LOCATING THE GENE THAT CONFERS RESISTANCE TO DEET IN *C. ELEGANS*

by

Preston Sawyer

Submitted in partial fulfillment
of the requirements for Departmental Honors in the
Department of Biology
Texas Christian University
Fort Worth, Texas

May 3, 2013

LOCATING THE GENE THAT CONFERS

RESISTANCE TO DEET

IN *C. ELEGANS*

Project Approved:

Phil Hartman, Ph.D.
Department of Biology
(Supervising Professor)

David Minter, Ph.D.
Department of Chemistry

Giri Akkaraju, Ph.D.
Department of Biology

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to take this opportunity to extend my sincerest thanks to my supervising professor Dr. Hartman. Thank you for all of your instruction, motivation, and patience throughout the duration of my senior project. I could not have accomplished something of this magnitude without the copious amount of time and effort that you invested in both my life and my educational endeavors. Finally, thank you for all of your kind instruction and advice throughout the duration of my time here at TCU. I will be forever grateful for all you have done to allow me to accomplish my goals.

Additionally, I would like to extend an equal amount of gratitude toward my committee members Dr. Minter and Dr. Akkaraju. Thank you both for your input and encouraging comments when editing my thesis! I could not have produced this paper without your kind input. Thank you for always being so willing to instruct me both in and out of the classroom. You have each blessed me with lessons of instruction, as well as served my needs as confidants, motivators, and encouragers. I have been truly blessed to have such inspiring educators and personal role models.

Finally, I would like to thank my family for their infinite support throughout the duration of my educational experiences. Without such loving, supportive, and incredible parents I could never have fulfilled so many of my collegiate goals and aspirations. Thanks you for all the emotional, spiritual, and financial support over the past years.

INTRODUCTION

The organic compound named N,N-diethyl-*m*-toluamide (commonly referred to as DEET) is the active ingredient in many popular insect repellents such as *OFF*. It is commonly regarded as the world's most comprehensively used functional insect repellent (Krajick *et al*., 2006). For the past fifty years it has been effectively utilized around the world to deter insects, as well as control vector-born diseases (Gupta and Rutledge, 1994; Osmitz and Grothaus, 1995; Mafong and Kaplan, 1997). The fundamental advantage of DEET, facilitating its high penetrance and associated popularity, is its inherent safety for topical application. Commercial DEET application manifests in a myriad of forms including aerosol spray, lotions, and even topical gelatinous substances. Once DEET is introduced within reasonable spatial proximity to a given recipient, it is quickly absorbed through the pores of the skin, efficiently metabolized, and ultimately excreted from the body (Koren *et al.,* 2003). However, despite widespread usage and high efficacy, the specific mechanism by which this compound actively repels insects (and *C. elegans* as well) remains elusive (Ditzen et al. 2008). It is currently ambiguous whether DEET repels the target organism by exuding a pervasive and unpleasant odor that acts upon the given organism's olfactory senses, or whether it indirectly inhibits the chemotaxis of an organism simply by masking the appeal of a given attractant.

Recent experimentation performed by Jacob Archer at TCU demonstrated that DEET does not operate as an active repellent in the nematode *C. elegans*. Instead, it diminishes an organism's positive response to a chemical attractant by some passive mechanism that nullifies the allure exuded by attractants. However, even this statement is ambiguous regarding the specific mechanisms underlying and simultaneously

facilitating this molecular interaction. The indeterminate nature of the specific molecular mechanism manifested by DEET resistance has prompted the effort to clone the gene that, when mutated, confers resistance. Regardless of the mechanism of action (which is discussed more in the subsequent paragraph), DEET has been proven, in a variety of laboratory settings, as an effective deterrent toward a specified attractant. For example, experiments run by Syed and Leal (2008) and Jahn et. al. (2010) demonstrated DEET's ability as an active repellent against the target organism *Aedes aegypti.* The research methodology employed by these individuals provided irrevocable evidence testifying to the continued application of DEET as the active ingredient for insect repellants.

Multiple levels of hierarchical data have been assimilated, all attesting to the ability of DEET to repel insects. However, as previously alluded to, the functional mechanisms underlying this phenomenon are not well understood. DEET has been shown in laboratory settings to reduce (to an unspecified degree) an insect's olfaction toward an attractant. Whether or not DEET's mode of action is strictly as a repellent to inhibit olfaction completely (Syed and Leal, 2008), or as a molecular block to incapacitate the ability of a given organism to sense and respond to attractants (Ditzen et. al. 2008), is unresolved. Syed and Leal (2008) were able to illustrate the fixative nature of DEET on the mosquito *A. aegypti*. Essentially, this means that the DEET compound physically mitigated the diffusion of chemical attractants, effectively masking their attractant ability. This experiment seems to lend credence to the idea that DEET acts by interfering with the attractant and *indirectly* inhibiting olfaction. However, when one changes the model organism used in the experiment from *A. aegypti* to *Drosophila melanogaster,* the conclusion differs. Ditzen *et al.* (4) wrote, ''here we show that DEET blocks

electrophysiological responses to olfactory sensory neurons to attractive odors in *Anopheles gambiae* and *Drosophila melanogaster.''*

In *Drosophila melanogaster,* the DEET compound was shown to inhibit responses toward a given attractant by *directly* acting upon the organism's olfactory senses. Specifically, the DEET was believed to adversely affect the olfactory sensory neurons' ability to sense, process, and respond to the attractant. Regardless of the specific nature of this functional paralysis of olfaction and response toward an attractant, the overarching effect of DEET has been tested upon a variety of organisms, with unanimous conclusions relating to its ability to act as a repellent. Furthermore, DEET has been shown to affect physiological functionality of the test subjects independent of obvious repellent properties. For example, exposure to DEET resulted in unusual feeding behaviors (Ditzen et. al. 2008). While DEET has been exhaustively researched and proven to be effective in its intended function as a repellent, the molecular pharmacology and associated molecules of interaction remain elusive.

Two specific elements need to come to fruition in order to move from a perfunctory understanding of DEET action to a pervasive one. The incipient portion of this process would be to isolate DEET-resistant mutants, and then clone the specific gene that codes for proteins facilitating DEET resistance. Upon completion of these procedures, there exists a real opportunity to understand exactly how DEET works upon organisms.

Thus, the seminal goal of experimentation culminating in this thesis is to follow said rationale and ultimately identify the location of the gene (*der-1*) coding for DEET sensitivity. Ideally, the data in this thesis will contribute to the determination of *der-1*'s

locale, and further sequencing of this region by scientists at the Rockefeller Institute will catalyze the resolution of the mechanism facilitating DEET's repellent properties.

RATIONALE FOR C. ELEGANS AS TEST ORGANISM

Caenorhabditis elegans (*C. elegans)* is a nematode that is well regarded as an ideal model organism. Model organisms are reasonably small creatures whose relatively short lifespan, small genome, and well-understood life histories make them ideal organisms for genetic experimentation. Also, in this era of widespread DNA sequencing, their genomes have been sequenced, which has invaluable ramifications for a variety of researchers working on a multitude of projects. A further advantage associated with C. elegans includes the fact that their unique molecular, genetic, and ontogenetic profiles are intimately understood. This further validates the current perception of *C. elegans* as ideal test organisms for a myriad of researchers since Sydney Brenner's seminal publication in 1974 (Brenner, 1974). Indeed, no less than three Nobel prizes have been awarded this millennium for work using *C. elegans.* One notable aspect of the *C. elegans'* genetic profile that is exceptionally useful in DEET bioassays is the manifestation of a particularly well-developed chemosensory system that has been examined and expounded upon (Bargmann, et. al. 2006). Approximately 5% of the genes comprising a given worm are allocated to the development, regulation, and maintenance of the chemosensory system (Bargmann, 1993). *C. elegans* has been known to be particularly responsive to both attractants and repellents. This fact, coupled with the previously mentioned advantages, makes *C. elegans* an ideal test organism for genetic experimentation examining the role of DEET. *C. elegans'* reaction to a particular reactant (attractant or

repellent) can observed via the implementation of a chemotaxis assay, whereby the DEET repellent is incorporated into the Nematode Growth Medium (NGM). This molten medium is allowed to cool and solidify into a gelatinous substance, upon which the worms are plated with an attractant, such as iso-amyl Alcohol (IAA). IAA acts in opposition to the DEET repellent, potentially persuading the worms to engage in chemotaxis and mobilize in its general direction.

ABSTRACT RATIONALE AND GOALS UNDERLYING THESIS

This project was designed to continue and verify the work done by Mr. Christopher Kim, who completed a senior honors research project May 2011. It attempts to prove the existence, and ultimately identify the relative location, of a particular gene in *C. elegans* that when mutated confers resistance to DEET. In the event that a mutant strain of *C. elegans* is successfully generated and isolated, these worms will positively respond to an attractant (IAA), despite spatial proximity to the active DEET compound within the medium. The goal is to essentially knock out (or functionally incapacitate) the gene coding for the phenotypic trait of DEET sensitivity. In order to formulate said mutagenesis, Mr. Kim first mutagenized *C. elegans* using ethyl methanesulfonate (EMS). Subsequently, the resulting progeny of the organisms were tested for the associated phenotypic manifestations of the mutation (specifically DEET resistance) via the previously described chemotaxis bioassay. Each mutant strain was re-assayed multiple times, always in comparison to a control wild-type strain, in order to rule out inconclusive negatives and false positives. Mr. Kim successfully employed this approach to isolate three mutants that were consistently DEET resistant, setting the stage for the project described in this thesis.

Following re-affirmation of the mutants isolated by Mr. Kim, a three-factor cross with two phenotypically distinguishable markers was used to map the specific location of the gene, which has been named *der-1*. Ms. Anh Nguyen had previously mapped other DEET-resistant mutants to a specific region of chromosome IV. This led to the prediction that the mutations isolated by Mr. Kim should, if in the same gene as those that were mapped by Ms. Nguyen, yield certain results in a specific three-factor cross. In fact, the results obtained in this study proved consistent with that prediction. Among other things, they gave credence to the notion that all three independently induced mutations are in the same gene. It is anticipated that the candidates will be sent to the Rockefeller Institute in New York to be sequenced. This should pinpoint the specific gene that was mutated to confer DEET resistance.

The recombinants utilized in this thesis were subjected to multiple levels of DEET testing for each of the 13 recombinant strains. As stated above, if the recombinant strains proved to be completely DEET resistant, then one could strongly infer that the *der-1* mutations isolated by Mr. Kim are in the same gene as those mutations previously mapped by Anh Nguyen. Furthermore, if the outcrossed mutant strains tested for in the third and final stage of the experimentation process are proven uniformly resistant, then that would be further affirmation that the mutations are in the same gene. The research methodology and associated data are discussed later in this thesis.

Further analysis of the mutants could potentially elucidate the specific mechanism of DEET upon the phenotype of *C. elegans* that has so eluded researchers. The ultimate goal underlying this research is to accumulate enough data to establish a cornerstone for constructing more efficient commercial insect repellents.

MATERIAS AND METHODS

A. **Strains**

1. **Incipient Mutants:**

There were multiple hierarchical levels of this thesis each utilizing a unique group of strains. The incipient stage of the project was simply to repeat the bioassays performed by Mr. Kim. The purpose of this repetition was to learn the procedure and simultaneously verify that the mutants were still DEET resistant. The strains used in this portion of the project were the wild-type control (N2), Mutant 1 (M1), M2, and M3. These were the strains isolated via EMS mutagenesis by Mr. Kim and provided by Dr. Hartman. These 4 strains were maintained in 60x15 mm petri dishes on Nematode Growth Medium (NGM), grown with *Escherichia coli* OP50 as a nutrient source, and incubated at 20 °C. The previously described methods of strain maintenance and growth are in direct accord with the methodology of Sydney Brenner (1974).

2. **Recombinant Mutants:**

In order to continue the assessment of the *der-1* region of *C. elegans*, hypothesized as the location of the gene coding for the DEET sensitive phenotype, the incipient mutants were subject to a three-factor cross using *dpy-13* and *unc-5*. Dr. Hartman prepared these recombinants. Furthermore, the nomenclature used to signify and identify a given strain is enumerated in the ensuing sentences. CB184 *(dpy-13)* was employed as the DEET-resistant control, and for the mutant recombinant strains the letter "D" was used to indicate the "Dumpy" (Dpy) phenotypic manifestation reflecting the small, stubby appearance of these mutated specimens. Worms labeled with a "D" identifier were additionally associated with limited mobility, when contrasted to the Wild

Type strain. The numerical delineation "13" was utilized to represent the thirteenth gene isolated by Sydney Brenner amidst thirty such genes. Additionally, "M plus a #" was used to demarcate which of Chris Kim's three mutants were currently undergoing experimental manipulation. Finally, regarding mutant nomenclature, a lower case letter was employed to further specify a given recombinant. The rationale for using "*dpy-13"* relates to its specific location. The strains isolated for subsequent testing in this phase of the project were: D13M1a, D13M1b, D13M1c, D13M1d, D13M1e, D13M2a, D13M2b, D13M2c, D13M3a, D13M3b, D13Mc, D13M3d, and finally D13M3e. These thirteen genetically recombined strains, at least three derived using each of the three mutants (M1, M2 and M3) described in the previous section, were each subjected to multiple rounds of DEET bioassays (and each assay was compared in temporal proximity to the WT control strain).

