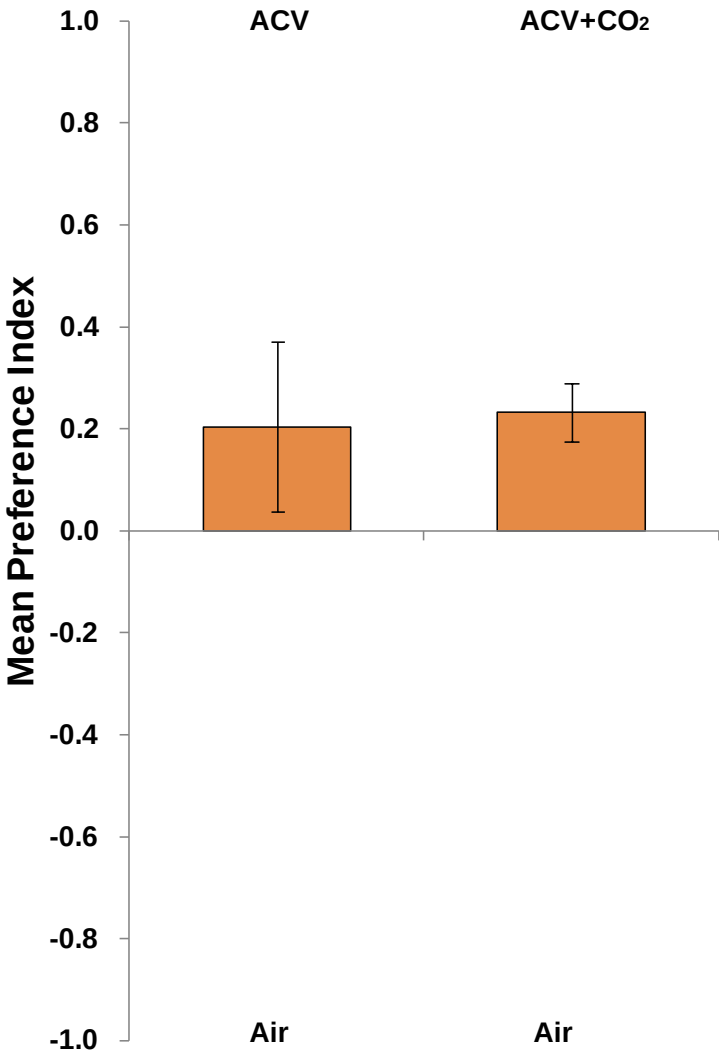

Figure 3.4: *D. suzukii* does not have enhanced attraction to CO_2 in the presence of a food source. Mean preference index of *D. suzukii* to 10% apple cider vinegar in the context of a choice with air or CO_2 (0.33%) in T-maze assay. N = 4 trials, 30 flies/trial, T-test is N.S. (P = 0.88).

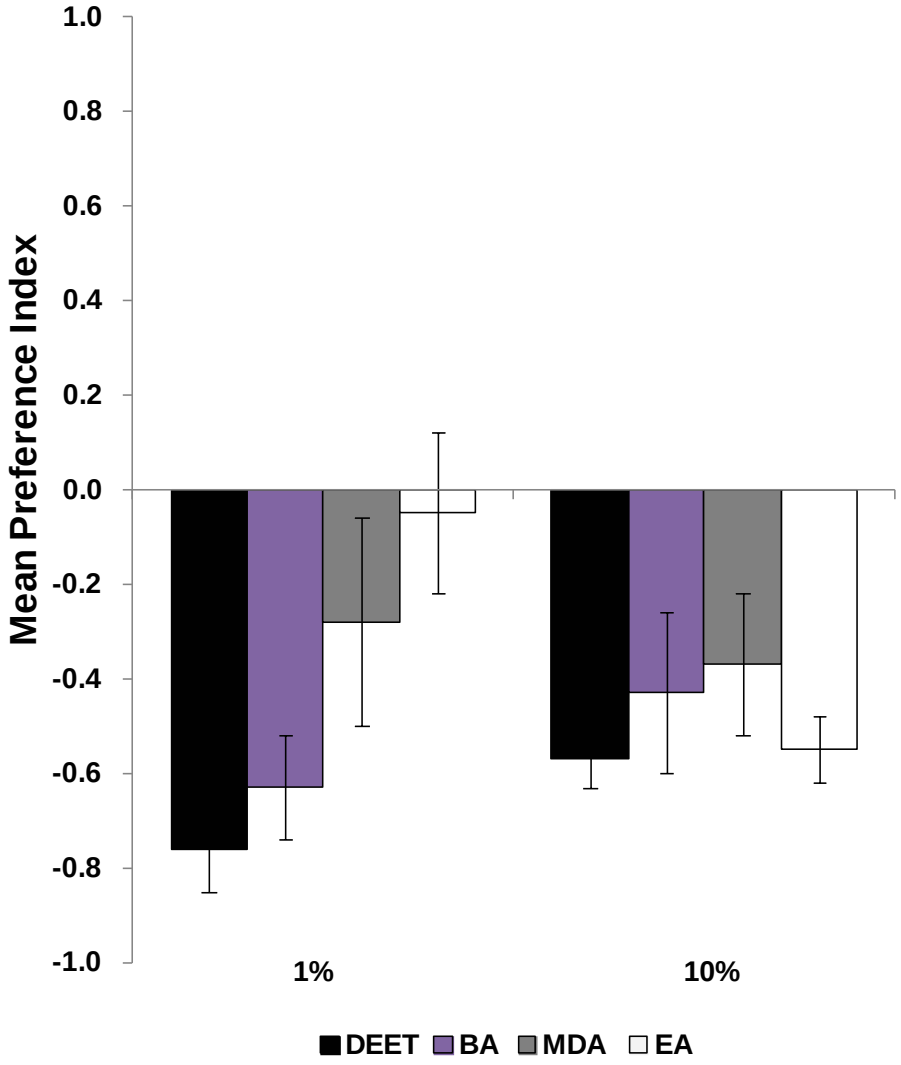

Figure 3.5: *D. suzukii* avoids traps emitting DEET or anthranilate compounds in a contact assay. Mean Preference Index of *D. suzukii* to 1% and 10% for DEET, BA, MDA and EA in a 48 hour two-choice contact trap assay in a plate. N = 4–8 trials, ~10 female flies/trial. Error bars = S.E.M. Kruskal-Wallis One Way Analysis of Variance on Ranks for 1% DEET ($P=0.027$) and for 10% DEET ($P = 0.926$). Dunn's test *($P < 0.05$).

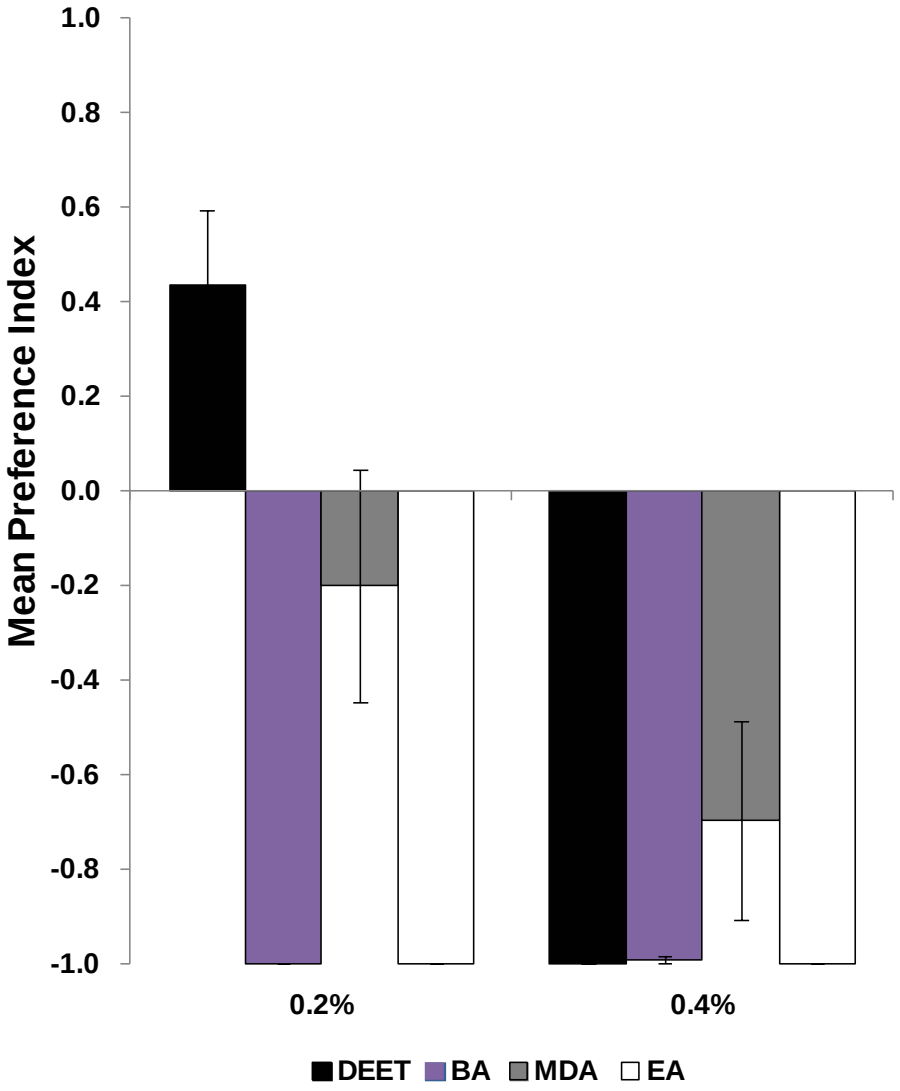

Figure 3.6: *Drosophila melanogaster* avoid BA and EA, but DEET and MDA only at a higher concentration in a two-choice oviposition assay. Mean preference index of *Drosophila melanogaster* to 0.2% and 0.4% for DEET, butyl anthranilate (BA), methyl N,N-dimethylanthranilate (MDA) and ethyl anthranilate (EA) in two-choice oviposition assay with grape juice plates. N = 5–10 trials, ~25 female flies and 15 male flies/trial. Error bars=S.E.M. , Kruskal-Wallis One Way Analysis of Variance on Ranks for 0.2% odorants is (P =<0.001) and for 0.4% odorants (P = 0.004), Tukey Test (*P < 0.05).

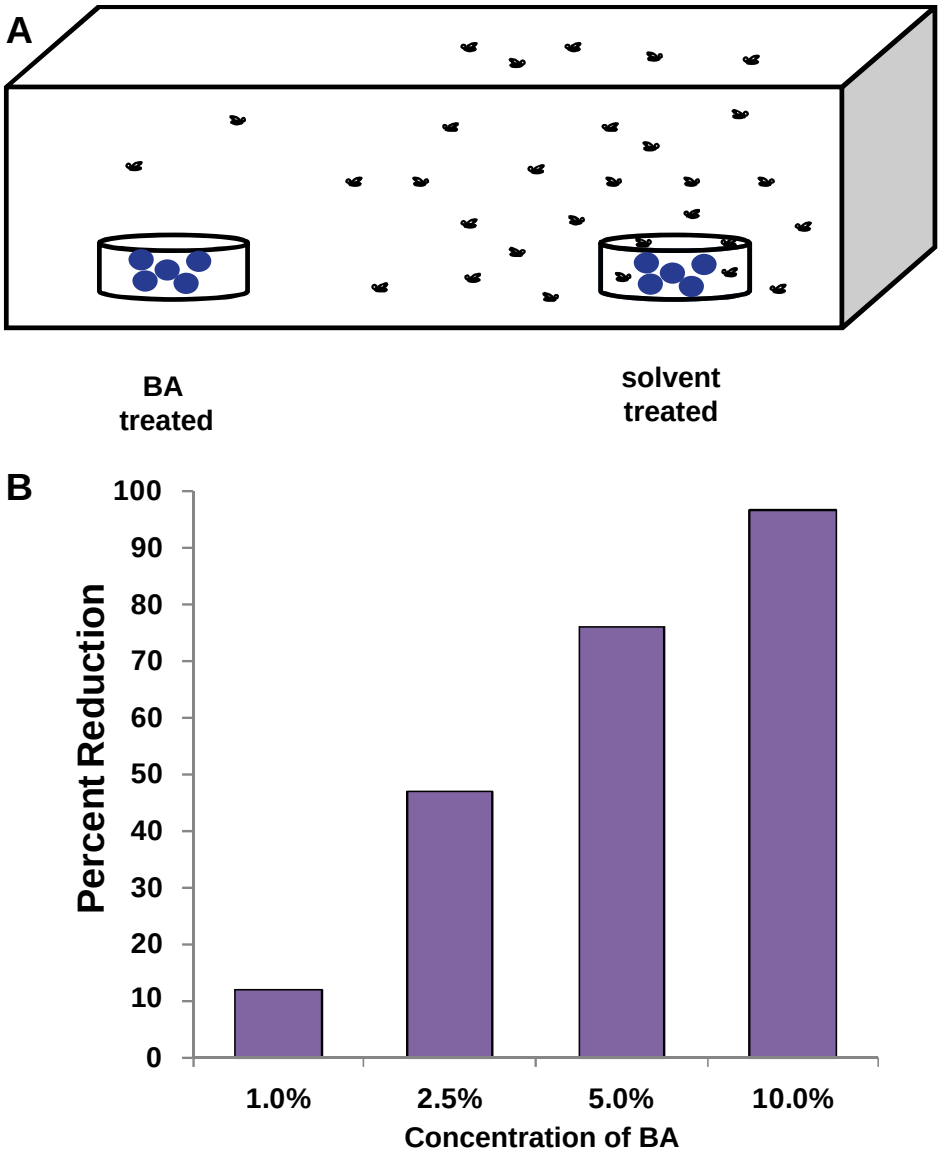

Figure 3.7: BA deters *D. suzukii* from ovipositing in treated blueberries in a two-choice assay in a glass chamber. (A) Schematic of the two-choice assay with blueberries. (B) Percent reduction of *D. suzukii* offspring (eggs, larvae and pupae) counted on 10% butyl anthranilate (BA)-coated fruit vs. solvent-coated fruit after 1 week of a free access to fruit in a glass chamber. Solvent is acetone. Approximately 30 (15 male/15 female) unstarved flies/ trial. Total number of eggs, larvae, pupae and adults were counted 6 days after adults were removed. N=6 trials per concentration.

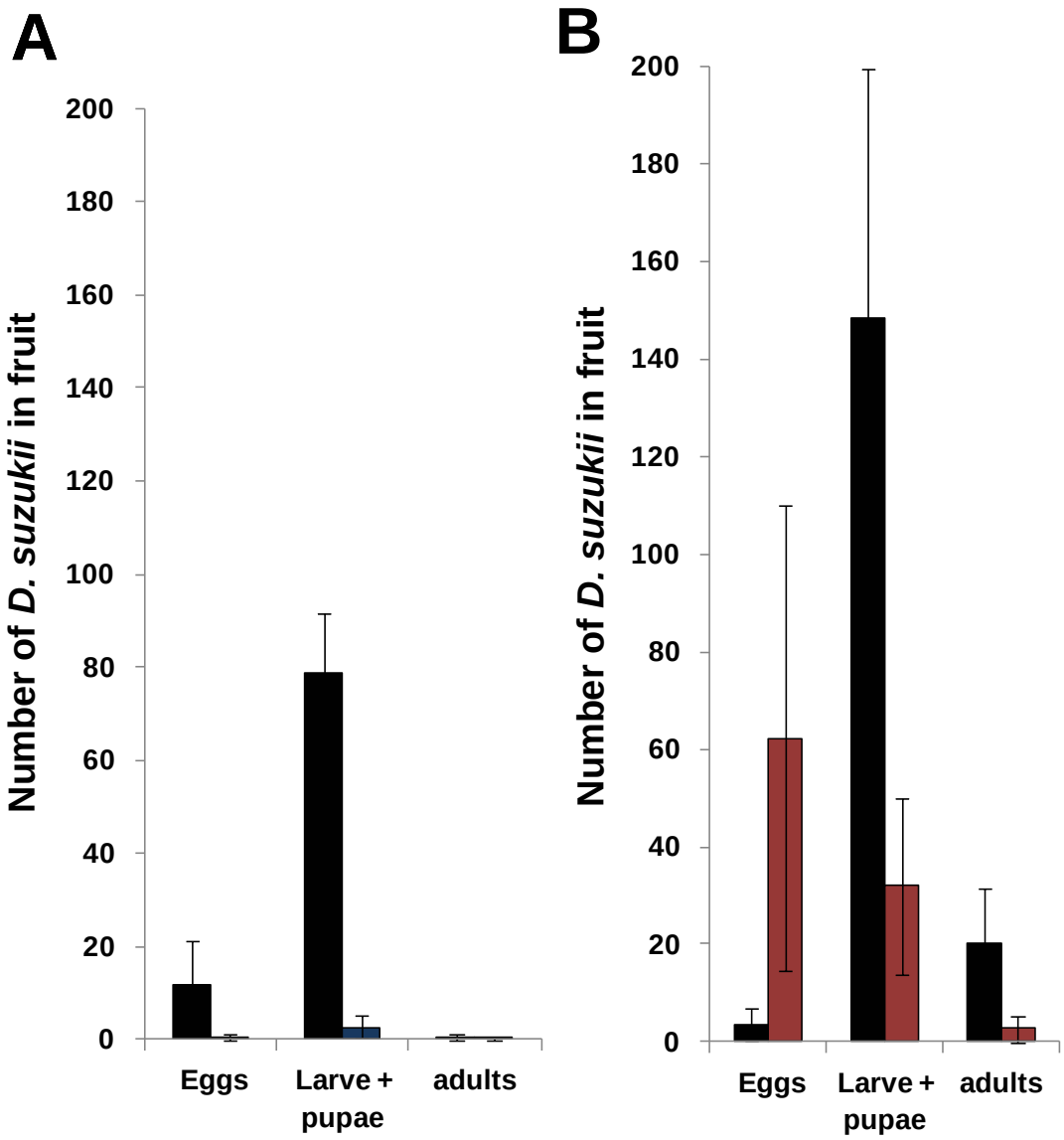

Figure 3.8: Reduced numbers of *D. suzukii* emerge from blueberries and raspberries coated with 10% BA in a two-choice assay in a glass chamber. Numbers of *D. suzukii* offspring (eggs, larvae, pupae and adult) counted on 10% butyl anthranilate (BA)-coated fruit vs. solvent-coated fruit after one week of a free access to fruit in a glass chamber. Solvent is acetone. Approximately 30 (15 male/15 female) unstarved flies/ trial. Total number of eggs, larvae, pupae and adults were counted 6 days after adults were removed. N=3-4 trials. (A) blueberries (B) raspberries.

Chapter 4: DEET receptors

Abstract

Harnessing insect avoidance behavior to reduce contact between pest species and hosts is an important goal. The mechanism of action of DEET, a widely used and effective insect repellent remains controversial. Mechanisms by which DEET triggers avoidance and the integration of avoidance signals in the *Drosophila* brain need further study. Our approach was to consider DEET as a ligand that activates receptors on neurons that result in avoidance behavior. Here we investigated the role of IR chemosensory receptor neurons by exposing *Drosophila melanogaster* co-receptor protein gene mutants for Ionotropic Receptors (IRs) to DEET. We found that *Ir25a*, *Ir76b*, *Ir93a*, and *Ir40a* are not necessary for *D. melanogaster* to avoid DEET. We also confirmed that the Olfactory Receptor (OR) co-receptor, Orco, is necessary for DEET avoidance, but not as a molecular confusant. Individual neurons containing OR family receptors need to be re-examined as possible DEET receptors. Flies deficient in one of these receptors, Or42a, were tested and found to avoid DEET. Identifying all chemosensory receptors activated by DEET will be the first step in understanding a complex *Drosophila* neuronal circuit that integrates sensory modalities. This information can then be used as a starting point to develop new ligands to nudge insects toward avoidance behavior to keep crops from being damaged and people safe from insects carrying disease.

Introduction

Insect avoidance cannot be fully understood without addressing the issue of DEET, a synthetic chemical created by the United States military in the 1940's and a repellent across a wide number of insect species (Travis et al., 1949; Syed and Leal, 2008; Leal, 2014). Previously, we showed DEET protected agricultural products such as blueberries from *Drosophila suzukii* (a pest species) of plants (Krause Pham and Ray, 2015). Despite this chemical also being highly effective at deterring insects from biting humans and offering protection against insect vectors of diseases (for example *Aedes aegypti, Anopheles gambiae, Culex quinquefasciatus*, etc) the pathway for avoidance is not completely understood (Bohbot and Dickens, 2010; DeGennaro et al., 2013; Klun et al., 2013; Leal, 2014; Sparks and Dickens, 2016).

The chemosensory mechanism used by *D.melanogaster* to avoid DEET has been proposed to involve odorant receptors such as Orco, (the Olfactory Receptor co-receptor) (Ditzen et al., 2008), Or42a a receptor found on *D. melanogaster* maxillary palps (Syed et al., 2011), and bitter taste receptors (Lee et al., 2010). How chemosensory systems, both olfactory and gustatory, work together to allow DEET avoidance and the possible role of other more recently discovered receptors, the ionotropic receptor class of chemosensory receptors (Abuin et al., 2011; Benton et al., 2009; Turner and Ray, 2009; Croset et al., 2010; Rytz et al., 2013) has not been considered. We hypothesized that a multimodal avoidance circuit may involve additional unknown taste and smell

inputs of a new class of chemosensory receptors, the ionotropic receptors. To fill this gap, we tested *D. melanogaster* with mutant co-receptors of odorant receptors and ionotropic receptors in behavioral assays to determine the degree to which flies avoid DEET. If we can narrow down the sets of possible ionotropic receptors playing a role in DEET avoidance, then we can proceed to test partners of those co-receptors to determine if specific IRs play a role in DEET avoidance. We then tested *Ir40a* mutant flies for a role in DEET avoidance and found they did not avoid DEET, suggesting *Ir40a* does not play a role in aversion to DEET.

