Uncovering The Molecular Basis for Pesticide Resistance in *Drosophila*

by

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4. Abstract

Over the past century, the harmful impact that pesticides have had both on our environment and on a wide range of species has become increasingly clear. Among these impacted species are a variety of insects, including many fruit flies in the genus *Drosophila*. As a result of pesticide exposure, *Drosophila* species have developed an evolved resistance to these man-made, highly toxic xenobiotics. This study looks at the resistance to Deltamethrin and Dibrom, two pesticides within two of the main classes of pesticides in four species of *Drosophila*. By drawing comparisons between the four recently diverged species *D. melanogaster*, *D. sechellia*, *D. simulans* and *D. mauritiana*, I identified significant differences in pesticide resistance between them. Then, through the use of synergists piperonyl butoxide (PBO) and diethyl maleate (DEM), I investigated the role of two gene families in *Drosophila* that are known to be responsible for detoxification: *Cytochrome P450s* (*CYP450s*), and *Glutathione-S-Transferases* (GSTs). When these gene families are knocked down, the differences in resistance between species supports a phylogenetic model where *D. sechellia* and *D. simulans* are the least diverged from one another. By continuing to accumulate findings such as these, it will be possible to investigate the role of these gene families in resistance to a vast array of pesticides. Through this research we continue to unfold the organismal and molecular impact of our agricultural and pesticide industries.
5. Introduction

5.1. *Drosophila* as a Model Organism

5.1.1. What is a Model Organism?

Over the course of the last century, the work in model organisms such as *Drosophila* has become ingrained in scientific methodology and experimentation. A model organism is described as a species whose reaction to experimentation is indicative of how other organisms would respond to similar stimuli. In the words of the IX Century Danish physiologist August Krogh, “For many problems there is an animal on which it can be most conveniently studied” (Markow and O’Grady 2007). It has become clear that all species are not the same in structure and function. Even still, many scientific models still rely on the fact that what occurs experimentally in one species can be minimally used as a baseline to predict processes that occur across both closely related and more divergent species (Powell 1997).

*Drosophila*, and more specifically *Drosophila melanogaster* is a species that has been capitalized on for wide-ranging scientific experimentation, predominantly in the fields of genetics, evolution, and ecology. It has also been used in experimentation that intertwines these interdisciplinary fields, as none of them can stand alone. For example, through describing *Drosophila*’s life history traits, and through sequencing its genome, it becomes possible to begin to uncover evolutionary and ecological models pertaining to the particular species and different species that surround it in a natural environment (Markow and O’Grady 2007).
5.1.2. The History of Drosophila Research

The first species of *Drosophila*, *Drosophila funebris* was described by J.C. Fabricius in 1787. After this original recording, more *Drosophila* species were catalogued by naturalists such as C.F. Fallen in the early and mid 1800’s. In 1830, *D. melanogaster* was described by the German entomologist Johann Wilhelm Meigen, and by the mid 1900s taxonomic and genetic work in *Drosophila* species became commonplace (Markow and O’Grady 2007). Specifically, once genetic mutations in a laboratory setting could be manipulated and isolated in order to pinpoint genes associated with traits (Morgan 1917) and evolutionary divergence, the laboratory analyses done on fruit flies grew exponentially (Powell 1997).

Much like any organism, the work done on *Drosophila melanogaster* provided limitations to experimentation. One limitation to *Drosophila* research is seen in the fact that while certain behaviors and processes, such as reproduction, have been thoroughly studied and explained in laboratory settings, the behaviors of these species in a natural ecosystem are less understood. It has been shown that reproductive behavior in *Drosophila* is governed by chemical, visual and auditory modes of communication. Mating preferences such as environmental light have been reproduced and studied in lab settings, and quantified through recording courtship dance and song. The use of chemosensory organs for targeted oviposition, the release of eggs onto a food medium, has also been studied in a lab (Markow and O’Grady 2007). Researchers often assume that the traits observed in the lab are representative of those seen in the natural world, but exceptions undoubtedly exist.
Another limitation to *Drosophila* research is the study of a single species. Because the evolutionary research was performed on *Drosophila melanogaster* alone, the analyses were “vertical” and not “horizontal”. The consistency that arose from research in a single species, disallowed for the breadth of analysis that evolutionary studies require in order to draw connections between a variety of species over time (Powell 1997). It was not until research in *Drosophila* expanded to include a variety of the species in the *Drosophila* genus, and more specifically other species in the *melanogaster* species subgroup, which includes *D. simulans*, *D. mauritiana*, *D. sechellia*, *D. melanogaster*, *D. orena*, *D. teissieri*, *D. yakuba* and *D. erecta*, that the work in evolutionary genetics became more inclusive (Powell 1997).

Differences between *D. simulans*, *D. mauritiana*, *D. sechellia*, *D. melanogaster*, the *melanogaster* species in the subgroup that are used in this thesis have been reported based on geographic and ecosystem characteristics. *D. melanogaster*, who has origins in western Africa has been coined a cosmopolitan species. This means that it thrives off the resources provided in human-dominant environments. *Drosophila melanogaster* has spread throughout the world to the Americas, Eurasia, Australia and Japan, not to mention the rest of the African continent. The continental spread, and spread to Eurasia occurred long ago in a natural manner. Conversely, the spread to the Americas and other continents is thought to have occurred in the last 500 years, with the help of human introduction. *D. simulans*, discovered in 1919, is also considered to be a cosmopolitan species. It exists across the globe, but is less commonly found in eastern Asia and western
Africa. It has been observed that *D. simulans* do not mate well in the dark, like other sister species such as *D. melanogaster*. *Drosophila mauritiana* was found on the island of Mauritius, where *D. simulans* and *D. melanogaster* are not found. *D. mauritiana* is also thought to be human-introduced to the Rodriguez island nearby. Morphologically, *D. mauritiana* and *D. simulans* are very similar. Also, it has been recorded that *D. mauritiana* is even more light sensitive in mating practices than *D. simulans*. Finally *D. sechellia* is perhaps the most unique of all. It is endemic to the Seychelles Islands and feeds exclusively on the fruit of the *Morinda citrifolia* tree, more commonly known as noni fruit. These fruits are toxic to other *melanogaster* species, due to their high octanoic acid concentration. The details of this host-specialist relationship between *D. sechellia* and *M. citrifolia* will be elaborated on in future sections of this introduction. If one were to distinguish between these species anatomically, the only quantitative way of ensuring their species identity is through observation of the male genitalia, specifically male genital arches (Figure 1). Changes in the bristle length and patterning, the relative size of the arch sub-pieces, and the notable changes in shape of sub-pieces allows for assured identification of each organism (Ashburner et al. 1989).
This expansion in both collection and depth of knowledge in *Drosophila* species other than *D. melanogaster* has proven to be critical in denoting difference in species genomes, as well as species ecological differences. From a genetics perspective, one difference between these ancestrally close species is the change in their structure and number of chromosomes. While this is less true within the *melanogaster* species subgroup, it has be observed in the change in chromosome number from three in *D. willistoni* to four in *D. melanogaster*, which are both found in the *Sophophora* subgenus (Figure 2). As species are more phylogenetically divergent, their genetic and phenotypic qualities tend to become more pronounced.
5.1.3. The *Drosophila* Lifecycle and Fly Sorting

Despite their subtle differences, all of the species in the *melanogaster* species subgroup, as well as the majority of all *Drosophila* species share distinctive life cycle events, as well as easy manipulability, which makes them ideal lab species. All *Drosophila* begin as an egg amongst a cluster of eggs laid by a female fly. The eggs are laid on a soft substrate such as a rotting fruit medium in a natural environment, or a food medium in a lab environment. The egg then hatches into a larvae moving into the first larval instar. The larvae feeds constantly, growing and molting into second and third larval instars. Upon reaching the third larval stage, the larvae begin to
wander about to prepare for pupariation. Once inside the pupal case, the organism begins to develop features that will prepare it for life outside of its enclosure. Two of the most notable structures that form during the pupal stage are wings and composite eye structures. The fly also forms an increasingly hard cuticle that will form it’s exoskeleton, and provide it with protection in its adult stage. It grows to be increasingly more pigmented and less sheer as the development progresses. After the pupal stage is complete, the adult fly ecloses (Figure 3). As a female fly, two to three days must pass before the fly reaches sexual maturity and is able to produce viable eggs. As a male, sexual maturity is reached more quickly, usually within the first day that the fly ecloses (Markow and O’Grady 2007). At the stage of sexual maturity, the life-cycle process begins anew with copulation and reproduction, leading to fertilization and eventual oviposition. The timing of these phases varies based on species and environmental context such as temperature, but generally a full cycle from egg to adult in ideal lab conditions occurs in a minimum of eleven days. When temperatures are colder and more varied, as they tend to be in natural settings, the prominent life cycle events may occur more slowly (Powell 1997).
The ability to sort *Drosophila* species by sex is another feature common to many *Drosophila* species within the *melanogaster* species subgroup. Only female flies were used in analyses presented herein. The clear dimorphism between male and female flies allows one to ensure that only females are being tested. It is possible to distinguish between fly sexes in many ways. Female flies are approximately 25% larger than male flies. They have similarly sized heads, wings, and thoraxes, but substantially larger abdomens. This is because the abdomen is where the eggs and reproductive organs are located in the female flies. Besides a simple size difference, one can differentiate male and female flies based on an anatomical and morphological differences in genitalia. Male genitalia tends to be darker in the genital region as well as harder and more scleratinized. In addition, males have sex combs, which allow the male to latch onto the females during mating. (Figure 4). Also, the posterior tergites (5 and 6) of the abdomen are darkly pigmented in the males of these species, whereas
the females lack this melanistic pigmentation pattern (Roeske et al. 2018). This can be seen through observing the dorsal side of the flies’ abdomens (Figure 5). A final way to distinguish male from female flies is that oftentimes there will be eggs emerging from the female genitalia. The anatomical difference between sexes is more clear in certain species such as *D. melanogaster* than in other species such as *D. simulans*, whose genitalia are not as darkly pigmented (Ashburner et al. 1989).

![Figure 4: A Comparison of Male and Female Drosophila melanogaster Genitalia](image)

The male (left) has darker more scleratinized genitalia and sex combs. The female has less pigmented genitalia. The female fly is also substantially larger than the male fly (Adapted from Barna et al. 2013).
5.2. *Drosophila sechellia*, a Resistant and Evolutionary Model

*Drosophila sechellia* is an island endemic species native to the Seychelles Islands, located off the eastern coast of the African continent. The Seychelles islands are coraline and granitic islands, newly inhabited by humans only 400 years ago (Jones 2005). As aforementioned, *D. sechellia* is known for its host specific relationship with the tropical fruit *Morinda citrifolia*, also known as the Indian mulberry (Legal et al. 1992), and most commonly known as the noni fruit. Besides its host-specific role, noni has historically been used in diabetes treatment and as an antiseptic. Its roots are also used as a natural dye, as they produce a deep red color (Farine et al. 1996). The noni fruit is grown along coastal shorelines in the Seychelles, as well as through coastal landscapes in the Indian Ocean and Malaysia (Jones 2005). It has been introduced to Australia, Hawaii, Moorea, Tahiti and the West Indies, where it has continued to thrive (Legal et al. 1994). Due to its pungent

**Figure 5: Tergite Pigmentation Patterning in Male and Female Drosophila**

Sexual dimorphism showing difference in tergite pigmentation patterns in female (Left) vs. male (right). Males have much darker pigmentation in tergites A5 and A6 than females do. (Adapted from Roeske et al. 2018).
and repulsive odor, the noni fruit has taken on less affectionate names such as the “vomit fruit” (Whiteman and Pierce 2008). When ripe, it is said to be reminiscent of rotting or decaying cheese, and has specifically been described as smelling like a mix of pineapple and blue cheese (Stensmyr 2009, and Higa and Fuyama 1993). The odor that permeates from this tropical treat originates in the secondary compounds produced by the fruits as natural deterrents to phytophagous herbivores, including a variety of insects such as *Drosophila* species. These deterrents do not halt all phytophagous activity, but rather limit the consumption of the plant (Chapman 2003).

