THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

GENETIC EFFECTS OF PINENE IN *DROSOPHILA PSEUDOOBSCURA* LARVAE

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SPRING 2018

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree in Biology
with honors in Biology

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ABSTRACT

Chromosomal inversion polymorphisms are common on the third chromosome in *Drosophila pseudoobscura* and are thought to be acted on by natural selection. Data from genome and transcriptome sequencing studies have suggested that genes involved in certain degradation pathways have been targeted by selection on some of the six most common third chromosome inversions. These inversions come from six southwest regions of the United States. It was found that differential expression and amino acid variation in genes linked to the degradation of α-pinene, a toxic chemical found in ponderosa pine trees, may provide an advantage in flies that carry one of the six inversion mutations. Pinene is a monoterpene that acts to repel insects and is found in coniferous trees in the southwest. We wanted to determine if the different inversion arrangements responded to the presence of pinene in similar or different ways. In this study we examined whether larvae that carried different chromosomal inversion types differed in their ability to compete with mutant larvae in the presence of pinene. The results of these experiments suggest that there is no difference in the ability to compete in the presence of pinene within the different inversion types.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Schaeffer for giving me the opportunity to work in his lab and providing me with the guidance and support to write my thesis. He was like a mentor to me because he not only advised me in my honors academics but taught me how to excel in my future endeavors. His advice and support are greatly appreciated. I would also like to thank Dr. Axtell for reviewing my thesis and my family for their unconditional support. Lastly, I would like to acknowledge data support from the NIH grant NIH_ROI_GM098478.

Chapter 1

Introduction

Polymorphisms

Genetic variation within a population is the raw material by which evolutionary change occurs over time. It is mainly brought about by mutations in the DNA that are either beneficial or harmful to a species. Types of genetic variations include small-scale mutations such as single nucleotide changes (SNP), insertions, and deletions, as well as large-scale structural variants such as inversions, duplications, deletions, insertions, and translocations (Korbel et al., 2007). Most of the time, rearrangement mutations are harmful because they cause disease in natural populations of humans (Pettenati et al. 1995; Sankaranarayanan, 1979). Alternatively, mutations can be beneficial in certain cases. Polymorphisms are variations in the DNA sequence of a species that are common in a population. Chromosomal inversions are a type of polymorphism commonly found in *D. pseudoobscura* populations and have suggested to be beneficial to the species. Chromosomal inversions modify the orders of genes by rearranging a segment of the chromosome, which preserves the genetic information in the DNA, unlike other rearrangement mutations such as duplications and deficiencies (Griffiths et al. 2005). An inversion occurs when a segment of a chromosome is broken into two pieces and then the central region is joined in reverse order. Some of the genes within the inverted regions of inversions of *D. pseudoobscura* has been suggested to be beneficial, but it is still unclear how the mechanism works (Fuller et al. 2016, 2017). In this study, we examined six different inversion types from six southwest regions inhabited by *D. pseudoobscura* to determine if there are differential benefits to the genes carried by each inversion type.

Pinene detoxification

Analysis of amino acid polymorphism and gene expression data from the inversions of *D. pseudoobscura* found that a number of inversions carry genes targeted by selection that are involved in the degradation of pinene, a chemical found in ponderosa pine trees (Fuller et al. 2016, 2017). Its toxicity is known to be a repellant to insects that inhabit or breed in the coniferous trees (Smith, 1977). Genes that encode proteins involved in the detoxification of pinene were found to be differentially expressed or had amino acid differences among inversions and had signatures of adaptive evolution (Fuller et al, 2016, 2017). *D. pseudoobscura* from the western region of the United States are exposed to α-pinene in their natural habitat. It was found that α-pinene levels in ponderosa pine vary among geographic regions with lower levels in trees from California and higher levels in trees from Arizona. This could explain why the frequencies of different inversions vary across the southwestern regions of the United States due to their differential abilities to detoxify pinene. To test if differential expression of genes within inversions influenced degradation of pinene, our study focused on the ability of larvae from different inversion strains to detoxify various levels of pinene in their food source. To do this, we set up larval competition experiments between wildtype larvae that carried the various chromosomal inversions and mutant strains that did not.