Methods

Drosophila stocks

Drosophila stocks were raised on a standard cornmeal diet in a humidified incubator at 25°Celsius on a 12-hour light/ 12-hour dark cycle. The most appropriate wild-type control line for each mutant was used and indicated in figure captions. Wild-type lines include Canton-S, wCs (which were lab stock Canton-S crossed to w^{1118} for five generations), w^{1118} and Oregon-R. The following mutant lines were used and Bloomington stock numbers noted, if relevant: (1) the *Or42a* flies were homozygoused from *Or42aINS/CyO;Or42aGal4,GFP/TM3*, (2) $Orco^{1}$ (Larsson et al., 2004) (3) $Ir25a^{2}$(BL41737) = *w[*]; $Ir25a^{2}$/CyO*, (Benton et al., 2009) (4) *Ir93a* (BL42090) is $y^{(1)}$ *w*;Mi{y[+mDint2]=mic}*$Ir93a^{[mio5555]}$ (5) *Drosophila* Genome Reference Panel

(DGRP) line 1 is BL28146 and (6) DGRP line 2 is BL28163. The *Ir40a* mutant was generated as part of this dissertation and is described later in methods.

Two-choice non-contact trap assay

The two-choice non-contact trap assay was performed to determine preference for an attractive food source in the context of a repellent odor by modification of a previous assay (Reeder et al., 2001). Briefly, ten male and ten female starved (4–7 day old) flies were placed in a cylindrical chamber containing two 10% apple cider vinegar traps, one with 10% DEET and the other with solvent. Apple cider vinegar (ACV) was placed in the snap-top lid of each microcentrifuge tube. To run the assay, 50 µl of DEET was pipetted into a piece of Whatman #1 filter paper (15 mm X16 mm) wedged between the cut opening of the microcentrifuge tube and the cut P1000 blue tip. Then, 125 µl of ACV was pipette into the snap-top lid. For all trials, the control trap had 50 µl of DMSO applied to the filter paper. The preference index was calculated as PI= (number of flies in test trap- number of flies in control trap)/ (number of flies in test trap + number of flies in control trap).

Two-choice contact trap assay in a plate

The two-choice contact trap assay in a plate tested less volatile odorants. Trials were performed as described (Syed et al., 2011). Ten female flies were placed in a Petri dish containing two traps. Traps were made with 1.5-ml micro centrifuge tubes (USA Scientific) with an opening cut in the bottom

of the tube. Both traps contained the fly's normal laboratory food at the base. The neck of one trap had a filter paper with test odorant, the other trap had solvent. Five microliters of hexane (control) and five microliters of 10% DEET or test compounds in hexane were applied to the stem part of filter paper inserted into upper part of pipette tip near entrance of trap to allow flies to walk over the treated surface. Traps were placed in chemical hood for 5 minutes to allow hexane to volatilize before being placed in the 1% agarose-treated Petri dish chamber.

One-choice contact plate assay

The one-choice contact plate assay was a variation of the two-choice contact assay in a plate where a single trap containing the test odorant and food was placed in the plate. Flies were not given the option of selecting a control trap with solvent.

T-maze assay with heated DEET

The T-maze assay with heated DEET was a modification of the T-maze assay (Turner and Ray, 2009). The DEET and paraffin oil (PO) control tubes were prepared as follows: (1) Aluminum foil circles (2-cm diameter) were positioned in the bottom of each test tube; (2) DEET (10µ) was pipette onto the foil into the experimental tube and PO (10µ) into the control tube; (3) Eight tubes (trials 1-4 DEET and PO) were rubber banded together and inserted into a water bath heated to 75-90°C; (4) Tubes were heated for 10 to 15 minutes; (5) Tubes

were removed from the water bath and cooled until the bottom of the tubes were at room temperature (25-26°C) measured by a Fisher Scientific Traceaole Calibration Device; (6) Unstarved flies (20 male + 20 female) aged to 3-8 days old were loaded into the T-maze elevator; (7) Test and control tubes were inserted and elevator opened. (8) Flies had one minute in the dark to choose DEET or paraffin oil. Trials were run in block design. Preference Index is calculated as PI= (number of flies in test arm- number of flies in control arm)/ (number of flies in test arm + number of flies in control arm).

Generation of Ir40a mutant fly lines

The CRISPR (Jinek et al., 2012) target site was chosen using tools available online at http://flycrispr.molbio.wisc.edu/tools. Targeting oligos Dmel-Ir40a-FwdC and Dmel-Ir40a-RevC were ligated directly into BbsI-digested pU6-BbsIchiRNA (Addgene #45946). Resulting clones were sequenced at the UC Riverside Core facility. The U6-Ir40a-chiRNA cassette was removed using NotI and XhoI digestion and cloned into plasmid pattB. The resulting pattB{U6-Ir40a-chiRNA} vector was verified by DNA sequencing. Injections were performed by Genetic Services and transgenic flies were created by injecting into *y,w;attP40* embryos, using the site-directed phiC31 integrase system. *Vas-Cas9* (BL# 51324) females were mated with attP40{Ir40a-chiRNA} males and the resulting attP40{Ir40a-chiRNA}/+; vas-Cas9/+ females were mated to balancer males to generate six isogenic lines. All six lines exhibited indels at the CRISPR target

site, three frame-shift alleles and three in-frame deletion alleles. CRISPR design and molecular cloning were done by Dr. Maria Irigoyen, PhD.

Results

Role of *Or42a* and *Orco* in DEET avoidance

First we tested the behavioral response of *Orco*^{1} and *Or42a* mutants in response to DEET to confirm previously published data (Ditzen et al., 2008; Syed et al., 2011). In a two-choice trap assay where flies can both smell and contact DEET (flies chose between two food containing traps, one with DEET and the other with solvent on filter paper at the entrance), there was no difference between avoidance by wild-type (PI=-0.96) and *Or42a* mutant flies (PI=-1.00). The olfactory neuron co-receptor mutant, *Orco*^{1} however showed a statistically significant reduced avoidance to DEET compared to wild-type flies. (PI=-0.67, P=0.006) (Figure 4.1). The palp-specific receptor, *Or42a*, is also dependent on Orco for functioning.

Next we used a more stringent test, a one-choice contact-trap assay (Syed et al., 2011), in which flies must go into the odor laced trap to access food or they starve. Over a three-day period, wild-type female flies starved to 30 hours avoided the traps laced with 10% DEET and to a lesser degree 1% DEET (Figure 4.2A). After a palpectomy, wild-type flies showed little difference in avoidance to DEET at the higher concentration (Figure 4.2B). At the 1% concentration, a few more flies overall entered the traps. We concluded *wCs* can sense 10% DEET

without palps, but are less deterred by the lower concentration of DEET when their palps are removed.

In the same assay, *Orco* was not necessary to avoid DEET at a concentration of 10%, but may play a role in avoidance of lower concentrations of DEET (Figure 4.3). *Orco* mutant flies responded similarly to wild-type flies by entering the food trap with 1% DEET. Interestingly, it seemed that additional receptors in the olfactory and taste systems may play a role at the higher concentrations, while the ORs play a role at lower concentrations. The flies may have also perceived the lower concentration of DEET as less bitter when they were extremely hungry (Sparks and Dickens, 2016).

Next we asked if *Drosophila* would avoid DEET in a short-term non-contact assay. We tested wild-type and *Orco* mutant flies in the T-maze assay and found that there was no preference to DEET in either genotype. The most likely explanation was that DEET is a low volatility compound (vapor pressure at 25°C and 1 atm is 0.005 mmHg). The flies may not have sensed the DEET volatiles in the decision-making zone of the T-maze in the 1 minute time allotted for the experiment. For example, flies may have been resting in the portion of the test odorant tube at a distance from the DEET and still not have sensed the DEET.

We then modified the T-maze assay by volatizing the DEET stimulus prior to the assay by increasing temperature. Tubes with DEET were then cooled to room temperature. When exposed to the tube with the volatized DEET, wild-type

flies avoided DEET (PI=-0.33), while *Orco* flies (PI=+0.02) showed no preference between the DEET-filled or paraffin oil-filled arms of the T-maze (P=0.001) (Figure 4.4). We concluded that *Orco* was necessary for avoidance to DEET in this non-contact, short-term assay that did not contain a food cue or lure.

Additionally, to determine if the bitter taste neurons are necessary for DEET avoidance, we silenced neurons containing the bitter co-receptor, *Gr89a* using the Gal4-UAS system (Figure 4.5). Briefly, we drove expression of Kir2.1, an inwardly-rectifying potassium ion channel, in Gr89a active cells, which caused these neurons to become hyperpolarized and prevented firing. The mean preference index to DEET in these bitter neuron silenced flies was PI=-0.74 (control PI=-0.9). There was a trend for slight reduction in repellency but it was not statistically significant. We concluded that bitter neurons are not necessary for DEET avoidance in this assay.

Role of Ionotropic Receptors (IR) in DEET avoidance

The discovery of Ionotropic Receptors(IR) led us to test for possible contributions by this class of chemosensory receptors for avoidance of DEET. In order to do so, we first analyzed flies mutant in co-receptors to narrow down the number of possible candidate IR neuron's playing a role in DEET avoidance. We tested behavioral responses to DEET in mutants of *Ir93a, Ir25a* and *Ir76b*.

In a non-contact two-choice trap assay, the participation rate of *Ir25a* mutant flies was very low (data not shown). This assay used apple cider vinegar

as a lure and required that the mutant insects were able to fly and to navigate toward it. We expected that the flies would be motivated to access the food source rather than die. The *Ir25a* mutant flies may have a compromised ability to sense apple cider vinegar and hence not able to find the traps. Next, we tested *Ir25a* mutants in a two-choice trap assay in a plate that allowed for flies to walk and to have contact with DEET. In this form of two-choice contact trap assay in a plate, *Ir25a* mutants had slightly lower avoidance to DEET (PI=-0.85 and $p<0.06$) compared to wild-type (PI=-1.00) and the heterozygous *Ir25a* flies (PI=-1.00), which had complete avoidance of DEET (Figure 4.6, black bars). In control experiments, all genotypes were able to find the fly food placed in control traps (Figure 4.6, colored bars).

Next, we tested flies mutant in the co-receptor, Ir93a. In a trap assay allowing for contact wild-type white-eyed flies were attracted to the food source and avoided 10% DEET (PI=-1.00). *Ir93a* mutant flies were strongly repelled by 10% DEET as well (PI=-0.92) (Figure 4.7). In the T-maze assay with heated DEET, *Ir93a* mutant flies avoided DEET (PI=-0.47) and there was no statistically significant difference between the wild-type (PI=-0.39) and *Ir93a* line tested ($P=0.51$) (Figure 4.8). *Ir93a* was not necessary for DEET avoidance in either contact or non-contact assays.

Flies mutant in Ir76b also avoided 10 µl DEET (PI=-0.33) in the T-maze with heated DEET assay. Additionally, there was not a statistical difference between these flies and the control line (PI=-0.25) (Figure 4.9). From this data,

we concluded that the ionotropic co-receptors *Ir25a*, *Ir93a* and *Ir76b* were not necessary for avoidance to DEET.

Hypothetical Ionotropic Receptor for DEET

We next considered the specific ionotropic receptor Ir40a, which is expressed in neurons housed in the first and second compartments of the sacullus on the fly antenna to play a role in an olfactory driven avoidance to DEET (Benton et al., 2009; Mackay et al., 2012). The sacullus is a cave-like chamber located on the fly antenna. Typical diagnosis tools such as single-sensellum electrophysiology cannot be used to monitor activity of these olfactory neurons. Hence, little is known about the olfactory neurons housed in the sacullus, specifically the Ir40a expressing neurons. A mutant line was not available; however, the recently collected population of flies in the *Drosophila* Genomic Research Panel (Mackay et al., 2012) had a number of fly lines with naturally occurring single-nucleotide polymorphisms in the coding sequence of Ir40a. We identified a subset of these fly lines with mutations expected to cause a change in the protein sequence of Ir40a. We selected two lines from the DGRP to sequence and confirmed a mutation in the Ir40a coding region. We tested these in behavioral assays with DEET to determine the behavioral output from mutations in this receptor protein.

The DGRP flies were collected from an outdoor market in North Carolina in 2003, isogenized and sequenced. The genetic backgrounds of these wild-caught *D.melanogaster* have not been well studied (Mackay et al., 2012). First,

we did preliminary behavioral tests to determine fitness of these fly lines. We found that these two lines were attracted to apple cider vinegar (ACV) to the same degree as our wild-type flies, in a two-choice non-contact assay when given the choice between ACV and water (Figure 4.10). In this assay, trials with less than 7 flies participating per assay were not included in the analysis. Of the trials analyzed, the overall participation rate of wild-type (wCs) was 62%, line 1 was 58% and line 2 was 47%. There was no statistically significant difference between line 1 (P=0.737) and line 2 (P=0.389) when each was compared with wild-type in a two-tailed student's t-test. The number of trials making our cutoff for participation was achieved in more than 90% of the trials for wild-type and line 1, whereas less than 50% of the trials for Line 2 were counted. There may have been something specific about the mutations that Line 2 flies have that reduce their fitness for participating in this assay.

Next, in a two-choice non-contact trap assay between 50 µl of DEET and 50 µl of solvent (DMSO), DGRP flies from line 1 showed reduced avoidance to DEET when compared with wild-type flies (PI=-0.16),(P=0.0004). For line 2 there was reduced avoidance overall (PI=-0.18), but the behavior was more variable and not statistically different from wild-type (P=0.0663). Here too, of the lines analyzed, the participation rate was 55% for wCs, 59% for line 1 and 42% for line 2. Again, we noted that Line 2 may have some other genetic variability that makes it difficult for these flies to participate in this assay (Figure 4.11).

In the meantime, a new technology, CRISPR-Cas9 genome editing, became available (Jinek et al., 2012). To validate our results from the DGRP mutant, we used this technology to create a knockout of the *Ir40a* gene. We generated several deletion alleles. We selected one allele *Ir40a(7),* which had 5 nucleic acids removed at the CRISPR cut site for further analysis. The resulting frame shift deletion created a stop codon near the beginning of the protein. The resulting truncated Ir40a protein contains a short piece of the extracellular portion of the protein and no membrane-bound portion. As a control we selected *Ir40a(6)*, which has the same genetic background as our CRISPR mutant, only it is missing three nucleotides (GTC) at the CRISPR cut site which generates an in-frame deletion allele for Ir40a (Figure 4.12). Next we repeated the two-choice non-contact trap assay (Figure 4.13). Surprisingly, we observed no difference in avoidance between our CRISPR mutant and our control line. Ir40a(7) had (PI=-0.81) and the control line, Ir40a(6) had (PI=-0.80). There was no significant behavioral difference between these two fly lines (P=0.97). As an additional precaution, siblings of these flies were sequenced a second time and confirmed to be the CRISPR generated lines. We then tested these mutants in the T-maze assay with heated DEET using two concentrations of DEET. At 50 µl of DEET, both lines avoided DEET with Ir40a(7) having a PI=-0.46 and control Ir40a(6) with a PI=-0.43. At a lower dose of 10 µl of DEET, both avoided at PI=-0.34 (control PI=-0.34) (Figure 4.14). From this data, we concluded that *Ir40a* is not necessary for DEET avoidance in *D. melanogaster*.

What is the natural ligand for the receptor that DEET activates?

We tested a number of concentrations of ammonia, which switches valence from an attractant at low concentrations to a repellent at high concentrations (Min et al., 2013). As expected wild-type flies showed attraction for low concentrations of ammonia but repulsion for concentrations greater than 1% ammonia (Figure 4.15). *Orco* mutant flies showed a similar pattern to wild-type. Interestingly, *Gr63a* mutant flies showed lower attraction at the low dose of 0.05% ammonia, attraction at 1% and less avoidance at 5% ammonia. The *Gr63a* mutant avoidance pathway may play a role in ammonia avoidance. However, an interesting note is the *Orco-Gr63a* double mutant had greater avoidance than wild-type at all concentrations. We would expect the double mutant to have less avoidance than the wild-type based on the results of the single mutant. This data implies that IRs make a large contribution to ammonia avoidance.

Next we tested $Ir76b^{1}$ co-receptor mutant and *Ir40a* mutant at our highest dosage of 5% ammonia (Figure 4.16). Ir40a mutants showed slight avoidance with a PI=-0.26 and control PI=-0.21. Interestingly, $Ir76b^{1}$ mutants had a reduced avoidance to 5% ammonia (PI=-0.08) and may play a role in avoidance to high concentrations of ammonia. Overall, our results suggested that ammonia sensing does not seem to require *Ir40a*.

Discussion

Two olfactory theories have been used to explain the repellency mechanism of DEET on insects. The first idea is that DEET acts as a ligand for chemosensory receptors (Lee et al., 2010; Syed et al., 2011) and the second that DEET interferes with the normal activation patterns of olfactory receptor neurons in response to their ligands acting as a “confusant” (Ditzen et al., 2008). We set out to determine whether DEET acts as a potential ligand for IR receptors. In our behavioral analysis, we found that all tested IR co-receptor (*Ir25a, Ir93a, Ir76b*) mutants as well as *Ir40a* were not necessary for DEET avoidance. Recently, Ir40a was shown to be a humidity sensor in behavioral assays allowing flies to avoid dry air (Enjin et al., 2016). In addition, the co-receptors Ir93a and Ir25a in the Ir40a containing neurons in chamber I and II of the sacculus were shown to be both humidity and temperature sensors (Knecht et al., 2016).

We found that *Orco* was partially necessary for DEET avoidance in long-term assays. However, we also observed that *Orco* flies avoided apple cider vinegar traps in the presence of high (10%), but not low (1%) concentrations of DEET. Interestingly, in the T-maze assay without food or another odor present, *Orco* flies were not repelled by DEET suggesting that the confusant model is likely incorrect, and that the chemical likely activated some ORs conveying aversion.

Orco may play a role in the degree to which avoidance occurs when flies approach DEET. In a short term non-contact assay, the *Orco* gene was

necessary for flies to avoid DEET. Our next question would be to determine specific OR genes required for DEET avoidance. We tested flies mutant in the maxillary palp specific olfactory receptor**,** Or42a, in a two-choice trap assay and conclude that *Or42a* was not necessary for DEET avoidance. This conflicts with the literature (Syed et al., 2011) that showed Or42a mutant flies were unresponsive to DEET. The published results may be due to the solvent DEET was dissolved in rather than DEET.

Perhaps DEET has triggered a complex avoidance pathway involving distinct olfactory receptors and gustatory receptors. This study tested some possible individual components. With new molecular tools such as CRISPR-Cas9, future studies may be able to create individual and multiple gene knock outs in a single fly genetic background. The currently available mutants in different genetic backgrounds likely reduced the clarity of results because of the effects of other genes on behavior. For example, the white gene present in many of our fly lines used caused these flies to produce less dopamine. This may have further impacted the ability of our experimental flies to participate fully in experiments.

Flies were attracted to low concentrations of ammonia, a waste product. Low concentrations of ammonia may have been a positive signal and higher concentrations a negative signal for flies. Some waste products may have signaled that other flies are around and this could be beneficial for finding mates. However, if too many flies were present and an excessive amount of waste was

in the food, then this would be less desirable for survival of both the adult and future offspring. Flies tested in the laboratory were under controlled conditions where the numbers and time flies were cultured were carefully controlled. If flies were raised at a greater density, then they may have been exposed to more waste products during development and it may have been beneficial to avoid them when present in high quantities. Fly waste products may have contained biologically relevant compounds; however, in *Drosophila melanogaster* responses to these natural substances did not follow the pattern of DEET response. Development of olfactory choice assays that used other innate characteristics such as phototaxis or geotaxis did not work in my hands. These innate responses seemed to be stronger than olfactory responses and may have involved activation of higher brain centers in flies that override olfactory signals. Fly fitness to participate in assays may have also resulted from defects in flying (we had more success with walking assays). Responses to odorants may have differed in flies while flying vs. walking.

Understanding the DEET detection pathway continues to be an important endeavor because it has wide implications for pest control and neuroscience of how different sensory modalities are integrated and weighed in the fly brain. We can learn a great deal about the wiring of information from disparate sensory modalities that are integrated.

Using flies from the DGRP collection, we identified a promising fly line (Line 1, BL28146) that avoids DEET and initially concluded that this was due to a

SNP in the *Ir40a* gene. A weaker, connection existed to DGRP line 2 (BL28163) due to its less consistent response and lower participation. Subsequently we showed by testing a CRISPR mutant of *Ir40a* that it was not necessary for DEET avoidance. In addition, co-receptor of the Ir40a containing neuron in the sacculus, Ir93a avoided DEET and Ir25a showed a slight reduction in DEET avoidance. These results were corroborated by a recently published report showing, Ir40a to be a humidity sensor in *D. melanogaster* and Ir93a a temperature sensor. Ir25a is our most likely co-receptor for contributing to an IR-related DEET avoidance pathway. Nevertheless, DGRP Line 1 is a good candidate for further investigation of factors necessary for avoidance to DEET. It may very well be that another naturally occurring SNP in this wild caught line is in a gene that is a player in DEET avoidance in *Drosophila melanogaster*.