While host-specialist relationships are common, particularly in biodiverse, tropical areas across the globe (Dworkin and Jones 2009), the nature of the *D. sechellia*-*M. citrifolia* relationship is of particular intrigue to evolutionary geneticists due to the fruit’s extreme toxic nature. Recently divergent *Drosophila* sister species experience paralytic or lethal responses to small doses of the fruit’s volatile compounds, but *D. sechellia* not only has shown immense resistance to the fruit’s toxins, but shows a behavioral and chemical affinity for the fruit. This is a phenomena seen in many phytophagous specialists. What was previously a deterrent to ancestral species becomes a means of attraction for the species in question. (Chapman 2003).

*D. sechellia* proves to be a convenient model for studying evolution, due to its relatively recent divergence from sister species *D. simulans* and *D. mauritiana*, and subsequent ability to form hybrid crosses with other species. Crosses between *D. sechellia and D. simulans* as well as *D. sechellia and D. mauritiana* form viable, fertile offspring. Crosses between the more diverged *D. melanogaster* and *D.
sechellia form infertile offspring. Through these hybrid crosses, recently adapted genes can be observed and identified (Jones 2005).

This section will first look at the volatile compounds in the noni fruit and describe D. sechellia’s unique resistance to them. It will then go into the detail of the genetic, chemoreceptive and behavioral qualities of resistance. Then it will describe how this resistance has led to specific and divergent evolutionary phylogeny of Drosophila sechellia within the melanogaster species subgroup. Finally, it will explain how the resistance mechanisms of D. sechellia to noni are similar to the mechanisms seen in other specialist species. Throughout this section we are left to ponder how resistance mechanisms in D. sechellia can be applied to other toxins, such as those found in pesticides.

5.2.1. Volatile Compounds and the Noni Fruit

*Drosophila sechellia* shows the ability to perform a process called “ratio-specific odor recognition”. By pinpointing the specific ratio of volatile chemical cues called kairomones released from the noni fruit, a D. sechellia individual can recognize the particular blend of volatile chemicals in the noni fruit, and pick the fruit out amongst the background of volatile kairomone blends being emitted from other plants. The unique blend of volatiles is more attractive to the flies than a single volatile presented alone would be (Bruce et al. 2005). After contemplating all potential toxins in noni, it was established that the volatile components that are most toxic to non-D. sechellia species are octanoic acid (OA), as well as the far less toxic, but prevalent hexanoic acid (HA). OA is a linear carboxylic
fatty acid with 8 carbons, which constitutes 58% of all volatiles found in noni fruit (Jones 2005), and HA is a linear carboxylic fatty acid with 6 carbons (Legal et al. 1994, Legal et al. 1999, and Amlou et al. 1997). While OA is lethal to *Drosophila* species, HA has proven to only be temporarily coma inducing, but not lethal (Farine et al. 1996). OA is toxic in both the larval and adult stages of *Drosophila* species that are not *D. sechellia*. The chemical is predominant in ripe fruit (Jones 1998), having been recorded to be 47 times less prevalent in green, or unripe fruit, and reduced by about half in rotten fruit (Legal et al. 1994). For use in laboratory experimentation, it has been calculated that 50 micrograms of OA in a lab-produced food medium is equivalent to 1.5 grams of ripe noni pulp (Legal et al. 1992).

Whereas most *Drosophila* species are saprophagous, that is they feed off rotting organic material, *D. sechellia* is unique in that it feeds off the ripe, unrotten fruit of *M. citrifolia* (Jones 2005). As the fruit rots, the volatile OA is degraded to ethyl octanoate, which is five times less toxic to non-resistant flies than OA is. This occurs through the process of ethyl esterification. During ethyl esterification a water molecule and a non-toxic product (ethyl octanoate) are produced from the acidic molecule upon introduction of an acid catalyst (Legal et al. 1999). Due to the fact that OA is volatile, noni must be stored in acetone in a lab environment to prevent evaporation or ethyl esterification of the chemical (Legal et al. 1994). Resistance to noni volatiles such as OA can be considered in a variety of ways.
5.2.2. *D. sechellia* resistance to Noni Volatiles

5.2.2.1. Genetic Resistance

It has been calculated that *Drosophila sechellia* is five to six times as resistant to OA than either *D. melanogaster* or *D. simulans* (Legal et al. 1994). Instead of acquiring new genes, resistance factors in *D. sechellia* show a decrease in expression of factors that exist in non-resistant sister species (Matsuo 2008). The genetic basis for OA resistance is likely not attributable to a single gene. Instead, many genes are responsible for resistance, making the trait oligogenic. The difference between a polygenic and a oligogenic trait refers to the number of genes associated with the trait. Polygenic traits are assumed to have a large number of associated genes and oligogenic traits are limited to a much smaller and more concrete number of associated genes (Jones 2001). In *D. sechellia*, toxin resistance increases as new mutations associated with resistance arise and are selected for. Simultaneously, existing mutations that further the fitness of the resistance trait will also be selected for (Matsuo 2008).

In adult flies, two resistance factors, loci associated with a trait, have been found on the X chromosome. In addition, at least one factor has been found on chromosome II and at least two factors on chromosome III. While all of these loci show partial dominance, the epistatic interactions between resistance factors show that about 25% of dominant resistance can be attributed to factors found on chromosome III (Jones 1998, Jones 2001, Early and Jones 2011, and Legal et al. 1999). The factors associated with larval resistance to OA may only be a subset of the
factors associated with resistance in adult flies. There is one resistance factor located on chromosome III and two located on chromosome II, but no recorded factors located on the X chromosome, like in their adult counterparts. Genetic resistance mechanisms appear to change over the course of a fly’s lifetime. The resistance factors may also change based on the sex of the fly. In male flies a resistance factor was located on chromosome 2R, for example, but this factor was not found to be present in female specimens (Jones 2001).

As mentioned, the specificity of the interactions between genes that form the resistance trait show dominant epistatic and also additive effects. These differences arise in such complexity due to the fact that the trait has adapted gradually over the course of thousands of years (Jones 1998). Resistance to OA has proven to be a dominant autosomal trait upon first glance, as \( D. \text{sechellia} \times D. \text{simulans} \) hybrids show increased resistance when compared to \( D. \text{simulans} \) alone. Through many back-crossing experiments that slowly reintroduce factors associated with resistance into non-resistant hybrids, the incrementally improving nature of the resistance trait confirms that dominance exhibited in \( D. \text{sechellia} \) is additive (Amlou et al. 1997).

Because there are multiple dominant factors, resistance becomes stronger when more factors are present in an individual (Jones 1998, Matsuo 2008, and R’Kha et al. 1991).

The complexity of the resistance trait is also seen in a variety of other ways. For example, in hybrid crosses, progeny from a \( D. \text{sechellia} \) mother are more resistant that progeny from a \( D. \text{simulans} \) mother. Proteins are shown to be passed down
through the maternal line that increase the dominant nature of the resistance trait (Jones 2001).

Even though the specific genes have not yet been identified, chromosomal patterning of resistance factors has shown that limited regions of the genome have large phenotypic effects on resistance. In addition to having large effects, the genetic regions or factors are described as pleiotropic. In this case, that means that the general trait “resistance”, can be broken down into a variety of more specific traits such as chemoreceptive tendencies, egg production, ovariole production and behavioral repulsion (Jones 2005). Certain aspects of the trait, such as repulsion seen in D. simulans in response to noni have begun to be better understood. In a 2011 study, Early and Jones found six loci associated with noni food preference, one of which, \textit{Obp56e}, was confirmed from past studies (Dworkin and Jones 2009). They also found loci which may further explain \textit{D. simulans}’s repulsion to noni including \textit{bubblegum}, a gene associated with fatty acid metabolism, \textit{black}, a gene associated with vision-directed behavior, \textit{nAcRAa-30D}, a gene associated with insecticide response and \textit{Smi35a}, a gene associated with olfaction-directed behavior (Early and Jones 2011). Next we look specifically at chemoreception in relation to resistance.

\subsection{5.2.2.2. Chemoreception: The Olfactory and Gustatory Systems}

Chemotaxis is the movement of an organism in response to a chemical stimulus. This process is dependent on sensory organs that partake in chemoreception (Jones 2005). The two systems observed with regard to \textit{D. sechellia} chemoreception are the olfactory and gustatory systems. A generalist has a complex relationship with
its environment, needing to sense its food sources and chemical threats with extreme accuracy. Specialists, on the other hand, have far less of a need to decipher danger and attractants, relying solely on the signal from a singular fruit. For this reason its chemoreception genes and chemoreceptive system accuracy are less complex (McBride 2007). In *D. sechellia* there is a reported enhancement of olfactory cues received, meaning that individuals can sense noni volatiles emitted from their hosts from distances at least 50 meters away (R’Kha et al. 1991 and Dworkin and Jones 2009). There is also a decrease in aversion to gustatory cues associated with noni. These traits are thought to be caused by a decrease in size or expression of the chemoreceptive gene families in *D. sechellia* (Matsuo 2008 and Whiteman and Pierce 2008). These families include the Odorant receptor gene family (*Ors*), the Gustatory Receptor gene family (*Grs*), and Odorant-binding protein gene family (*Obps*) (Matsuo 2008).

The *Ors* and *Grs* gene families are large with upwards of sixty genes per gene family distributed throughout the chromosomes in the *Drosophila* genome. These genes are almost identical to those found in other insect species, such as bees, mosquitoes or aphids (McBride and Arguello 2007). Loss of genes in the *Gr* and *Or* gene families has been reported as occurring at rates five times faster in *D. sechellia* than in *D. simulans*. Not to mention, the loss of *Or* and *Gr* genes occurs at a much faster rate than that of other genes found in the *D. sechellia* genome (McBride 2007). Specifically, the loss of *Gr* genes occurs more rapidly in specialist species such as *D. sechellia* or *D. erecta* than in generalist species such as *D. simulans* or *D.
melanogaster. For example, subfamily E of the Gr gene family is associated with putative bitter sensing, a process heavily prevalent in noni recognition and repulsion. In D. sechellia, three disparate genes associated with bitter sensing have been reportedly lost (McBride and Arguello 2007). The general patterns of gene loss and species divergence can be seen in Figure 6 below. The figure distinguishes between generalists and specialists, and also considers when genes have been lost or duplicated in different species.

Figure 6: Ors and Grs Gene Family Loss in the melanogaster Species Subgroup Phylogeny
In this phylogeny duplications of genes are marked with a blue dot. Loss of genes are marked with a red line. Generalist species are marked with a solid black line and specialist species are marked with dotted black lines. Sim = D. simulans, sec = D. sechellia, mel = D. melanogaster, yak = D. yakuba, and ere = D. erecta. (Adapted from McBride and Arguello 2007).

Obps, commonly expressed in insect antennae or other sensory organs help to detoxify chemicals by sending signals to either odor or gustatory sensilla via Ors or Grs in insects such as Drosophila (Figure 7) (Whiteman and Pierce 2008). Odorants or gustatory chemicals first pass through pores in the cuticles of hair-like antennal
sensilla (McBride and Arguello 2007). Once inside, Obps deliver the odorants to receptors on the surface of neurons, causing firing of the cells and stimulation of the gustatory and olfactory systems (Chapman 2003). Obps have been attributed to the loss of toxin avoidance seen in host specialists (Whiteman and Pierce 2008).

Figure 7: A Simplified Depiction of the Role of Odorant Binding Proteins
Odorant binding proteins and the relay system they mediate between odorants or tastants and the Ors or Grs. Within the sensillum, Obps carry and modify chemicals received from the outside environment to the awaiting Ors and Grs on the dendritic membrane of receptor neurons. (Adapted from Whiteman and Pierce 2008).

Like Grs, the evolution and lack of expression of Obps is more commonly seen in specialists than in generalists. Due to the loss and evolution of these genes, what might have once been repulsive becomes a means of attraction in order for the specialist species to escape competitive environments. It has also been seen that while
expression of $Obp$s, such as $Obp56e$ may be downregulated normally, once a $D. sechellia$ fly reaches the noni food medium, expression is upregulated (Whiteman and Pierce 2008).