Larvae Competition

According to a migration selection balance model evaluated in Schaeffer (2008) model, *D. pseudoobscura* larvae are thought to experience the environment in a coarse-grained as opposed to fine-grain manner. Experiencing a coarse-grained environment means that an organism will spend their life in only one of several niches or habitats as opposed to moving throughout different niches throughout its life time. It was concluded that polymorphisms are more likely to be stable throughout a population that experiences coarse-grained environments (Levins and MacArthur, 1966). Flies experiencing coarse-grained environments are less likely to breed with flies from other niches, reducing the mixing of genes. This results in the fixation of one allele and stable polymorphisms. Because the egg, larval, or pupal stages of *D. pseudoobscura* are more sedentary within their habitat compared to adult flies, we assumed that egg, larval and pupal stages experience coarse-grained environments while adults experience fine-grained environments. We chose to conduct competitions between wildtype and mutant larvae because they experience a coarse-grained environment.

Because little is known about the biotic factors that are involved in the differentiation of the six niches, we focused on abiotic factors that could contribute to the variation in gene arrangements. Abiotic factors such as climate, altitude, and food resources could potentially affect gene expression. *D. pseudoobscura* are known to be exposed to pinene but little is known how the chemical is involved in the selection of certain gene arrangements. Pinene could be an abiotic factor that alters gene expression in *D. pseudoobscura*. To test this, we incorporated different levels of α-pinene in the medium to determine if strains carrying different inversions differ in their ability to compete with common mutant type larvae. We found that there was no significant difference in larval competition between five of the inversion types. Larvae from one inversion type, Cuernavaca, was able to compete significantly better against mutant type larvae in the presence of pinene.

Chapter 2

Materials and Methods

Hypothesis

This study examined whether *D. pseudoobscura* larvae that carry different chromosomal inversion arrangements differ in their ability to compete with mutant type larvae in the presence of pinene. Through competition between wildtype inversion types and mutants, we can determine how well each strain was able to deal with pinene in their environment. It was hypothesized that the six third chromosome inversion types will compete similarly in the presence of different levels of pinene.

Experimental Design

The goal of this research was to study the effects of pinene, known to be toxic to insects, on the competitiveness of fly larvae that carry different chromosomal inversions. The independent variables in this study were the various inversion types (Arrowhead, AR; Standard, ST; Pikes Peak, PP; Chiricahua, CH; Cuernavaca, CU; and Tree Line, TL, where the names are derived from the locality where the inversion type was first discovered (Dobzhansky and Sturtevant, 1938)) and the amount of pinene in the fly food (0.0, 0.1% and 0.2%). The control groups lacked pinene and the experimental groups each had either 0.1% or 0.2% of α-pinene added to the fly media. We used Acros Organics alpha pinene (98%) and observed that when pinene was present, the larvae tended to move away from the media compared to the control. This suggests that the pinene was active in the fly medium. Previous experiments found that exposing flies to higher doses (>0.4%) of pinene was lethal. Exposing flies to less than 0.1% pinene had no effect on fly survival. Therefore, we put less pinene than the lethal amount but enough into the fly media to induce competition. The variable of interest was the survival rate of the wild-type flies relative to the white-eyed flies. This variable shows us the result of competition between flies of the wild-type allele and the mutant allele. The wildtype flies carried the six inversion types while the mutant flies did not carry any third chromosome inversion. Three biological replicates were used for ST, PP, CH, and TL strains and two biological replicates were used for the AR strain. The biological replicates used for each strain are shown in Table 1. Each biological replicate had three technical replicates unless noted otherwise.

Table 1. ***D. pseudoobscura*** **strains and their locations**

Working Name	***Arrangement***	***Population***
AR_DM1015	Arrowhead	Davis Mountains, TX
AR_DM1050	Arrowhead	Davis Mountains, TX
PP_DM1020_B	Pikes Peak	Davis Mountains, TX
PP_DM1038	Pikes Peak	Davis Mountains, TX
PP_DM1049	Pikes Peak	Davis Mountains, TX
ST_JR72	Standard	James Reserve, CA
ST_MSH177	Standard	Mount St. Helena, CA
ST_MSH217	Standard	Mount St. Helena, CA
CH_JR4_L	Chiricahua	James Reserve, CA
CH_JR20	Chiricahua	James Reserve, CA
CH_KB888	Chiricahua	Kaibab National Forest, AZ
TL_MSH76_B	Tree Line	Mount St. Helena, CA
TL_MSH130	Tree Line	Mount St. Helena, CA
TL_SCI12-2	Tree Line	Santa Cruz Island, CA
CU_SPE123_1-2	Cuernavaca	San Pablo Etla, Mexico
CU_SPE123_4-1	Cuernavaca	San Pablo Etla, Mexico
CU_SPE123_5-2_B	Cuernavaca	San Pablo Etla, Mexico

Fly Strains

Wild *D. pseudoobscura* were collected from different southwest localities of southwestern United States. These locations include Davis Mountains, TX, Mount St. Helena, CA, James Reserve, CA, Kaibab Natl. Forest, AZ, San Pablo Etla, Mexico, and Santa Cruz Island, CA (Table 1). Iso-female lines were prepared and the six most common third chromosome inversion arrangements used from the southwest niches were: Arrowhead (AR), Chiricahua (CH), Pikes Peak (PP), Cuernavaca (CU), Standard (ST) and Tree Line (TL) (Schaeffer et al., 2003).

