

Isolating an Olfactory-related Gene in *Drosophila melanogaster*

INTRODUCTION

Vector-borne diseases such as malaria kill over 1 million people every year. DEET (Diethyl-meta-toluamide) is the most widely used topical insect repellent used to avoid disease vectors such as mosquitoes, but it has neurotoxic effects (1) and its molecular mechanism of action is unknown (2). Understanding the mechanism of action of DEET may lead to the development of safer and more effective insect repellents.

Researchers previously isolated a mutant strain of *Drosophila melanogaster* that was largely insensitive to the repellent effects of DEET, but had normal avoidance responses to other odorants (3). They mapped this trait to the X chromosome. We obtained this mutant strain and used recombination mapping of visible traits and a simple behavioral assay to further isolate a major locus of this trait to a specific region on the X chromosome.

The gene for the voltage-gated potassium ion channel *Shaker* mapped to this region, and we observed that the molecular structure of DEET is similar to the potassium channel blockers used to characterize *Shaker* conductivity, both having N-diethyl 'heads' and hydrophobic tails. In DEET, the hydrophobic region is a 7 carbon toluene ring, and studies show that TEA blockers with a 6 carbon hydrophobic tail (triethylhexylammonium, TEHA) have an increased blocking affinity with *Shaker* ion channels (4). Altering the conductivity of ion channels would likely disrupt neuronal signaling. In repellency trials using DEET analogs, both the N-diethyl head and the size of the hydrophobic tail are critical to the effectiveness of DEET as a repellent against mosquitoes (5).

Based on this hypothesis, we developed and ran behavioral assays designed to test whether a mutation to *Shaker* could be responsible for the DEET-insensitive trait. The data generally supported this hypothesis, and so we determined the DNA sequence of a region in the *Shaker* gene most likely to be involved interactions with DEET, in both the mutant and wild-type strains.

METHODS

Recombination Mapping with Visible Marker Strains

See panel to the right for an experiment design overview. We assayed the DEET response of 2258 recombinant F2 males with the following protocol: in each assay, we place 20-50 flies in a 100mm Petri dish. Yeast paste is placed within two microfuge tubes as an attractive odorant. Each tube has a one-way entrance (a 200 uL micropipette tip cut 11mm from the tip). At the larger entrance into the micropipette tip is a 5mm x 5mm piece of filter paper with 4 uL of 100% DEET. We collected data on their visible phenotypes relative to their response to DEET in an attempt to map this trait to a specific region of the X chromosome. Results in Figure 1 and 2.

Behavioral Assays of *Shaker* Mutants and using TEHA as a Repellent

We tested the avoidance response of *Shaker*¹⁴ and EAG (ether-a-go-go) mutants against our wild-type strain to DEET. We also tested the avoidance response of *Shaker*¹⁴, DEET-insensitive, and wild-types strains to the potassium channel blocker TEHA. Results in Figure 3 and 4.