In comparison to sister species, the olfactory system of $D. sechellia$ is rearranged. Differences in $D. sechellia$ chemosensory morphology have been described in order to specify its particular response to the noni plant. Whereas in most $Drosophila$ species ab2 type sensilla dominate the antennae, in $D. sechellia$ there is a large portion of ab3 type sensilla that replace the ab2 sensilla. Ab3 sensilla are more positively sensitive to noni volatiles such as the byproducts of HA, and are lacking in other $Drosophila$ species. The generation of these new sensilla are due to the unusual increase in expression of $Or22a$ in $D. sechellia$ compared to sister species (Stensmyr 2009 and McBride 2007). Also, distinct neurons are responsible for interpreting input from particular odorants with speed and precision. When odorants are presented in specific proportions as they are in host plants like noni, specific neurons are fired, triggering detoxification and attraction responses (Bruce et al. 2005). The neurons in the ab3 type sensilla of $D. sechellia$ are organized in such a way that they are more equipped to sense the odorants being emitted from the noni fruit than other species are (Linz et al. 2013).

The gustatory cues and corresponding taste receptors associated with noni resistance, as well as olfactory cues and olfactory receptors are thought to probe the behavioral resistance traits also seen in $D. sechellia$, such as oviposition site preference and food preference (Matsuo 2008). Two genes in particular, $Obp57e$ and
*Obp57d* are associated with preferential food-seeking behavior and taste perception (Dworkin and Jones 2009). Mutations in the upstream regulatory regions of these genes occur in *D. sechellia* but not in *D. simulans* or *D. melanogaster* (Stensmyr 2009). These examples are not associated with a mutation in the gene itself, but are rather indicative of changes in regulation of gene expression that lead to the specific trait differences (Whiteman and Pierce 2008).

5.2.2.3. Behavioral Resistance

An understanding of resistance through gene expression and regulation, as well as the evolution and anatomy of chemoreceptive organs was established before resistance was considered as the behavioral trait “preference” (Whiteman and Pierce 2008). Instead of thinking of the behavior “preference” in *D. sechellia*, it can instead be thought of as a “lack of avoidance” (Matsuo 2008). If a species is less resistant to OA, this ‘lack of avoidance’ will be the less extreme (Legal et al. 1992). *D. sechellia* exhibited the most ‘lack of avoidance’ of noni fruit, followed by *D. simulans* and then *D. mauritiana* (Legal et al. 1992).

A specific “lack of avoidance” behavior that is altered by OA is oviposition. The difference in ‘lack of avoidance’ of ripe noni between male and female *D. sechellia* confirms that, due to their need for a suitable oviposition site, females are more likely to prefer ripe noni fruit than males, and are also more resistant to the toxins in noni. In an oviposition trap assay, 98% of all *D. sechellia* females tested chose to oviposit on either OA or noni food medium. Both *D. simulans* and *D. melanogaster* females actively avoided the noni food medium (Higa and Fuyama
When OA is not present, the rate of oviposition in non-
*D. sechellia* females like *D. melanogaster* far exceeds *D. sechellia’s* oviposition rate. When OA is present however, *D. sechellia* oviposition rates increase, and *D. melanogaster* oviposition rates decrease (Dworkin and Jones 2009). Because *D. sechellia* innately produce five to ten times fewer eggs than *D. melanogaster* (Jones 2005) and *D. simulans* (Amlou et al. 1997) due to decreased ovariole production, when oviposition in *D. sechellia* increases, the results are considered to be more significant than in sister species. This is especially true when *D. sechellia* is bred on a noni food medium. Inversely, when noni is present for oogenesis in *D. simulans*, rates of egg production are decreased to equal those of *D. sechellia* (R’Kha et al. 1991). I next question why and how these genetic, chemoreceptive and behavioral tendencies came to be.

5.2.3. **Evolutionary Divergence of *D. sechellia***

5.2.3.1. **Claiming a New Ecological Niche**

The adaptive evolution and specialization of *D. sechellia* to noni is thought to have happened for many reasons. The first is attributed to sympatric speciation, that is speciation that occurs between two species that exist in the same environment. In a biologically rich environment, such as a tropical climate, the interspecies resource competition is great (Amlou et al. 1997). Species that exist within similar ecological niches must find a way to establish individuality in order to reduce competition for resources (Jones 1998). By specializing to feed on a plant that is toxic to other species, *D. sechellia* would experience far less competition, (R’Kha et al. 1991), as well as avoid predation from parasitic wasps that inhabit other potential food sources.
such as the Pandanus fruit (Jones 2005). Not to mention, by feeding on ripe instead of rotten fruit, *D. sechellia* has a temporal advantage in comparison to other *Drosophila* species. The competition for food and oviposition location is reduced before the fruits have rotted and become less toxic to other species (R’Kha et al. 1991). In choosing a ripe, toxic host, *D. sechellia* experience prezygotic isolation from sister species, ensuring further behavioral and evolutionary divergence (Jones 1998). Because of toxicity, individuals of the specialist species must be able to survive exposure to the ripe host’s toxins at all phases of life, from egg to adult in order for the species to show evolutionary success (Higa and Fuyama 1993). While there is still plausible gene flow between *D. simulans* and *D. sechellia*, species that exist in the same environment, specialization of *D. sechellia* negates any reverse speciation that may occur in natural populations. *Drosophila sechellia* has also undergone allopatric speciation from species such as *D. mauritiana* due to geographic isolation inherent in these species’ island endemic natures. (Garrigan et al. 2012).

Because *D. sechellia* populations are much smaller than *D. simulans* populations, the effects of selection that lead to specialization are magnified and occur rapidly. When a population is small and also isolated, mutations and genetic drift have a much greater effect on the population and the species as a whole (Whiteman and Pierce 2008). These mutations are further selected for during positive selection. Positive selection occurs when mutations that lead to a given trait or trait change are favorable, making individuals carrying them more evolutionarily successful. In this case, the trait considered is increased resistance to noni fruit as
seen in *D. sechellia*. Positive selection likely drove *D. sechellia* to be highly resistant to noni toxins (McBride 2007).

5.2.3.2. *D. sechellia* Phylogeny

It is believed that *D. sechellia* and *D. simulans* diverged 0.4 to 2 million years ago (Matsuo 2008). Previous to the *D. sechellia-D. simulans* divergence, it has been calculated that the *simulans* clade, composed of *D. simulans*, *D. sechellia* and *D. mauritiana*, split from *D. melanogaster* approximately 3 million years ago. It is also estimated that the split between *D. sechellia* and *D. mauritiana* occurred only 500,000 years ago when the island endemic species originated (Garrigan et al. 2012).

It has been most commonly postulated that *D. sechellia* is the most divergent from *D. mauritiana* and *D. simulans*. Albeit, through mismatch alignment, a process that compares the genomes of these species by lining them up and recognizing explicit difference in genomes, the phylogeny within the clade has become less definitive. Most of the “mismatches” between the species compared occurred on the X chromosome. By means of this X-chromosome “mismatch” system, it is now thought that *D. simulans* and *D. sechellia* are in fact the least divergent of the three species.

The phylogeny of *D. sechellia* and its sister species is still highly debated, and various models of the phylogeny have been proposed, showing combinations of all three species of the *simulans* clade as the most diverged of the three species (Figure 8). The dispute over exact phylogenies may be attributable to gene flow between species in natural populations such as *D. sechellia* and *D. simulans* that may have occurred as recently as 2,400 years ago (Garrigan et al. 2012).
One thing that is unanimously agreed upon amongst scientists in the field is that the speciation of these three species did not occur in one simple bifurcation, but instead was the product of a series of speciation events. Another evolutionary event that is unanimously agreed upon is the split of the *simulans* clade from *D. melanogaster* (Garrigan et al. 2012). When looking at *D. sechellia* as an evolutionary model, it is also valuable to compare it to other specialists with similar toxin resistance mechanisms.
5.2.4. Resistance Phenomena in Other *Drosophila* Species Specialists

Other species of *Drosophila* besides *D. sechellia* that also have host-specialist relationships include the cactophillic species *D. pachea*, and also *D. erecta*. *D. pachea* has adapted resistance to unusual alkaloids and sterols in the Lophocereus cactus. These chemicals are toxic to other species in the phylogenetically close *repleta* species subgroup (Legal et al. 1992).

*Drosophila erecta*, a facultative, seasonal specialist found in west-central Africa inhabits the Pandanus fruit as a host. Three species of Pandanus fruit are found in regions similar to that of *M. citrifolia* (noni). The fruits are another example of host plants that are toxic to recently divergent non-*D. erecta* species. While the Pandanus fruit has been described as having an “offensive and pungent odor”, the chemical properties of the fruit are distinct from those of *M. citrifolia*. The predominant volatile found in the Pandanus fruits is 3-methyl-2-buten-1-yl acetate (3M2BA). 3MB2A triggers the behavioral trait of oviposition in *D. erecta*, and can also be detoxified most efficiently by this species. Much like in *D. sechellia*, *D. erecta* shows an increase in ab3 sensilla, which allow for detoxification of 3MB2A. Unlike in *D. sechellia*, *D. erecta* does not replace ab2-type sensilla with ab3-type sensilla, but rather just shows an increase in the number of ab3 sensilla in odorant receptors. In seeing similar phenomena arise in *D. erecta*, one can ensure that patterns recognized in *D. sechellia* are not simply attributable to a small population size and the resulting bottleneck-induced speciation (Linz et al. 2013 and Stensmyr 2009). The morphology associated with resistance are common to many specialist species. Next I move into a
discussion of how resistance patterns to natural toxins in species such as *D. sechellia*,
*D. erecta* and *D. pachea* can be applied to non-naturally occurring toxins: pesticides.

5.3. **Human Impact on the Environment through Agriculture and the History of Pesticide Use**

5.3.1. **Modern Agriculture, Pesticides, and Their Undesired Effects**

By the 1960’s, high input farming became normalized and heavily implemented in the United States. A high input farming system is the production of a limited number of cash crops that rely on a highly mechanized and machine-based agricultural model for increased crop yield. With this model, pesticides, as well as herbicides and fungicides began to be used in mass quantity. This is because as more of a crop is grown repeatedly over time in the same field, a microclimate that allows for pests, weeds, and the soil microbiome to ascertain resistance to the toxins being used to kill them is magnified. As resistance grows, more toxins are introduced into these agricultural systems to counterbalance these dilemmas (Sultan 2017). Pesticide resistance is an example of man-made natural selection. This inherently means that it has occurred only in the time that humans have dominated the Earth (Ffrench-Constant et al. 2004). Much like in the OA resistant *D. sechellia*, when considering pest resistance resistance, it is essential to juxtapose the behavioral, the morphological and the genetic mechanisms of resistance to form a complete understanding of how the trait is acquired.
Before doing this, I focus away from the effects on the pests themselves. Most of the directly damaging effects of pesticides are seen in the fact that they are more inclusive or “broad spectrum” than initially anticipated upon production. More species are affected than just the intended pest. An example of this is seen in the undesired effects on honey bees (*Apis mellifera*) and their response to neonicotinoids, a class of insecticides used to control agricultural pests such as aphids in field settings (Pesticide Network Action UK 2017). In the 2014 study, Lu et al. compared the success of *Apis mellifera* in wintering-over hives when exposed to the neonicotinoids imidacloprid and clothianidin. Winterization is a process that involves producing food and insulation for the colony to survive the winter. Their results concluded that at sublethal levels, bees exposed to neonicotinoids showed equal survival rates to non-neonicotinoid exposed groups up until the winter season. At this point, however, mortality by the end of the winter season increased, and neonicotinoid-exposed bees were increasingly likely to be subject to the mysterious and harmful event called colony collapse disorder (CCD). CCD causes the bee population to dwindle and die out (Lu et al. 2014).

A less contemporary example of unwanted effects of pesticides is seen in the use of DDT (*Dichlorodiphenyltrichloroethane*) within and outside the U.S. post World War Two. DDT was advertised as a toxic and efficient insecticide that was not poisonous to humans (Insecticida Fogo 1947). DDT was successful in killing mosquitoes and other pests, and its effects were also shown to be toxic to animals, such as large carnivores and humans, species at higher trophic levels. DDT at high
concentrations may cause tremors, seizures, or vomiting (Center for Disease Control and Prevention 2009). DDT is also shown to have non-biotic implications. Because it is non-volatile, it remains in bodies of water and the atmosphere for extended periods of time. This reminds us how interconnected ecosystems both biotic and abiotic can be. Due to the harmful effects of DDT, its use has been strictly regulated and controlled in the U.S. and Europe (Goudie 2000).