Egg-laying Chambers

Male and female flies from each strain were placed in different egg-laying chambers. Chambers were kept in room temperature and in the dark. As seen in Figure 1, the egg-laying chambers were composed of three pieces. The agar plate, the plastic chamber with wire netting, and a plastic piece to secure the chamber to the agar plate. The chambers were stored with the agar plate laying down. These chambers allowed males and females of the same strain to mate. Females then laid their eggs onto an agar plate containing wet yeast as a food source for the adults and larvae. The agar plates were replaced after 24 hours and larvae collected after an additional 24 hours. A microscope was used to collect larvae that were at the first instar stage. Larvae were picked off the agar plates with a small paint brush in groups of five until 15 larvae of the same stage were collected and placed into competition chambers. This process was done for each biological and technical replicate.

Figure 1. Assembly of egg-laying chamber. The chambers were made from three pieces: an agar plate, a plastic chamber, and a plastic cap to secure the two together.

Competition Chambers

Competition chambers were made from plastic vials and cotton stoppers (Figure 2). Each competition chamber (1.5 ml microfuge tube) was filled with 15 wild-type and 15 white-eyed larvae from each fly strain. Three biological replicates were made for CH, PP, CU, ST, and TL and two replicates were made for AR. Each biological replicate was then replicated three times. Three sets of chambers were created. For each chamber, the food source contained either no pinene, 0.1% pinene, or 0.2% pinene, where the no pinene condition served as the control. For each chamber, 0.4g of fly food was placed in a 1.5 ml microcentrifuge tube. This was found to be the ideal amount of food and set up in order to reduce surface area and drying out of the food. The chambers were kept at 24°C until flies emerged.

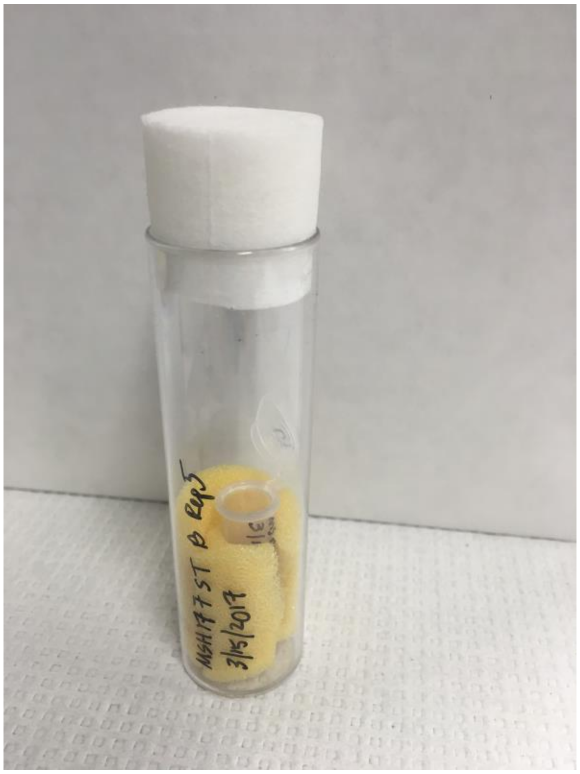

Figure 2. Competition chambers. A plastic vial with cotton stopper and a small plastic vial secured in place with cotton.

Counting Flies

Newly emerged flies were anaesthetized using diethyl ether, sorted as either wild type or white-eyed, and counted. Flies were then discarded. Flies were collected from 5-10-2017 to 5-26-2017 for the control group and from 6-8-2017 to 6-28-2017 for the 0.1% and 0.2% pinene groups.