3. Outcrossed Mutants:

The terminal portion of experimentation required the synthesis and isolation of another group of mutants. Outcrossed mutants were prepared and tested in order to determine whether the DEET resistant trait was linked or unlinked to *dpy-13*. The strains utilized in this portion of experimentation had associated nomenclature as follows: D13M# (for the same reasons alluded to previously) + CO# (the number corresponds to either a 1,2,3 and its presence indicates a specific outcrossed strain). The individual strains utilized in this portion of the experimental methodology were: D13M1CO1, D13M1CO2, D13M1CO3, D13M2CO1, D13M2CO2, D13M2CO3, D13M3CO1, D13M3CO2, D13M3CO3. The DEET bioassay was applied multiple times to each respective strain in order to establish if the trait in question is linked or unlinked.

B. **Assay Plates**

1. Initial Phase of Re-Testing Mutants:

A separate and distinct medium was used in DEET bioassays as compared to the medium used for the storage and maintenance of stock strains. In order to test the ability of mutants to move toward an attractant, despite the presence of DEET, an alternative medium was required. This test medium (DEET bioassay medium) was autoclaved and DEET was then added. The DEET bioassay medium was prepared as follows: 17 g agar and 3 g NaCl were added to 1 L of deionized (DI) water, autoclaved, then cooled to 50 °C in a water bath. The solution was then supplemented with 25 mL of 1 M $KHPO_4$ (pH 6.0) and 2 mL of 1 M DEET in ethanol. 250 mL of the DEET agar solution was then poured into 100 x 15 mm Petri dishes, allowed to sit uncovered at room temperature for 3 hours to partially dry before being stored at 4 °C. The assay plates were then stored for subsequent use.

2. DEET Assays of Recombinants:

Upon completion of the initial phase of retesting mutants to ensure the persistence of their mutagenesis, it was necessary to move into the portion of experimentation that specified whether or not the genetic region in question was linked or unlinked to *dpy-13*. In order to prepare agar bioassay plates with an ideal concentration and dispersal of DEET throughout the test medium, several different levels with increasing concentrations of DEET were prepared. All other aspects regarding the preparation of this medium were identical. At this juncture, the research trajectory veered in a tangential direction and attempted to delineate the effectiveness of the mutagenesis when varying concentrations of DEET were mixed into the agar bioassay plates. The previous concentration that was

implemented to test the efficacy of the mutagenesis upon the mutant strains was 2 mL of 1 M DEET; however, in order to test the respective effects of increased and decreased concentrations of DEET upon the recombinant strains three different concentrations of DEET were prepared and utilized: 0 ml/L, 2 ml/L, and 3 ml/L, and ultimately 1.5 ml/L.

3. **DEET Assays of Outcrossed Mutants:**

In order to amass further experimental data regarding the nature and location of the *der-1* region of *C. elegans* genome, DEET assays of outcrossed mutants were utilized. DEET bioassays of these strains were conducted to provide further evidence that the mutations were in the same region as those analyzed by Anh Nguyen. Bioassay agar plates were prepared using the same methods as previously enumerated; however, the concentration of DEET that was added to the medium in its molten stage corresponded to that used in the testing of the resistance of the recombinants (1.5 ml/L). All results and extrapolative interpretations for these procedures can be found in subsequent sections.

C. **DEET Assay**

While the specific nuances of each experimental procedure exhibited variance in DEET concentration, the fundamental methodology of the DEET assay procedure remained consistent throughout the project. In each assay, 100 mm DEET agar plates were selected from storage at 4 °C. While these dishes were allowed to acclimate to the temperature of the room, 10^{-2} M isoamyl alcohol (IAA) was prepared. The IAA served as the attractant chemical compound that would compete with and attempt to override the repellent forces of DEET present in the medium. The desired concentration of IAA was prepared by mixing the following reagents in a test tube: 8.8 microliters of IAA and 10.0 ml of 95% ethanol. Subsequently, test tubes were labeled with consecutive integers

corresponding to the strain to be tested using that tube. For example, the test tubes containing the M1, M2, M3, and N2 mutants were labeled 1,2,3, and N2, respectively. These tubes acted as transport vessels to transfer the given strain from the storage agar dish to the 100 mm DEET agar plates used in the DEET bioassays.

The petri dish containing the stored stock to be used was then selected from the refrigerator. The worms were transferred from the stock petri dish to the appropriately labeled test tube by washing the surface of the stock petri dish with DI water. Approximately 3 ml of DI water was pipetted onto the surface of the storage petri dish outside of the bacterial lawn, the water was gently swished around the desired surface area of the storage petri dish and re-pipetted away with the nematode worms coming along for the ride. Adding additional DI water from the faucet then mixed the worms and the DI water further. At this point, the total amount of DI water in a given test tube was 10 ml. This step was done in order to separate the worms through suspension in liquid. The worms were separated all throughout the 10 ml of DI water and given 3-5 minutes to settle. Upon the completion of the 3-5 minute time allotment (dependent on the amount of worms suspended in a given test tube), the worms that did not pellet at the bottom of the tube, along with the excess DI water were aspirated to approximately 0.5 ml. The reasoning underlying this process was to take advantage of gravity to separate the larger worms in a pellet from the smaller worms remaining in suspension.

Immediately following the aspiration step, the worms were allowed to sit in the test tube for an additional couple of minutes to allow them to re-pellet at the bottom of the test tube. This ensured the greatest concentration of mature worms. In order to transfer the worms in the pellet from the test tube to the 100 mm DEET agar plate, a P20

micropipeter was selected and set to 15 microliters. The plunger was depressed, its tip placed into the center of the pellet of DI water and worms, and the plunger was released, effectively "sucking up" all the worms into its tip. In order to complete the transfer of the worms, 3-4 drops from the P20 were placed at one end of each 100 mm DEET agar plate. This resulted in approximately 50-100 worms being placed on the surface of the DEET medium in a given 100 mm DEET agar plate.

Now that the worms were successfully transferred, 5 microliters of the attractant IAA was placed inside a small circle on the side of the petri dish opposite that of the worms. The worms (currently existing in a drop of DI water and thus unable to achieve locomotion) were then spread around the base of the DEET-containing dish with a flame sterilized platinum wire until they were evenly dispersed across the medium's surface. This step resulted in an even distribution of the strain being tested and prevented clumping, which would inhibit the worms' pursuing the attractant regardless of mutant state. The lid of the DEET-containing petri dish was then replaced and the worms were allowed to pursue the IAA if genetically capable.

The 100 mm DEET agar plates used were marked with a circle at one end (where the IAA would be placed) and a line was drawn bisecting the center of the plate perpendicular to the center of the circle. After the experimental setup was completed, the worms were monitored at 15, 30, 45, and 60 minutes. The number of worms that crossed the ½ way point (denoted by the line bisecting the 100 mm DEET agar plate) were counted at each temporal checkpoint, as were those worms that made it all the way to the dime-sized circle at the apex of the dish. These numbers were then divided by the total number of worms plated on the 100 mm DEET agar plate dish to come up with a

proportion of mobile worms for each strain tested. This percentage was used as a metric to quantify and compare the WT control to the mutant strain being tested at each hierarchical level of experimentation.

After each DEET assay was completed, the untested mutants for a given strain, or those left over from the storage petri dish, were plated onto fresh petri dishes containing the same NGM as previously described. These dishes contained a fresh lawn of 0P50 bacteria and were used to maintain the mutant strain stock by allowing them to produce more and more progeny to be tested and re-tested. The worms were picked off the NGM surface and transferred from a given dish using a small wire and microscope.

RESULTS

A. **Reaffirmation of Mutants (M1, M2, & M3)**

In the incipient stage of experimentation, the EMS mutants isolated by Mr. Kim were subjected to DEET bioassays in order to confirm that the mutants were still DEET resistant. The mutant strains tested in this phase were M1, M2, and M3. These strains were tested against a WT control (N2) known to be DEET sensitive. A group of N2 worms were also tested concomitantly in order to rule out the possibility of false positives. The results of each bioassay were graphed in relation to one another and the N2 control. There are two data sets with associated graphs for each DEET bioassay performed, one depicting the number of worms that crossed the line delineating the halfway point at each time increment, and one representing the number of worms that reached the circle containing the attractant IAA per unit time. Those mutant organisms that managed to make it all the way from their starting point to the circle were considered to be somewhat less susceptible to the DEET present in the bioassay medium. Thus, the

magnitude of displacement and manifestation of resistance are believed to be directly proportional. The y-axis illustrates the percentage of worms that managed to travel past the halfway point, or into the circle, depending on the specific graph. The X-axis is labeled with 15-minute increments used to evaluate the temporal progression of the experiment. Lines with contrasting colors are used to trace a given strain's performance relative to the other strains involved. In addition to the graphical representation, all experimental data were summarized in a qualitative fashion via tables presenting each strain's performance relative to the control on individual assays. The mutants were characterized with designations from one of three qualitative categories: No Difference ("No Diff"), Clear Difference ("Clear Diff"), or Marginal Difference ("Marginal"). These qualitative labels are all relative to how a given strain compared to the N2 strain. The data described here are consistent throughout the duration of experimentation.

Representative graphs of data gleaned from DEET bioassays purporting the effectiveness of EMS mutagenesis and associated mutant phenotypes are presented below:

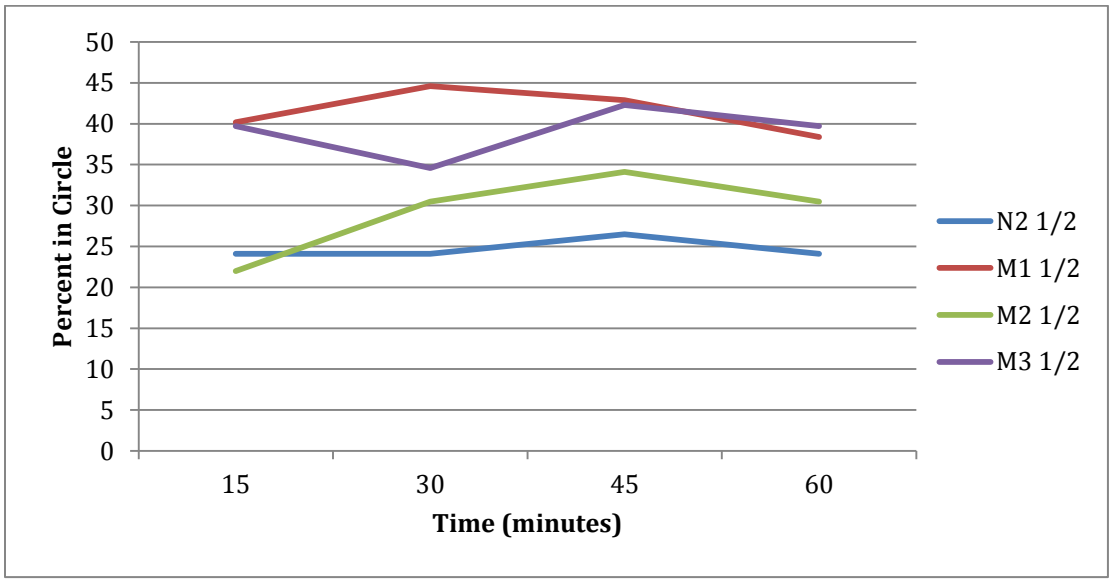

Figure 1. The "1/2" represents the # of worms that crossed the line bisecting the petri dish at the halfway point. Y-axis signifies the percentage of worms that crossed ½ way line. X-axis specifies the temporal progression of the experiment reported in minutes.

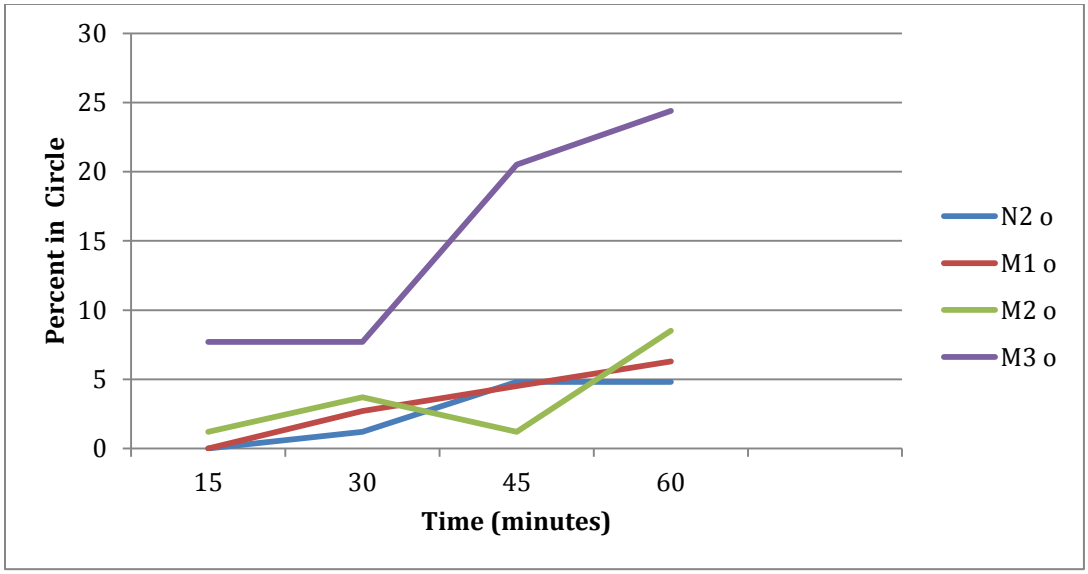

Figure 2. The "o" represents the # of worms that made it all the way to the circle at the apex of the petri dish. Y-axis signifies the percentage of worms that crossed into the circle. X-axis specifies the temporal progression of the experiment reported in minutes.