The effects of agriculturally used chemicals are clearly long-lasting and intensive. This is seen in the fact that even after it has been taken out of commission, the residual effects of a pesticide such as DDT remain pressing and present. It is also seen in that species’ resistance to such chemicals is maintained over time. Although human produced pest management is occasionally successful, the resistance to pesticides seen in pests seems to be an unstoppable force. Because of this, it would be the most environmentally responsible decision to cease the production of new insecticides. They only seem to further contaminate the environment as they continue to be used in more massive quantities (Feyereisen 1995). While this is a unrealistically optimistic solution, we can first begin to deconstruct the implications of the pesticides that are commonly used today, and how species have acquired resistance to them.

5.3.2. Pyrethrins, Organophosphates and Other Pesticides on the Market Today

Pesticides are examples of man-made xenobiotics, substances that are described as foreign and potentially toxic to an organism. Insecticides are pesticides that target insects in particular. These terms will be used interchangeably throughout
this thesis. The majority of pesticides target a pest’s nervous system, causing a
decrease in movement known as paralysis, and sometimes death. Targeting the
nervous system is the most direct way to eliminate mobility and activity of pests in an
agricultural system. The main pesticide classes that are found in high-input
agricultural systems include cyclodienes, neonicotinoids, ivermectins, pyrethroids,
organophosphates (OPs), carbamates, insect growth regulators, and DDT, which
stands alone, as it originated before there were major pesticide classifications
(Bonizzoni et al. 2012 and Ffrench-Constant et al. 2004). The role of pyrethroids and
OPs will be described in detail below. Carbamate function is almost identical to that
of OPs. Cyclodienes target ligand-gated ion channels that bind to the
neurotransmitters GABA or acetylcholine, disturbing neural activity and
neurotransmitter binding function. Neonicotinoids act on the nicotinic acetylcholine
receptor. Ivermectins target the glutamate-gated chloride channel, yet another
neurological activity targeter. Unlike other pesticide classes, insect growth regulators
cause a decrease in development and lead to eventual death of the targeted pest. As
resistance to pesticides evolves, the intended target of pesticides must change to avoid
already resistant systems, such as the nervous system. For example, targets for a new
pesticide may be hormonal instead of neurological, as is seen in insect growth
regulators. (Ffrench-Constant et al. 2004).

The pesticides, both broad-spectrum insecticides that are used in this thesis are
Deltamethrin and Dibrom. Both of these insecticides are used in household and
agricultural settings for general pest control.
5.3.2.1. Pyrethroids and Deltamethrin

Deltamethrin is a pyrethroid. Pyrethroids are synthetic insecticides that mimic and magnify the chemical structure of pyrethrins. Pyrethrins are found in chrysanthemum flowers in the Asteraceae family and are a natural plant defense that target an insect’s nervous system, causing temporary paralysis (National Pesticide Information Center, 2010). Pyrethroids are said to have low toxicity and high efficacy rates in pest management. They are so low, in fact they are amongst the only class of pesticides used in insecticide nets and indoor residual sprays, both of which humans come into contact with frequently. They have historically been used as a mosquito repellent for malaria prevention (Bonizzoni et al. 2012 and Zhu et al. 2014). Because pyrethroids are far more potent than their naturally found analogs, pyrethrins, they cause rapid paralysis or death in a variety of insects when there is persistent exposure to the toxin over time. This persistent state is one in which a pyrethroid would exist in the vast majority of agricultural systems. This is due to the fact that the insecticide is sprayed frequently and in mass quantities.

Pyrethroid resistance is at least partially attributed to a polymorphism of the gene \textit{para}, which acts on the \textit{Na$^+$} voltage gated channels found throughout the nervous system (Feyereisen 1995). The \textit{para} gene encodes for the alpha subunit of the \textit{Na$^+$} voltage-gated channel, the action site of pyrethroids and other pesticides such as DDT (Pittendrigh et al. 1997). \textit{Na$^+$} voltage gated channels are in part responsible for the depolarization of a neuron, allowing the action potential, the electrical signal used by neurons, to be propagated through a neuron and transferred from one cell
body to another throughout an organism. Na⁺ voltage-gated channels work alongside K⁺ voltage-gated channels to propagate depolarization within the cell. When the Na⁺ channels are open and K⁺ channels are closed, membrane potential becomes positive, allowing the cell to become depolarized. When the Na⁺ voltage-gated channels close, K⁺ channels open, repolarizing the cell (Figure 9).

![Diagram of ion channels](image)

**Figure 9: A Simplified Depiction of Depolarization in a Neuron**

When depolarization occurs, Na⁺ voltage-gated channels open, causing membrane potential to rise up to about 120 mV. When they close and K⁺ channels open, the neuron is repolarized back to a resting membrane potential of -65 mV, and the AP formed by depolarization continues to travel down the neuron. (Adapted from MacMillan Higher Education)

When pyrethroids disallow the passage of action potential in neurons, electrochemical signals cannot be passed on, and nerve cells will ultimately fail. If species are pyrethroid resistant, there are recorded mutant alleles of the *para* genes, which allow for the Na⁺ voltage-gated channels to propagate action potentials as they would in normally functioning cells that are not being targeted by pesticides. These alleles include both *knockdown resistant (kdr)*, as well as *super knockdown resistant (super kdr)*. These alleles arise when there is a single base mutation from TTG to TTT, leading to a change in amino acid sequence from leucine to phenylalanine in
*kdr*, or the single base mutation from TTG to TTC, leading to the change in amino acid sequence from leucine to cysteine in *super kdr* (Pittendrigh et al. 1997 and Zhu et al. 2014). These mutations allow the effects of pyrethroids to be nullified, without altering the rest of the Na$^+$ voltage-gated channel complex.

Deltamethrin, the most potent of the pyrethroids, has been found to be most effective in moths, aphids, mosquitoes, fleas and, of course, *Drosophila*. Inversely, Deltamethrin has been found to be less efficient in ticks. Commercial products that include Deltamethrin include Butoflin, Butoss, Butox, Cislin, Crackdown, Cresus, Decis, Decis-Prime, K-Othrin, and K-Otek. While effects in small mammals such as mice are far less extreme than those seen in insects, they experience type II motor symptoms such as writhing and extreme salivation observed upon Deltamethrin exposure (Extension Toxicology Network, 1995). This is an example of how a broad-spectrum chemical that is intended to have a focused pest effect works up the chain of beings to cause harm to non-targeted organisms.

Much like any pesticide the effects of Deltamethrin on the atmosphere must be taken into account. Due to its stable, or non-volatile nature, Deltamethrin remains in the environment for extended periods of time. It may degrade in soil environments, but Deltamethrin has shown to be extremely stable in atmospheric oxygen, causing perpetuation of the toxin in the atmosphere far beyond its intended focus and use (Extension Toxicology Network, 1995).

Previously, resistance to pyrethroids has been associated with a single mutation to the *para* gene. That being said, more recent literature has shown that
resistance to pyrethroids is polygenic. In their 2012 study, Bonizzoni et al. showed how exposure and resistance to Permethrin also lead to the resistance of local mosquito populations to other pyrethroids, such as Deltamethrin. This is because they require similar resistance mechanisms. Because pyrethroids target one of the same genes, *para*, and act on the same structure, the Na$^+$ voltage gated channel, resistance to one led to resistance to the other. Seen in the above described *kdr* mutation, resistance to both of these pyrethroids arose in Kenyan mosquito populations. It was recorded that the *kdr* mutation rate arose in 94% of mosquitoes tested. There are shown to be seventeen loci associated with resistance to both Deltamethrin and Permethrin. Apart from just the *para* gene, there were also changes in *Cytochrome p450s (CYP450s)*, *Glutathione S-transferases (GSTs)*, and *carboxyl esterases*. The resistance mechanisms behind the gene families *CYP450s* and *GSTs* will be discussed in the section “Synergists and Gene Families Implicated in Detoxification: Cytochrome P450s and Glutathione S-Transferases”, below.

Expression of these genes is more complex than the single mutations associated with other genetic factors involved in resistance. Also, the up and down regulation of specific genes in these gene families will differently alter resistance (Zhu et al. 2014). On a morphological level, resistance genes have been associated with thickening of cuticle tissue to mediate permeation of the toxin into the organism’s body, as well as strengthening modifications to the digestive tract lining of the mosquitoes (Bonizzoni et al. 2012). The Bonizzoni et al. study exemplifies the multifaceted nature of resistance to a pesticide.
5.3.2.2. Organophosphates and Dibrom

Since the 1960’s Dibrom has proven effective in pest control on a large-scale agricultural level. Both in examples of lemon storehouse use (Gojmerao and Fox 1962) and tomato fields (David et al. 1960) Dibrom has reduced the Drosophila population, allowing crop production to flourish. Sprayed from large aircrafts over large fields of crops, a major concern has been whether or not Dibrom will degrade in the environment, losing its toxic nature over time. The consensus is that Dibrom is more volatile than other pesticide counterparts such as Deltamethrin, degrading to become chemicals such as 2,2-dichlorovinyl dimethyl phosphate (DDVP) (Virginia Department of Health, 2017). That being said, the undesired effects of Dibrom beyond its Drosophila intended focus are clear. Dibrom is highly toxic to honeybees, one of the main pollinators that the crop system relies so heavily upon, and has also proven to be toxic to aquatic invertebrates and fish at high levels of exposure (Environmental Protection Agency, 1983).

Dibrom, also known as Naled, is an organophosphate (Environmental Protection Agency, 1983). Like pyrethroids, organophosphates target the nervous system. Specifically, they attack the enzyme acetylcholinesterase (AChE) by phosphorylating the enzyme, inhibiting its function. When this occurs, nerve impulse cannot propagate as it normally would. Under normal conditions, AChE breaks down the neurotransmitter acetylcholine (ACh). ACh either works to excite or inhibit nerve impulses throughout the nervous system, binding to receptors on postsynaptic neurons (Figure 10).
Organs that are targeted by organophosphates have malfunctioning AChEs. When AChE is inhibited, it causes the continual binding of ACh to receptors on the postsynaptic neuron. Though this could either continuously excite or inhibit the receiving neuron, we consider the case of over-excitation. If over-excited, the receiving neuron will eventually be damaged or destructed (Environmental Protection Agency 2013). Resistance to organophosphates has been attributed to a mutation in the gene *Ace*. This mutation causes a “fix” in the malfunctioning AChE binding site. There is a decrease in ACh overstimulation and a decrease in over-excitation on postsynaptic neurons (Feyereisen 1995). Like in most cases, when pests show these resistance traits, more pesticide is used to overcome evolutionarily powerful mechanisms. Through depicting neurological and cellular processes, such as those involved in both Dibrom and Deltamethrin resistance, one’s understanding of the trait resistance becomes more complete.
5.3.3. Agricultural Pest Control Beyond Pesticides

As we have begun to get more proactive about working towards a less polluted environment, and away from a high-input agricultural system, other options besides pesticide use for treating agricultural pests, such as *Drosophila* have arisen. Integrative pest management including intercropping and crop rotation are two potential mechanisms for such a solution. An example of the intercropping system is being used in the Yunnan region of China. Intercropping is the integration of various species of crops to decrease pest’s resistance build-up and maximize product yield. It has been recorded that the necessity for both fungicide and pesticide use decreased, and the yield of high-demand and high-value rice increased (Yoon 2000). Another example of this is seen in the intercropping of soybeans and sunflowers, leading to a 96% increase in sunflower yield and a 36% increase in soybean yield from original crop production (Fuente et al. 2014). Although this knowledge is becoming more common, it will take many years to move away from a high input agricultural system. In the immediate future, it would be beneficial to implement pesticide use in a less massive and more focused way. This would entail identifying the specific genes or gene families that are associated with resistance, and that are specific to particular pests, such as *Drosophila* and other insects. If the resistance mechanisms are understood and chemical control of insects is focused, the need for dumping massive quantities of pesticides into agricultural settings, risking toxin runoff and buildup decreases.
5.4. Synergists and Gene Families Implicated in Detoxification: Cytochrome P450s and Glutathione S-Transferases

I define molecular synergists as chemicals capable of inhibiting the function of enzymatic proteins derived from the gene families that are associated with toxin resistance in insects. Previously synergists have been used as insecticides, due to the detrimental effects they have in knocking down the functional products of detoxifying gene families (Feyereisen 1995). In this project, the goal of the synergists was to target enzymes associated with particular gene families in order to investigate the genetic basis for pesticide resistance in Drosophila. In the case of this experimentation, the synergists used alone do not affect fly survival (Peyser et al. 2017). Thus, if the flies’ resistance to a pesticide decreases upon introduction of the synergist to a food medium, I conclude that the function of the gene family is somehow influencing traits associated with resistance.