Statistical Analysis

A two-level nested ANOVA test was done where inversion type and biological replicates effects were the two treatments (Sokal and Rohf, 1981). A nested analysis of variance was used because there was a hierarchy of variables in this study. In nested ANOVA, each level in the hierarchy has its own error term and each of the lower variables occurs in only one level of the higher variables. In this study, technical replicates are nested within biological replicates while biological replicates are nested within each inversion type. This test calculates the variance between those levels. Two technical replicate vials from the control group and one from the 0.1% pinene group had more than 15 wildtype flies emerge and counted. These replicates all came from the CU inversion type. These counts were excluded from the data and statistical analyses.

Chapter 3

Results

The results suggest that there is no significant difference between inversion types on competition in the presence of pinene. The two-way nested ANOVA test revealed a significant effect of the biological replicates, but no significant inversion effect in the control, 0.1% pinene, and 0.2% pinene groups. Tables 2-4 show the ANOVAs. An additional ANOVA test was done for the Cuernavaca inversion type because there seemed to be a difference in competition among pinene levels. The test revealed that there was no significant difference among pinene levels in the CU inversion type. There were three outliers where more than 15 flies were counted. These outliers were excluded from the data and statistical analyses.

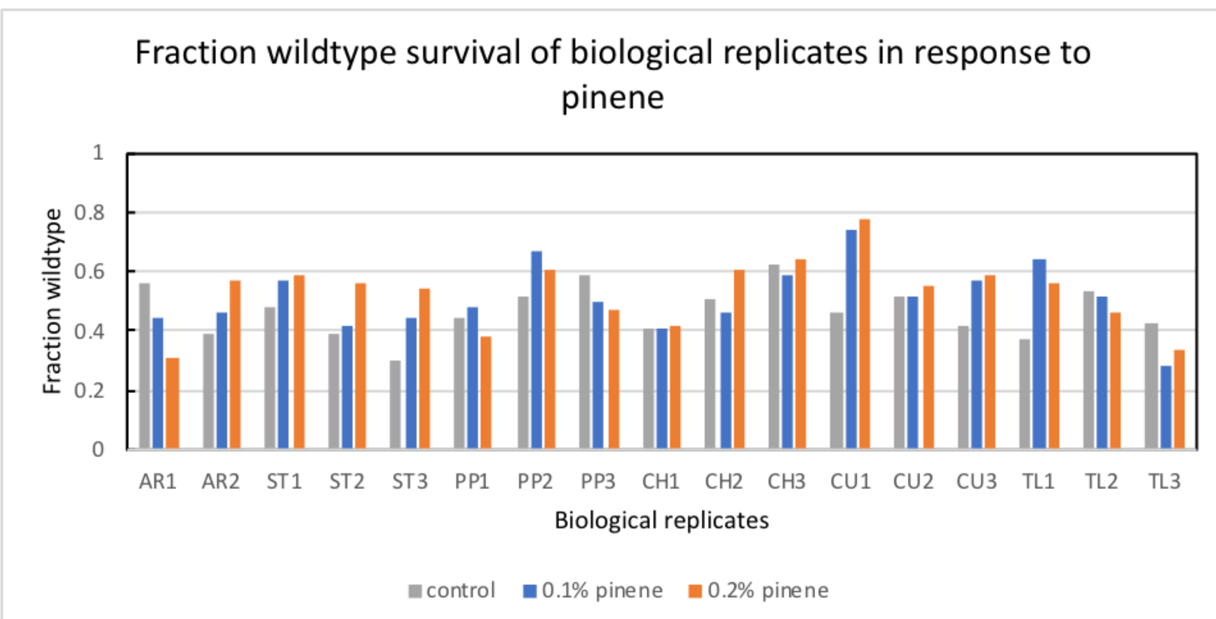

Figure 3. Fraction wildtype survival of biological replicates in response to pinene. This graph shows the fraction of wildtype flies of each biological replicate from the control, 0.1% pinene, and 0.2% pinene group.

The results show that the CU strain wildtype flies out-competed the mutant flies more than any other inversion type, however, not by a significant amount. The CU inversion had the highest survival rate for both levels of pinene. Figure 4 shows that CU wildtype flies in response to 0.1% pinene levels survived at a fraction of 0.64 compared to mutant flies and survived at a fraction of 0.61 in response to 0.2% pinene. The first biological replicate of CU (SPE123_1.2) in the 0.2% pinene group had the highest survival rate of 0.78 (Figure 3). When looking at the trend of responses to increasing pinene levels, results varied within the two levels. For ST, CH, and CU, the survival rates dropped as the pinene level increased. For AR, PP, and TL, the survival rates increased as pinene levels decreased. Wildtype flies of inversion type AR and TL were out-competed by mutant flies in both levels of pinene. In the absence of pinene, the survival rate was similar for all inversion types, with ST having the lowest survival rate. PP and CH survival rate was the about the same for the control group. None of the inversion types wild type survival rate exceeded 0.5 in the control group. The results were quite varied between inversions and none amounted to a significant difference.