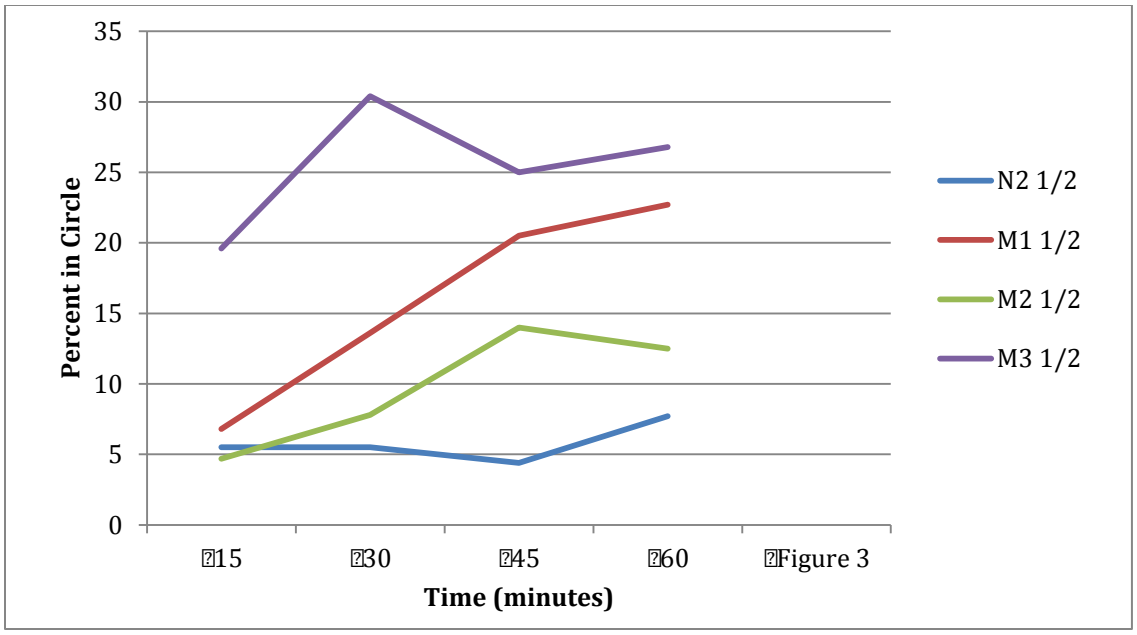

Figure 3 The "1/2" represents the # of worms that crossed the line bisecting the petri dish at the halfway point. Y-axis signifies the percentage of worms that crossed ½ way line. X-axis specifies the temporal progression of the experiment reported in minutes.

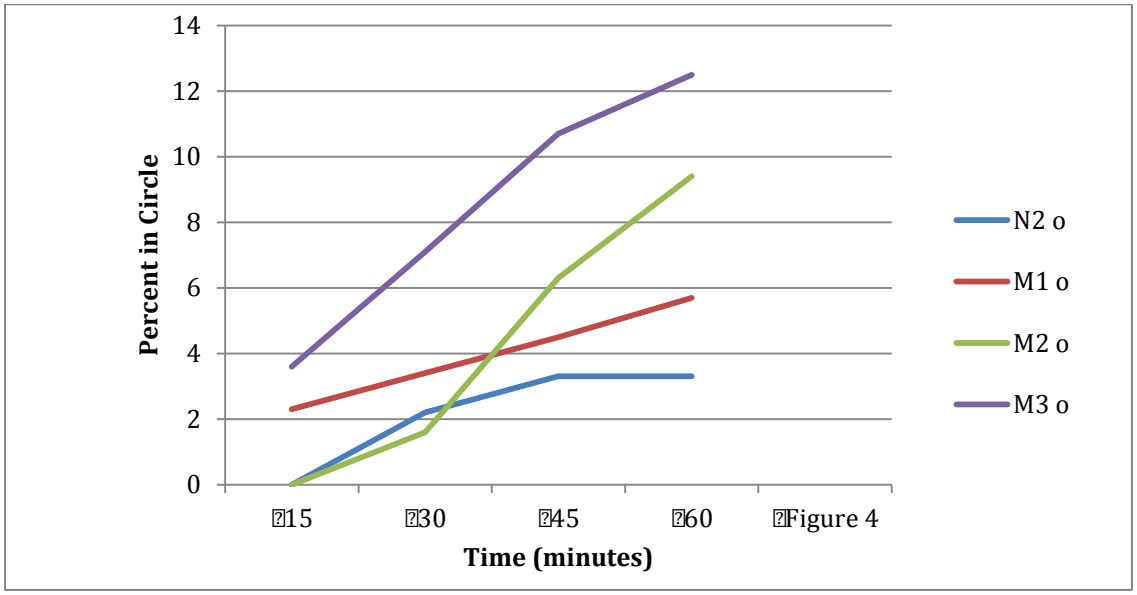

Figure 4. The "o" represents the # of worms that made it all the way to the circle at the apex of the petri dish. Y-axis signifies the percentage of worms that made it into the circle. X-axis specifies the temporal progression of the experiment reported in minutes.

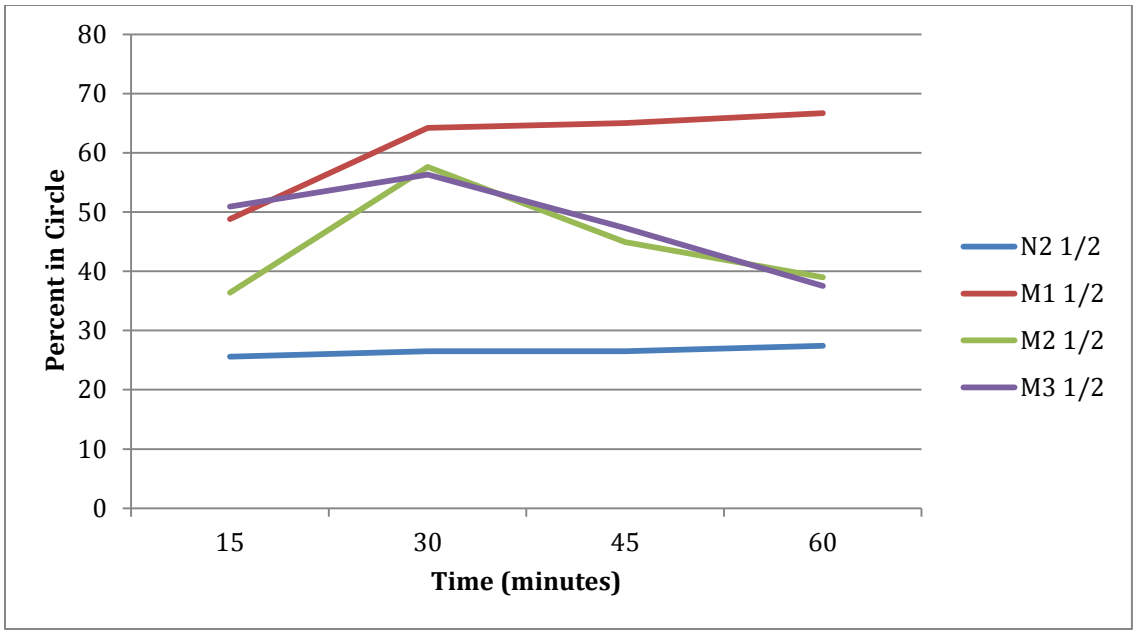

Figure 5. The "1/2" represents the # of worms that crossed the line bisecting the petri dish at the halfway point. Y-axis signifies the percentage of worms that crossed ½ way line. X-axis specifies the temporal progression of the experiment reported in minutes.

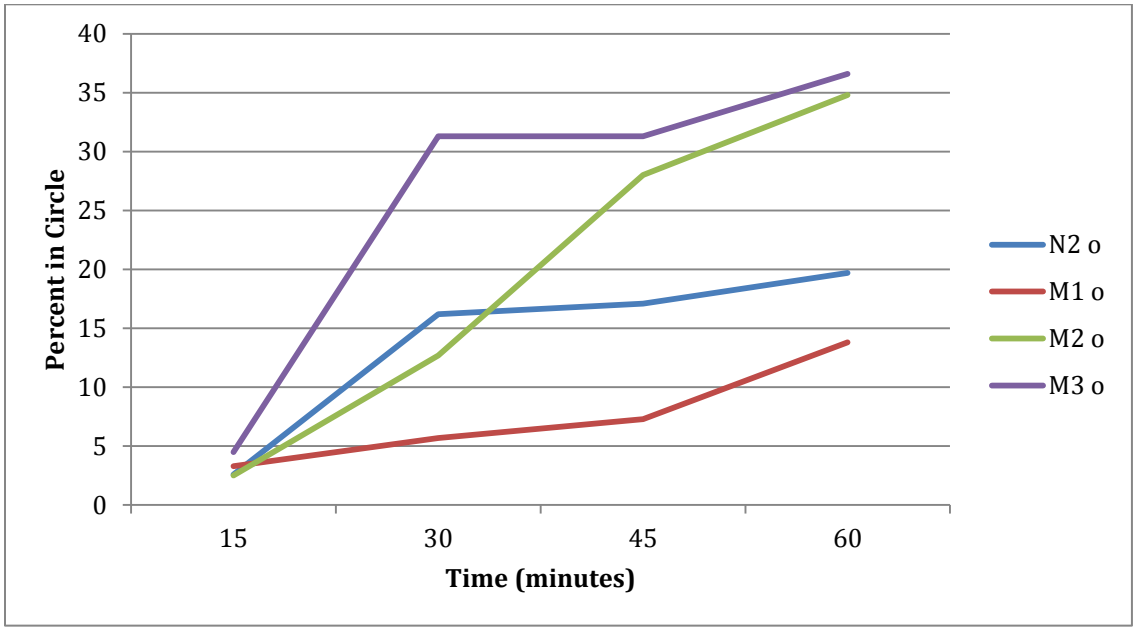

Figure 6. The "o" represents the # of worms that made it all the way to the circle at the apex of the petri dish. Y-axis signifies the percentage of worms that made it into the circle. X-axis specifies the temporal progression of the experiment reported in minutes.

Careful examination of the graphs depicted above indicates a clear difference between the mutants M1, M2, M3 when compared to the wild-type strain N2 in the

presence of DEET. As alluded to previously, this positive correlation was expected and confirms that the mutants isolated by Mr. Kim, whose progeny were manipulated throughout the experimentation described here, were in fact mutants whose phenotypes exhibited some level of resistance to DEET.

Data Set A shows that the WT N2 strain maintained some level of locomotion in the presence of 2 ml/L concentration of DEET, the rate of displacement of the control strain was significantly depreciated relative to the mutant strains. Another interesting aspect of the graphical representations of this data set can been seen in the sudden decline of the number of worms that crossed the half way point as the experiment progressed beyond the 45 minute mark. However, when the first graph is supplemented with information in the second graph of the data set, one can see that there was a spike in the number of mutant worms that made it to the circle containing the IAA. This makes sense because the worms that traversed to the circle had to cross the halfway line before making their way to the circle. Worms whose locomotion took them into the circle are no longer represented in the percentage of worms that crossed the halfway marker, and this fact explains any potential paradox. This is true for the remainder of graphs purported in this paper, and illustrates the importance of synthesizing the “halfway” graphs in combination with the “circle” graphs.

Data Set B and Data Set C each reflect similar information. Interpreting these graphs leads one to conclusions consistent with those drawn from Data Set A, as is the case with all the DEET assays juxtaposing incipient mutants (M1, M2, and M3) with WT N2 controls. The data set forth in the following table qualitatively summarizes and encompasses all the experiments in the first phase of the project:

Table 1. A qualitative comparison of mutants M1, M2 and M3 versus wild type.

Strain	**Assay #1**	**Assay #2**	**Assay #3**	**Assay #4**	**Assay #5**	**Assay #6**	**Assay #7**
M1	Clear Diff	Clear Diff	Clear Diff	Clear Diff	Clear Diff	Clear Diff	Clear Diff
M2	No Diff	Clear Diff	Clear Diff	Clear Diff	Clear Diff	Clear Diff	Clear Diff
M3	Clear Diff	Clear Diff	Clear Diff	Clear Diff	Clear Diff	Clear Diff	Clear Diff

As one can see, this table only exhibits one instance of inconsistency regarding the ability of the mutants' displacement to clearly differentiate itself from that of the WT control. This is a testament not only to the accuracy of the experimental methodology utilized here, but to the persistence of the mutagenesis performed by Dr. Hartman and Mr. Kim prior to current experimentation. Such conclusive positive results provided confidence that all background work prior to this thesis was accurate and would provide a reliable basis for any extrapolative determinations made from the work described in this thesis regarding the nature of the *der-1* region of *C. elegans* and specifically its ability to mutate in the DEET resistance phenotype.

Once the EMS Mutagenesis performed by Mr. Kim was verified, the project's focus settled on the main goal, to determine whether or not the *der-1* region believed to code for the DEET sensitive phenotype was genetically linked or unlinked to *dpy-13*, the position determined by Anh Nguyen's mapping efforts.

B. **Recombinant Testing**

In order to determine if the mutations isolated by Chris Kim mapped to the same region as those characterized by Anh Nguyen, an optimal DEET concentration for the chemotaxis assay needed to be determined. To do this, the recombinant strains listed previously were tested using varying concentrations of DEET. The DEET bioassay used was the same as that used on the incipient mutants.