Deficiency Mapping with Gene/Region Knockout Strains

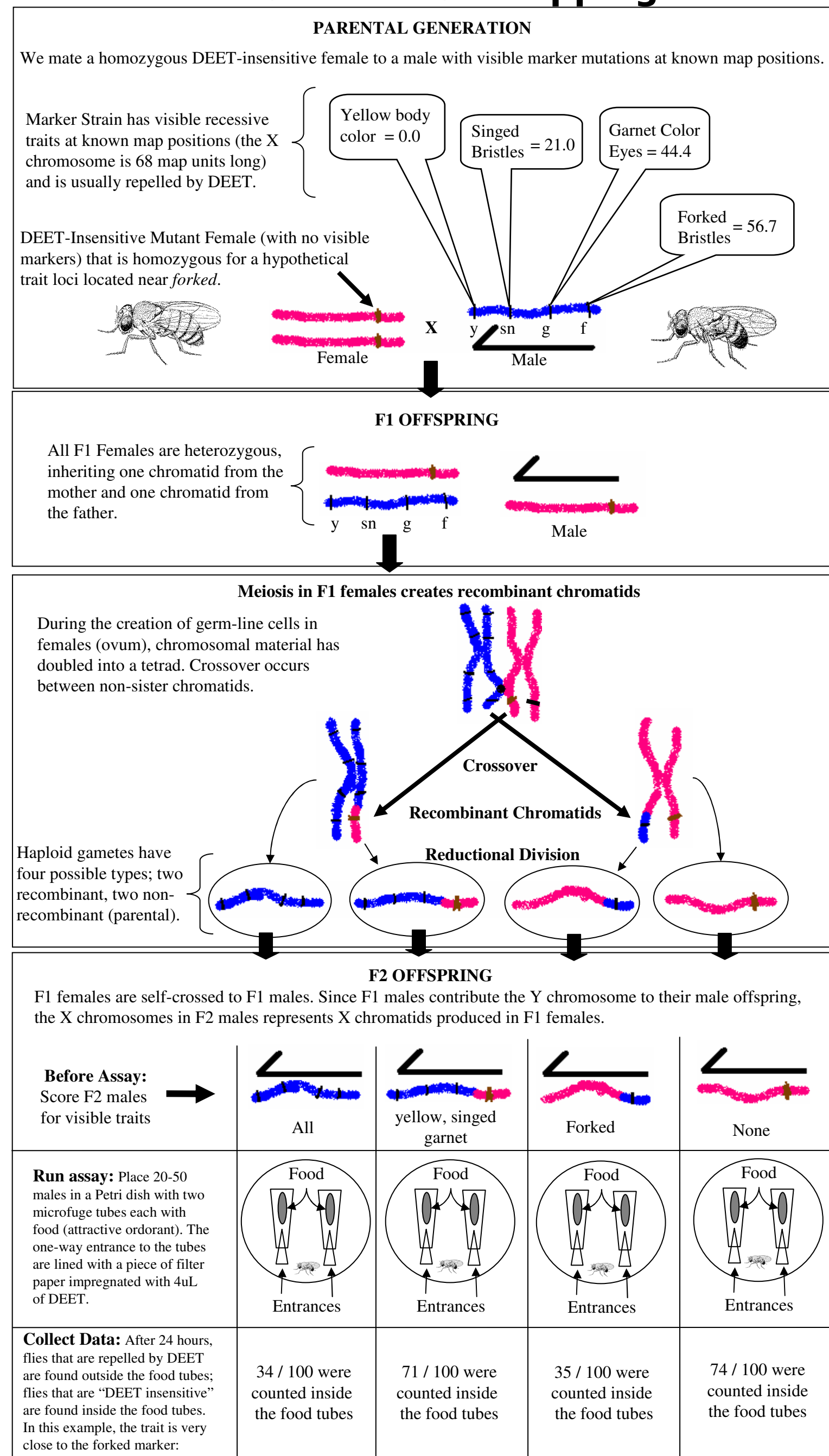
We obtained mutants strains with gene or genomic region deficiencies (created by P-transposon insertions) in the region of *forked* (map position 56.7), including a *Shaker* knockout strain. A cross between the mutant and the deficient strains will produce heterozygous F1 females; if the deficiency is in the same region as the mutation, the recessive trait will be uncovered, and should be expressed phenotypically in assays using the F1 females. Results in Figure 5.

DNA Sequencing of *Shaker* Pore Region

We did a DNA extraction of three wild-type strains and the DEET insensitive mutant and PCR amplified the exon that contained amino acids 418-449 of the *Shaker* gene, corresponding to the pore region of the ion channel, and obtained the DNA sequence using ABI Big Dye terminator method and an ABI 310 Sequencer. Results in Figure 6.