I consider two synergists: diethyl maleate (DEM) and piperonyl butoxide (PBO). These synergists target the proteins products of the equally large gene families Glutathione S-transferases (GSTs) and Cytochrome P450s (CYP450s) respectively. In most insect species there are approximately 80 to 120 CYP450s alone (Daborn et al. 2007). The expression of both GSTs and CYP450s have been discovered to play major roles in both toxin metabolism and toxin resistance, processes that define the trait detoxification within Drosophila both for naturally occurring and man-made toxins, such as pesticides (Feyereisen et al. 1995, Daborn et al. 2007, Ffrench-Constant et al. 2004).
Much like any model organism, the patterns and experimentation performed in *Drosophila* species can be used as an indication of how these gene families may function in other organisms. GSTs and CYP450s, in particular, are thought to metabolize potentially harmful chemicals within almost every organism’s body (Daborn et al. 2007 and Feyereisen 1995). For example, in humans CYP450s have been attributed to the catalysis of many drugs and lipophilic (lipid-loving) xenobiotics. This information is essential in unpacking and understanding the roles of drug metabolism in humans (Zanger and Schwab 2013). Once the role of CYP450s in drug metabolism is understood, the adverse effects on CYP450s that are drug induced can be altered and improved upon (Lynch and Price 2007).

It has been determined that a general upregulation of *CYP450s* and *GSTs* will increase the amount of metabolic enzyme accessible to an organism, therefore increasing detoxification rates. It is logical, then that the use of PBO and DEM to limit the function of CYP450s and GSTs respectively have been shown to decrease pesticide resistance in *Drosophila*. There has also been research to show that upregulation of *CYP450s* only occurs in the presence of harmful toxins, but that in the absence of these toxins, expression levels remain low. By evolving plasticity to environmental stimuli, the organism will decrease wasted metabolic processes and nullify the effects of harmful and counterproductive enzymes that may also be produced through mass upregulatory processes (Daborn et al. 2007). This plastic response was also observed by Willoughby et al. in their 2006 study. This study showed that as insects are confronted with naturally-occurring xenobiotics such as
phenobarbital, the upregulation of \textit{CYP450s} and \textit{GSTs} are induced in both \textit{Drosophila} larvae and adults in the tissues of the midgut, the malpighian tubules, and the gastric caeca, all of which constitute major components of the digestive tract of the fly.

While trends of regulation have proven substantive, due to the fact that these gene families are so large, it becomes difficult to generalize about their regulatory processes (Feyereisen 1995). Given that human-induced selection pressures are relatively new and generally unadapted to, there is a greater chance that resistance traits will be attributed to a single, or few genes, associated with a single or few newly arising mutations (Ffrench-Constant et al. 2004).

Whereas one \textit{CYP450} or \textit{GST} may be upregulated for resistance to a toxin, such as an insecticide, another in the same family may be downregulated. There have been a multitude of studies that attempt to focus on particular genes in these families that may be responsible for resistance behaviors. \textit{Cyp6g1}, for example, is overexpressed in various \textit{Drosophila} populations with regard to the resistance of pesticides DDT (Ffrench-Constant et al. 2004), the neonicotinoid, nitenpyram, and the insect growth regulator, dicyclanil (Daborn et al. 2007). Resistance was also observed by Bonizzoni et al. (2012) where \textit{Cyp450 CM6} was downregulated as other \textit{CYP450s} were upregulated, and when \textit{GSTE2} was upregulated, other \textit{GSTs} were downregulated. In addition to focusing on specific genes associated with resistance, the study also showed that specific gene regulation, which causes resistance in one population may inhibit resistance in another population. This proves that an entire gene family is not implemented in pesticide resistance in a unanimous manner.
The more specific the research about regulation of particular \textit{CYP450s} becomes, the easier it will be to understand molecular interactions and particular genes associated with resistance (Daborn et al. 2007).
6. Methods

6.1. Maintenance and Strains

Multiple species of *Drosophila* were used in this study. The species include the *D. melanogaster* line W1118 from the Vienna *Drosophila* Resource Center (VDRC, #60000), the genome sequenced line of *D. mauritania* (14021-0241.60), the genome sequenced line of *D. simulans* (14021-0251.195), and genome sequenced line of *D. sechellia* (14021-0428.25). All lines used in this study are easily bred and maintained in a lab environment.

Within the *melanogaster* species subgroup, controlled lab settings of the four species allowed for repeatability and reliability. The regulation and maintenance of lab settings ensures that any differences between species were due to innately organismal distinctions, not environmental ones. All species utilized in these experiments were maintained in the lab and fed the commonly used Bloomington recipe media (LabExpress, Food M). Flies were stored in a temperature and light mediated room. The temperature remained at 20°C and light settings followed a diurnal cycle of 16 hours on and 8 hours off. This cycle mimicked a typical day of light that a fly might experience in a natural environment, therefore maintaining the organisms’ innate circadian rhythm. Every two to three weeks, adult flies were transferred to new bottles of food in order to prevent overlapping generations from cohabitating the same bottles. This procedure also reduces crowding.
6.2. *Drosophila* Survival Bioassay

Flies that had been allowed to age for three to five days after eclosion from the pupal case were “knocked out” with carbon dioxide gas on a carbon dioxide fly pad. This ensured that the flies remained immobile while they were sorted according to sex.

The female flies were sorted into groups of 10 in each vial for resistance assay testing. A total of 6 vials or “reps” of 10 flies for each species in each assay were tested in the survival bioassay. This produced a total experimental sample size of \( N = 60 \) flies per treatment. Once ten female flies were sorted into each of the empty vials, they were set on their side in order to allow for fly revival after carbon dioxide exposure. After fifteen to twenty minutes, the flies were mobile once again, and the vials were set upright. The flies were then ready to be transferred onto a food medium for the survival bioassay.

In a control assay the flies were placed onto a food medium for a pre-exposure of 1 hour prior to the start of the experiment. While the flies revived, 2.5 mL of purified water was mixed with 0.75g of Instant Drosophila Medium (Formula 4-24, Carolina Biological Supply Company) in each pre-exposure vial. The food was then tamped down with a falcon tube, consolidating the mixture at the bottom of the vial. Then, the inside of the vial was wiped clean with kimwipes to ensure that no food was stuck to the vial walls. If food remains were left on the walls of the vial, flies could get stuck in the food particles and die before or during the assay. When the pre-exposure food tube was prepared, the now-revived flies were transferred into the
pre-exposure tubes, labelled with species and rep number (Ex: D. sechellia 1). The flies were left to feed on the food medium for one hour.

In a synergist assay, there is an addition of the synergist to the pre-exposure food media. For piperonyl butoxide (PBO) testing, 19.5 microliters were added to the food and water medium, creating a 0.6% concentration PBO food medium. For diethyl maleate (DEM) testing, 9.75 microliters of DEM were added to the food and water medium to produce a 0.3% concentration DEM food medium. In synergist testing, the flies were transferred onto vials with synergist food instead of onto vials containing only water and Formula 4-24 food. This exposed the flies to the synergists before they were exposed to the pesticide. The effects of the synergist in knocking down enzymatic function could begin to occur before the hour of pesticide exposure began.

After an hour either on a control food medium or a synergist medium, the flies were transferred onto food that contains either food plus the addition of a pesticide, or food plus the addition of a synergist and a pesticide. For Dibrom pesticide resistance testing a 1.1% Dibrom concentration food medium was prepared. This was done by adding 35.75 microliters of Dibrom to a food-water medium or a food-water-synergist medium. For Deltamethrin testing, 39 microliters of Deltamethrin were added to a food-water medium or a food-water-synergist medium to produce a 1.2% Deltamethrin concentration food medium. For both of these pesticide assays, the food was tamped down with a falcon tube and cleaned off the vial sides with kimwipes. For pesticide testing it is essential to use new falcon tubes for each assay. This is
because many pesticides are non-volatile, and remain on the plastic of the falcon tube cap. If there is residual pesticide on a tube that is repeatedly used, the concentration of the food may exceed the concentration intended for the assay.

Once transferred onto the pesticide food medium the hour-long bioassay began. Every five minutes the flies were tapped down onto the food. This ensured that there would be contact made between the flies and the pesticide in the food. Starting with 10 live flies, given that all 10 flies survived during the sorting and pre-exposure phases, the flies that remained alive in each vial were counted and recorded in a bioassay chart (Table 1). The number recorded in the bioassay chart in each row corresponded to the number of flies that were still alive after every five minutes in each vial. If the pesticide was successful in killing the flies, the number of live flies decreased throughout the hour. Fly “death” is considered to be the state in which the fly is no longer walking, moving, or spasming, and remains immobile on the food surface of the vial. At the end of the assay the number of flies alive at 60 minutes showed the outcome of the assay.
6.3. Statistical Methodology

In order to convert the bioassay chart into a format that could be statistically analyzed using Cox regression models (Cox 1972 and Fox 2002) for survivorship data, the data from the chart was first transformed by a Python script into the correct format for Cox-regression analysis. Instead of showing a decreasing number of flies from 10 to 0, as seen in the bioassay collection chart, the Cox-formatted chart indicated the status of each individual fly. It did this by assigning each fly in the vial a status of 0 or 1. The number 0 indicated that nothing happened to the fly, or that it remained “living”, and 1 indicated that the fly was “dead”. A simplified example of how one vial of flies would correspond to a Cox-formatted python output is seen below in Figure 11. In a standard bioassay there would be six times as much data per trial, testing 60 flies distributed in groups of 10 into in six vials, not just 10 flies in one vial.

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>Vial 1</th>
<th>Vial 2</th>
<th>Vial 3</th>
<th>Vial 4</th>
<th>Vial 5</th>
<th>Vial 6</th>
</tr>
</thead>
<tbody>
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<td></td>
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</tr>
</tbody>
</table>

Table 1: A Bioassay Collection Chart Sample
In this collection chart flies that remain alive would be counted down from 10 to 0 in each vial, or counted down until the 60 minute assay was complete. This would determine how many of the 10 flies in each vial survived a 60 minute exposure period.
After this original conversion, the data could then be analyzed in an R program that generated a Cox proportional hazards model in order to draw pairwise comparisons between different *Drosophila* species and different pesticide treatments. The goal of this test was to measure how different “hazards”, or “risks” would alter the effects of the pesticides on the flies’ resistance. The “hazards” in this project were the various synergists being used to knock down different groups of genes associated with pesticide resistance. The model compared the values of survival without any use of synergist, to the values of survival with ‘hazards’, or synergists, present. It was also effective in comparing species to one another when exposed to different treatments. This model generated two values that were used in the graphical representations of this project. The first was a $\beta$ coefficient value, which was used as
a measure of relative survival across all time points. It is used in order to plot
different Cox comparisons on the same bar graph (Ex: Results Figure 13b). This
means that the control values, or values without ‘hazards’ collected from flies tested
with pesticide alone, were the baseline for the models. They were then compared to
values collected from flies exposed to pesticides and synergists, or ‘hazards’. In order
to see how the synergists negatively affected survival rate, β coefficient values were
made negative to make graphical representations more intuitive.