1. DEET Concentration Gradient

As mentioned in the previous paragraph, the correct concentration of DEET needed to be established in order to properly separate the recombinant strains from the WT control (CB184 strain). If too little DEET were incorporated into the medium, then the WT control would be rendered too active. This would result in an insufficiently stringent separation pressure. However, too great a concentration of DEET in the medium would functionally incapacitate all strains on the surface of the assay medium and would not allow for any distinctions between the strains. Another reason to run assays of recombinants with three different concentrations of DEET was to establish a concentration gradient and to see if the recombinants exhibited resistance that mirrored the spectrum of concentrations. Essentially, the question to be answered was whether or not the recombinants' resistant phenotype would persist regardless of DEET concentration, or whether that phenotype was concentration dependent.

1. Highest DEET Concentration:

The first step perform was to run the DEET bioassay at the highest concentration of DEET chosen (3 ml/L). The graphical representation of the data is shown for worms

that crossed the halfway point and for those who made it to the circle for the specified strains:

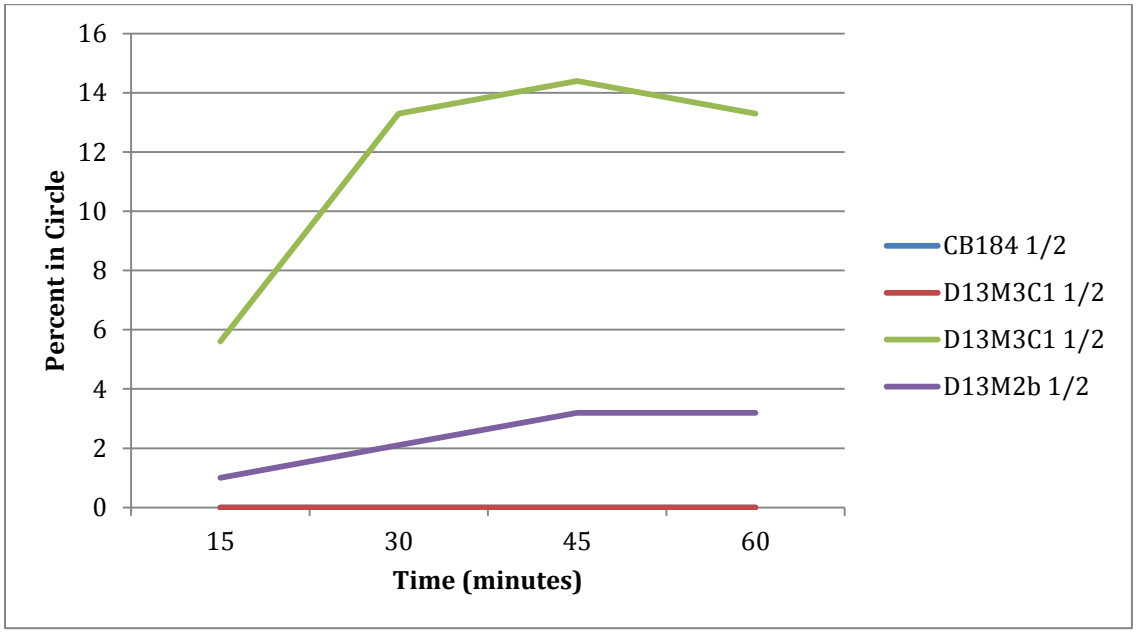

Figure 7. The "1/2" represents the # of worms that crossed the line bisecting the petri dish at the halfway point. Y-axis signifies the percentage of worms that crossed ½ way line. X-axis specifies the temporal progression of the experiment reported in minutes.

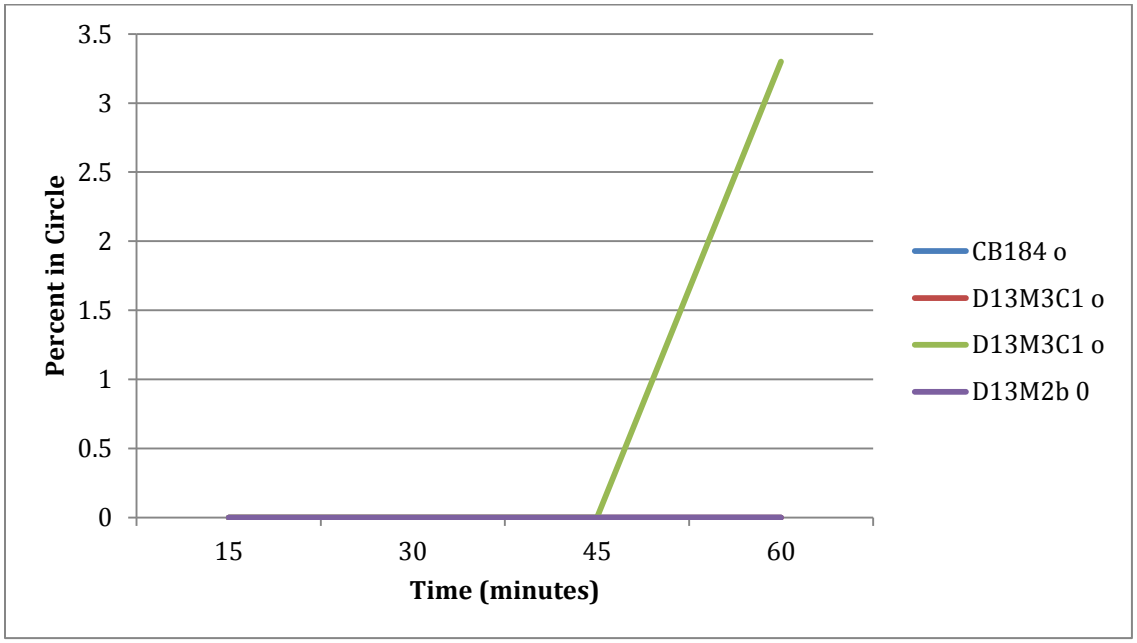

Figure 8. The "o" represents the # of worms that made it all the way to the circle at the apex of the petri dish. Y-axis signifies the percentage of worms that made it into the circle. X-axis specifies the temporal progression of the experiment reported in minutes.

A perfunctory examination of the tables positioned above illustrates the inability of the recombinant mutants to demonstrate any meaningful locomotive separation when contrasted with the displacement percentages of the WT control strain. Thus, when synthesized within the context of the information presented below, it appears that the 3 ml/L concentration was too potent to display accurately the mutant phenotype. The significance of this is enumerated further in the discussion section.

There is a multitude of graphical data synthesized both to establish the upper echelon of the concentration dependency of the recombinant strains and to help pinpoint the most effective concentration of DEET to be used in establishing whether or not the "Kim mutations" are the same as the "Nguyen mutations." The following table summarizes the data involved in making the determination that the 3 ml/L of DEET was too stringent of a repellent pressure:

Table 2. A qualitative summarization of recombinant mutants versus wild type at the highest DEET concentration.

Strain	**Assay #1**	**Assay #2**	**Assay #3**
D13M1a	No Diff	X	X
D13M1b	Clear Diff	X	X
D13M1c	No Diff	Clear Diff	X
D13M1d	No Diff	Clear Diff	X
D13M2a	Marginal	X	X
D13M2b	Marginal	No Diff	X
D13M3a	No diff	Marginal	X
D13M3b	Clear Diff	Clear Diff	X
D13M3c	Clear Diff	No Diff	Marginal

Representative strains of each recombinant groups M1, M2, M3 were chosen and assayed in order to see if there was any level of consistency between assays of different strains as well as between subsequent assays of the same strains. A quick glance at this table makes it clear that 3 ml/L of DEET was not the ideal concentration to be implemented in future assays of these recombinants. A cursory glance at the data presented in subsequent paragraphs is more than enough to establish the truth of the previous statement. There are too many instances where the result of a given recombinant strain yielded a “no difference” verdict when compared to the WT CB184 strain.

Further graphical samples of the results from testing the recombinants at the highest DEET concentration chosen are shown below:

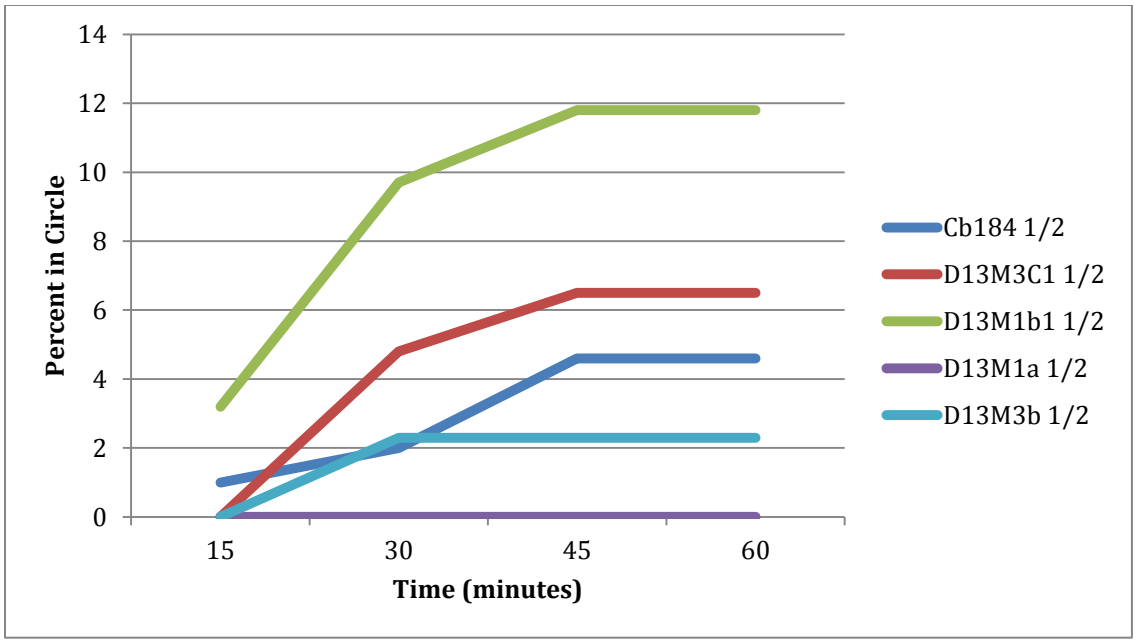

Figure 9. The "1/2" represents the # of worms that crossed the line bisecting the petri dish at the halfway point. Y-axis signifies the percentage of worms that crossed ½ way line. X-axis specifies the temporal progression of the experiment reported in minutes.

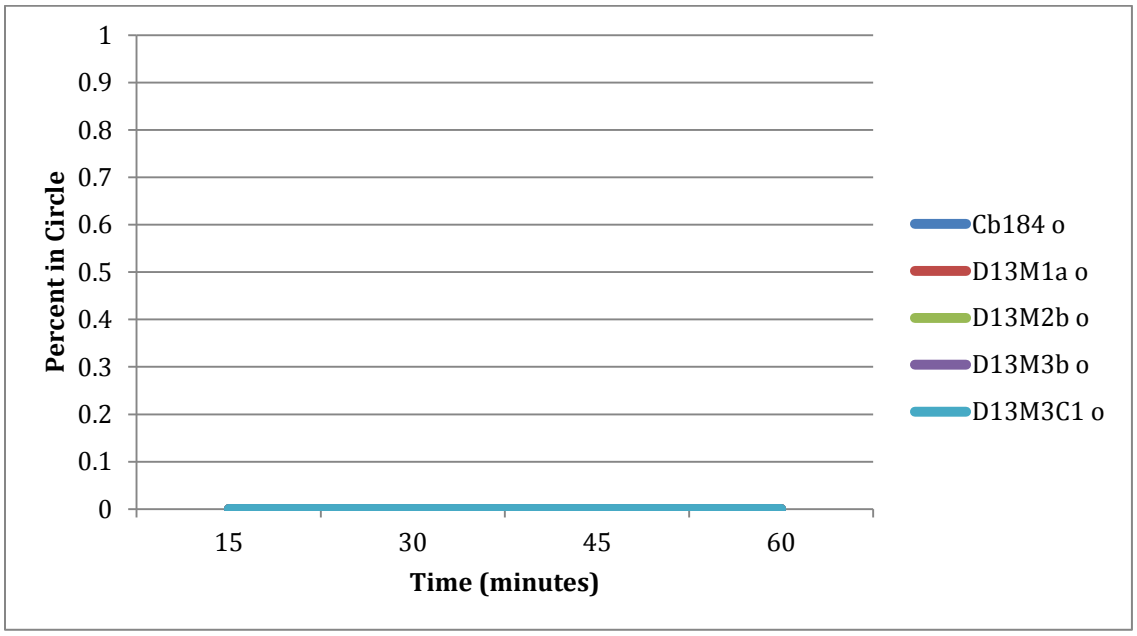

Figure 10. The "o" represents the # of worms that made it all the way to the circle at the apex of the petri dish. Y-axis signifies the percentage of worms that made it into the circle. X-axis specifies the temporal progression of the experiment reported in minutes.