Alan Raetz Faculty Advisor: Jeff Bell
Department of Biological Science, California State University, Chico

Recombination Mapping



RESULTS

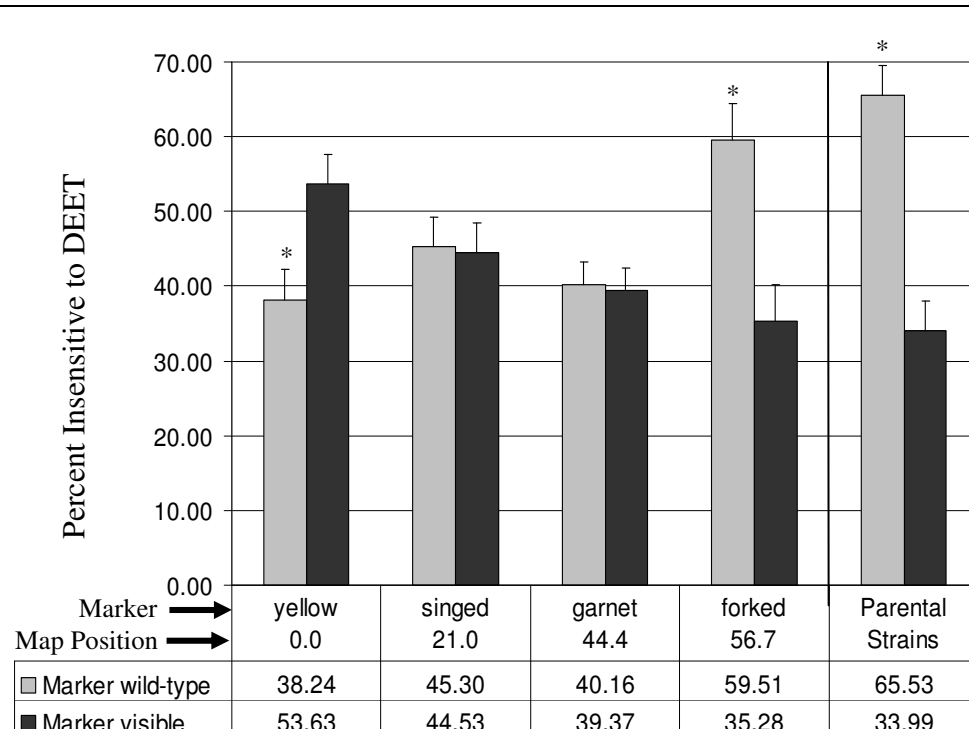


Figure 1. Percent of F2 males that were DEET-insensitive versus presence of visible markers. This is the data on recombinant flies compiled on a per-marker basis. Error bars represent a 90% confidence interval based on a binomial distribution. The rightmost bars are the DEET response of parental types for comparison. The two rightmost groups show that the presence or absence of forked bristles is tightly linked to the trait of DEET insensitivity. Recombinant male flies with a yellow body are also less repelled by DEET, suggesting that genes on the marker strain close to *yellow* contribute to this trait. (*) above bars denotes that DEET insensitivity was significantly different than the marker strain ($p < 0.0001$).

Response in Behavioral Assay	DEET-Insensitive	Total Tested	Percent Insensitive
<i>Shaker</i> ¹⁴ Mutant	39	75	52.00
Ether-a-Go-Go ¹	21	142	14.79
Wild-type Control	38	171	22.22

Figure 3. Testing Mutants of Candidate Genes for Sensitivity to DEET. The *Shaker*¹⁴ mutant was significantly less repelled to DEET versus the wild-type control (y,sn,g,f marker strain), $p < 0.001$. In contrast, the EAG mutant response to DEET did not significantly differ from wild-type.

Response in Behavioral Assay	TEHA Insensitive	Total Tested	Percent Insensitive
DEET Mutant	88	95	92.63
<i>Shaker</i> ¹⁴ Mutant	74	80	92.50
Wild-type Control	20	57	35.09

Figure 4. Testing Mutant Strain Response to Triethylhexylammonium. Both the DEET insensitive mutant and *Shaker*¹⁴ were significantly less repelled by this potassium channel blocker molecule than the wild-type strain ($p < 0.001$).