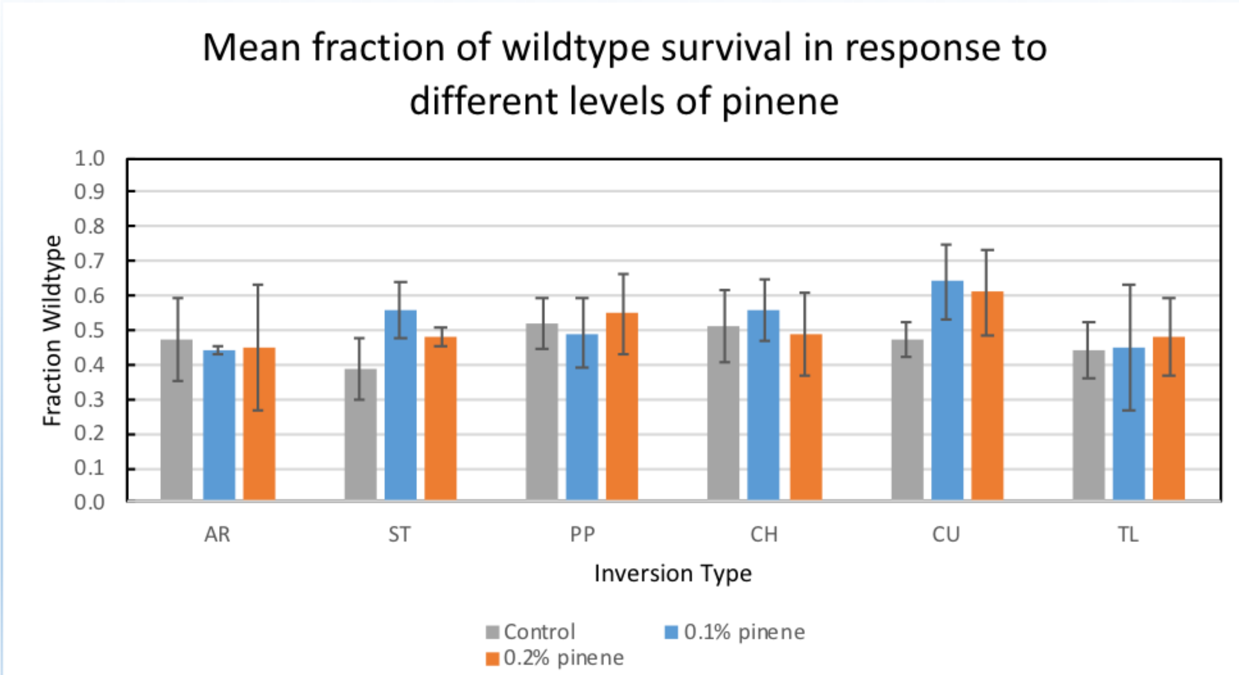

Figure 4. Mean fraction of wild type survival in response to different levels of pinene. This graph shows the mean fraction of wild type survival in the biological replicates of each inversion type with no pinene, 0.1% pinene, and 0.2% pinene. The fraction of wild types was averaged across biological and technical replicates. Standard error is shown.

To determine the significance of an inversion effect, a two-way nested ANOVA test was done. Table 2 below shows the results of the control group. The percent wildtype survival rate values were used to calculate the p-values. There was a significant effect of the biological replicates but no significance between inversions. Tables 3-4 show the results for the 0.1% pinene group and the 0.2% pinene group. Similar to the control, the test revealed a significant difference among biological replicates but no significant inversion effect on competition.