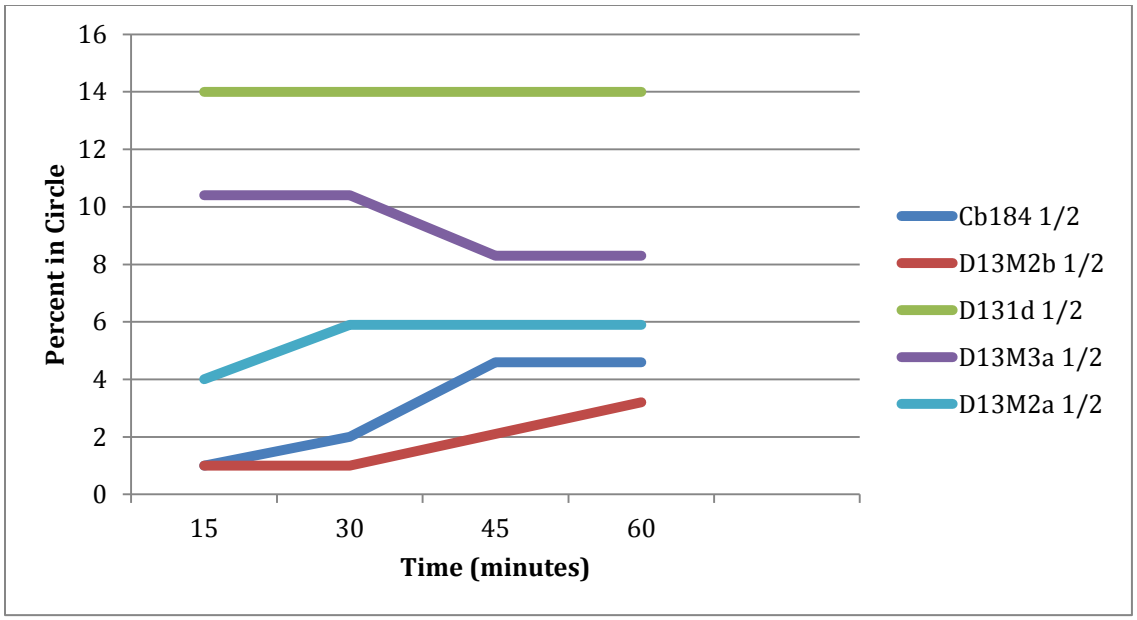

Figure 11. The "1/2" represents the # of worms that crossed the line bisecting the petri dish at the halfway point. Y-axis signifies the percentage of worms that crossed ½ way line. X-axis specifies the temporal progression of the experiment reported in minutes.

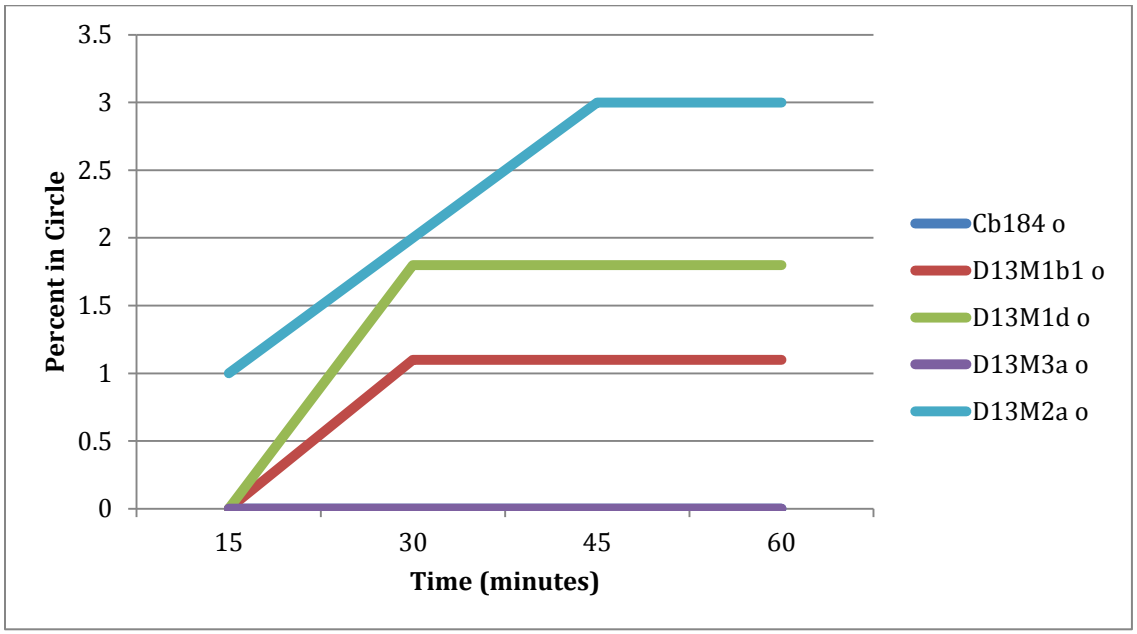

Figure 12. The "o" represents the # of worms that made it all the way to the circle at the apex of the petri dish. Y-axis signifies the percentage of worms that made it into the circle. X-axis specifies the temporal progression of the experiment reported in minutes.

2. DEET-free Assay:

The experiments run with no DEET were performed in order to make sure that the recombinant strains were capable of vigorous locomotion in the absence of DEET. This was an across-the-board negative control that was done to add validity to the findings of the 3 ml/L and 2 ml/L DEET assays. The recombinant strains tested were expected to move in a manner similar, if not identical, to the WT control strain CB184. The results of the DEET assays run in the absence of DEET yielded results that were completely anticipated. There was functionally no difference the WT strains and the recombinant strains, and graphical representation of data sets illustrating this conclusion were selected and presented below:

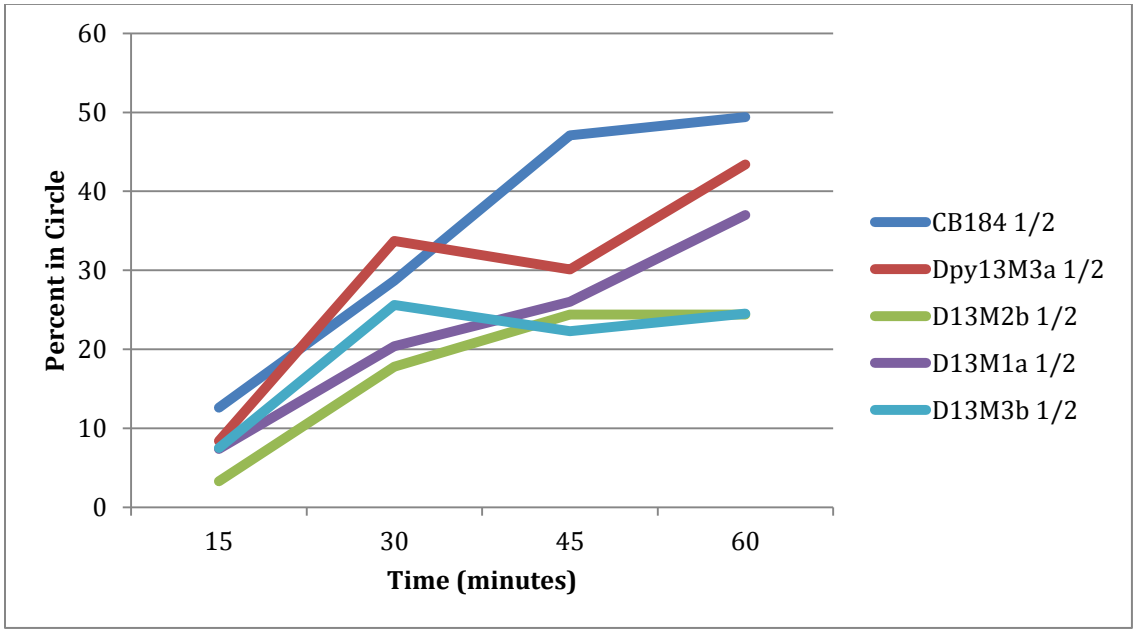

Figure 13. The "1/2" represents the # of worms that crossed the line bisecting the petri dish at the halfway point. Y-axis signifies the percentage of worms that crossed ½ way line. X-axis specifies the temporal progression of the experiment reported in minutes.

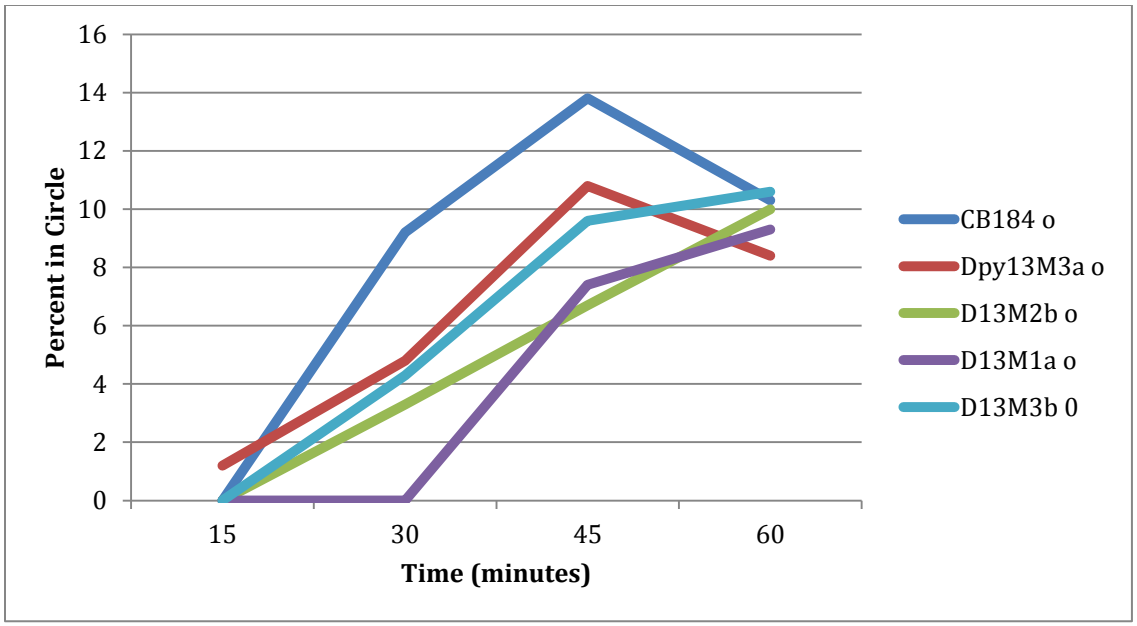

Figure 14. The "o" represents the # of worms that made it all the way to the circle at the apex of the petri dish. Y-axis signifies the percentage of worms that made it into the circle. X-axis specifies the temporal progression of the experiment reported in minutes.

According to Figures 13 and 14, the CB184 WT control is more than capable of exhibiting locomotion similar or surpassing that of the mutant phenotypes. This provides a semi-obvious, but nevertheless important, distinction that the induced mutations do not inherently increase the locomotive ability of the recombinant strains. Thus, any differentials in displacement between the recombinants and the WT control are directly influenced by the presence of DEET in the test medium.

All relevant experimental data gleaned from numerous DEET bioassays in this capacity were synthesized and qualitatively recorded in the table below:

Table 3. A qualitative summarization of recombinant mutants versus wild type at the zero DEET concentration.

Strain	**Assay #1**	**Assay #2**	**Assay #3**
D13M1a	No Diff	No Diff	X
D13M1b	No Diff	X	X
D13M1d	No Diff	Clear Diff	X
D13M2a	No Diff	X	X
D13M2b	No Diff	No Diff	X
D13M3a	No Diff	Clear Diff	No Diff
D13M3b	No Diff	Clear Diff	No Diff
D13M3c	No Diff	No Diff	No Diff

As one can see, the data presented in the preceding table show that there is largely no qualitative difference in the displacement rates of recombinant strains and that of the WT control

3. Intermediate DEET Concentration:

Once the upper and lower ceilings of the DEET concentration gradient were established, it was time to move past the relative extremes and settle on a DEET concentration that would facilitate the desired constraints. It was decided to split the difference and continue testing the recombinant strains at an intermediate concentration. The proverbial “Goldilocks” point utilized was 2 ml/L. As previously enumerated, the process of trial and error suggested that this would be the ideal DEET concentration that would provide a realistic and accurate depiction of the mutant vs. non-mutant dichotomy.

The majority of the data and results using this concentration will be discussed in the subsequent section. However, a representative data sampling is included below to illustrate the ideal nature of using this specific concentration of DEET. Contrast the tabulated qualitative data presented below with Table 2 and Table 3, respectively.

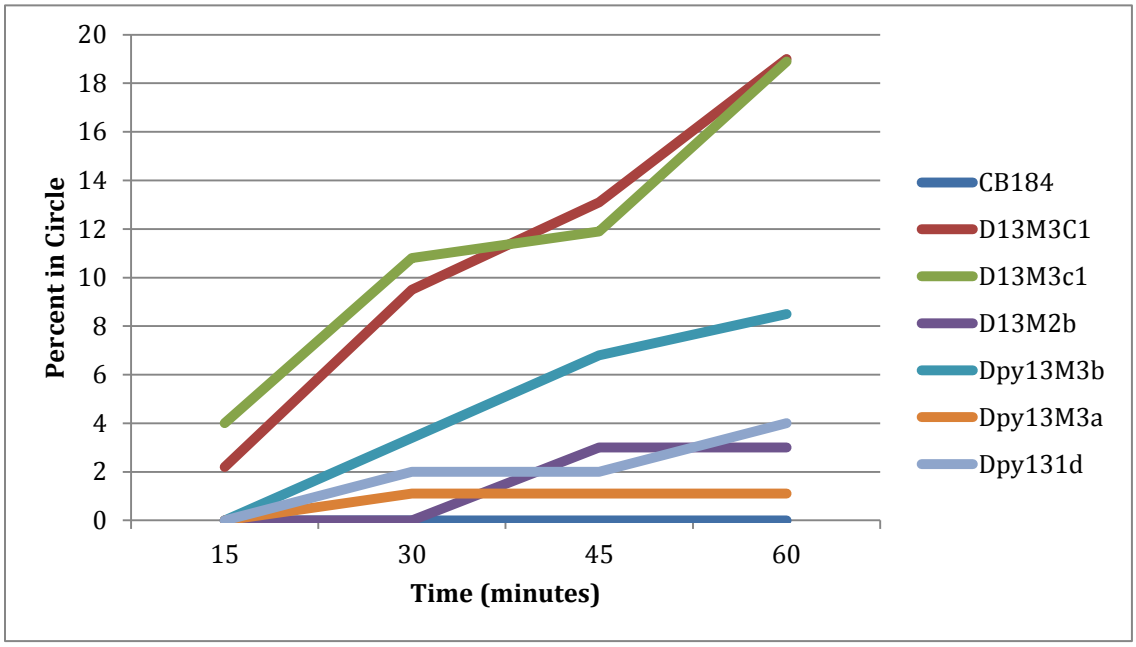

Figure 15. The "1/2" represents the # of worms that crossed the line bisecting the petri dish at the halfway point. Y-axis signifies the percentage of worms that crossed ½ way line. X-axis specifies the temporal progression of the experiment reported in minutes.