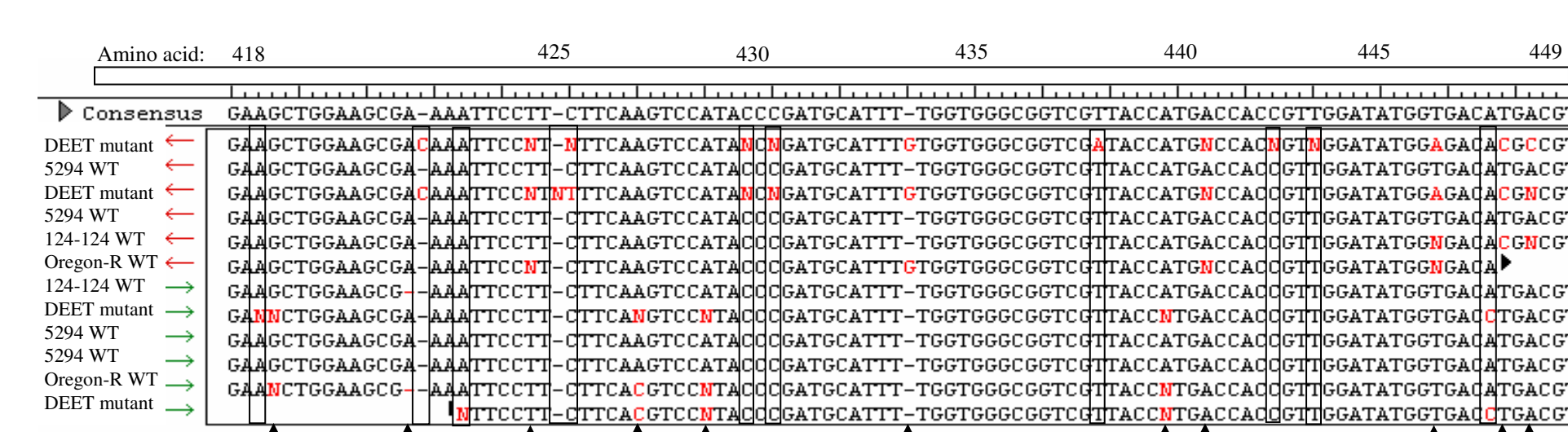


Figure 6. *Shaker* amino acids 418-449, obtained by DNA sequencing the 3 wild-type strains (5294 marker, Oregon-R WT, and 124 WT) and the DEET insensitive mutant strain. Boxes highlight base changes that only occurred in the DEET mutant sequences, while arrows (bottom) show base polymorphisms that occurred in both the DEET insensitive mutant and in at least one of the wild-type strains. The consensus sequence obtained matches the published drosophila genome sequence exactly, if you discount the gaps added to align the sequences together.

DISCUSSION

The recombination mapping experiments show that DEET insensitivity is significantly linked to the visible marker *forked*. A survey of genes in that region led to identifying *Shaker* as a reasonable candidate gene. *Shaker* is one map unit away from *forked*, and encodes a voltage-gated potassium channel that has been shown to be involved in olfactory responses (8). Immunocytochemistry has shown that *Shaker* products are most highly expressed in the mushroom bodies of the drosophila brain, which accepts neuronal input from olfactory receptors (9).

If the DEET-insensitive trait was caused by a mutation to *Shaker*, we hypothesized that other mutations to the *Shaker* gene may also cause altered responses to DEET. Using the same behavioral assay as our recombination experiments, we found that *Shaker*¹⁴ mutants are significantly less repelled by DEET than our control strain, but a mutant of the potassium ion channel *ether-a-go-go* (also in the region near *forked* at map position 50.0) shows no difference in response compared to the control strain.

Using the *Shaker* potassium channel blocker TEHA as an odorant in our behavioral assay, we found that both the DEET-insensitive mutants and *Shaker*¹⁴ mutants are significantly less repelled by this compound compared to the wild-type strain, thus these responses are similar to their responses to DEET. Although these results can be interpreted as supporting the hypothesis that the DEET-insensitive trait is due to a mutation in *Shaker*, there are reasonable alternate explanations for these results; for example, it may be that *Shaker* and DEET-insensitive mutations simply share this olfactory response profile by random chance, or that the similarity in response is only because DEET and TEHA are molecular analogs.