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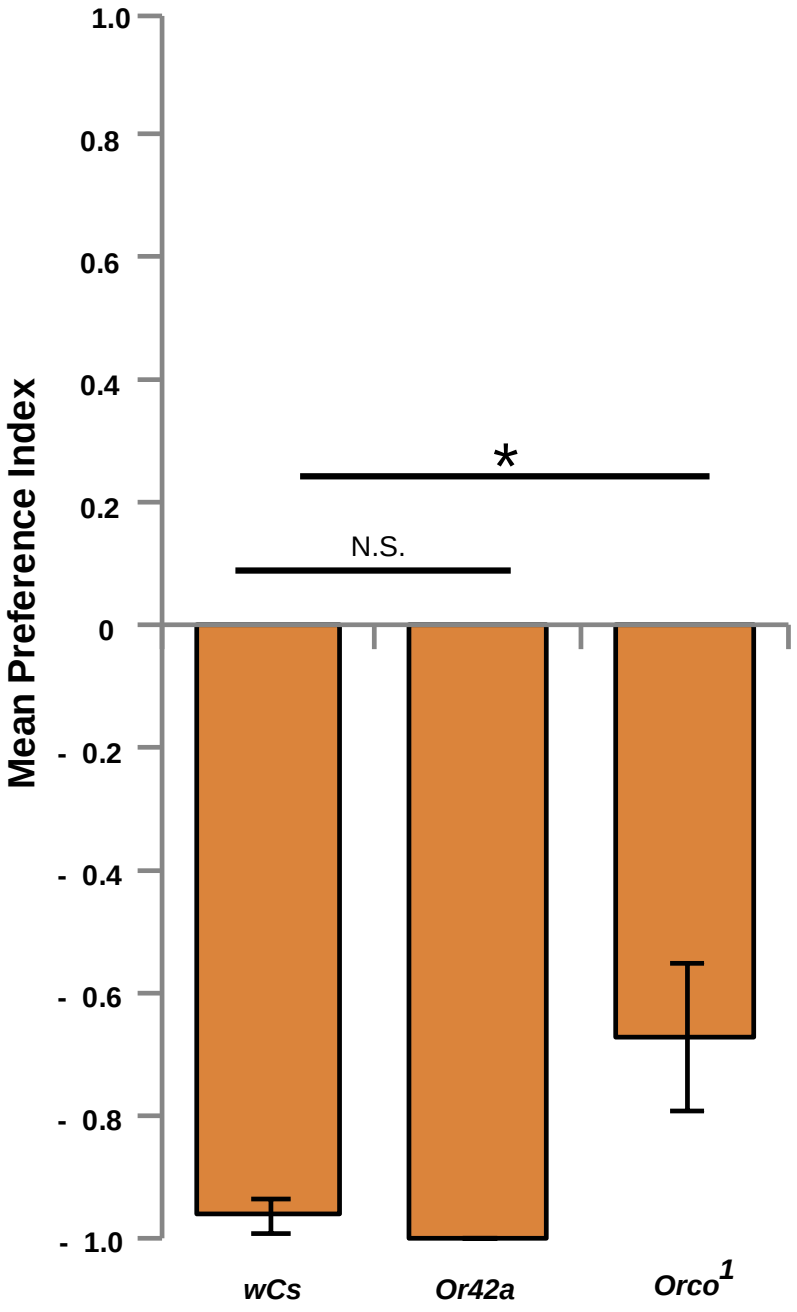

Figure 4.1: *D. melanogaster* mutant in *Or42a* shows complete avoidance and *Orco*^{1} shows reduced avoidance to 10% DEET in two-choice contact plate assay. Mean Preference Index for wCs (PI= -0.96), *Or42a* (PI= -1.00) and *Orco*^{1} (PI= -0.67). Ten female flies per trial were given 48 hours to choose, N=3-10 trials, error bars = S.E.M., student's t-test value for wCs and *Orco*^{1} (P=0.006).

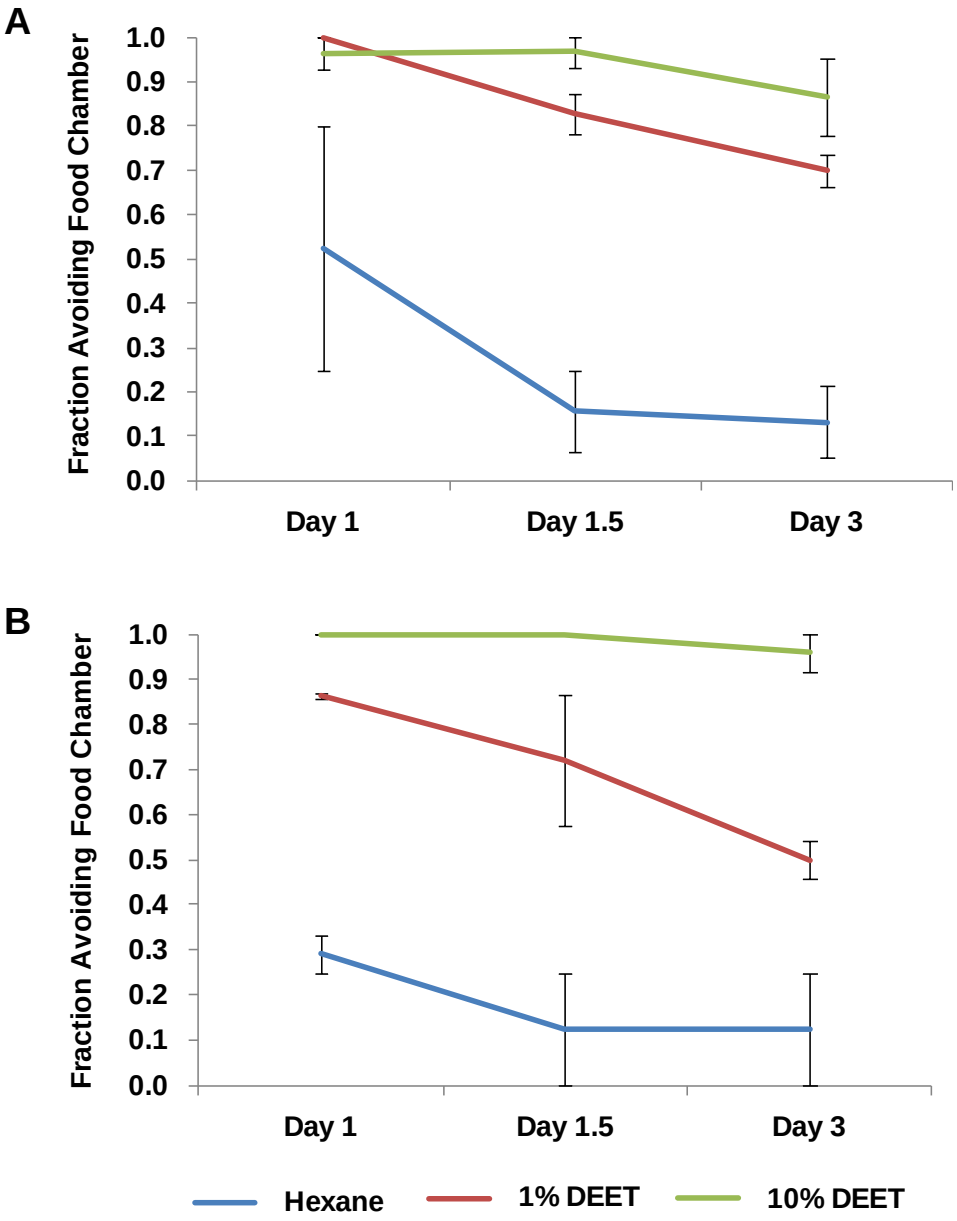

Figure 4.2: Wild-type *D. melanogaster* avoid DEET in the one-choice contact plate assay. (A) wCs flies avoid 1% and 10% DEET in hexane solvent. Ten female flies per trial, N= 3-4 trials, error bars = S.E.M. (B) wCs flies with maxillary palps removed avoid 1% and 10% DEET in hexane solvent. N= 3 trials, error bars = S.E.M

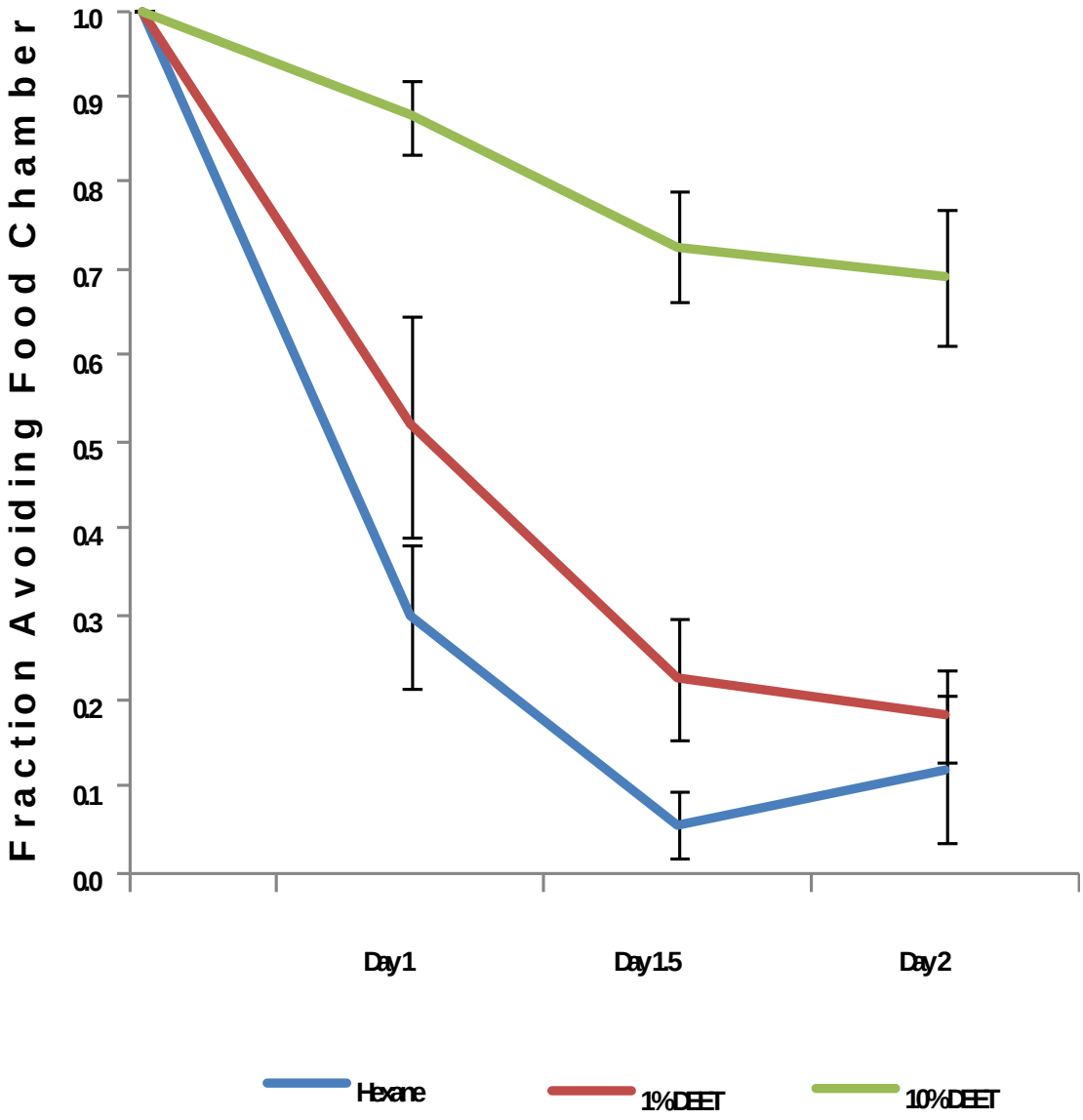

Figure 4.3: *D. melanogaster Orco*[1] mutant avoid DEET in the one-choice trap assay to a lesser extent than wild-type flies. DEET was presented at 1% and 10% in hexane solvent. Ten female flies per trial, N=6 trials, error bars = S.E.M.

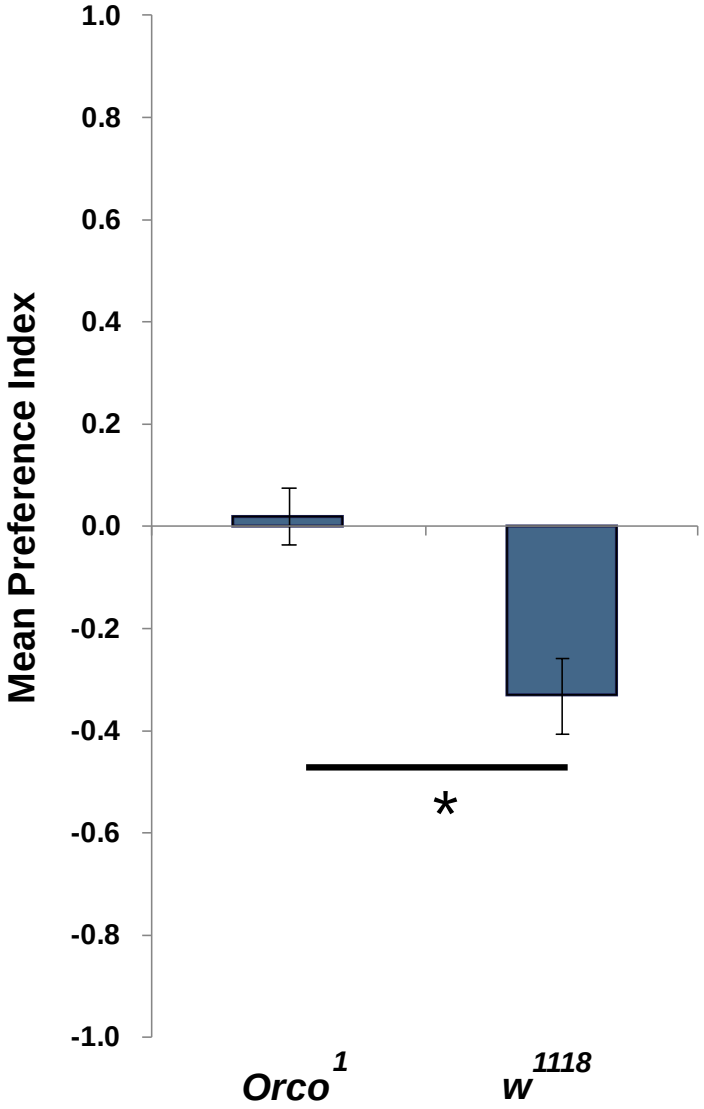

Figure 4.4: *D. melanogaster Orco* mutant flies show no preference in T-maze assay to DEET heated. Mean preference index of white eyed mutant flies and *Orco* mutant flies to 10 µl of pure DEET heated. N=12, error bars = SEM. Two-tailed student's t-test (P=0.001).

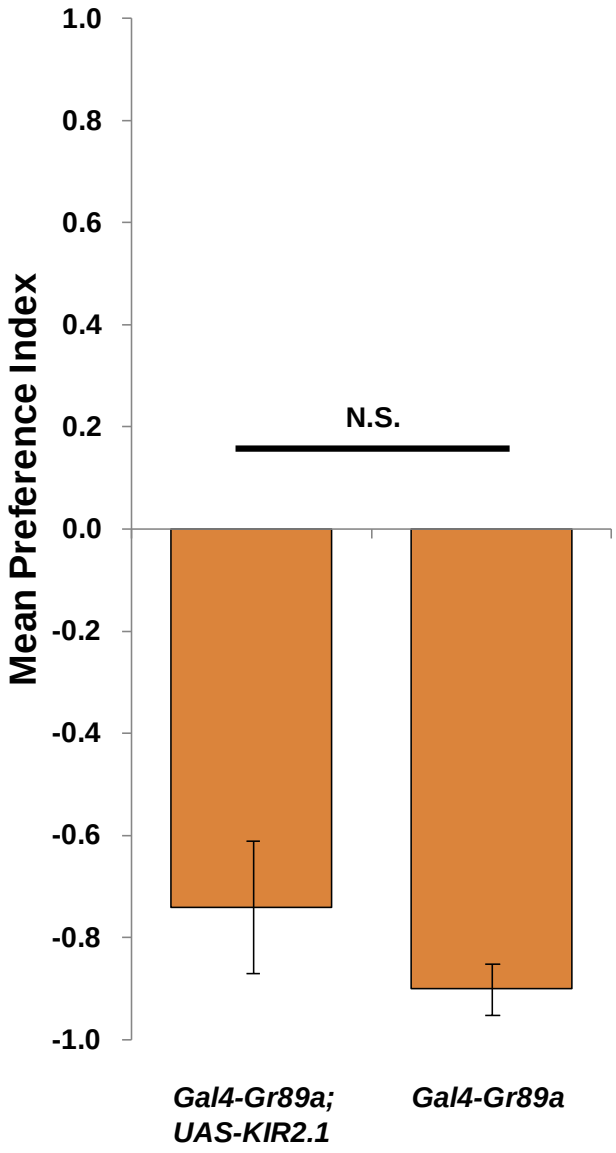

Figure 4.5: *D. melaogaster* with inactivated neurons containing the bitter co-receptor Gr89a avoid DEET in a two-choice contact plate assay. DEET was presented at 10% and dissolved in hexane, N=8 trials, Ten female flies per trial, error bars = S.E.M., two-tailed student's t-test was N.S. (P=0.18).

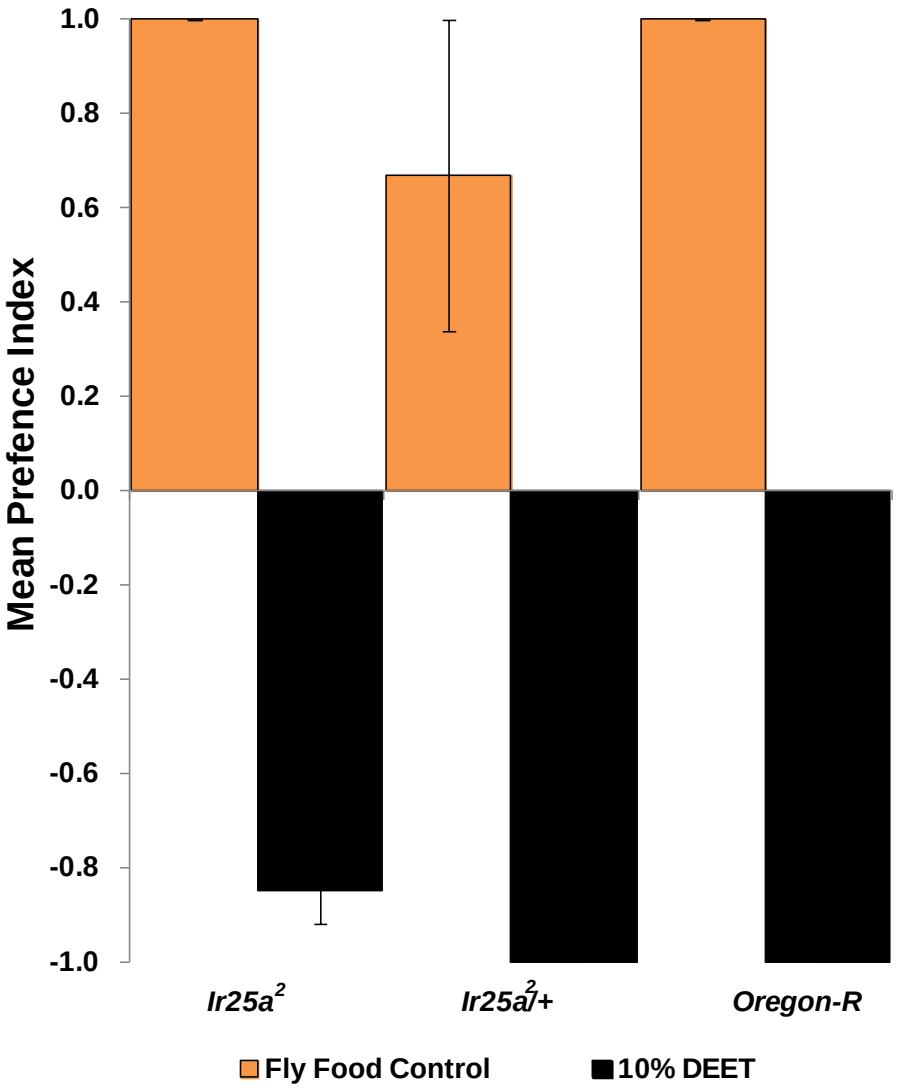

Figure 4.6: *Ir25a*^{2} mutant flies do not show statistically significant avoidance to DEET in the two-choice contact trap assay in a plate. Mean Preference Index of 20 (10 male/10 female) flies to 5 µl of 10% DEET in hexane vs. 5 µl hexane (food in both traps), (PI=-0.85) (P=0.06). All flies were able to find the food traps when 5 µl of hexane was placed on both filter papers. Test trap had food and control trap had no food. The *Ir25a*^{2} allele was tested. Wild-type background is Oregon-R, N=6-9.