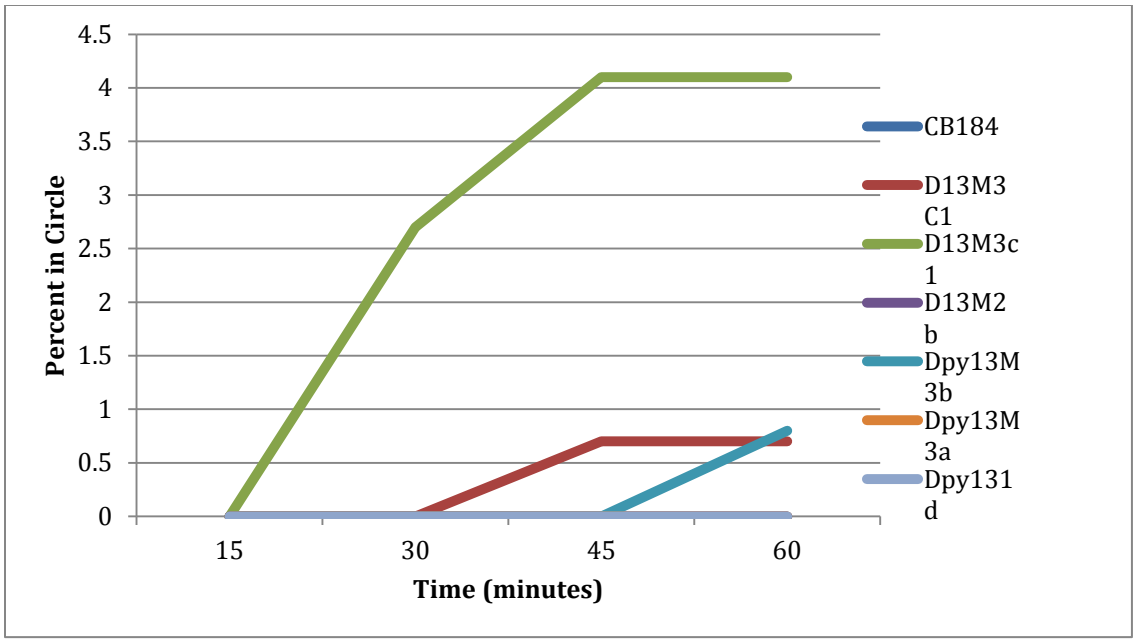

Figure 16. The "o" represents the # of worms that made it all the way to the circle at the apex of the petri dish. Y-axis signifies the percentage of worms that made it into the circle. X-axis specifies the temporal progression of the experiment reported in minutes.

As one can see, the 2 ml/L concentration of DEET successfully suppressed the locomotion of the WT control strain, and simultaneously allowed the mutant phenotype to manifest in displacement despite the presence of the DEET repellant. The myriad of tests run with these mutants under the intermediate DEET concentration is summarized and tabulated below:

Table 4. A qualitative summarization of recombinant mutants versus wild type at intermediate DEET concentration.

Strain	Assay #1	Assay #2	Assay #3	Assay #4	Assay #5	Assay #6
D13M1a	Clear Diff	Clear Diff	X	X	X	X
D13M1b	Clear Diff	X	X	X	X	X
D13M1c	Clear Diff	X	X	X	X	X
D13M1d	Clear Diff	X	X	X	X	X
D13M1e	Clear Diff	X	X	X	X	X
D13M2a	No Diff	X	X	X	X	X
D13M2b	Clear Diff	Clear Diff	Marginal	Clear Diff	Marginal	Clear Diff
D13M2c	Clear Diff	X	X	X	X	X
D13M3a	Clear Diff	Marginal	Marginal	Marginal	Clear Diff	X
D13M3b	Clear Diff	Clear Diff	Clear Diff	Clear Diff	Clear Diff	X
D13M3c	Clear Diff	Clear Diff	Marginal	No Diff	Clear Diff	Clear Diff
D13M3d	Clear Diff	Marginal	X	X	X	X
D13M3e	Clear Diff	X	X	X	X	X

2. Actual Testing Using Established DEET Concentration (1.5 ml/L)

Once the appropriate DEET concentration was established, it was slightly adjusted to 1.5 ml/L and utilized throughout the duration of remaining experimentations. Now that the relative distributions and associated outcomes of the DEET concentration gradient were assayed and reported, it was time to move into the most pertinent portion of this inquiry and look at how each recombinant strain behaved relative to one another in the presence of 2 ml/L of DEET. Most conclusions and salient distinctions presented in the Discussion section deals with analyzing and synthesizing the results from this portion of the process. Once again, if each of the 13 recombinant strains manifested a DEET-resistant phenotype that was markedly different than the WT control under constraints inherent to the chemotaxis assay previously portrayed, then one can infer that the "Kim mutants" are in the same gene as the "Nguyen mutants." However, if even some of the "Kim mutants" are DEET sensitive, then at least these are in a different gene than that defined by the "Nguyen mutations."

A representative tabulated sample, with corresponding graphical presentation, of data purporting the results of experimentation are presented below:

Table 5. Example of reporting methodology and graph used to record the percentage of recombinants that crossed the ½ waypoint during DEET bioassays at 1.5 ml/L.

	CB184 1/2	D13M1a ½	D13M1 b 1/2	D13M1c ½	D13M1 d 1/2	D13M1e ½	D13M2a 1/2
15	0	7.00	3.6	4.6	8.03	8.2	0
30	0	15	21	37.1	33.7	9.8	7.4
45	1	17	25.9	34.4	36.9	11.5	17.9
60	0.00	14	15.1	39.7	39.3	13.1	19.4

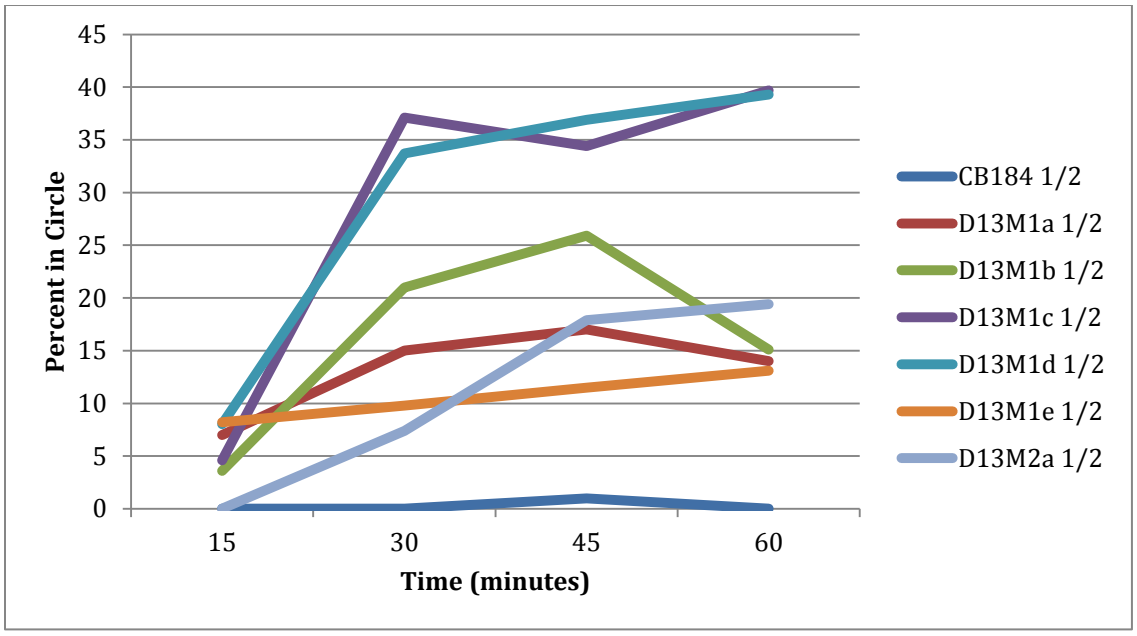

Figure 17. The "1/2" represents the # of worms that crossed the line bisecting the petri dish at the halfway point. Y-axis signifies the percentage of worms that crossed ½ way line. X-axis specifies the temporal progression of the experiment reported in minutes

Table 6. Example of reporting methodology and graph used to record the percentage of recombinants that made it to the circle at the apex during DEET bioassays at 1.5 ml/L.

	CB184 o	D13M1a o	D13M1b o	D13M1c o	D13M1d o	D13M1e o	D13M2a o
15	0	0.00	0	0	0	0	0
30	0	2	4.8	4	10.4	9.8	0
45	0	7	12	13.9	25.7	11.5	1.5
60	0.00	12	23.5	14.6	28.9	14.8	11.9

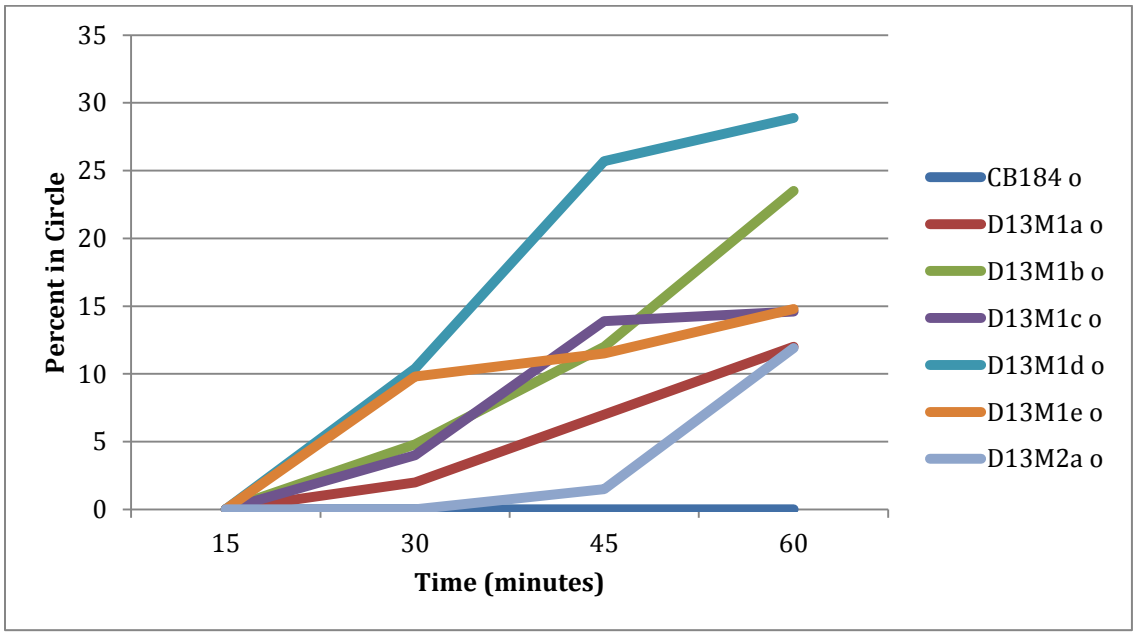

Figure 18. The "o" represents the # of worms that made it all the way to the circle at the apex of the petri dish. Y-axis signifies the percentage of worms that made it into the circle. X-axis specifies the temporal progression of the experiment reported in minutes.

The previously illustrated table and associated graphs were pulled from two bioassays and reported together in one table and graph (one for those worms that crossed the ½ line and one for those worms that made it all the way to the apex circle). As one can see, each of the recombinant strains included in the representations above showed marked difference from their WT counterparts. For all practical purposes, each of the

recombinant strains from the bioassay reported in figures 15 and 16 would be denoted with the qualitative specification of "clear difference."

All DEET bioassays performed with the recombinant strains under the 1.5 ml/L DEET concentration are tagged with a qualitative denotation and reported in the following table:

Table 7. A qualitative summarization of all assays of recombinant mutants versus wild type at 1.5 ml/L DEET concentration.