The second value used for statistical analysis in this project was a P value,
which allowed us to interpret if the Cox comparison yielded a significant outcome. If
the value was significant, (P ≤ 0.05), it indicated that the hazard or synergistic effect
was substantial enough to alter pesticide resistance.
7. Results

Pesticides Deltamethrin and Dibrom were tested on *D. melanogaster*, *D. sechellia*, *D. simulans* and *D. mauritiana* independently and in conjunction with synergists piperonyl butoxide (PBO) or diethyl maleate (DEM). This testing will help to determine how the gene families associated with pesticide resistance differ in relevance among the species in the *melanogaster* species subgroup. Using survivorship curves, and calculated relative survival seen in -β values generated by the Cox proportional hazards test (CPHT), I was able to determine the effects of these synergists on each species alone. I then compared these results in order to analyze how the synergists affected different species to varying degrees. When the -β value increased in magnitude, there was a larger change in survival rates of a species in the presence of a synergist when compared to the pesticide alone. The sign of the -β value is indicative of whether survival increased (+) or decreased (-). In this section I will explain the results of Deltamethrin resistance testing in conjunction with both PBO and DEM. Then I will explain the results of Dibrom resistance testing in conjunction with both PBO and DEM.

7.1. Deltamethrin

I define a 60 minute 1.2% Deltamethrin food medium bioassay as the control assay for Deltamethrin testing. Results show that in this control trial, *D. sechellia* was the most resistant to the pesticide, followed by *D. simulans*, *D. mauritiana*, and *D.
*melanogaster* respectively (Figure 12a). The difference in resistance to Deltamethrin between species was significant for all comparisons except *D. sechellia* and *D. simulans* (Table 2). PBO and DEM have also been tested alone to ensure that the synergists being used did not have toxic effects on the species (Peyser et al. 2017). Results from Peyser et al. 2017 have been converted into survivorship curves to indicate the success of the species under synergists DEM and PBO control conditions. 100% of flies survived these assays (Figure 12b).
Figure 12: Deltamethrin, PBO and DEM Control Survivorship Curves
(a) The relative survival of *D. melanogaster* (green), *D. sechellia* (red), *D. mauritiana* (yellow) and *D. simulans* (blue) when exposed to 1.2% Deltamethrin food medium. Survivorship is measured as the mean percentage of flies alive across trials at each 5-minute interval. The trials ran for 60 minutes. (b) The relative survivorship curve of *D. melanogaster* when exposed to 0.6% PBO food medium and 0.3% DEM food medium. Survivorship is measured as the mean percentage of flies alive across trials at each 5-minute interval. The trials ran for 60 minutes.
**Table 2: Interspecies Comparison P-Values for the Deltamethrin Control Assay**

Table 2 shows the significance of interspecies comparisons for the Deltamethrin control assay. If p<0.05, we consider the difference between species compared to be significant. MEL= *D. melanogaster*, SIM= *D. simulans*, SEC= *D. sechellia*, and MAU= *D. mauritiana*. NS= Not Significant.

<table>
<thead>
<tr>
<th>Species</th>
<th>MEL/SIM</th>
<th>MEL/SEC</th>
<th>MEL/MAU</th>
<th>SEC/SIM</th>
<th>SEC/MAU</th>
<th>SIM/MAU</th>
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<tr>
<td>P-Value</td>
<td>5.60x10⁻⁹</td>
<td>1.60x10⁻⁸</td>
<td>0.012</td>
<td>0.17 (NS)</td>
<td>1.00x10⁻⁵</td>
<td>4.40x10⁻⁵</td>
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</table>

I then looked at how survivorship changed when *D. melanogaster* was exposed to DEM and PBO in the presence of 1.2% Deltamethrin in 60 minute bioassays. As previously stated, when 0.3% DEM is implemented, the gene family *Glutathione-S-transferases (GSTs)* are inhibited, and when 0.6% PBO is implemented, *Cytochrome P450s (CYP450s)* are inhibited. In comparison to the control 1.2% Deltamethrin assay, where below 40% of flies survived (Figure 13a blue), the survival of *D. melanogaster* when exposed to DEM increased to above 50% (Figure 13a, red). I can therefore infer that the presence of GSTs do not play a role in Deltamethrin resistance in *D. melanogaster* due to the fact that when they were knocked down, survival rates increased. When exposed to PBO, the survival of *D. melanogaster* decreased to below 30% survival (Figure 13a, green). I can establish that CYP450s may be necessary for Deltamethrin resistance in *D. melanogaster*. The survivorship curve results were then analyzed with a CPHT. When exposed to DEM, relative survival of *D. melanogaster* compared to the 1.2% Deltamethrin control assay increased significantly (-β=0.289, p=0.011). When exposed to PBO, relative survival of *D. melanogaster* compared to the control assay decreased, but not in a significant way (-β=-0.278 p=0.172) (Figure 13b).
Figure 13: The Relative Survival of *D. melanogaster* when exposed to Deltamethrin and DEM or PBO

(a) The relative survival of *D. melanogaster* when exposed to 1.2% Deltamethrin food medium alone (blue), 1.2% Deltamethrin + 0.6% PBO food medium (green), and 1.2% Deltamethrin + 0.3% DEM food medium (red). Survivorship is measured as the mean percentage of flies alive across trials at each 5-minute interval. The trials ran for 60 minutes. (b) A measure of the relative survival of *D. melanogaster* when exposed to 0.6% PBO + 1.2% Deltamethrin food medium, or 0.3% DEM + 1.2% Deltamethrin food medium compared to 1.2% Deltamethrin alone. Relative survival is measured as the -β value generated by the Cox proportional hazard test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.2% Deltamethrin (See Figure 13a). Error bars represent 2SD. SD was calculated by the Cox proportional hazard model.
Next I looked at how survivorship changed when *D. simulans* was exposed to DEM and PBO in the presence of 1.2% Deltamethrin. In comparison to the control 1.2% Deltamethrin assay, where 90% of *D. simulans* flies survived (Figure 14a, blue), the survival of *D. simulans* when exposed to DEM decreased to below 70% (Figure 14a, red). Thus, the presence of GSTs contribute to Deltamethrin resistance in *D. simulans* because when they were inhibited, survival rates decreased. When exposed to PBO, the survival of *D. simulans* decreased to 0% survival 50 minutes through the 60 minute assay (Figure 14a, green). This indicates that CYP450s are likely necessary for Deltamethrin resistance in *D. simulans*. The survivorship curve results were then further analyzed with a CPHT. When exposed to DEM, relative survival of *D. simulans* compared to the 1.2% Deltamethrin controls decreased significantly (-β=-0.675, p=0.0004). When exposed to PBO, relative survival of *D. simulans* compared to the control assay decreased even more significantly (-β=-4.298 p=1.50E-14) (Figure 14b).
Figure 14: The Relative Survival of *D. simulans* when exposed to Deltamethrin and DEM or PBO
(a) The relative survival of *D. simulans* when exposed to 1.2% Deltamethrin food medium alone (blue), 1.2% Deltamethrin + 0.6% PBO food medium (green), and 1.2% Deltamethrin + 0.3% DEM food medium (red). Survivorship is measured as the mean percentage of flies alive across trials at each 5-minute interval. The trials ran for 60 minutes. (b) A measure of the relative survival of *D. simulans* when exposed to 0.6% PBO + 1.2% Deltamethrin food medium, and 0.3% DEM + 1.2% Deltamethrin food medium compared to 1.2% Deltamethrin alone. Relative survival is measured as the -β value generated by the Cox proportional hazard test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.2% Deltamethrin (See Figure 14a). Error bars represent 2SD. SD was calculated by the Cox proportional hazard model.
After, I looked at how survivorship changed when *D. sechellia* was exposed to DEM and PBO in the presence of 1.2% Deltamethrin in a 60 minute bioassay. When compared to the control 1.2% Deltamethrin assay, where more than 90% of *D. sechellia* flies survived (Figure 15a, blue), the survival of *D. sechellia* when exposed to DEM decreased to below 90% (Figure 15a, red). The presence of GSTs may therefore play a role in Deltamethrin resistance in *D. sechellia* due to the fact that when they were knocked down, survival rates decreased. When exposed to PBO, the survival of *D. sechellia* decreased to below 10% survival by the end of the 60 minute assay (Figure 15a, green). This indicates that CYP450s are likely needed for Deltamethrin resistance in *D. sechellia*, due to the magnitude of change in survival relative to the control assay. The survivorship curve results were then further analyzed with a CPHT. When exposed to DEM, relative survival of *D. sechellia* compared to the 1.2% Deltamethrin control assay decreased significantly (-β=-0.614, p=0.032). When exposed to PBO, relative survival of *D. simulans* compared to the control assay decreased significantly as well (-β=-3.882 p=3.90E-13) (Figure 15b).
Figure 15: The Relative Survival of *D. sechellia* when exposed to Deltamethrin and DEM or PBO
(a) The relative survival of *D. sechellia* when exposed to 1.2% Deltamethrin food medium alone (blue), 1.2% Deltamethrin + 0.6% PBO food medium (green), and 1.2% Deltamethrin + 0.3% DEM food medium (red). Survivorship is measured as the mean percentage of flies alive across trials at each 5-minute interval. The trials ran for 60 minutes. (b) A measure of the relative survival of *D. sechellia* when exposed to 0.6% PBO + 1.2% Deltamethrin food medium, and 0.3% DEM + 1.2% Deltamethrin food medium compared to 1.2% Deltamethrin alone. Relative survival is measured as the -β value generated by the Cox proportional hazard test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.2% Deltamethrin (See Figure 15a). Error bars represent 2SD. SD was calculated by the Cox proportional hazard model.
Finally, I looked at how survivorship changed when *D. mauritiana* was exposed to DEM and PBO in the presence of 1.2% Deltamethrin bioassay. Compared to the control 1.2% Deltamethrin assay, in which 50% of *D. mauritiana* flies survived (Figure 16a, blue), the survival of *D. mauritiana* when exposed to DEM decreased to 0% by 50 minutes of the 60 minute assay (Figure 16a, red). This suggests that the presence of *GSTs* play a major role in Deltamethrin resistance in *D. mauritiana*. When they were inhibited, survival rates decreased. When exposed to PBO, the survival of *D. mauritiana* decreased to 0% survival by 55 minutes of the 60 minute assay (Figure 16a, green). This provides a strong indication that *CYP450s* are also necessary for Deltamethrin resistance in *D. mauritiana*, due to the decrease in survival compared to the control assay. The survivorship curve results were substantiated by performing a CPHT. When exposed to DEM, relative survival of *D. mauritiana* compared to the 1.2% Deltamethrin control assay decreased significantly (-\(\beta=-1.424, p<2E-16\)). When exposed to PBO, relative survival of *D. mauritiana* compared to the control assay decreased significantly, too (-\(\beta=-2.05\ p=2.20E-16\)) (Figure 16b).
Figure 16: The Relative Survival of *D. mauritiana* when exposed to Deltamethrin and DEM or PBO

(a) The relative survival of *D. mauritiana* when exposed to 1.2% Deltamethrin food medium alone (blue), 1.2% Deltamethrin + 0.6% PBO food medium (green), and 1.2% Deltamethrin + 0.3% DEM food medium (red). Survivorship is measured as the mean percentage of flies alive across trials at each 5-minute interval. The trials ran for 60 minutes. (b) A measure of the relative survival of *D. mauritiana* when exposed to 0.6% PBO + 1.2% Deltamethrin food medium, and 0.3% DEM + 1.2% Deltamethrin food medium compared to 1.2% Deltamethrin alone. Relative survival is measured as the -β value generated by the Cox proportional hazard test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.2% Deltamethrin (See Figure 16a). Error bars represent 2SD. SD was calculated by the Cox proportional hazard model.
Using the results from individual species’ bioassays, I was able to construct a bar plot that compares the relative survival (-\( \beta \)) of the four species to one another, using their control 1.2% Deltamethrin assays as a baseline for each relative survival point in each species. For 1.2% Deltamethrin + 0.6% PBO, the relative survival of *D. melanogaster* (-\( \beta \)= -0.278 p=0.172), *D. simulans* (-\( \beta \)= -4.298 p=1.50E-14), *D. sechellia* (-\( \beta \)= -3.882 p=3.90E-13) and *D. mauritiana* (-\( \beta \)= -2.05 p=2.20E-16) are included in Figure 17. I also compared species in order to determine if they were significantly different from one another when exposed to Deltamethrin and PBO. I found that *D. simulans* (CPHT, p=1.5E-11), *D. sechellia* (CPHT, p=2.60E-07) and *D. mauritiana* (CPHT, p=5.4E-10) had significantly greater reductions in resistance in response to PBO exposure than *D. melanogaster* did to the same treatment. No significant differences in Deltamethrin resistance were observed between *D. simulans* and *D. sechellia* (p=0.73), *D. sechellia* and *D. mauritiana* (p=0.82), or *D. simulans* and *D. mauritiana* (p=0.52) in response to PBO exposure (Figure 17).
Figure 17: Comparing the Relative Survival of All Four Species When Exposed to Deltamethrin and PBO
The relative survival of *D. melanogaster* (MEL), *D. simulans* (SIM), *D. sechellia* (SEC) and *D. mauritiana* (MAU) when exposed to a 1.2% Deltamethrin + 0.6% PBO food medium compared to 1.2% Deltamethrin alone. Relative survival is measured as the -β value generated by the Cox proportional hazard test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.2% Deltamethrin for each individual species. Error bars represent 2SD. SD was calculated by the Cox proportional hazard model. Brackets represent the interspecies relationship also measured by the Cox proportional hazards model. Each bracket corresponds to the two species of *Drosophila* being compared. * = p<0.05. NS = not significant, p>0.05.