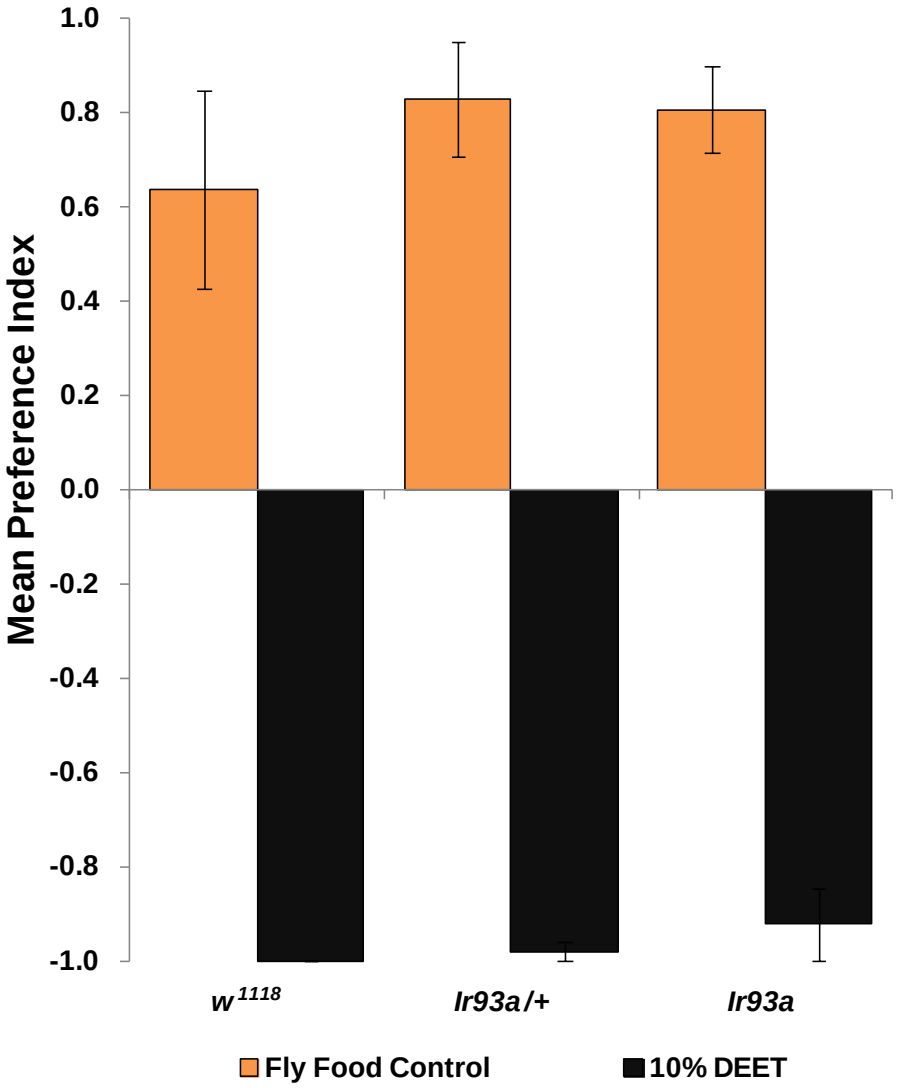

Figure 4.7: *Ir93a* mutant flies avoid DEET in two-choice contact trap assay in a plate. Mean Preference Index of 20 (10 male/10 female) white-eyed flies and mutant *Ir93a* flies to 5 µl of 10% DEET in hexane vs. 5 µl hexane (food in both traps). All flies were able to find the food traps when 5 µl of hexane was placed on both filter papers. Test traps had food and control traps had no food N=9-26.

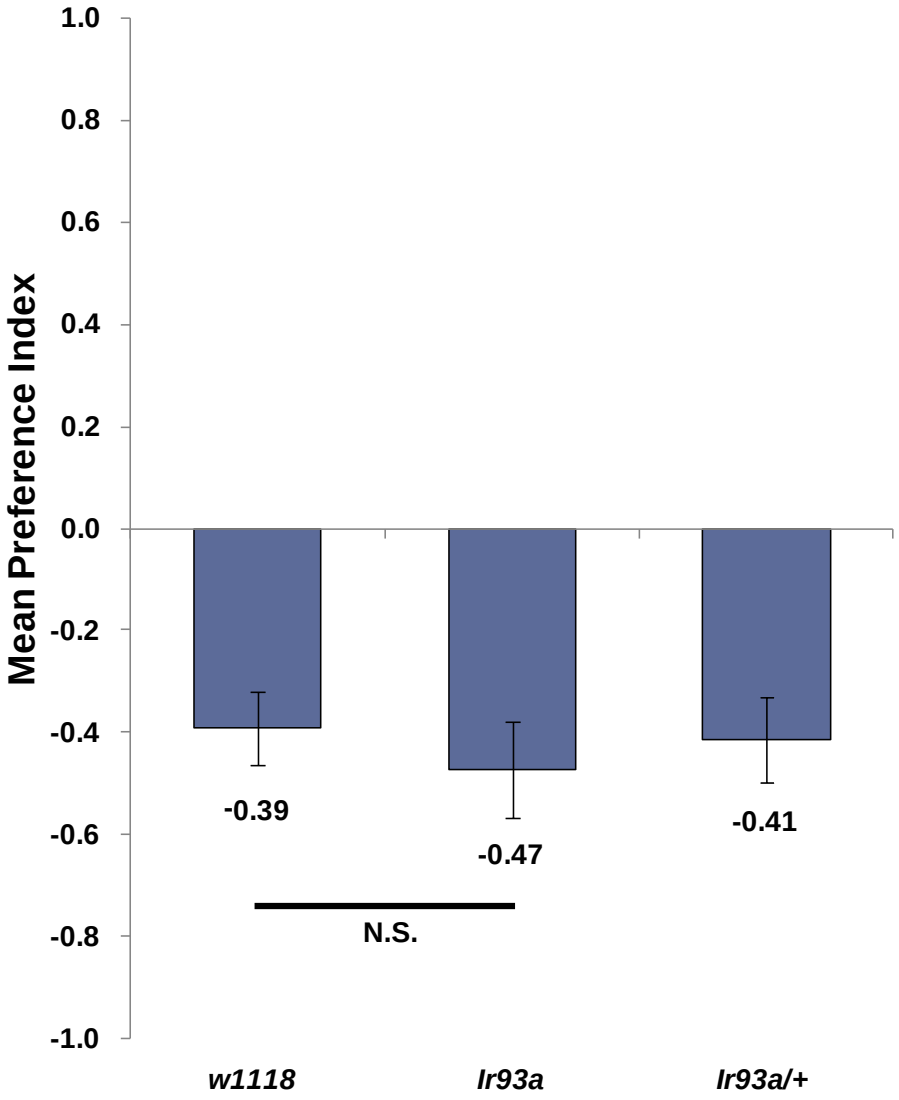

Figure 4.8: *Ir93a* mutant flies avoid DEET in T-maze assay with heated DEET. Mean preference index of 40 unstarved wild-type w1118 flies and *Ir93a* mutant flies to 50 µl of pure DEET heated. N=9-11, error bars = SEM. Two-tailed student's t-test between *w1118* and *Ir93a* mutant is N.S. (P=0.507).

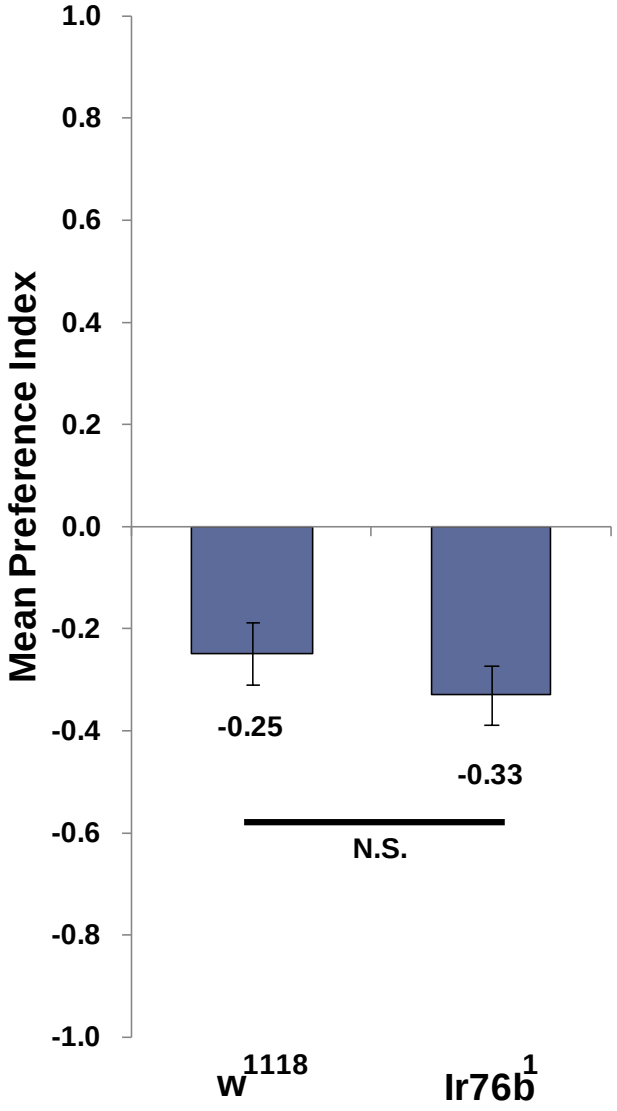

Figure 4.9: *Ir76b*^{1} mutant flies avoid DEET in T-maze assay with heated DEET. Mean preference index of 40 white-eyed flies and *Ir76b*^{1} mutant flies to 10 µl of pure DEET heated. N=6-12, error bars = SEM. Two-tailed student's t-test was N.S. (P=0.387).

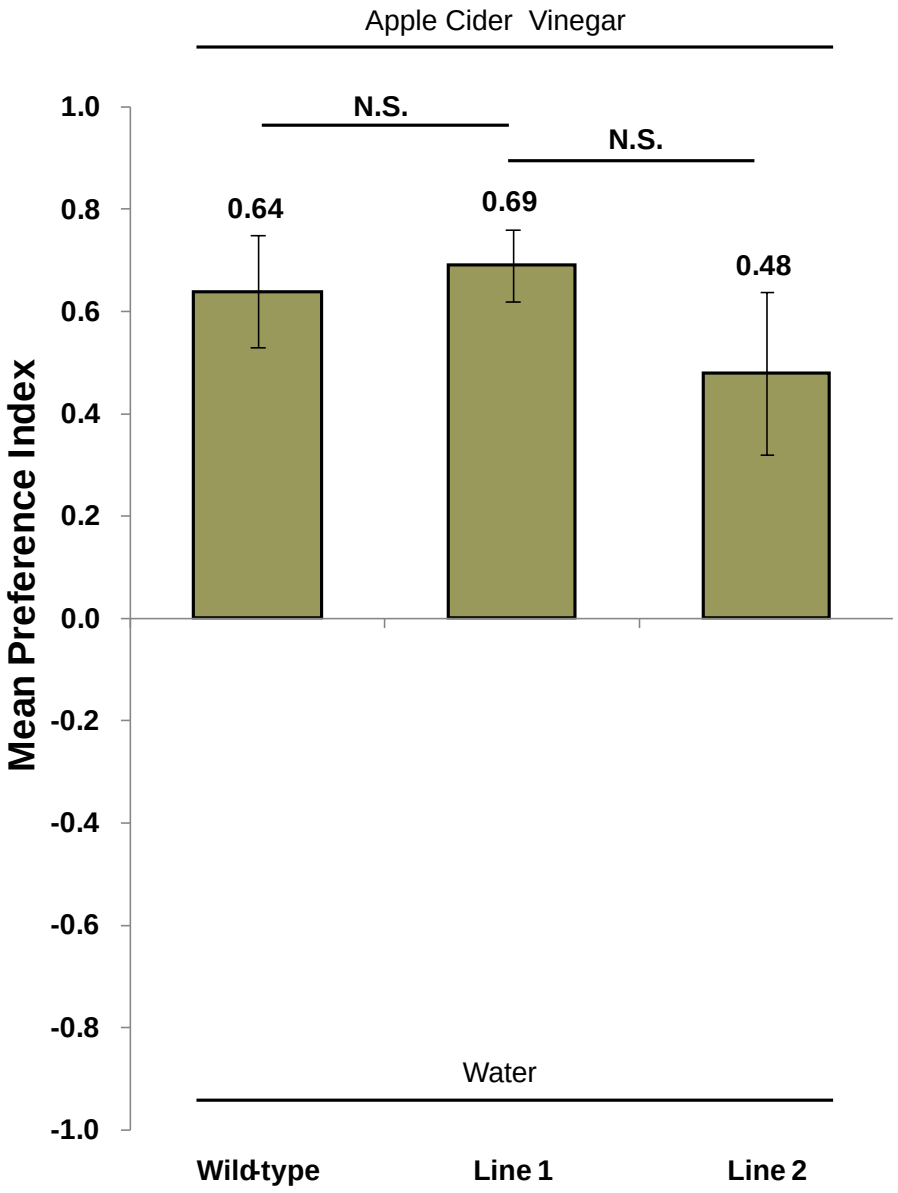

Figure 4.10: DGRP Lines 1 and 2 are attracted to apple cider vinegar (ACV) in two-choice non-contact trap assay. Flies were given 24 hours to choose between two traps: one with ACV, the other with water. Both traps had filter paper coated with solvent (DMSO) at the neck of trap. Wild-type was *wCs*. Line 1 was BL28146. Line 2 was BL28163. Twenty flies per trial. N=6-9, error bars= S.E.M. Two-tailed student's t-test was N.S. For wild-type vs. Line 1 (P=0.947) and for wild-type vs. Line 2 (P=0.389). Trials with less than 7 flies participating were not included.

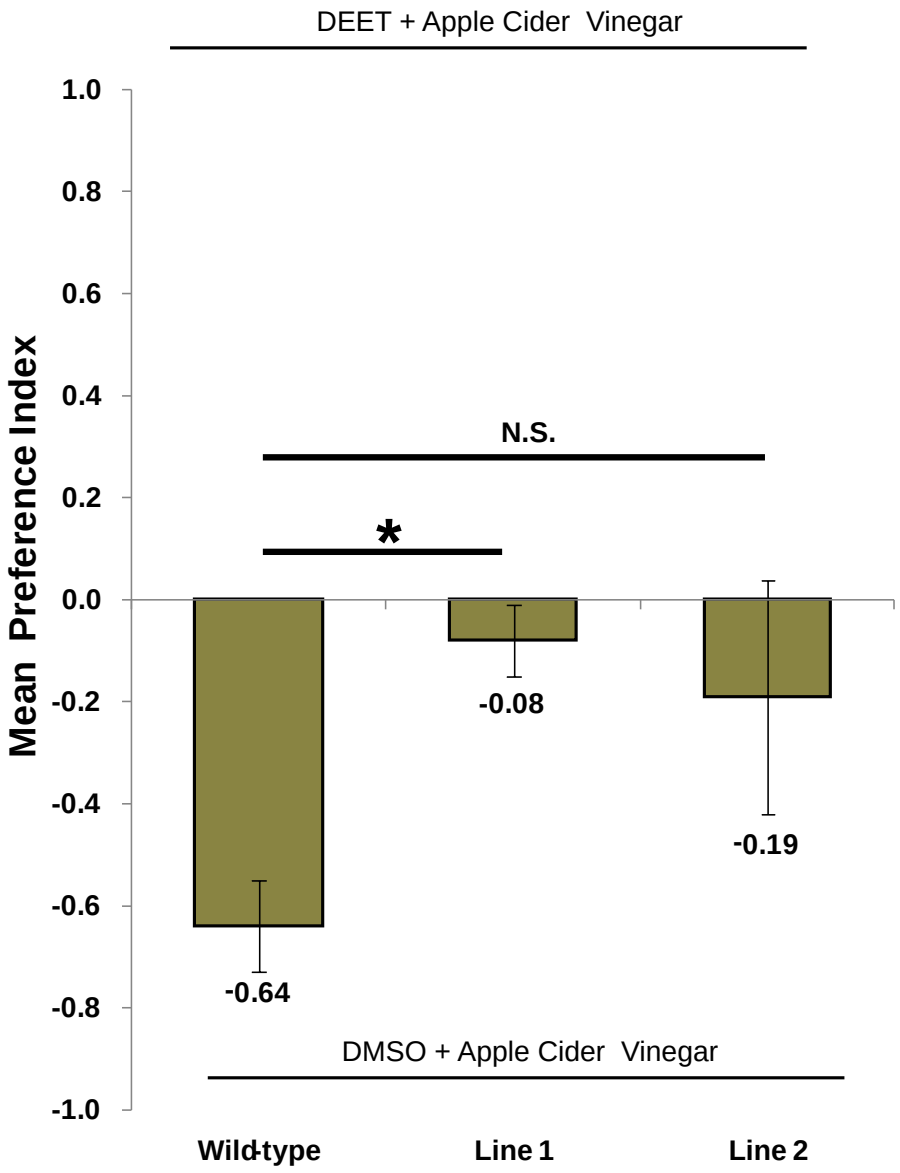

Figure 4.11: DGRP lines 1 and 2 do not avoid DEET in two-choice non-contact trap assay. Flies were given 24 hours to choose between with two apple cider vinegar (ACV) traps: one with 50% DEET on filter paper, the other with solvent on filter paper. Wild-type was *wCs*. Line 1 was BL28146. Line 2 was BL28163. Twenty flies per trial. N=6-8, error bars= S.E.M. Two-tailed student's t-test for wild-type vs. Line 1 is (P=0.006) and for wild-type vs. Line 2 is (P=0.066). Trials with less than 7 flies participating are not included.