Strain	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5
D13M1a	clear diff	clear diff	clear diff	X	X
D13M1b	clear diff	clear diff	X	X	X
D13M1c	clear diff	clear diff	X	X	X
D13M1d	clear diff	no diff	clear diff	X	X
D13M1e	clear diff	clear diff	X	X	X
D13M2a	no diff	clear diff	X	X	X
D13M2b	clear diff	clear diff	clear diff	clear diff	X
D13M2c	clear diff	clear diff	clear diff	clear diff	clear diff
D13M3a	marginal	clear diff	clear diff	marginal	
D13M3b	clear diff	no diff	clear diff	clear diff	clear diff
D13M3c	clear diff	clear diff	clear diff	no diff	clear diff
D13M3d	clear diff	clear diff	X	X	X
D13M3e	clear diff	clear diff	clear diff	clear diff	clear diff

The data reported in Table 7 indicates that there was a consistent correlation amongst the recombinant strains as exemplifying "clear diff" phenotype profiles when contrasted with the phenotype of the WT control. The minimum number of assays needed to confidently render the qualitative verdict of "clearly different" was two separate instances of marked separation in the displacement rates of a given recombinant strain versus the WT. Once that threshold was crossed, that strain was considered DEET resistant at 1.5 ml/L concentration of DEET. Obviously, some strains were subjected to additional tests beyond this lower limit, and the determinations as to which strains were partially arbitrary and partially due to marginally conclusive results. However, the data presented in Table 7 is conclusive evidence that all of the recombinant strains are DEET resistant and that all of the mutations are linked to *dpy-13* as were the Nguyen mutations.

C. **Outcrossed Mutants**

Finally, in order to substantiate linkage to *dpy-13* (and therefore provide even stronger circumstantial evidence that the "Kim mutations" are in the same gene as the "Nguyen mutations"), it was necessary to repeat the previous bioassays using outcrossed mutant strains. As was the case with recombinant mutants, if the outcrossed mutant strains tested for in the third and final stage of the experimentation process are proven uniformly resistant, then that would be further affirmation that the mutations are in the same gene as those mapped by Anh Nguyen.

All relevant data for this portion of the experimental proceedings is synthesized and tabulated in the following table and associated graph:

Table 8. Example of reporting methodology and graph used to record the percentage of outcrossed mutants that crossed the ½ waypoint during DEET bioassays at 1.5 ml/L.

	CB184 1/2	D13M1 C01 1/2	D13M1 C02 1/2	D13M3 C03 1/2	D13M1 C03 1/2	D13M2 C01 1/2	D13M2 C02 1/2	D13M2 C03 1/2
15	0	2	0	0	0	2.9	7	0
30	0	3.9	1.7	0	2.2	2.9	10.5	2.5
45	0	7.8	5	0	3.3	4.3	14	2.5
60	0	9.8	8.3	3.2	3.3	5.7	15.8	3.8

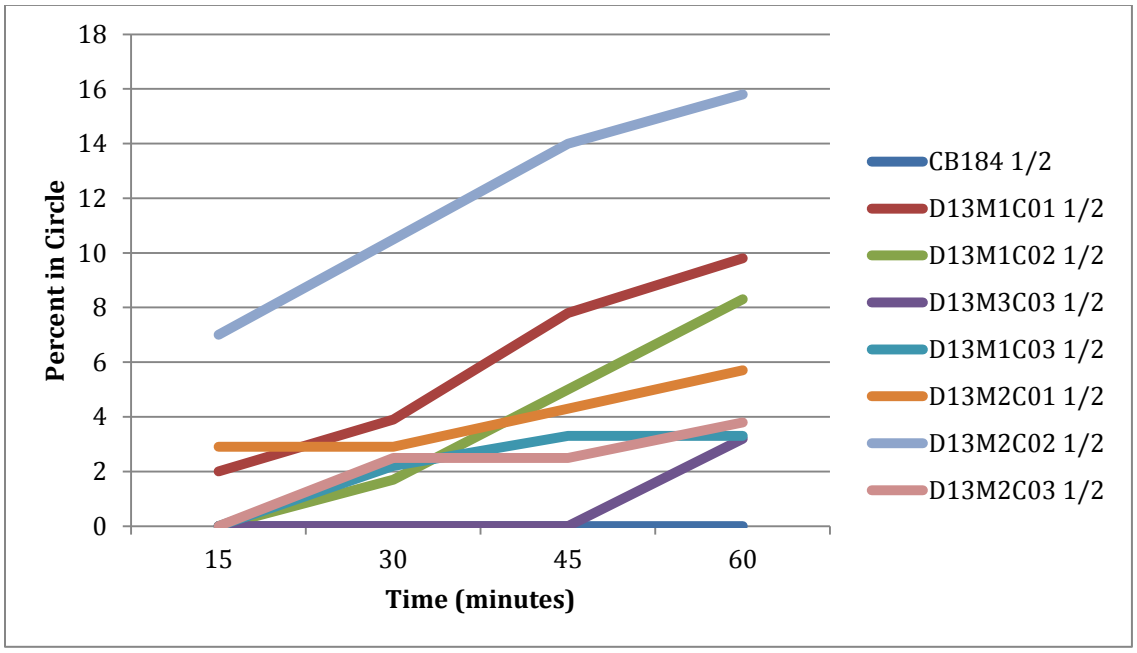

Figure 19. The "1/2" represents the # of worms that crossed the line bisecting the petri dish at the halfway point. Y-axis signifies the percentage of worms that crossed ½ way line. X-axis specifies the temporal progression of the experiment reported in minutes

Table 9. Example of reporting methodology and graph used to record the percentage of outcrossed mutants that made it to the circle at the apex during DEET bioassays at 1.5 ml/L.

	CB184 o	D13M1 C01 o	D13M1 C02 o	D13M3 C03 o	D13M1 C03 o	D13M2 C01 o	D13M2 C02 o	D13M2 C03 o
15	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0

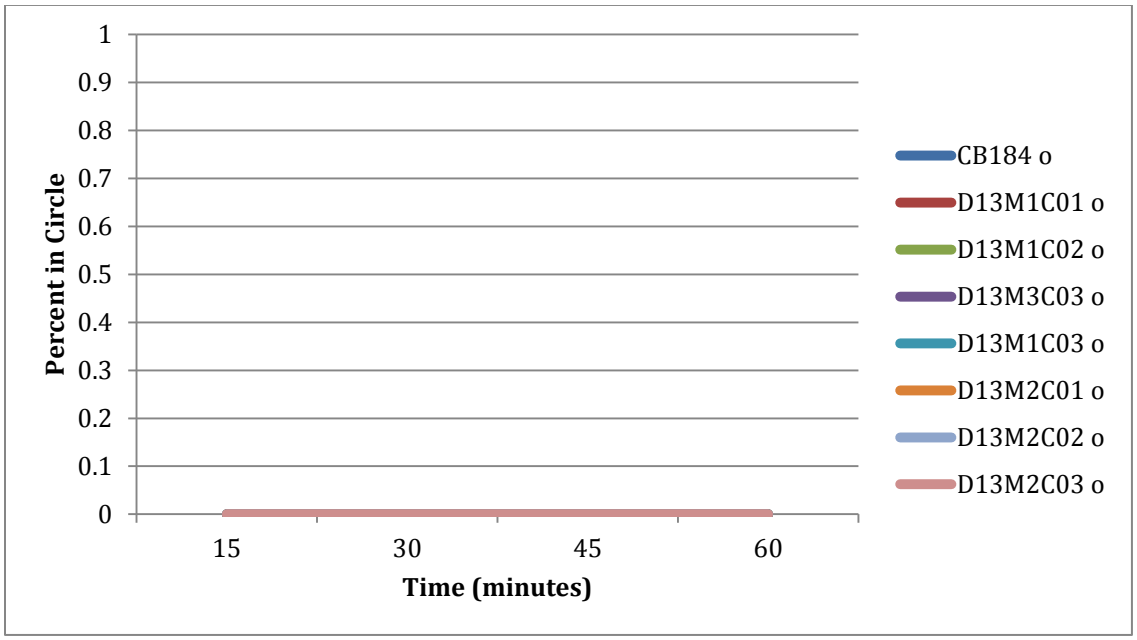

Figure 20. The "o" represents the # of worms that made it all the way to the circle at the apex of the petri dish. Y-axis signifies the percentage of worms that made it into the circle. X-axis specifies the temporal progression of the experiment reported in minutes.

As is made apparent in the previous data excerpts; tables 8, 9 and associated graphs, there seems to be a persistence of the DEET-resistance phenotype in each of the outcrossed mutants. However, the previously alluded to data sets are merely

representative excerpts clearly illustrating this separation between outcrossed strains and the WT. Thus, many bioassays were performed to substantiate the claim that the gene coding for resistance to DEET is indeed linked to *dpy-13*. In the interest of continuing to adhere to the pattern of reporting paradigms utilized in this thesis, the following table effectively summarizes the qualitative results of all DEET bioassays run at 1.5ml/L DEET concentration using the outcrossed mutants:

Table 10. A qualitative summarization of all assays of outcrossed mutant strains versus the wild type strain at 1.5 ml/L DEET concentration.

Strain	**Assay #1**	**Assay #2**	**Assay #3**
D13M1C01	No Diff	Clear Diff	Clear Diff
D13M1C02	Clear Diff	Clear Diff	Clear Diff
D13M1C03	Clear Diff	Clear Diff	Clear Diff
D13M2C01	Clear Diff	Clear Diff	Clear Diff
D13M2C02	Clear Diff	Clear Diff	Clear Diff
D13M2C03	Clear Diff	Clear Diff	Clear Diff
D13M3C01	Clear Diff	Clear Diff	Clear Diff
D13M3C02	Clear Diff	Clear Diff	Clear Diff
D13M3C03	Clear Diff	Clear Diff	Clear Diff

Almost completely homogeneous outcomes across all three assays for each strain itemized in the table above were simultaneously anticipated and desired. Table 10, when combined with previously enumerated lines of logic, makes a strong case that the Kim mutations maps to the same region as the Nguyen mutations.

DISCUSSION

I. General Points of Clarification:

The only inconsistency present in Table 10 is the qualitative label of "No Diff" for the initial assay of the D13M1C01 outcrossed strain. However, there is a myriad of possible explanations for this singular discrepancy. Furthermore, while one divergent result amidst twenty-six consistent ones is highly favorable, there were multiple assays that showed no qualitative difference when compared to the WT control. Potential explanations include human error, too much DI water on the surface of the medium in the assay plate (which would incapacitate the worms and result in inhibited locomotion), and the simple concept that the sampling of worms was statistically unrepresentative of the mutant phenotype in general. Additionally, fluctuations in temperature can negatively impact the effectiveness of DEET (Hongchun *et al.*, 1998). If the integrity of the DEET compound is compromised, then the CB184 control strain will not be effectively mitigated, ultimately rendering an incorrect "No Diff" determination. Regardless of previous speculation, the data presented in Table 10, as well the others posited throughout the duration of this thesis, is highly conclusive.

In the interest of resolving all potential discrepancies, several aspects of the graphical depictions in the results section merit further discussion. There is a logical explanation for those instances where the positively sloped linear graphs, reflecting a greater percentage of worms crossing the ½ waypoint for a given strain, would make a sudden, drastic change and drop into a sharp negative slope. In order to conceptualize properly he information being presented in said instances, one needs to understand that worms that continued moving all the way into the circle at the apex of the petri dish were

no longer represented in the percentage of worms that crossed the halfway marker. This fact explains any potential paradox arising from sudden drastic declines in the graphical representations of worms that made it past the line ascribing the ½ waypoint. This is true for all of the graphs reported in this paper, and illustrates the importance of synthesizing the "halfway" graphs in conjunction with the "circle" graphs. The graphs are presented in pairs, neither of which is meant to be mutually exclusive or analyzed in isolation.

Table 1 contains only one instance of inconsistency regarding the ability of the mutants' displacement to differentiate itself clearly from that of the WT control. While 100% consistency amidst results is ideal, none of the bioassays were performed in a vacuum, and all could have suffered one or more of the potentially detrimental, forces discussed above. Thus the data in Table 1 possess a high degree of consistency, and should be viewed as compelling data. As alluded to previously, the small amount of divergence is a not only a reflection of the accuracy of the experimental methodology utilized here, but to the persistence of the mutagenesis performed by Dr. Hartman and Mr. Kim prior to current experimentation. Such conclusive positive results provide confidence that all background work outlying this thesis was accurate and would provide a reliable basis for any extrapolative determinations made from the work described in this thesis regarding the nature of the *der-1* region of *C. elegans* and specifically its ability to mutate and manifest in the DEET resistant phenotype.

II. Discussion of DEET Concentration Gradient

Turning the focus of this discussion to the crux of the question posed in the "DEET Concentration Gradient" portion, one finds another opportunity to garner meaningful conclusions from the data. The fundamental question underlying the "DEET

Concentration Gradient" portion of the thesis was whether or not the recombinants' resistant phenotype would persist regardless of DEET concentration, or whether that phenotype was concentration dependent. The data support the notion that there exists some spectrum of DEET concentration dependency. As one diminishes the amount of DEET to which a given mutant is exposed, said mutant displays displacement rates that are inversely proportional to changes in DEET concentration. Thus, as DEET concentration goes up, locomotion goes down, and vice-versa.

A. ***Highest DEET Concentration Discussion (3ml/L)***

One element of the "DEET Concentration Gradient" portion was to test and document how recombinant mutants responded to the highest DEET concentration of 3 ml/L. It was decided that this was too repressing of a concentration to use. Careful examination of Figures 7-12 shows both the inconsistency inherent to using such a high concentration of DEET for the bioassays. The data reflected in the previous graphs demonstrates the tendency of the highest DEET concentration to stifle the magnitude of separation between the displacement of recombinant strains and that of the WT control. This illustrates that the recombinant mutant phenotypes are concentration dependent and the effectiveness of their resistance is sensitive to DEET concentration. Additional information can be deduced from the two graphs presented. There seems to exist some threshold at which the high DEET concentration is able to overwhelm the mutated *der-1* gene, ultimately rendering the mutant phenotype either diminished or in some cases superficially incapacitated. Furthermore, not only are the recombinants mutant phenotypes concentration dependent, but also this dependency manifests itself in varying ways from one strain to the other. It seems this threshold for DEET resistance can vary

from one recombinant group to the other, and even within the same strain. Future experimentation may be warranted in order to attempt to uncover the molecular/genetic mechanisms underlying the complex interactions involved in concentration dependency.