I then used the results from 1.2% Deltamethrin + 0.3% DEM bioassays for all four species in order to observe the difference in relative survival between species.

Much like in the PBO bar plot, -β values generated from a CPHT, which measure relative survival of the species when exposed to Deltamethrin and DEM in comparison to a 1.2% Deltamethrin control bioassay were used to construct a Deltamethrin + DEM bar plot across all four species. The bar plots of *D. melanogaster* (-β=0.289 p=0.011), *D. simulans* (-β=-0.675 p=0.00044), *D. sechellia* (-β=-0.614 p=0.032) and *D. mauritiana* (-β=-1.424 p<2E-16) are included in Figure
Next I compared species to determine if the species were significantly different from one another when exposed to Deltamethrin and DEM. Using a CPHT to compare *D. melanogaster* to *D. simulans*, I found that there was no significant difference in the effect of DEM on Deltamethrin resistance between these species (CPHT, p=0.3). When comparing *D. melanogaster* to *D. sechellia* (CPHT, p=0.00011) and *D. melanogaster* to *D. mauritiana* (CPHT, p<2E-16), I found that both *D. sechellia* and *D. mauritiana* had greater reductions in Deltamethrin resistance in response to DEM exposure than *D. melanogaster* did. Additionally, when I compared *D. simulans* to *D. sechellia* I found these species were not significantly different (CPHT, p=0.73). Finally, I found that *D. mauritiana* had a significantly greater reduction in Deltamethrin resistance in response to DEM exposure as compared to both *D. sechellia* (CPHT, p<2E-16) and *D. simulans* (CPHT, p<2E-16) (Figure 18).
Figure 18: Comparing the Relative Survival of All Four Species When Exposed to Deltamethrin and DEM

The relative survival of *D. melanogaster* (MEL), *D. simulans* (SIM), *D. sechellia* (SEC) and *D. mauritiana* (MAU) when exposed to a 1.2% Deltamethrin + 0.3% DEM food medium compared to 1.2% Deltamethrin alone. Relative survival is measured as the \(-\beta\) value generated by the Cox proportional hazard test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.2% Deltamethrin for each individual species. Error bars represent 2SD. SD was calculated by the Cox proportional hazard model. Brackets represent the interspecies relationship also measured by the Cox proportional hazards model. Each bracket corresponds to the two species of *Drosophila* being compared. * = p<0.05. NS = not significant, p>0.05.

7.2. Dibrom

I next performed pesticide resistance assays using the pesticide Dibrom. Like for Deltamethrin, I define a 60 minute 1.1% Dibrom food medium bioassay as the control assay for Dibrom testing. Results show that in this control trial, *D. sechellia*
was the most resistant to the pesticide, followed by *D. melanogaster*, *D. mauritiana*,
and *D. simulans* respectively (Figure 19). I found the comparisons between species to
be significant between all species except *D. melanogaster* and *D. sechellia* (Table 3).
I use the same results from Peyser et al. 2017 as a synergist control to ensure that the
synergists alone have no toxic effect on the flies (Figure 12b).

![Graph](image)

**Figure 19: Dibrom Control Survivorship Curve**
The relative survival of *D. melanogaster* (green), *D. sechellia* (red), *D. mauritiana* (yellow) and *D. simulans* (blue) when exposed to 1.1% Dibrom food medium. Survivorship is measured as the mean percentage of flies alive across trials at each 5-minute interval. The trials ran for 60 minutes.

<table>
<thead>
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<th>MEL/SEC</th>
<th>MEL/MAU</th>
<th>SEC/SIM</th>
<th>SEC/MAU</th>
<th>SIM/MAU</th>
</tr>
</thead>
<tbody>
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<td>0.15 (NS)</td>
<td>0.05</td>
<td>3.20x10⁻¹²</td>
<td>0.0014</td>
<td>0.0096</td>
</tr>
</tbody>
</table>

**Table 3: Interspecies Comparison P-Values for the Dibrom Control Assay**
Table 3 shows the significance of interspecies comparisons for the Dibrom control assay. If p<0.05, we consider the comparison between species to be significant. MEL = *D. melanogaster*, SIM = *D. simulans*, SEC = *D. sechellia*, and MAU = *D. mauritiana*. NS = Not Significant.
After the control Dibrom assay, I first looked at how survivorship changed when *D. melanogaster* was exposed to DEM and PBO in the presence of 1.1% Dibrom in 60 minute bioassays. In comparison to the control 1.1% Dibrom assay, in which less than 50% of *D. melanogaster* flies survived (Figure 20a, blue), the survival of *D. melanogaster* when exposed to DEM decreased to 10% by the end of the 60 minute assay (Figure 20a, red). I can therefore infer that the presence of GSTs likely contribute to Dibrom resistance in *D. melanogaster* due to the fact that when they were inhibited, survival rates decreased. When exposed to PBO, the survival of *D. melanogaster* decreased to 0% survival at 55 minutes of the 60 minute assay (Figure 20a, green). CYP450s are also likely necessary for Dibrom resistance in *D. melanogaster*, which is seen in the decrease in survival from the control assay. The survivorship curve results were analyzed with a CPHT. When exposed to DEM, relative survival of *D. melanogaster* compared to the 1.1% Dibrom control assay decreased significantly (-\(\beta=-0.508\) p=2.00E-06). When exposed to PBO, relative survival of *D. melanogaster* compared to the control assay also significantly decreased (-\(\beta=-1.787\) p=3.30E-14) (Figure 20b).
Figure 20: The Relative Survival of *D. melanogaster* when exposed to Dibrom and DEM or PBO
(a) The relative survival of *D. melanogaster* when exposed to 1.1% Dibrom food medium alone (blue), 1.1% Dibrom + 0.6% PBO food medium (green), and 1.1% Dibrom + 0.3% DEM food medium (red). Survivorship is measured as the mean percentage of flies alive across trials at each 5-minute interval. The trials ran for 60 minutes. (b) A measure of the relative survival of *D. melanogaster* when exposed to 0.6% PBO + 1.1% Dibrom food medium, and 0.3% DEM + 1.1% Dibrom food medium compared to 1.1% Dibrom alone. Relative survival is measured as the -β value generated by the Cox proportional hazard test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.1% Dibrom (See Figure 20a). Error bars represent 2SD. SD was calculated by the Cox proportional hazard model.
Then I looked at how survivorship changed when *D. simulans* was exposed to DEM and PBO in the presence of 1.1% Dibrom. In comparison to the control assay, where 0% of *D. simulans* flies survived the 60 minute trial (Figure 21a, blue), some *D. simulans* remained living when exposed to DEM (Figure 21a, red). That being said, the rate at which the flies died when exposed to DEM was much greater than the control group. I thus interpret that the presence of GSTs may contribute to Dibrom resistance in *D. simulans*. When exposed to PBO, the survival of *D. simulans* decreased to 0% survival after only 40 minutes through the assay (Figure 21a, green). There is a strong indication that CYP450s may be necessary for Dibrom resistance in *D. simulans*, due to the rapid decrease in survival rates as seen in the control assay. I confirmed the treatment effects observed in the survivorship curve results with CPHTs. When exposed to DEM, relative survival of *D. simulans* compared to the 1.1% Dibrom control assay decreased significantly (-\(\beta=-0.3445\) p=0.00036). When exposed to PBO, relative survival of *D. simulans* compared to the control assay also significantly decreased (-\(\beta=-2.308\) p<2E-16) (Figure 21b).
Figure 21: The Relative Survival of *D. simulans* when exposed to Dibrom and DEM or PBO
(a) The relative survival of *D. simulans* when exposed to 1.1% Dibrom food medium alone (blue), 1.1% Dibrom + 0.6% PBO food medium (green), and 1.1% Dibrom + 0.3% DEM food medium (red). Survivorship is measured as the mean percentage of flies alive across trials at each 5-minute interval. The trials ran for 60 minutes. (b) A measure of the relative survival of *D. simulans* when exposed to 0.6% PBO + 1.1% Dibrom food medium, and 0.3% DEM + 1.1% Dibrom food medium compared to 1.1% Dibrom alone. Relative survival is measured as the -β value generated by the Cox proportional hazard test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.1% Dibrom (See Figure 21a). Error bars represent 2SD. SD was calculated by the Cox proportional hazard model.
Next I observed how survivorship changed when *D. sechellia* was exposed to DEM and PBO in the presence of Dibrom. In comparison to the control 1.1% Dibrom assay, where 50% of *D. sechellia* flies survived the 60 minute trial (Figure 22a, blue), the survival of *D. sechellia* when exposed to DEM decreased to 0% survival after 50 minutes (Figure 22a, red). The presence of GSTs therefore likely plays a role in Dibrom resistance in *D. sechellia* due to the fact that when inhibited, survival rates decreased compared to the control assay. When exposed to PBO, the survival of *D. sechellia* decreased to 0% survival after 55 minutes of the assay (Figure 22a, green). This indicates that CYP450s may be necessary for Dibrom resistance in *D. sechellia*. The survivorship curve results were next analyzed with a CPHTs. When exposed to DEM, relative survival of *D. sechellia* compared to the 1.1% Dibrom control assay decreased significantly (-β=-1.282 p<2E-16). When exposed to PBO, relative survival of *D. sechellia* compared to the control assay also significantly decreased (-β=-2.16 p<2E-16) (Figure 22b).
Figure 22: The Relative Survival of *D. sechellia* when exposed to Dibrom and DEM or PBO