A

B

```
Flybase      GATAACCTTG GTCTGCAACG AATTCCCCTC CTCCCGACTG CATTATCCGT
Ir40a-6      GATAACCTTC ---TGCAACG AATTCCCCTC CTCCCGACTG CATTATCCGT
Ir40a-7      GATAACCTTG -----CAACG AATTCCCCTC CTCCCGACTG CATTATCCGT
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C

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Flybase VVT RPRKKAFRIY YNQARPCSDS QLQLVNWYDG DNLGLQRIPL LPTALSVYAN FKGRTFRVPV
Ir40a-6 VVT RPRKKAFRIY YNQARPCSDS QLQLVNWYDG DNL-LQRIPL LPTALSVYAN FKGRTFRVPV
Ir40a-7 VVT RPRKKAFRIY YNQARPCSDS QLQLVNWYDG DNLATNSPP- -PDCI----- -----IRVRQ
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Figure 4.12: CRISPR-Cas9 generated *Ir40a* mutant. (A) *Ir40a* gene spans chromosome locations 22189526 to 22214265 in *Drosophila melanogaster*. The CRISPR cut site was between the two guanine bases at 22213615 and 22213616 (designated by arrow head). Yellow bars indicate locations of two large introns (6276 bp and 15969 bp). Light blue box represents UTR. (B) Nucleic acid sequence for *Ir40a* from Flybase, Ir40a-6 (control) and Ir40a-7 (mutant). (C) Protein sequence for Ir40a from Flybase, Ir40a-6 (control) and Ir40a-7 (mutant).

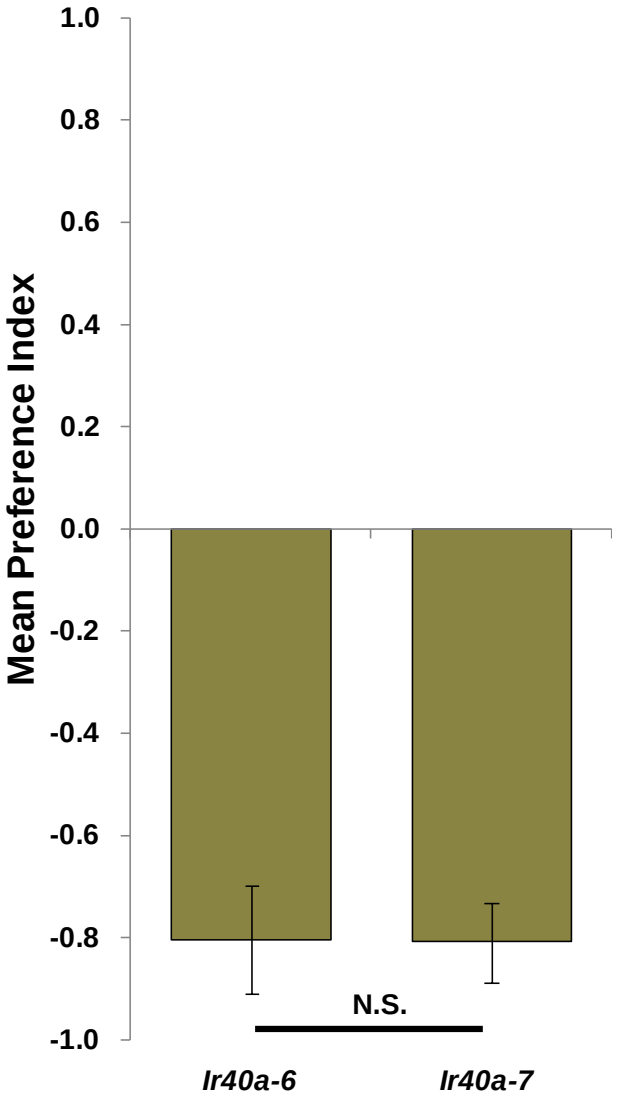

Figure 4.13: *Ir40a* mutant flies avoid DEET in two-choice non-contact trap assay. Mean Preference Index of *Ir40a* mutant flies and control exposed to 50% DEET in DMSO. Twenty flies were given 48 hours to select DEET trap or solvent trap. *N* = 5-11 trials, 20 flies per trial, error bars represent S.E.M. Two-tailed student's t-test (P= 0.97). Trials with greater than 35% participation were included.

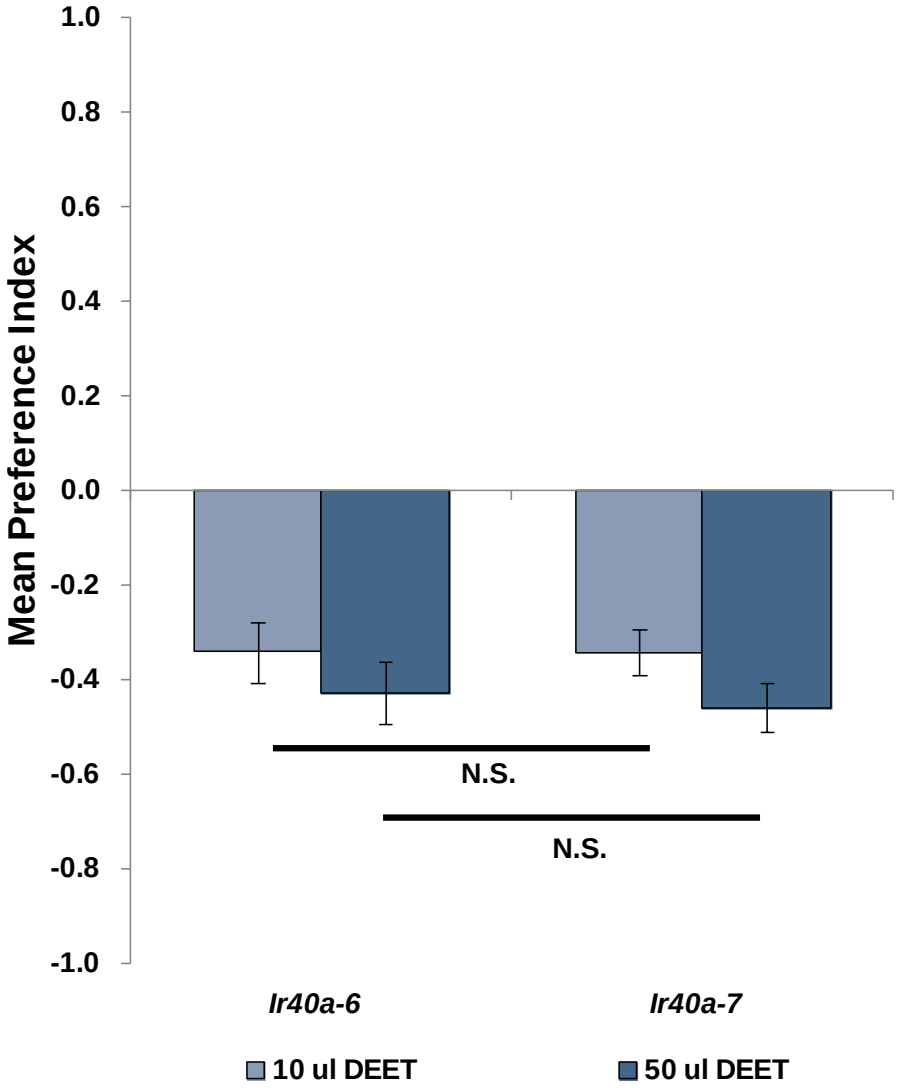

Figure 4.14: *Ir40a* mutant flies avoid DEET in T-maze assay with heated DEET. Mean preference index of 40 unstarved flies exposed to 10 µl or 50 µl of pure DEET heated. Flies were given one minute to choose. N=11-13, error bars = SEM. Two-tailed student's t-test at 10µ dosage is (P=0.986) and at 50µ dosage is (P=0.726).

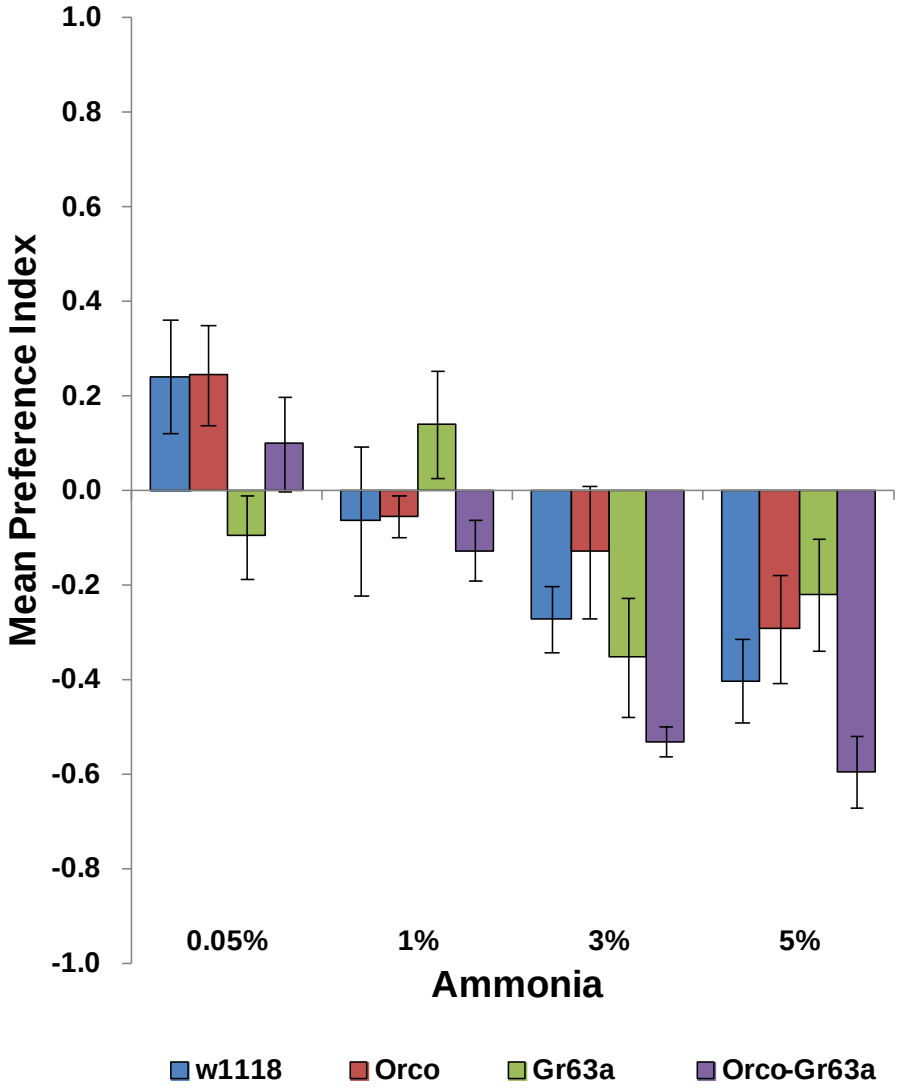

Figure 4.15: *Drosophila* avoids ammonia in a dose-dependent manner in the T-maze. Mean Preference Index to 0.05%, 1%, 3% and 5% ammonia dissolved in water. N=5-6. ~40 flies per trial, error bars =S.E.M.

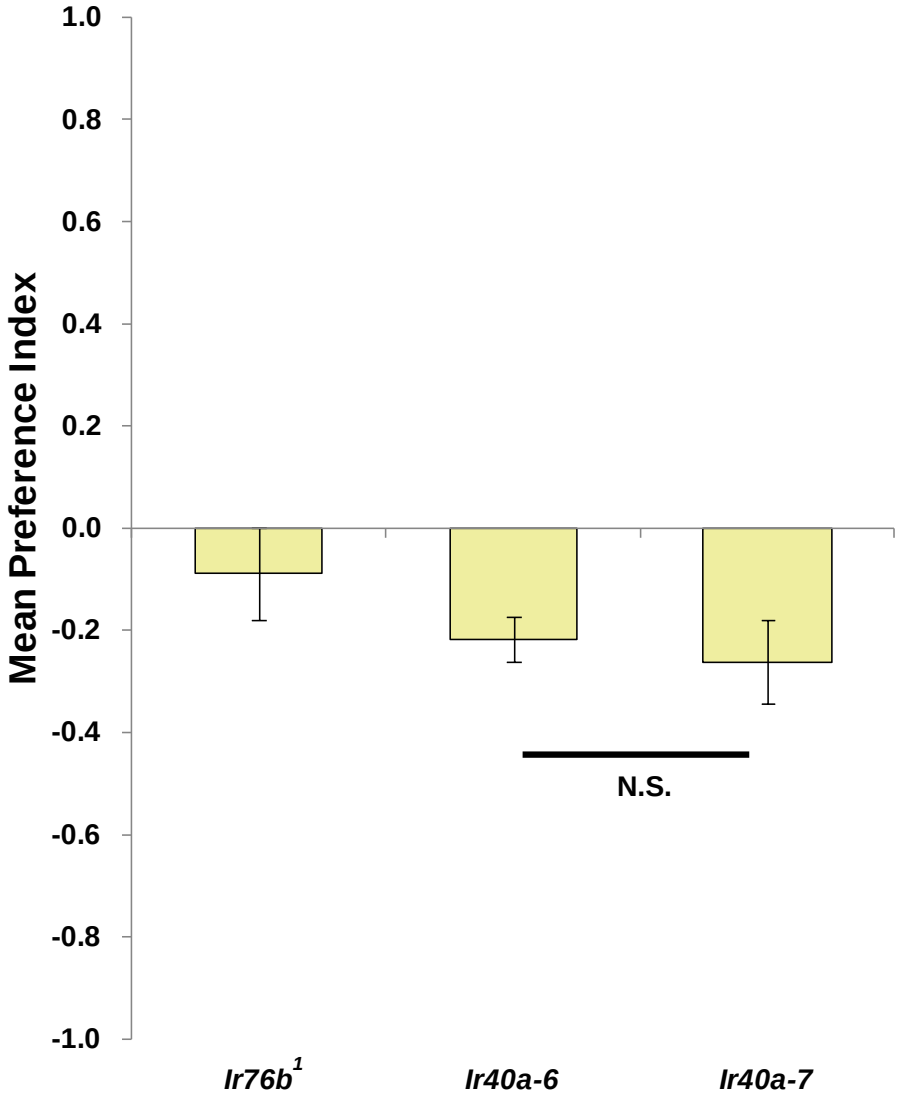

Figure 4.16: *Drosophila* avoids 5% ammonia in the T-maze. Mean preference index to ammonia dissolved in water. N=10 trials, ~40 flies per trial, error bars =S.E.M. Two-tailed student's t-test between *Ir40a-6* (wild-type) and *Ir40a-7* (mutant) is (P= 0.638).

Chapter 5: The role of odorant VP in *Drosophila melanogaster* avoidance

Abstract

In *Drosophila melanogaster*, a few specific olfactory cues can elicit an innate avoidance behavior. Recent research has identified a number of olfactory receptor neurons that may play a role in an innate avoidance response by adult *D. melanogaster*. Whether chemical structure alone or in combination with vapor pressure plays a role in avoidance behavior has not yet been tested. Here we tested a variety of odorants identified in a chemical informatics screen that were predicted to activate repellant receptors and circuits. We first ranked odorants by low and high vapor pressure and utilized two different behavior paradigms to screen for repellency. We demonstrated that 18% of the newly tested odorants caused avoidance, whereas only 2% of the compounds were attractants. We then compared the vapor pressure distribution of these compounds. Of the new repellents identified, we examined the behavioral response for different dosages of the chemicals and the response when dissolved in different solvents. We then determined whether the Odorant Receptor gene family was involved in repellency by testing the response of *Orco* mutant flies. The identification of additional structurally dissimilar chemicals that acted as ligands for neurons sensing repellents provided a foundation for better understanding of the olfactory-repellent pathways.

Introduction

One hypothesis we wanted to test was that the vapor pressure of repellents impacts the fly's avoidance to a chemical independent of the odorant structure. Alternatively, chemical structure alone could be the determinant of repellency. Thus far, I have studied a small number of repellents. These chemicals activated different avoidance pathways. First, the DEET activated pathway, which may also be activated by DEET-like compounds and 4-methyl piperidine (4-MPD) (Guda et al., 2015). Second, pyridine activated the CO_2 pathway. Third, citronellal activated ab11A and ab12A (Kwon et al., 2010). These chemicals have vapor pressures that range from 0.003 to 22.8 mmHg at 25° Celsius and 1 atmosphere pressure (Figure 5-1).

In order to test this hypothesis, we needed to identify more repellents. Typically, pharmaceutical companies will develop new chemicals that act as drugs by modifying existing known bioactive compounds. By adding a functional group or making a single modification to the molecule they can develop different drugs. Our approach was to use predictions from a chemical informatics screen that utilizes combinations of multiple properties of compounds.

Using a chemical informatics screen, we obtained a list of odorants to test for repellency (Table 5.1). Compounds insects have been shown to avoid were used as a training set for a computational analysis similar to that published in Kain et al. (Kain et al., 2013). The predictions were made from a set of ~400,000

different chemicals (with ~12,000 natural ones). The computational algorithm evaluated and ranked compounds for expected repellent activity.

Materials and Methods

The T-maze assay

Trials were conducted based as in prior experiments (Turner and Ray, 2009). Briefly, approximately 40 flies were released from an elevator into the horizontal intersection of a T-shaped apparatus. A test odorant was applied to one arm of the T-maze and the solvent to the opposite arm as a control. Specific solvents used are listed in Tables 5.1 and 5.2. Flies were given one minute to choose an arm before the elevator closes. Orientation of arms for test and control were switched between trials. Preference index was calculated as = (number of flies in test arm - number of flies in control arm)/(number of flies in test arm + number of flies in control arm).

Two-choice contact trap assay in a plate

The two-choice contact trap assay in a plate tested less volatile odorants. Trials were performed as described (Syed et al., 2011). Ten female flies were placed in a Petri dish containing two traps. Traps were made with 1.5-ml micro centrifuge tubes (USA Scientific) with an opening cut in the bottom of the tube. Both traps contained the fly's normal laboratory food at the base. The neck of one trap had a filter paper with test odorant, the other trap had solvent. Five microliters of solvent (control) and five microliters of test

compound were applied to the stem part of filter paper inserted into upper part of pipette tip near entrance of trap to allow flies to walk over the treated surface. Traps were placed in a 1% agarose-treated Petri dish chamber.

Results