Perhaps the best indication of the unreliability of the concentration of DEET used to obtain this data can be found by examining the qualitative results of the strain D13M3C. In three consecutive assays, this strain was labeled as clearly different, no different, and marginally different than the control strain that was known to be DEET sensitive. Contrast this table to the previous one tabulating the resistance of the incipient mutants relative to their WT strain of N2. This table is considerably more irregular. Thus, it was determined that 3 ml/L of DEET in the bioassay medium was too great a concentration to determine the nature of the linkage group of the genetic region coding for DEET sensitivity in un-mutated *C. elegans.*

B. Lowest DEET Concentration Discussion (0 ml/L)

Again, the purpose of running bioassays with no DEET present in the test medium was to establish that the recombinants would behave similarly to the WT control in the absence of experimental manipulations via DEET repellency. As one can see, both Figures 13 and 14 effectively illustrate the phenotypic similarities, with respect to locomotion, of the recombinant and the WT strains. These data sets portray the CB184 WT control strain to be particularly active regarding their gravitation toward the attractant. This acts as definitive proof that the WT negative control is an accurate control and that in the absence of DEET, the CB184 strain is more than capable of pursuing the attractant. Not all of the data sets showed CB184 locomotion as leading all other

recombinant strains, but they did unequivocally portray that all strains involved, mutant and WT alike, are predisposed to move toward the attractant IAA.

Another useful fallout of this semi-tangential aspect of the experimental process was the empirical proof showing IAA to be an effective attractant to all strains involved. This was useful information to gather and understand because it removed the possibility of any faulty results predicated upon an insufficient or ineffectual attractant. Thus, the concentration and amount of IAA was maintained throughout the duration of experimentation.

C. Intermediate DEET Concentration Discussion (2ml/L)

If one examines the data excerpts presented in Tables 15 and 16, it becomes apparent that the 2 ml/L concentration was the most appropriate application of DEET, at least compared to the other concentrations that were tested. The data sets incorporating these results show a clear separation in net movement between the WT strain and the mutant strains. Thus, this concentration was ultimately tweaked to 1.5 ml/L in order to facilitate the most realistic precipitations of the mutant phenotype.

Perhaps the most dynamic manifestation regarding the accuracy of the bioassay methodology, at this concentration and in in general comes from a careful examination of the results in Table 15. Specifically examine the red and green lines relating the displacement rates of the D13M3C1 recombinant strain. This identical, yet duplicitous, strain experienced simultaneous lifecycles, were tested according the constraints of an identical experimental methodology, were exposed to identical stressors and, were taken from different dishes, yet showed almost mirrored displacement rates at 2 ml/L

concentration. This is perhaps the best indication for why this DEET concentration facilitates the most realistic portrayal of the mutant phenotype.

These two samples of recombinant strain D13M3C1 were intentionally tested simultaneously in order to illustrate the reliable nature of the results obtained using this concentration of DEET in the medium. Furthermore, the D13M3C1 strains used in this bioassay were taken from the same petri dish to make sure they were truly similar and were exposed to identical stimuli and stressors throughout their respective lifecycles. Ultimately, this is a positive reflection of the reliability of the conclusions predicated on the data gleaned from this type of bioassay.

III. Discussion of "Phase One" of Experimentation

The incipient portion of experimentation was again to re-assay the phenotypic character of the M1, M2, and M3 mutants as they relate to the N2 wild type control strain. The reason for this endeavor was to ensure that the mutants utilized for the remainder of experimentation were DEET resistant, and that the Wild Type control strain was DEET sensitive. While one may believe that this step could have been avoided using the mutants isolated by Nguyen, these mutants from previous experiments were fundamentally flawed due to contamination from incorporation of genetic elements from varying strains. Thus, they were judged to be unsuitable for whole-genome sequencing, which will hopefully be conducted by Dr. Cori Bargman at the Rockfeller Institute. It was necessary to start fresh and establish a new lineage from a fresh stock. Thus, a portion of this project was to establish a novel line of mutants and essentially start from scratch. In accord with Mr. Kim's research, we were able to cultivate, interrelate, and isolate a pristine N2 background that could be utilized during subsequent experimentation. I

continued his experimental legacy by showing the incipient mutants to be DEET resistant, and this provided a cornerstone upon which we could base the entire project.

At this point in the project, we utilized the previously described assay methodology and concluded that the mutants picked were in fact DEET resistant. Furthermore, based on previous experimentation performed by Nguyen and Kim, we believed that the mutation resulting in DEET resistance manifests either in the same gene or in an alternate gene of close proximity. All of Nguyen's mutants mapped to the same relative location. This experimental process was an attempt to posit a positive indication that the DEET specific mutation of these mutants also mapped to the same location as that of Nguyen's. The primary goal of this thesis was to verify that the location of Kim's mutants would still correspond to the location as ascertained by Nguyen. The proverbial litmus test confirming or denying our hypothesis would be whether or not the Dpy recombinants were ultimately all DEET resistant.

IV. Discussion of "Phase Two" of Experimentation

In order to continue the inquiry into the nature of *der-1* region and how it relates to the DEET resistant phenotype, mutant C. elegans were subjected to genetic recombination. Double mutants possessing the *dpy-13* and *unc-5* gene were crossed with organisms possessing the *der-1* mutation in order to allow for a crossing over phenomenon and produce recombinant mutant strains. Subsequently, these mutant progeny were screened and Dpy non-Unc phenotypes were selected by visual inspection. As illustrated in the Results section of this thesis, these recombinants were reported to be DEET resistant relative to their WT counterparts. The underlying genetics behind this phenotype suggests that a crossing over event took place and produced genetically variant

offspring. The chiasmas formed during this natural process functionally bisected the DNA sequence effectively separating the *dpy-13* from the *unc-5* gene.

The progeny produced from the previously described occurrence had allelic profiles of either “Unc Non-Dpy”or “Dpy Non-Unc.” Again, these determinations were a result of the crossing over phenomenon inherent to recombinant strain production. At this point in the experimental progression it was germane to determine if the F2 generation was homozygous or heterozygous. Non-recombinants would be three quarters either Dpy or Unc and one quarter Dpy Unc phenotype, while recombinants would be of the genetic profile alluded to at the beginning of this paragraph. The fact that DEET bioassays of these test subjects produced results illustrating that a majority of the mutant progeny were DEET resistant was taken as an indication that the offspring were homozygous and that a crossing over event occurred that captured the *der-1*, as predicted by Nguyen’s mapping data. This recombinants were labeled (*e.g.,* D13M1R) and were propagated and stored for the duration of experimentation.

In order to understand the significance of this information it is necessary to synthesize the information alluded to previously with the data in the Results section. If each of the 13 recombinant strains manifested a DEET-resistant phenotype that was markedly different than the WT control under constraints inherent to the chemotaxis assay, then one can infer that the “Kim mutants” are in the same gene as the “Nguyen mutants.” However, if even some of the “Kim mutants” are DEET sensitive, then at least these are in a different gene than that defined by the “Nguyen mutations.” Associating this biological statute with the data gathered and presented in the results section, one can see that the recombinant mutant strains unanimously manifested a DEET resistant

phenotype; thus, the "Kim mutants" are decidedly in the same gene as those of the "Nguyen mutants."

V. Discussion of "Phase Three" of Experimentation

In the wake of the seminal advancement that the mutation we are tracking was mapped to the same gene as both the "Kim mutants" and "Nguyen mutants," it was necessary to elucidate the nature of this genetic interaction. Essentially, the terminal portion of this thesis was to confirm that *der-1* region was linked to *dpy-13*. This consideration was the final piece of the genetic puzzle and would constitute further proof that the "Kim mutations" were in the same gene as the "Nguyen mutations."

In order ascertain this, we took the recombinants discussed in phase two and crossed them with a pure WT N2 strain, thus producing two generations of outcrossed mutants. As alluded to previously in this paper, these outcrossed mutants were subjected to chemotaxis assays in order to determine the kind of interaction with a DEET medium. According to the figures reported in the Results section, the outcrossed mutants depicted a strong propensity to displacement in the presence of DEET. Because the outcrossed mutants were quantitatively all DEET resistant, this is a strong indication that the *der-1* region is linked.

Consequently, in the event that the results would have come back differently, the extrapolated interpretation would have also differed. This means that if the outcrossed mutants would not have been totally DEET resistant then we would not be able to infer that the *der-1* region is linked to *dpy-13*. If the ratio of DEET resistant outcrossed mutants to DEET sensitive outcrossed mutants would have been reported as 1:4, then we would have confidently classified the der-1 region as unlinked.

REFERENCES

Bargmann, C.I. Chemosensation in *C. elegans* (October 25, 2006), *WormBook*, ed. The *C.elegans* Research Community, WormBook, doi/10.1895/wormbook.1.123.1, http://www.wormbook.org.

Bargmann, C., Hartwieg, E., Horvitz, R. H. (1993). Odorant-selective genes and neurons mediate olfaction in C. elegans. *Cell*, Vol. 74: 515-527.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77: 71-94.

Ditzen, M., Pellegrino, M., Vosshall, L.B. (2008). Insect odorant receptors are molecular targets of the insect repellent DEET. *Sciencexpress*. <http://www.sciencexpress.org>.

Gupta, R.K. and L.C. Rutledge. 1994. Role of repellents in vector control and disease prevention. Am. J. Trop. Med. Hyg. (Suppl.). 50: 82-86.

Hongchun Q., H.W. Jun, and J.W. McCall. 1998. Pharmacokinetics, formulation, and safety of insect repellent N,N-diethyl-3-methylbenzamide (deet): a review. J. Amer. Mosq. Cont. Assoc. 14**:** 12-27.

Jahn, A., Kim, S.Y., Choi, J.H., Kim, D.D., Ahn, Y.J., Yong, C.S., Kim, J.S. (2010). A bioassay for mosquito repellency against Aedes aegypti: method validation and bioactivities of DEET analogues. *Journal of Pharmacy and Pharmacology*. 62: 91-97.

Koren, G., D. Matsui, and B. Bailey. 2003. DEET-based insect repellents: safety implications for children and pregnant and lactating women. Canadian Med. Assoc. J. 169**:** 209-212.

Krajick, K. (2006). Medical Entomology: Keeping the Bugs at Bay. *Science*. 313: 36-38.

Mafong, E.A., and L.A. Kaplan. 1997. Insect repellents: what really works? Postgrad. Med. **102:**63-69.

Moore, S.J., Davies, C.R., Hill, N., Cameron, M.M. (2007). Are mosquitos diverted from repellent-using individuals to non-users? Results of a field study in Bolivia. *Tropical Medicine and International Health*. 12 (4): 532-539.

Osmitz, T.G., and R.H. Grothaus. 1995. The present safety assessment of deet. J. Am. Mosq. Control Assoc. 11**:** 274-278

Syed, Z., Leal, W. (2008). Mosquitoes smell and avoid the insect repellent DEET. *PNAS Early Edition*: 1-6.

ABSTRACT

This project was designed to continue and verify the seminal work done by Mr. Christopher Kim. It attempts to prove the existence, and ultimately identify the relative location, of a particular gene in *C. elegans* that when mutated codes for protein products resistant to the DEET repellant compound. In the event that a mutant strain of *C. elegans* is successfully generated and isolated, these worms will positively respond to an attractant (IAA), despite special proximity active DEET compound within the medium. This is the criterion implemented in order to correctly determine if a potential mutagenesis transformation was successful. The goal of this transformation is to essentially knockout (or functionally incapacitate) the region of the genome coding for the phenotypic trait of DEET sensitivity. In order to formulate said mutagenesis, the *C. elegans* of interest were exposed to ethyl methanesulfonate (EMS). Subsequently, the resulting progeny of the organisms exposed to EMS genetic alterations will be tested for the associated phenotypic manifestations of the mutation (specifically DEET resistance) via the previously expounded upon chemo-taxis bioassay. Each mutant strain was re-assayed multiple times, always in comparison to a control wild-type strain, in order rule out inconclusive negatives and false positives. The results of all the subsequent assays were synthesized in order to accurately determine the effectiveness of the mutagenesis by EMS upon a given potentially mutant strain. Following re-affirmation of the mutants isolated by Mr. Kim, 3-factor cross with two phenotypically distinguishable markers were used to map out the specific location of the gene *der-1*. The *der-1* gene is the genetic region hypothesized that when mutated, confers resistance to the repellant powers of DEET. The recombinants prepared were subject to multiple levels of DEET testing for

each of the 13 strains in order to determine if DEET resistance in Der-1 is inked or unlinked. If all of the recombinant strains come back unanimously DEET resistant then one could infer that the Der-1 region is indeed unlinked. Furthermore, if the mutant strains tested for in the third and final stage of the experimentation process ends up being uniformly resistant, then that is further affirmation that the Der-1 region is linked. Once several 3-factor crosses have been completed with multiple mutant candidates and DEET resistance is still accounted for in the progeny from the crosses, the candidates will hopefully be sent to the Rockefeller Institute in New York to have their DNA sequenced. Further analysis of the mutants could potentially elucidate the specific mechanism of DEET upon the phenotype of *C. elegans* that has so eluded researchers. The ultimate goal underlying this research is to accumulate enough data to establish a cornerstone for constructing more efficient commercial insect repellents.