Our initial testing using genetically deficient strains shows that this X chromosome recessive trait of DEET insensitivity is 'uncovered' in a cross with a *Shaker* deficient strain. Although this supports our hypothesis that *Shaker* is responsible for this mutation, we also find that crosses with other deficiencies have varying levels of response to DEET. Olfactory responses in this assay involve complex behaviors and many genes may alter behavioral responses. Differences in response may be due to genes unrelated to the region of the genomic deficiency, since these strains come from different genetic backgrounds. Thus we interpret these preliminary results with caution, and are continuing to collect data with this method.

We thought the most likely region of *Shaker* that would cause an alteration in the response to DEET was the ion channel pore, corresponded to be amino acids 418 to 449. Previous research has shown that single amino acid mutations in this region using site-directed mutagenesis altered the affinity of *Shaker* channels expressed in *Xenopus laevis* oocytes to TEHA (4). The results of the DNA sequencing of the pore region of the *Shaker* gene in the mutant versus wild-type strains were inconclusive. The duplicate DEET-insensitive mutant samples differed from the six wild-type transcripts in ten separate locations, but there was also a total of eleven polymorphisms between wild-type strains, putting the accuracy of this sequence data in doubt. Interestingly, there are no instances of where a wild-type strain has a single base mutation without a corresponding change in at least one of the DEET insensitive mutants, whereas there are 10 instances of single base mutations in the DEET insensitive strain that are not in any of the wild-type strains.

REFERENCES

- Abdel-Rahman A, Shetty AK, Abou-Donia MB. 2001. Subchronic dermal application of N,N-diethyl m-toluamide (DEET) and permethrin to adult rats, alone or in combination, causes diffuse neuronal cell death and cytoskeletal abnormalities in the cerebral cortex and the hippocampus, and Purkinje neuron loss in the cerebellum. *Exp Neurol*. 172(1):153-71.
- McIver SB. 1981. A model for the mechanism of action of the repellent DEET on *Aedes aegypti* (Diptera: Culicidae). *J Med Entomology*. 18(5):357-61.
- Reeder NL, Ganz PJ, Carlson JR, Saunders CW. 2001. Isolation of a deet-insensitive mutant of *Drosophila melanogaster* (Diptera: Drosophilidae). *J Econ Entomol*. 94(6):1584-8.
- Choi KL, Yellen G. 1993. The internal quaternary ammonium receptor site of *Shaker* potassium channels. *Neuron*. 10(3):533-41.
- Suryanarayana MV, Pandey KS, Prakash S, Raghuvveeran CD, Dangi RS, Swamy RV, Rao KM.1991. Structure-activity relationship studies with mosquito repellent amides. *J Pharm Sci*. 80(11):1055-7.
- Lander ES, Botstein D. 1989. Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics*. 121(1):185-99.
- Doerge RW, Churchill GA. 1996. Permutation Tests for Multiple Loci Affecting a Quantitative Character. *Genetics*. 142(1):285-294.
- Cowan T, Siegel RW. 1986. *Drosophila* mutations that alter ionic conduction disrupt acquisition and retention of a conditional odor avoidance response. *J Neurogenetics*. 3:187-201.
- Schwarz TL, Papazian DM, Carretto RC, Jan YN, Jan LY. 1990. Immunological characterization of K⁺ channel components from the *Shaker* locus and differential distribution of splicing variants in *Drosophila*. *Neuron*. 4(1):119-27.

Acknowledgements

- DNA sequencing was done in the lab of Dr. Gordon Wolfe.
- Nancy Reeder of Proctor and Gamble, and John Carlson of Yale University provided the DEET-insensitive mutant.
- All other fly stocks were obtained Bloomington Stock Center, <http://flystocks.bio.indiana.edu/>.
- Financial support by the Phillip A. Cothern Memorial Scholarship and the Megan Lee Hudzik Scholarship is gratefully acknowledged.