I tested 44 compounds (Tables 5.2 and 5.3) in one of two behavioral assays, which were selected depending on the volatility range of the compounds to be tested as predicted by vapor pressure (VP). We divided our candidate repellent list into compounds with vapor pressures greater and less than 0.05 mmHg. The T-maze assay (a short-term one-minute assay) was used for more volatile compounds (Table 5.1) and the two-choice contact trap assay in a plate (a residence assay) for less volatile compounds (Table 5.2). Our rationale for using two behavioral assays resulted from an observation we made with *Drosophila melanogaster* in the T-maze assay. Wild-type flies were not repelled by DEET, a strong repellent.(Ditzen et al., 2008; Syed et al., 2011), but a very low volatility compound (VP= 0.005mmHg). Our explanation was that flies were unable to get close enough to the DEET to detect it in the one-minute duration of the T-maze assay. Flies randomly dispersing may have rested on the DEET containing arm outside of the zone where DEET was sensed.

A strong repellent of *D.melanogaster* with a higher vapor pressure is 4-methylpiperidine (4-MPD), which we tested in the T-maze assay as a positive control. We found wild-type flies (wCs) avoided 4-MPD at two concentrations, 5%

and 10% (Figure 5.1). In proceeding with this screen, we tested all higher vapor pressure compounds at 5% concentration along with 5% 4-MPD as a control in the T-maze. Lower vapor pressure compounds were tested at 1% with 1% DEET as a control in the two-choice contact trap assay in a plate.

Testing of higher volatility compounds

The computational analysis was somewhat predictive of repellency. In the T-maze assay, our experimentally observed preferences were weighted towards avoidance (Figure 5.2). However, the relationship between preference and VP was not clear (Figure 5.3). For example, in the T-maze assay strongly repellent compounds had preference indexes that ranged from -0.6 and -0.8 and VP between 10 to 80 mmHg (Figure 5.3). For compounds with no observed preference there was a wide-range of VP (Figure 5.3). A clear relationship between VP and avoidance was not observed when we plotted VP versus Preference Index (R^2=0.01) based on the T-maze assay (Figure 5.3). In this assay, two strong repellents were identified: 3,4-dihydro-2H-pyran and 1-methyl-1,4-cyclohexadiene.

Testing of lower volatility compounds

In the two-choice trap assay (Figure 5.4), tested odorants were more skewed toward repellents, and four compounds tested were stronger repellents than DEET: 1,2,3,4-tetrahydroquinoline, cis-2,4,5-trimethoxypropenylbenzene, phenethyl isothiocyanate and methyl dihydrojasmonate . These lower volatility

compounds have a distribution of vapor pressures from 0 to 0.003 and again there was little correlation between VP and preference (Figure 5.5). Of the compounds with VP close to zero, many were less repellent and some attractant. In conclusion, of the 44 compounds tested, there was not a clear relationship between VP and repellency. Nevertheless, we have identified at least six possible strong repellents.

Multiple dose testing of strong repellents

Our next goal was to determine if these compounds repel in a dose-dependent manner. We found wild-type flies avoided 3,4-dihydro-2H-pyran dissolved in water in a dose-dependent manner (Figure 5.6A). Interestingly, when 3,4-dihydro-2H-pyran was dissolved in ethanol, the effect was different. We observed reduced avoidance (PI=-0.20, approximately) in ethanol and this avoidance was observed over three orders of magnitude (0.1, 1 and 10%) (Figure 5.6B). In Figure 5.7A we tested 1-methyl-1,4-cyclohexadiene in a dose response, which was repellant at 5% when dissolved in water, but not lower dosages of 1% and 2.5%. In this case the trend was similar when the compound was dissolved in ethanol, but the lower dosages of 0.1% and 1.0% of 1-methyl-1,4-cyclohexadiene were slightly preferred by wild-type flies.

Next we did dose response for our identified repellents in the trap assay. For 1,2,3,4-tetrahydroquinoline, an inverted v-shaped dose response pattern was observed; strong avoidance at 1%, attraction at 0.1% and avoidance at 0.01% (Figure 5.8A).Methyl jasmonate showed a dose response; however, the

avoidance at 1% was observed at about PI =-0.3 (Figure 5.8B). Our S.E.M. values showed the aversion to methyl jasmonate was highly variable. Further evaluation of methyl jasmonate is necessary to rule out the possibility of a weak response. Three additional compounds (1) β-asarone, (2) phenethyl isothiocyanate and (3) isobutyl anthranilate continued to show avoidance at 1% concentrations; however, no dose response was observed with phenethyl isothiocyanate and isobutyl anthranilate (Figure 5.9). At lower concentrations results for β-asarone were ambiguous. At the intermediate dosage of 0.10 % attraction to β-asarone and at 0.01% avoidance of β–asarone was observed. Both of these dosages showed wide variability in the S.E.M.

Behavioral testing of *Orco* mutant flies with repellents

Next, for the compounds in which avoidance was observed with dose response, we tested compounds using *Orco*^{1} mutant flies to determine if avoidance was mediated by an Olfactory Receptor neuron pathway. In Figure 5.10, *Orco*^{1} flies avoided 5% 3,4-dihydro-2H-pyran, but not 5% 1-methyl-1,4-cyclohexadiene in the T-maze assay. Orco^{1} mutant flies had reduced avoidance to 4-MPD (PI=-0.25) compared with wild-type flies (PI=-0.46). This difference was statistically significant (P=0.0532) (Figure 5.11). In the two-choice contact trap assay in a plate, *Orco* mutant flies avoided β-asarone and to a small degree methyl dihydrojasmonate. Methyl jasmonate, phenethyl isothiocyanate, and isobutyl anthranilate did not repel *Orco* mutant flies (Figure 5.12). *Orco* flies were slightly attracted to 1,2,3,4-tetrahydroquinoline.

Next we further evaluated 4-MPD, a cyclic amine that we hypothesize may be sensed by an ionotropic receptor (Silbering et al., 2011). We tested flies mutant in IR co-receptors *Ir93a, Ir25a,* and *Ir76b* with 4-MPD. We first examined flies mutant for $Ir25a^2$, one of the most ancient and widely expressed co-receptors for chemosensory receptors of the ionotropic class (Croset et al., 2010). Short term (one minute) exposure to 10% 4-methylpiperidine in the T-maze assay did not show statistically significant reduced avoidance for homozygous $Ir25a^2$ mutant flies generated by Benton (Benton et al., 2009) in an Oregon R background (Figure 5.13). This data implied *Ir25a* is not necessary for avoidance of 4-MPD.

$Ir76b^1$ mutants showed reduced avoidance to 4-MPD at three concentrations. Avoidance was statistically significant at 5% and 20% concentrations of 4-MPD.

Another ionotropic co-receptor mutant, *Ir93a,* avoided 4-MPD. Interestingly, *Ir93a* mutants avoided 4-MPD to a greater extent than our wild-type w^{1118} control flies (Figure 5.15). *Ir40a* mutant flies avoided 10% 4-MPD (Figure 5.16), as did *Ir8a* mutants (data not shown).

Discussion

Here, I considered repellents as a whole in investigating a relationship between odorant vapor pressure and avoidance behavior in *Drosophila melanogaster* and found that there was no correlation. Since the precise odorant receptor pathways mediating aversion was not known for these compounds, we

did not know whether a more specific analysis of individual pathways by comparing a group of repellents activating the same receptor, may have shown a relationship with vapor pressure that was not apparent from this study.

We also began an analysis to determine the receptor family detecting the repellent compounds identified.