Table 2. Two-way nested ANOVA for control group

Sources of variation	*Df*	*SS*	*MS*	*F_s/F'_s*	*P value*
Among inversions	5	0.0983	0.0197	0.9127	0.5073
Among biological reps.	11	0.2371	0.0216	2.7952	0.0119
Among technical reps.	31	0.2391	0.0077		

Table 3. Two-way nested ANOVA for 0.1% pinene

Sources of Variation	*Df*	*SS*	*MS*	*F_s/F'_s*	*P value*
Among inversions	5	0.1830	0.0366	0.9747	0.4723
Among biological reps	11	0.4231	0.0376	3.1004	0.0058
Among technical reps	33	0.3998	0.0121		

Table 4. Two-way nested ANOVA for 0.2% pinene

Sources of variation	*Df*	*SS*	*MS*	*F_s/F'_s*	*P value*
Among inversions	5	0.1317	0.0263	0.6313	0.6803
Among biological reps.	11	0.4588	0.0417	3.6585	0.0017
Among technical reps.	34	0.3876	0.0114		

An additional two-way nested ANOVA test was done for the Cuernavaca inversion types. Three data points were excluded from this analysis because they were outliers. These outliers came from the CU inversion types (SPE123 1.2 CU) and (SPE123 5.2 CU) in the control and (SPE123 1.2 CU) in the 0.1% pinene group. The wildtype survival rate values were used to calculate the p-values. The ANOVA test revealed that there was no significance among biological replicates and among inversions.

Table 5. Two-way nested ANOVA for Cuernavaca

Sources of variation	*Df*	*SS*	*MS*	*F_s/F'_s*	*P value*
Among inversions	2	0.1019	0.0509	2.0563	0.2089
Among biological reps.	6	0.1487	0.0248	1.1181	0.3977
Among technical reps.	15	0.3324	0.0222		

Chapter 4

Discussion

Previous studies show evidence that there is a link between differential chromosomal inversions and environmental factors in *D. pseudoobscura* habitat (Schaeffer et al., 2017). This study's goal was to determine if there was a difference in larval competitive among inversion types and their ability to deal with the toxic chemical pinene found in their environment. Competition was set up between wildtype larvae that carried one of the six inversion types and a common mutant type larval. Competition between wildtype and mutant larvae of the six most common third chromosome inversion types revealed that there was no significant difference in competitive ability to deal with α-pinene. ANOVA results showed that different levels of pinene (0.1% and 0.2%) had no effect on competition (Table 2-4). This suggests that each inversion type was able to deal with different levels of pinene in similar ways. One reason that larvae of different inversion types compete similarly could involve the migration selection balance model (Schaeffer 2008). Because flies experience a fine-grained environment, it could be beneficial for each inversion type to be able to deal with different levels of pinene in similar ways. Adult flies can live their life throughout all six niches and therefore are exposed to different levels of pinene. Because flies can encounter pinene in different niches across the geographic range, it could beneficial for adult flies to be able to degrade different levels of pinene.

An issue that we might want to further study is the expression and regulation of these third chromosome inversions at different stages of life. If the level of gene expression changes from the larval stage to the adult fly stage, then we might want to look at how that effects their ability to deal with pinene. If there is a significant difference in gene expression at the various stages of the life cycle, then that could further explain the results. We do not know if the ability to deal with pinene could affect competition differently in adult flies compared to larvae.

Although we tested for significance within the CU inversions, there was no significant difference in competition within the CU inversion strain. The slight increased competition seen in the results however may be attributed to environmental boundaries or other abiotic or biotic factors (Figure 4). The Cuernavaca niche is found in San Pablo Etla, Mexico, which was the southernmost location out of the all the niches we studied. The location of this niche could be a contributing factor to the increased competition between wildtype and mutant larvae compared to other locations. The exclusion of some data points from this inversion type could have affected the statistical analysis. If we were able to keep those data for analysis, we might have gotten different results. Further studies would have to be conducted to isolate the cause of the competition between wildtype and mutants.

This study suggests that the inversion types act similarly in their ability to degrade different levels of pinene. Because flies live in a fine-grained environment and could potentially inhabit all six niches in their lifetime, it could be beneficial for flies to be able to deal with various levels of pinene. If the expression and regulation of these genes acts the same throughout their lifetime then the migration selection balance model could explain our findings. Statistical analysis of Cuernavaca inversion type revealed no significant difference in competition between pinene groups and the control group (Table 5). A number of factors such as location and other abiotic and biotic factors could explain the slight difference in CU's competitive ability. Further studies could test whether abiotic factors like moisture, temperature or different chemicals present in the environment affect larval competition. Because we had a rather small data set, there was a lot of variation in the results of this study. Therefore, we still do not know the competitive ability of different inversion strains. To improve this study, we could add more biological replicates. Adding more replicates will increase the sample size and provide more accurate results to make conclusions from.

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- Tutor two students with disabilities in math and history

Penn State University Biology Department — Sept. 2015 – Dec. 2015
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- Prepared lessons and study guides every week to organize topics taught in the course lecture
- Taught weekly lessons that assisted students in understanding course materials and provided test-taking and studying skills

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