We were able to identify eight new repellent compounds. Of these we can say that three are in neurons requiring *Orco*. It would be interesting to test these compounds electrophysiologically to determine whether these compounds activate known avoidance pathways like ab3A (ethyl 3-hydroxybutyrate), ab4B (geosmin) and ab12A (citronellal). Likewise, compounds showing strong avoidance in *Orco* mutants (3,4-dihydro-2H-pyran, β-asarone and methyl dihydrojasmonate) should be tested electrophysiologically in ab1C for a possible response from the Gr21a/Gr63a receptor. As repellents are mapped to their activating ORN, a receptor-specific relationship between VP and avoidance may be observed. A relationship between avoidance and compound vapor pressure may have been obscured here by noise in our data when the responses were compared across all repellents.

Methods used to assess fly behavior each have inherent limitations. Here, we conducted two assays, both of which require flies to walk and to make their choice. In their natural environment, flies are able to locomote by flying and walking. Results may differ in experimental set-ups where *Drosophila* can freely fly instead of walk. It would also be beneficial to test these odorants in related

insect species such as mosquitoes where we can examine behavior in an experimental set-up that allows for flying insects to fly. As we learn and better characterize olfactory avoidance pathways in *D. melanogaster*, we may be able to determine a precise relationship between vapor pressure and avoidance.

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Table 5.1: Compounds tested in trap assay

Chemical Name	**CAS #**	**solvent**	**VP (mmHg)**
methyl cyclopentenolone anhydrous	80-71-7		*
L-tryptophan	73-22-3	1% in water	0
capscaisin	404-86-4	ethanol	0
epsilon-caprolactam	105-60-2	water	0
(z)-3-hexen-1-yl anthranilate	65405-76-7	ethanol	0.000019
cyclohexyl anthranilate	7779-16-0	acetone	0.000037
methyl jasmonate	39924-52-2	DMSO	0.000337
1-(2,6,6- trimethyl-1-cyclohex-2-enyl)hepta-1,6-dien-3-one	79-78-7	ethanol	0.000498
methyl dihydrojasmonate	25851-98-7	ethanol	0.001
isopropyl anthranilate	18189-02-1	ethanol	0.001670
isobutyl anthranilate	7779-77-3	ethanol	0.002
ethyl cyclopentenolone	21835-01-8	ethanol	0.002
isobutyl anthranilate	7779-77-3	ethanol	0.002
β-asarone (aka Cis-2,4,5-trimethoxypropenylbenzene)	5273-86-9	ethanol	0.0026
formanilide	103-70-8	1% in water	0.00357
DEET (N,N-diethyl-meta-toluamide)	134-62-3	hexane	0.005
phenethyl isothiocyanate	2257-09-2	ethanol	0.007
acetoacetanilide	102-01-2	ethanol	0.007501
methyl 2-(4-tert-butylphenyl)acetate	3549-23-3	ethanol	0.008
ethyl 2-aminobenzoate	87-25-2	ethanol	0.01
methyl dimethyl anthranilate	10072-05-6	ethanol	0.01
isopropyl quinoline	135-79-5	ethanol	0.011
5-ethyl-4-hydroxy-2-methyl-3 (2H) furanone	27538-09-6	water	0.012
trithioacetone	828-26-2	ethanol	0.017
geraniol	106-24-1	ethanol/water	0.03
strawberry furanone solution (aka 4-hydroxy-2,5-dimethyl-3(2H)-furanone)	3658-77-3	ethanol	0.032

Table 5.1: Low vapor pressure compounds predicted to be repellents in a chemoinformatic screen. Solvent used was experimentally determined. VP was predicted to be less than 0.05 mmHg. These compounds were referred to as low VP compounds in the text and tested in the two-choice contact trap assay in a plate. Repellency was compared to DEET.

Table 5.2: Compounds tested in T-maze assay

Chemical Name	**CAS #**	**solvent**	**VP (mmHg)**
1,2,3,4-tetrahydroquinoline	635-46-1	ethanol	0.07
ethyl chrysanthemate	97-41-6	ethanol	0.0724
polyvinyl pyrrolidone	9003-39-8	water	0.0900
1-ethyl-2-pyrrolidone	2687-91-4	ethanol	0.103
2-isobutyl-3-methoxypyrazine	24683-00-9	water	0.273
(+/-)-tetrahydrofurfuryl alcohol	97-99-4	ethanol	0.326
nerol oxide	1786-08-9	ethanol	0.478
hexyl alcohol	111-27-3	ethanol	0.947
2,2,4,6,6-pentamethylheptane	13475-82-6	ethanol	1.420
2-isobutyl-4-methyl-1,3-dioxolane	18433-93-7	ethanol	3.53
1,2-epoxyhexane	1436-34-6	ethanol	6.83
4-methylcyclohexene	591-47-9	ethanol	10.3
(E)-diazene-1,2-dicarboxamide	123-77-3	DMSO	10.7
2-methylpiperidine	109-05-7	water	16.395
propylene glycol acetone ketal	1193-11-9	ethanol	17.5
1-methyl-1,4-cyclohexadiene	4313-57-9	water	20.6
1-methylpiperidine	626-67-5	water	27.7
1-ethyl pyrrolidine	7335-06-0	ethanol	31.5
3,4-dihydro-2H-pyran	110-87-2	water	76.1
DL-1,2-isopropylidene glycerol	100-79-8	water	107

Table 5.2: High vapor pressure compounds predicted to be repellents in a chemoinformatic screen. Solvent used was experimentally determined. VP was predicted to be greater than 0.05 mmHg. These compounds were referred to as high VP compounds in the text and tested in the T-maze assay. Repellency was compared to 4-MPD.

Table 5.3: Compounds previously tested behaviorally in Chapter 2**.**

Chemical	**VP (mmHg)**	**Mean Preference Index**
DEET (N,N-diethyl-meta-toluamide)	0.003	-0.78
ethyl anthranilate	0.010	-0.05
methyl N,N-dimethylanthranilate	0.019	-0.25
ethyl 3-hydroxybuytate	0.400	-0.80
butyl anthranilate	1.290	-0.63
4-methylpiperidine (4-MPD)	10.000	-0.70
pyridine	22.800	-0.75
citronellal	0.200	*

Table 5.3: Experimentally verified *Drosophila melanogaster* repellents and their vapor pressures. Values for mean preference index were from testing in two-choice contact trap assay in a plate with N,N-diethyl-meta-toluamide (DEET), ethyl anthranilate (EA), methyl N,N-dimethylanthranilate (MDA) and butyl anthranilate (BA). Values for mean preference index from testing in T-maze assay were for 4-methylpiperidine (4-MPD) and pyridine. Citronellal was tested in DART assay (Krause Pham and Ray, 2015).

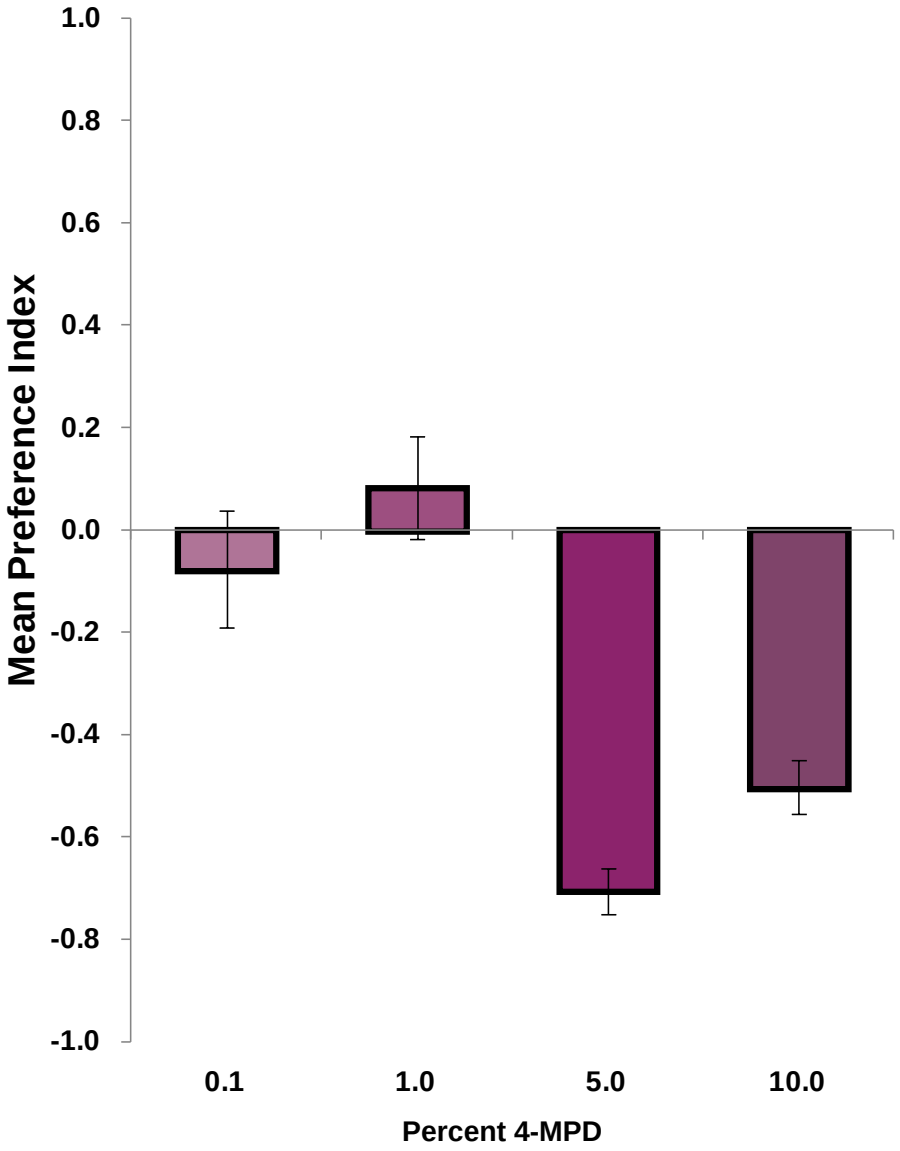

Figure 5.1: *Drosophila melanogaster* avoid 5% and 10% 4-methylpiperidine (4-MPD) in T-maze assay. Mean preference index of wCs flies given a choice between water and 4-methylpiperidine at four concentrations (0.1 %, 1.0%, 5.0% and 10.0%). Approximately 20 male and 20 female flies per trial, N=7-8 trials, error bars = S.E.M.

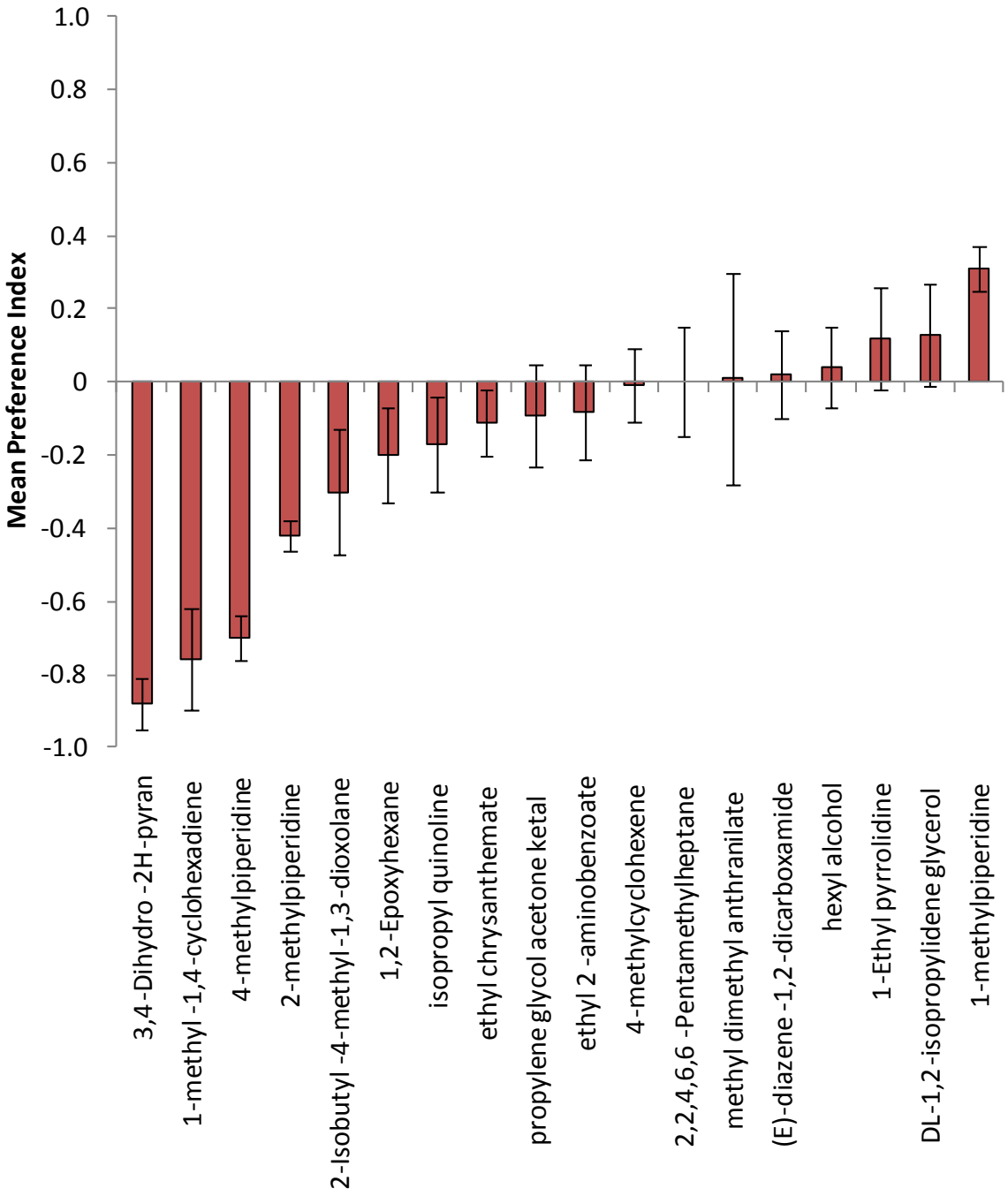

Figure 5.2: Avoidance behavior in *Drosophila melanogaster* to compounds with vapor pressure greater than 0.05 mmHg in T-maze assay. Mean preference index of starved wCs flies given a choice between solvent and 5% proposed repellent compounds. N=3, error bars = S.E.M.

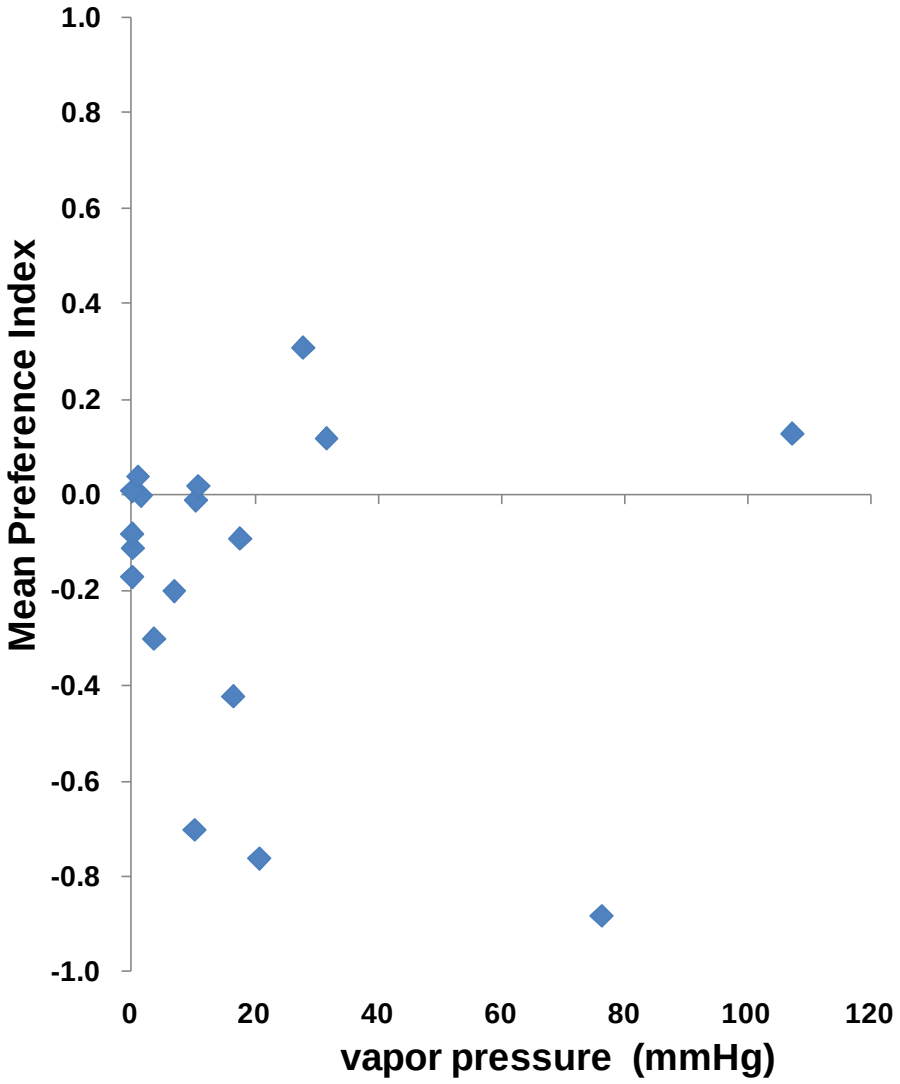

Figure 5.3: Higher volatility compounds show little correlation between avoidance behavior and compound vapor pressure. Avoidance index from T-maze assay plotted with compound vapor pressure for 18 compounds. (R^2=0.01).

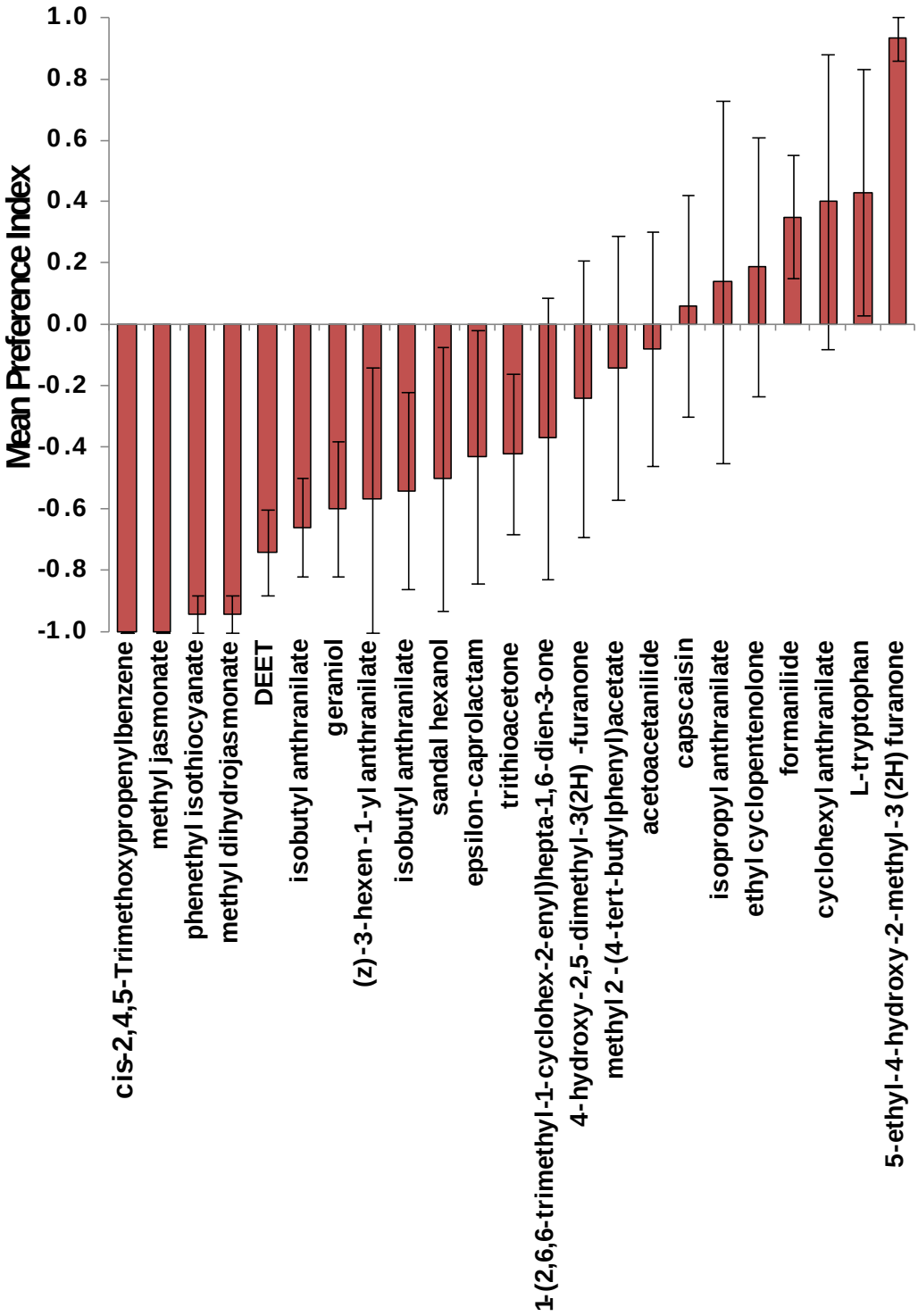

Figure 5.4: Avoidance behavior in *D. melanogaster* to compounds with vapor pressure less than 0.05 mmHg. Two-choice trap assay in a plate assay: Mean preference index of wCs given a choice between solvent and 1% proposed repellent compounds at 48 hour point. n=3-4, error bars = S.E.M.

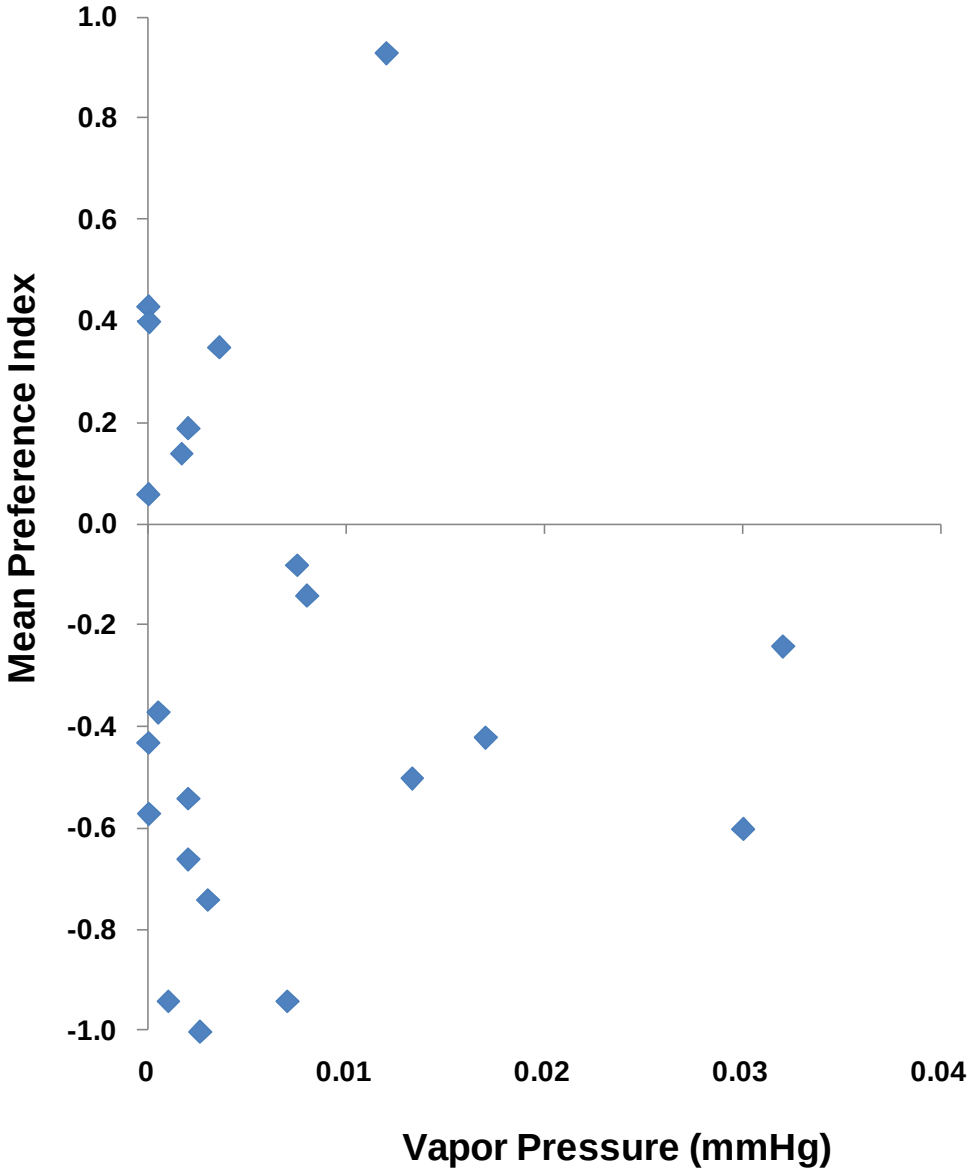

Figure 5.5: Lower volatility compounds show little correlation between avoidance behavior and compound vapor pressure. Avoidance index from two-choice trap assay in a plate assay plotted with compound vapor pressure for 22 compounds.

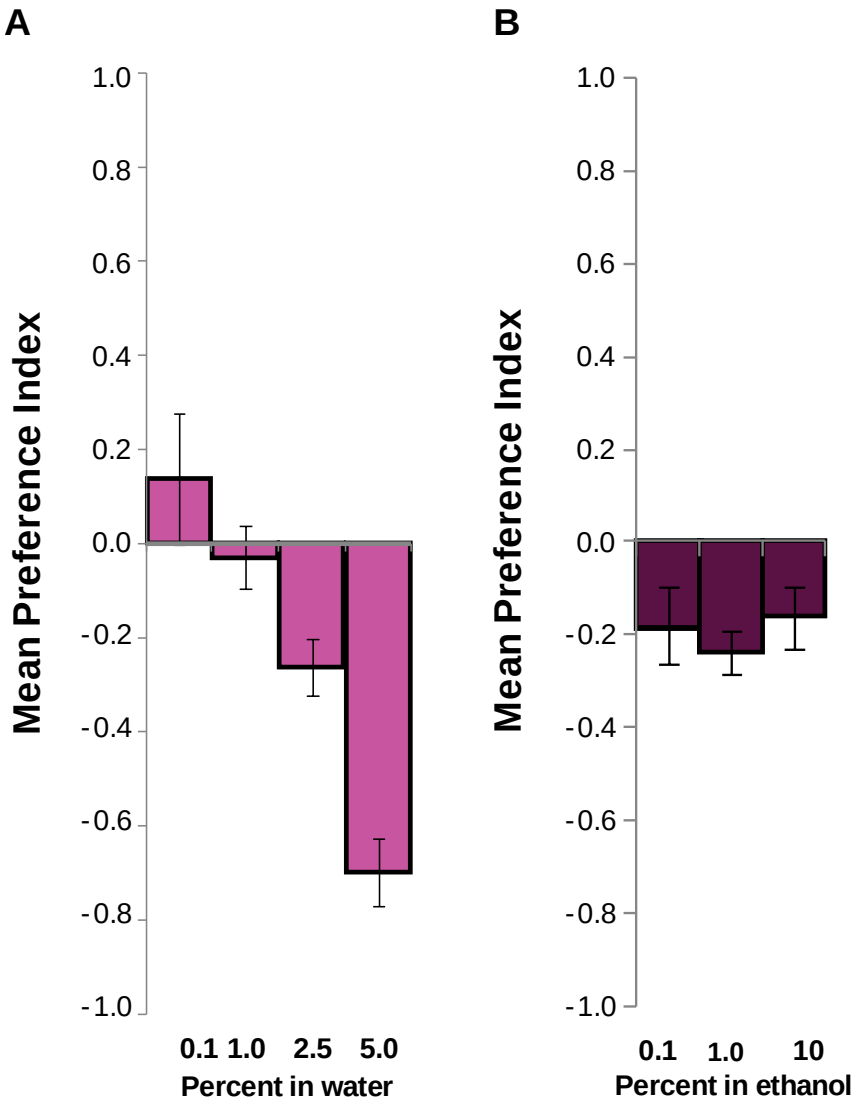

Figure 5.6: Avoidance behavior in *Drosophila melanogaster* to dose response of 3,4-dihydro-2H-pyran in T-maze assay. Mean preference index of starved wCs flies given a choice between different concentrations of 3,4-dihydro-2H-pyran in (A) water as solvent, N=4-8, (B) ethanol as solvent. N=6, error bars = S.E.M.

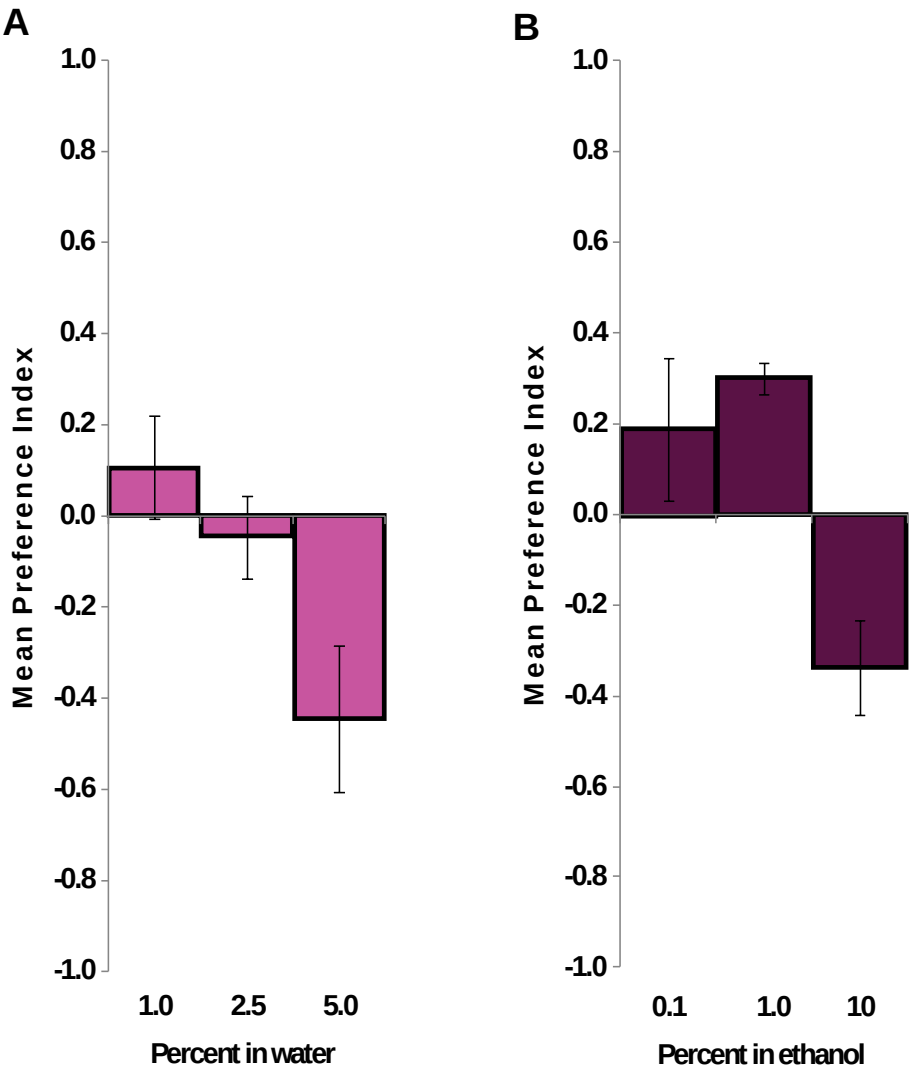

Figure 5.7: Avoidance behavior in *Drosophila melanogaster* to dose response of 1-methyl-1,4-cyclohexadiene in T-maze assay. Mean preference index of starved wCs flies given a choice between solvent and different concentrations of 1-methyl-1,4-cyclohexadiene in (A) water as solvent, N=7 trials (B) ethanol as solvent. N=2-3 trials, error bars = S.E.M.

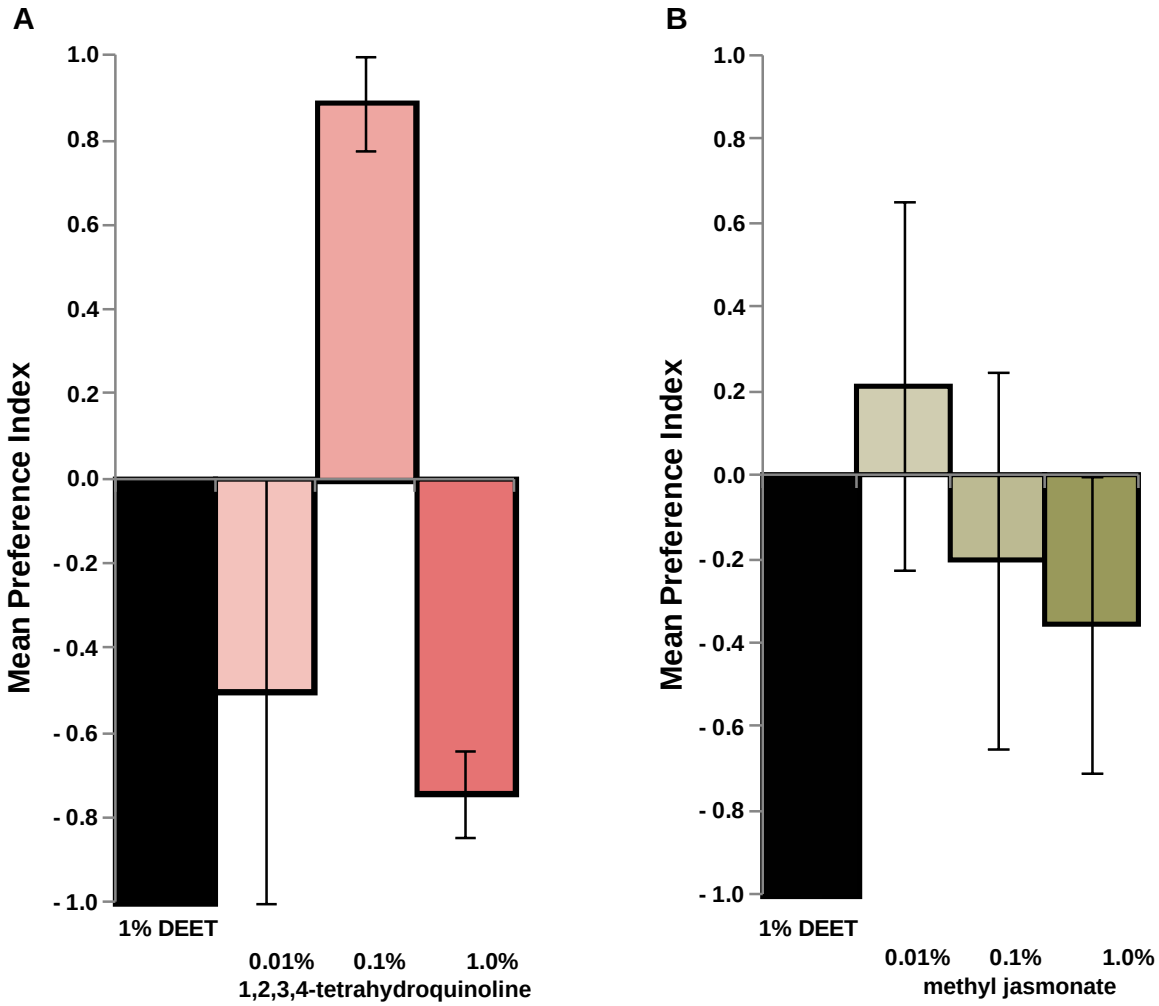

Figure 5.8: Avoidance behaviors in *Drosophila melanogaster* to dose response of repellents in two-choice contact trap assay in a plate. Mean preference index of wCs flies given a choice between solvent and 0.01%, 0.1% and 1% concentrations of repellent compounds at 48-hour point. (A) 1,2,3,4-tetrahydroquinoline (THQ), N=2-4 trials, (B) methyl jasmonate (MJ), N=3-5, error bars = S.E.M.

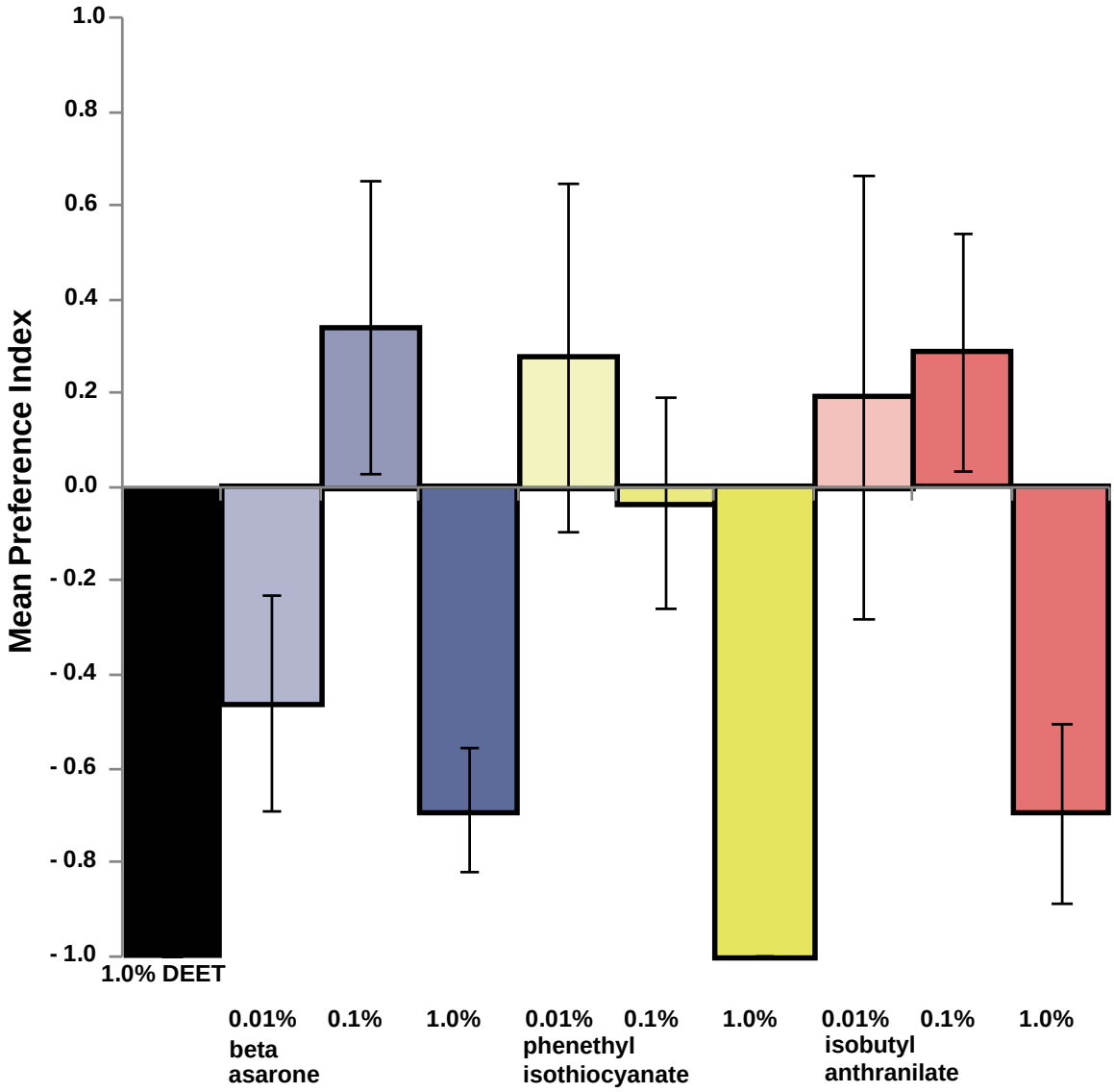

Figure 5.9: Avoidance behaviors in *Drosophila melanogaster* to dose response of repellents in two-choice contact trap assay in a plate. Mean preference index of wCs flies given a choice between solvent and 0.01%, 0.1% and 1% concentrations of repellent compounds at 48 hour point. Compounds are β-asarone, phenethyl isothiocyanate and isobutyl anthranilate. N=4-5 trials, error bars = S.E.M.

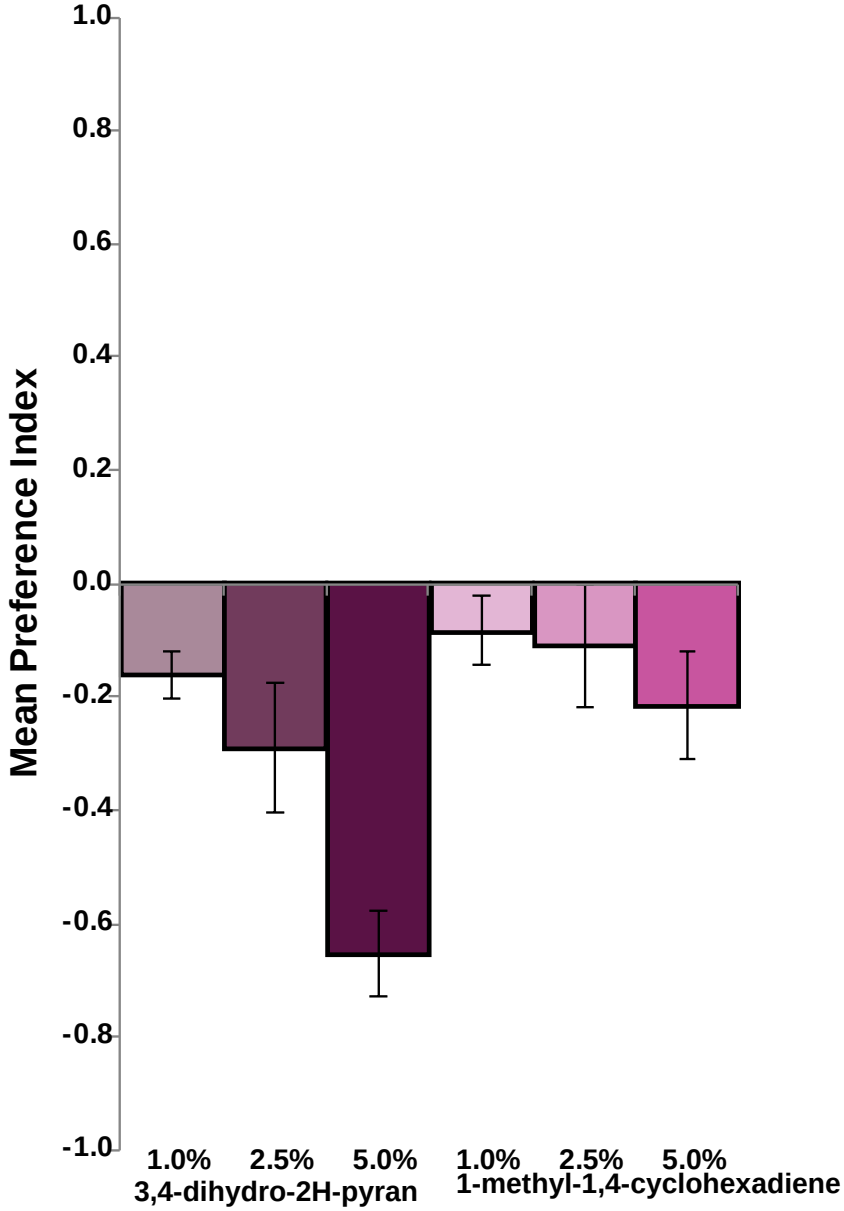

Figure 5.10: *Orco*^{1} mutant avoided 3,4-dihydro-2H-pyran in a dose dependent manner in T-maze assay. Mean preference index of solvent (water) and 1.0%, 2.5% and 5.0% concentrations of 3,4-dihydro-2H-pyran and 1-methyl-1,4-cyclohexadiene. N=6-8, error bars = S.E.M.

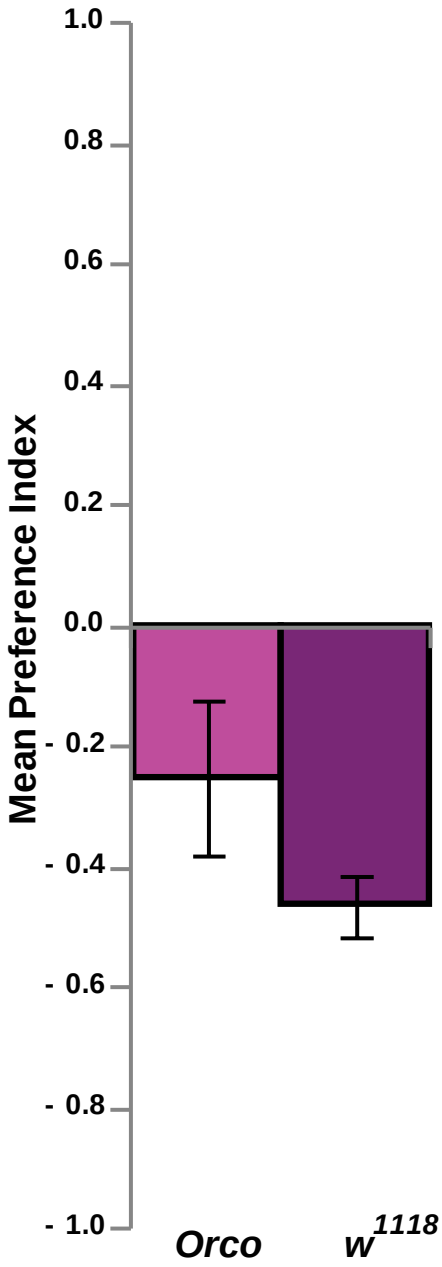

Figure 5.11: *Orco*^{1} mutant has reduced avoidance to 4-methylpiperidine in T-maze assay. Mean preference index between 10% 4-methylpiperidine and solvent (water) for *Orco*^{1} (PI=-0.25) and for *w*^{1118} (PI= -0.46), N=5-10, error bars = S.E.M. For two-tailed student's t-test (P=0.053).

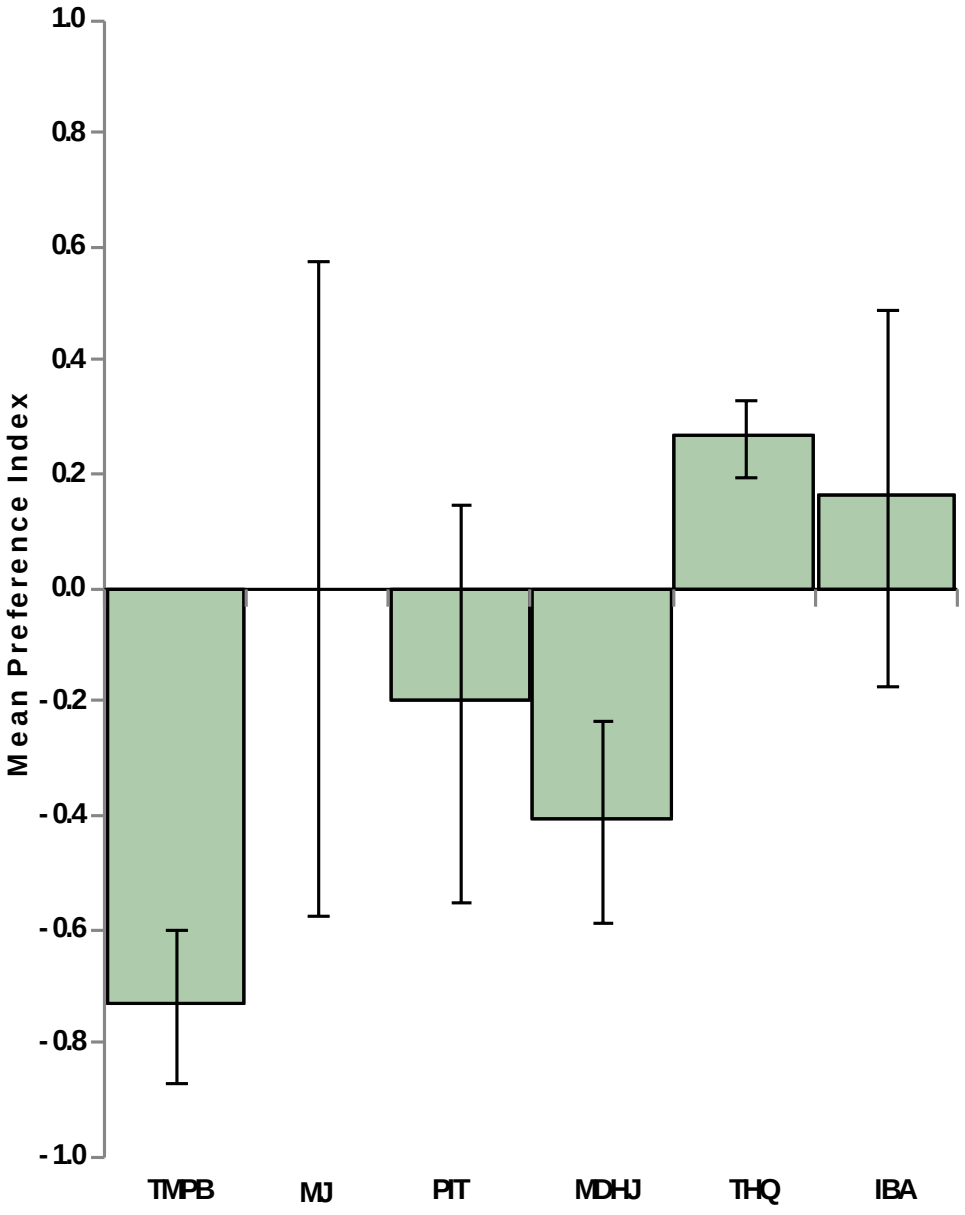

Figure 5.12: *Orco*[1] mutant behavioral response to 1% of repellent varies in two-choice trap assay in a plate. Mean preference index at 48-hour exposures to solvent and 1% concentrations of cis-2,4,5-trimethoxypropenylbenzene (TMPB), methyl jasmonate (MJ), phenethyl isothiocyanate (PIT), methyl dihydrojasmonate (MDHJ), 1,2,3,4-tetrahydroquinoline (THQ) and isobutyl anthranilate (IBA). N=2-3 trials, error bars=S.E.M.

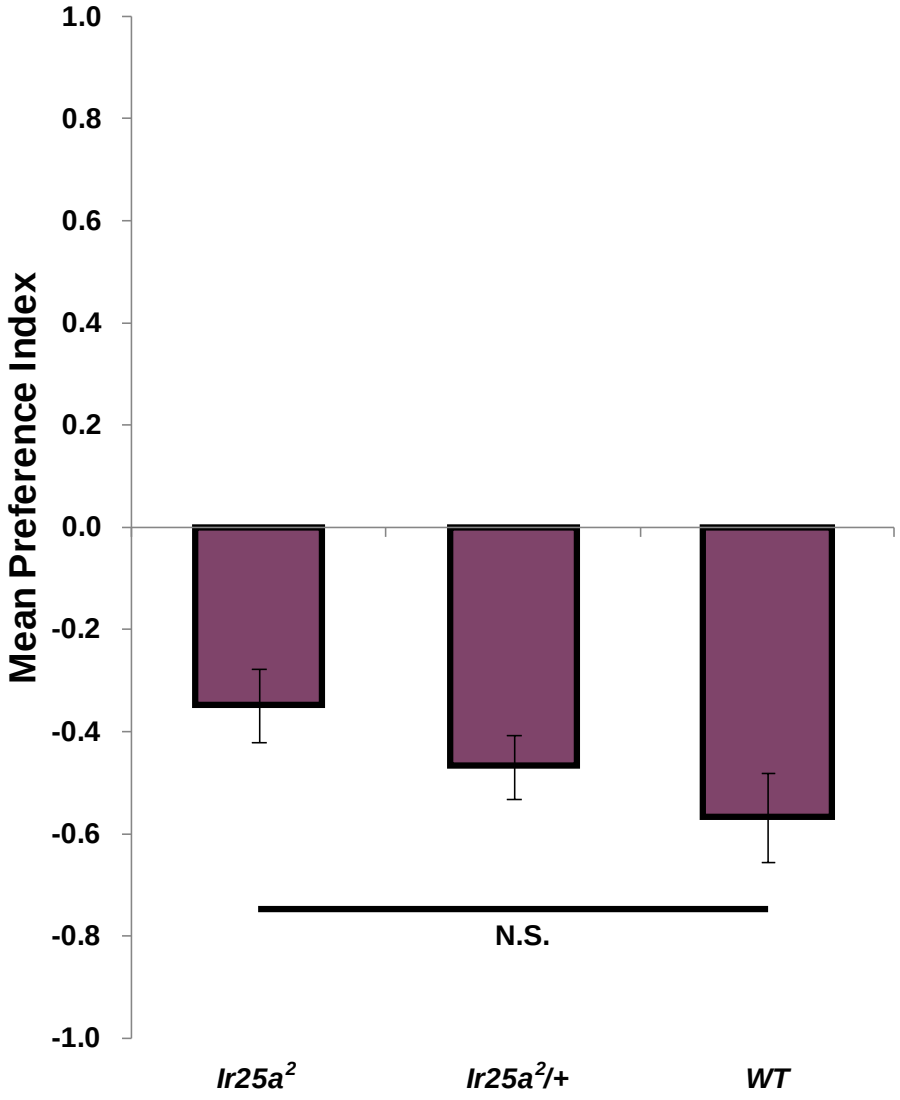

Figure 5.13: *Ir25a*^{2} mutant avoids 4-methylpiperidine in T-maze assay. Mean preference index between 10% 4-methylpiperidine and solvent (water) for *Ir25a*^{2} (PI=-0.35), *Ir25a*^{2}/OreR (PI=-0.47) and OreR (PI=-0.57) flies, N=5, error bars = S.E.M. Two tailed student's t-test between homozygous *Ir25a*^{2} mutant and wild-type (p=0.09). *Ir25a*^{2} mutant line was BL41737.

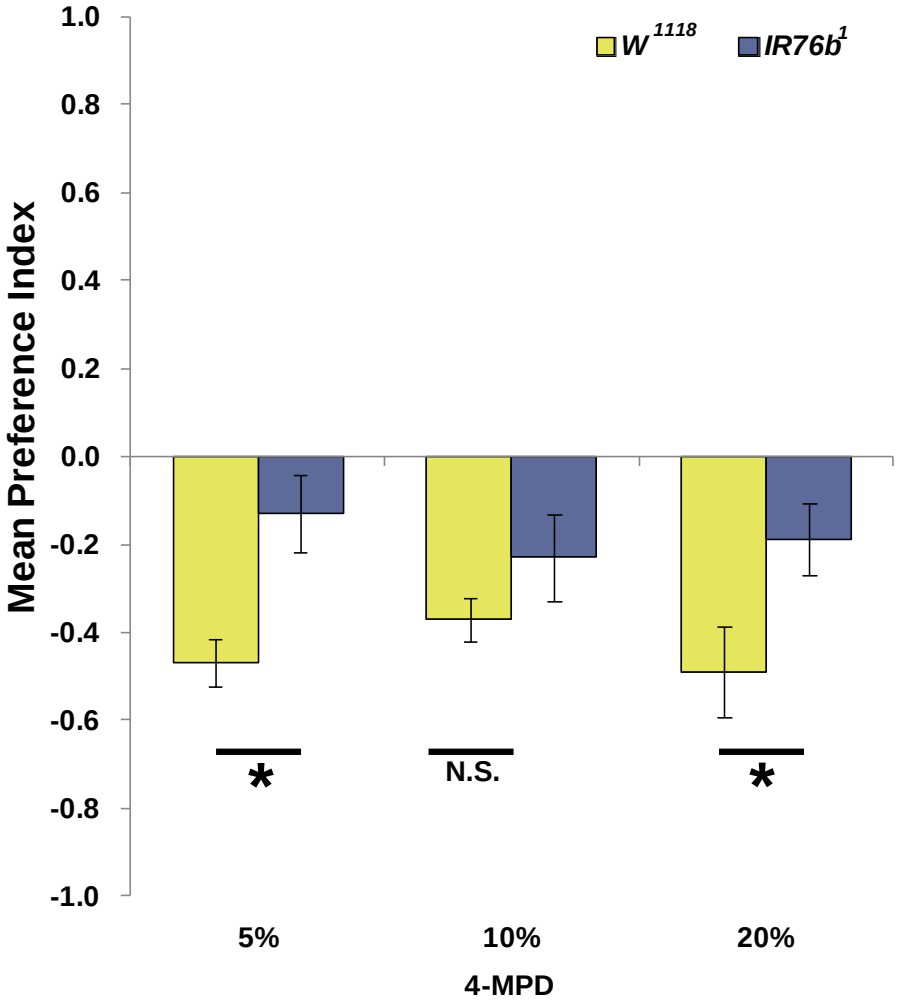

Figure 5.14: *Ir76b*^{1} mutant flies show reduced avoidance to 4-MPD compared with wild-type over multiple dosages in T-maze assay. Mean preference index of solvent (water) and 5%, 10% and 20% 4-MPD. N= 4-14, error bars = S.E.M. Two tailed student's t-test at 5% (p=0.006), at 10% (p=0.162), and at 20% (p=0.042). *Ir76b*^{1} was BL51309 and wild-type was w^{1118}.

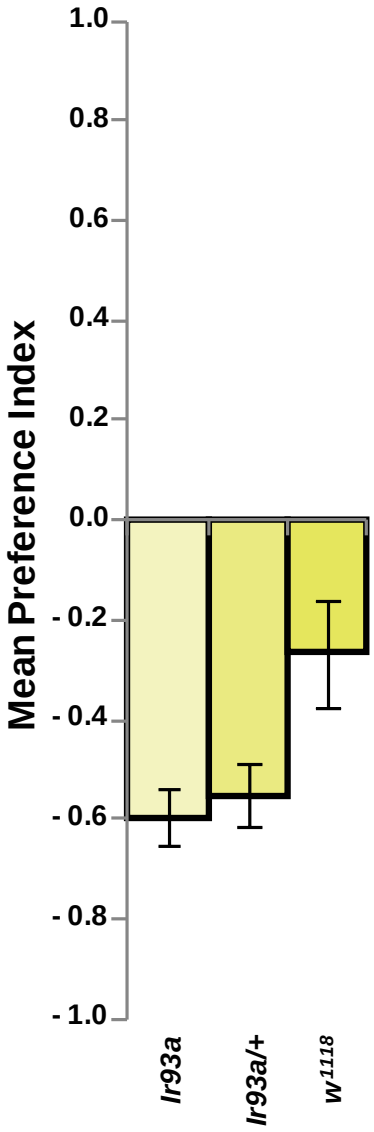

Figure 5.15: *Ir93a* mutant flies avoid 10% 4-MPD more strongly than the wild-type in T-maze assay. Mean preference index of solvent and 10% 4-MPD. N= 8-9, error bars = S.E.M. Two tailed student's t-test between w^{1118} and *Ir93a-/-* (p=0.01). *Ir93a* mutant was BL42090 and in a yellow, white background.

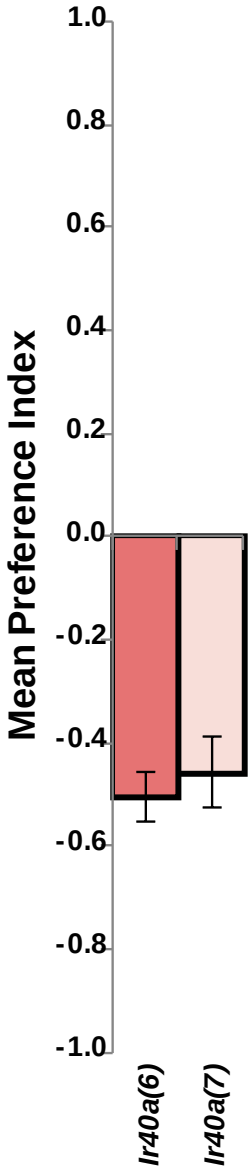

Figure 5.16 *Ir40a* mutant flies avoid 10% 4-MPD in the T-maze assay. Mean preference index of solvent and 10% 4-MPD. N= , error bars = S.E.M.

Chapter 6: Conclusion

Previously, some repellency pathways were identified in *D. melanogaster*. This study has expanded our knowledge of conserved avoidance pathways in other *Drosophila* species. In chapter 2, we showed that some avoidance responses were conserved across the species we tested: *D. yakuba*, *D. suzukii*, *D. pseudoobscura*, and *D. virilis*. Specifically, all species avoided DEET. Other odorants revealed different degrees of behavioral conservation between species. *D. melanogaster* and *D. yakuba,* two species more closely related evolutionary, avoided ethyl-3-hydroxybutyrate, whereas *D. suzukii*, *D. pseudoobscura*, and *D. virilis* did not. Interestingly, *D. suzukii* and *D. virilis* did not avoid CO_2, but in an electrophysiological analysis, we observed strong activation of a CO_2-sensing neuron in these species. This brought up an intriguing question. Because these flies were able to sense CO_2 and did not respond to it, have *D. suzukii* and *D. virilis* evolved a change in the neuronal circuit activated by CO_2? Other insects such as mosquitoes sense CO_2 and exhibit attraction behavior. Processing of olfactory cues may have evolved to allow different insects to adapt to specific ecological niches. Further studies need to be done to determine if the higher level targets of the olfactory neurons responsible for CO_2 detection differ in *D. suzukii* and *D. virilis*.

In chapter 3, we observed *D. suzukii* avoidance of three anthranilate compounds: ethyl anthranilate, butyl anthranilate and methyl N,N-

dimethylanthranilate. More importantly, *D. suzukii* avoided ovipositing on blueberries and raspberries protected by butyl anthranilate in a residence assay lasting one week. Further studies need to be done to test the feasibility of delivering butyl anthranilate and related compounds in field studies to protect crops from this pest species adapted to lay its eggs in fresh, ripe fruit ready for market. In addition, by applying a push-pull technique using these compounds in combination with *D. suzukii* attractants, further studies may be able to create highly effective, safe methods to deter this pest insect from destroying crops world-wide.

In understanding the mechanism by which DEET causes avoidance, several competing models have been proposed: an olfactory driven model, a gustatory model and DEET acting as a general “confusant”. In chapter 4, our negative results have shown that DEET is not processed via *Ir40a*. Future work needs to be done to better understand how DEET works and to take advantage of this strong repellency pathway that is conserved across a wide variety of insects.

In chapter 5, we identified eight compounds that repel *D. melanogaster*. These compounds are: 3,4-Dihydro-2H-pyran, 1-methyl-1,4-cyclohexadiene, cis-2,4,5-trimethoxypropenylbenzene (β-asarone), methyl jasmonate, phenetyl isothiocyanate, methyl dihydrojasmonate, 1,2,3,4-tetrahydroquinoline, and isobutyl anthranilate. For the new compounds tested in *D. melanogaster*, three repellents (3,4-dihydro-2H-pyran, β-asarone and methyl dihydrojasmonate)

where then shown to not require *Orco*. Additional experiments need to be done to understand specifically which sensory neurons are necessary for avoidance to these compounds. These compounds may activate existing known avoidance pathways such as Or85a, Gr63a/Gr21a, Or59a, or ab12, which should be tested first by electrophysiology. Alternatively, these compounds may trigger other unknown avoidance pathways, which would require further investigation.

Ideally, we would like to know how higher order brain circuits integrate the repellent input from specific olfactory receptor neurons. By studying the circuits these new repellents activate, and interactions between circuits in the fly brain, greater understanding of neuronal processing of aversive environmental cues may be achieved. This could lead to advances in neuroscience by bringing further light onto how environmental cues are processed in insect brains. In turn, this basic scientific understanding could also identify new higher order targets triggering insect avoidance behavior, which could lead to development of novel techniques for protecting humans from disease-transmitting insects and crops from pests.