(a) The relative survival of *D. sechellia* when exposed to 1.1% Dibrom food medium alone (blue), 1.1% Dibrom + 0.6% PBO food medium (green), and 1.1% Dibrom + 0.3% DEM food medium (red). Survivorship is measured as the mean percentage of flies alive across trials at each 5-minute interval. The trials ran for 60 minutes. (b) A measure of the relative survival of *D. sechellia* when exposed to 0.6% PBO + 1.1% Dibrom food medium, and 0.3% DEM + 1.1% Dibrom food medium compared to 1.1% Dibrom alone. Relative survival is measured as the -β value generated by the Cox proportional hazard test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.1% Dibrom (See Figure 22a). Error bars represent 2SD. SD was calculated by the Cox proportional hazard model.
Lastly, I observed how survivorship changed when \textit{D. mauritiana} was exposed to DEM and PBO in the presence of Dibrom. In comparison to the control 1.1\% Dibrom assay, in which 30\% of \textit{D. mauritiana} flies survived the 60 minute trial (Figure 23a, blue), the survival of \textit{D. mauritiana} when exposed to DEM decreased to 0\% survival after 55 minutes (Figure 23a, red). I therefore infer that the presence of \textit{GSTs} may lead to Dibrom resistance in \textit{D. mauritiana}. When exposed to PBO, the survival of \textit{D. mauritiana} decreased to 0\% survival after only 25 minutes of the assay (Figure 23a, green), indicating that \textit{CYP450s} may be necessary for Dibrom resistance in \textit{D. mauritiana}. The survivorship curve results were then further analyzed with a CPHT. When exposed to DEM, relative survival of \textit{D. mauritiana} compared to the 1.1\% Dibrom control assay decreased significantly (-\(\beta=-0.822\ p=5.4\times10^{-14}\)). When exposed to PBO, relative survival of \textit{D. mauritiana} compared to the control assay also significantly decreased (-\(\beta=-2.726\ p=4.4\times10^{-16}\)) (Figure 23b).
Figure 23: The Relative Survival of D. mauritiana when exposed to Dibrom and DEM or PBO
(a) The relative survival of D. mauritiana when exposed to 1.1% Dibrom food medium alone (blue), 1.1% Dibrom + 0.6% PBO food medium (green), and 1.1% Dibrom + 0.3% DEM food medium (red). Survivorship is measured as the mean percentage of flies alive across trials at each 5-minute interval. The trials ran for 60 minutes. (b) A measure of the relative survival of D. mauritiana when exposed to 0.6% PBO + 1.1% Dibrom food medium, and 0.3% DEM + 1.1% Dibrom food medium compared to 1.1% Dibrom alone. Relative survival is measured as the -β value generated by the Cox proportional hazard test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.1% Dibrom (See Figure 23a). Error bars represent 2SD. SD was calculated by the Cox proportional hazard model.
Once these analyses were complete, I used the results from 1.1% Dibrom + 0.3% DEM bioassays for all four species in order to observe the difference in relative survival between species. The bar plots showing the relative survival of *D. melanogaster* (CPHT, \(-\beta=-0.508\) p=2.00E-06), *D. simulans* (CPHT, \(-\beta=-0.3445\) p=0.00036), *D. sechellia* (CPHT, \(-\beta=-1.282\) p<2E-16) and *D. mauritiana* (CPHT, \(-\beta=-0.822\) p=5.40E-14) when exposed to Dibrom and DEM compared to the Dibrom control assays are included in Figure 24. I was then able to compare species to one another to determine if the species differed significantly in the effect of DEM on Dibrom resistance. Exposure to DEM lead to a greater reduction in Dibrom resistance for *D. melanogaster* than *D. simulans* (CPHT, p=7.00E-05). Exposure to DEM led to a less severe reduction in Dibrom resistance in *D. melanogaster* compared to *D. sechellia* (CPHT, p=7.40E-10) and *D. mauritiana* (CPHT, p=1.20E-13). Both *D. sechellia* (CPHT, p=0.003) and *D. simulans* (CPHT, p=0.0022) had significant differences in DEM effects on Dibrom resistance as compared to *D. mauritiana*. No significant differences were observed between *D. simulans* and *D. sechellia* (CPHT, p=0.96) when testing for the effect of DEM on Dibrom resistance (Figure 24).
If they were significantly different from one another when exposed to Dibrom and
PBO are depicted in Figure 25. Then I compared species to determine D. melanogaster
survival between species. The bar plots that compare relative survival (-\(\beta\) values) of
PBO bioassays for all four species in order to observe the difference in relative

![Graph](image)

**Figure 24: Comparing the Relative Survival of All Four Species When Exposed to Dibrom and DEM**

The relative survival of D. melanogaster (MEL), D. simulans (SIM), D. sechellia (SEC) and D. mauritiana (MAU) when exposed to a 1.1% Dibrom + 0.3% DEM food medium compared to 1.1%
Dibrom alone. Relative survival is measured as the -\(\beta\) value generated by the Cox proportional hazard
test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.1% Dibrom for
each individual species. Error bars represent 2SD. SD was calculated by the Cox proportional hazard
model. Brackets represent the interspecies relationship also measured by the Cox proportional hazards
model. Each bracket corresponds to the two species of Drosophila being compared. * = \(p<0.05\). NS =
not significant, \(p>0.05\).

To conclude testing and analysis, I used the results from 1.1% Dibrom + 0.6%
PBO bioassays for all four species in order to observe the difference in relative
survival between species. The bar plots that compare relative survival (-\(\beta\) values) of
D. melanogaster (-\(\beta\)=-1.787 \(p=3.30E-14\)), D. simulans (-\(\beta\)=-2.308 \(p<2E-16\)), D.
sechellia (-\(\beta\)=-2.16 \(p<2E-16\)) and D. mauritiana (-\(\beta\)=-2.726 \(p=4.40E-16\)) in response
to Dibrom and PBO are depicted in Figure 25. Then I compared species to determine
if the they were significantly different from one another when exposed to Dibrom and
PBO using a CPHT. This test found that *D. melanogaster* had a significantly less severe reduction in Dibrom survival in response to exposure to PBO than either *D. simulans* (CPHT, p=2.90E-14) or *D. mauritiana* (CPHT, p<2E-16). When comparing *D. melanogaster* to *D. sechellia*, the CPHT found no significant difference between these species (CPHT, p=0.7). The effect of PBO exposure on Dibrom resistance for *D. sechellia* was significantly less severe than for *D. simulans* (CPHT, p=8.50E-15) and also for *D. mauritiana* (CPHT, p<2E-16). Finally the CPHT found no significant difference in survival rate decrease between *D. simulans* and *D. mauritiana* (p=0.29) (Figure 25).
Figure 25: Comparing the Relative Survival of All Four Species When Exposed to Dibrom and PBO
The relative survival of *D. melanogaster* (MEL), *D. simulans* (SIM), *D. sechellia* (SEC) and *D. mauritiana* (MAU) when exposed to a 1.1% Dibrom + 0.6% PBO food medium compared to 1.1% Dibrom alone. Relative survival is measured as the -β value generated by the Cox proportional hazard test. The baseline (x-axis) represents the baseline hazard associated with exposure to 1.1% Dibrom for each individual species. Error bars represent 2SD. SD was calculated by the Cox proportional hazard model. Brackets represent the interspecies relationship also measured by the Cox proportional hazards model. Each bracket corresponds to the two species of *Drosophila* being compared. * = p<0.05. NS = not significant, p>0.05.

The results found in Dibrom and Deltamethrin testing will be discussed and analyzed in the Discussion and Further Directions section below.
8. Discussion and Further Directions

In this section I will describe species differences in pesticide resistance, while considering the gene families that may determine the changes in resistance patterning. In describing gene families, I will also consider how targeting entire gene families may limit the analysis of pesticide resistance, given the gene-specific nature of detoxification. Next I will contemplate further directions for the continuation of this project. This will include testing other genes and gene families that may play a role in pesticide resistance, and will also include the use of different synergists to target such gene families. The project would also involve testing survivorship on a variety of different pesticides to complete the full range of pesticide classes in testing. Lastly, I will broach the subject of environmentalism, with the hopes of informing the decision to decrease the quantity of pesticides pollutants that we put into a high-input agriculture system.

8.1. Gene Families Associated with Resistance and Their Limitations

Before approaching the gene families that contribute to pesticide resistance, it is useful to consider how the species’ resistance differs in the pesticide control assays. In Deltamethrin, for example, *D. sechellia* was the most resistant to the pesticide alone, followed by *D. simulans, D. mauritiana* and lastly, *D. melanogaster* (Figure 12a and Table 2). I used these comparative survivorship curves as a parallel to potential phylogenetic models of divergence in *Drosophila*. This pattern proposes that *D. melanogaster* is the most divergent from the other three species, a fact that has
been confirmed (Garrigan et al. 2012), and also that *D. sechellia* and *D. simulans* are the most closely related species pair. Given that *D. sechellia* and *D. simulans* are not significantly different from one another when exposed to Deltamethrin alone (p=0.17), this proposition is further substantiated. This phylogenetic model was supported by full genome analyses presented by Garrigan et al. in 2012 (See Figure 8b in Introduction).

The same phylogenetic hypothesis can be considered for the Dibrom control assay (Figure 19 and Table 3). Figure 19 showed that *D. sechellia* was the most resistant to Dibrom, followed by *D. melanogaster*, *D. mauritiana*, and finally *D. simulans*. Dibrom resistance was not as clearly correlated with a substantiated phylogenetic model. There are two hypotheses for why *D. sechellia* is the outlier in this series of curves. The first is due to potential historical environmental exposure to the pesticide. Given geographic isolation of this species, this is unlikely to be the case (Linz et al. 2013 and Stensmyr 2009). Alternatively to pesticide exposure, one can also consider the genes that have developed for noni detoxification in *D. sechellia*. As previously postulated, the same families associated with resistance to a toxin, such as OA, may be responsible for resistance to other toxins, such as pesticides (Jones et al. 1998).

The oversimplified intent behind drawing a parallel between phylogeny and general resistance to a pesticide is made more complex when particular gene families are examined. I now look at how species’ resistance change in comparison to one another when certain gene families are considered. For the pesticide Deltamethrin, I
tested how the absence of two detoxifying gene families affected resistance to pesticides in four *Drosophila* species. *Drosophila melanogaster* experienced an increase in survival when *GSTs* were knocked down, and a decrease in survival when *CYP450s* were knocked down (Figure 13). As previously stated, this suggests that perhaps *GSTs* are not related to resistance in *D. melanogaster*. That being said, it also may suggest the complexity of the gene family. While the overarching effect of the family’s inhibition may have positive effects on survival, if the inhibition process were more precise, targeting one *GST*, the effects on survival rates may differ. This has been described in experiments such as the Bonizzoni et al. 2012 study, where individual *CYP450s* and *GSTs* were regulated to test for gene-specific resistance.

*Drosophila simulans* experienced a decrease in survival when both gene families were knocked down (Figure 14), as did *D. sechellia* (Figure 15) and *D. mauritiana* (Figure 16). It would seem that both of these gene families are needed for pesticide resistance in these three species. All things considered, one must not omit the possibility that these trends may, too, be obscured by the intricacies of individual gene contributions within these large gene families (Bonizzoni et al. 2012).

I next separated the data to look at the relative effects of only one gene family in all four species. In Figure 17, for example, it is clear that *D. melanogaster* experiences the least decrease in survival upon *CYP450s* inhibition, whereas *D. simulans* experiences the most detrimental effects. *Drosophila melanogaster* is the outgroup from all three species in the *simulans* clade, which may suggest that due to species divergence, the resistance mechanisms in *D. melanogaster* are different than
those in the other three species. The species within the simulans clade are not significantly different from one another, a pattern that is consistent with the phylogenetic relationships that have been established between species.

Figure 18 looks at effects on survival when GSTs are knocked down. Like Figure 17, Figure 18 shows that *D. melanogaster* is once again the outlier of the four species. *Drosophila melanogaster* shows an increase in survival, while the other species show moderate to extreme levels of decrease in survival. The comparison between species identified them as significantly different from one another, except *D. melanogaster* and *D. simulans*. Even though GSTs present minor complications for clear associations with phylogenetic relationships of these species, as seen in the lack of significantly different relative survival between *D. melanogaster* and *D. simulans*, the general patterns observed in this figure remain consistent with Garrigan’s model shown in Figure 8b in the Introduction. *Drosophila simulans* and *D. sechellia* seem to experience toxicity to Deltamethrin to similar intermediate levels, while *D. mauritiana* appears to be the most sensitive to the toxin. In order to summarize the effects of the gene families on each species, one can take Garrigan’s phylogeny and compare how species depend on gene families for detoxification within it (Figure 26). If survival decreases upon gene family inhibition, I consider the gene family to be necessary for resistance. If survival increases upon gene family inhibition, I have chosen to omit the data point. This is only seen once in all of the data when considering the effects of GSTs on *D. melanogaster*. 
Figure 26: A Phylogeny to Consider the Gene Families Associated with a Decrease in Resistance to Deltamethrin

Figure 26 considers the phylogeny proposed by Garrigan et al. 2012 (See Figure 8b in Introduction). It then assigns the significance of both CYP450s and GSTs in Deltamethrin resistance for each species. I will define the magnitude of the -β values as indicators for how detrimental each gene family is to resistance. *= the gene family has a moderate effect on resistance (0.00< -β< |-1| ). **= the gene family has an intermediate effect on resistance (|-1|<(-β)<|2.5| ). ***= the gene family has a large effect on resistance ( |2.5| <-β≤ |5.5|). GSTs are disregarded for D. melanogaster due to the fact that resistance increases, not decreases.

Then I analyzed interspecies effects when the flies were exposed to Dibrom and the two synergists, knocking down both GSTs and CYP450s. All four species experienced a decrease in survival rate upon exposure to the two synergists in conjunction with Dibrom (See Figure 20 for D. melanogaster, Figure 21 for D. simulans, Figure 22 for D. sechellia and Figure 23 for D. mauritiana). Thus, both gene families are considered to be necessary for resistance to the pesticide Dibrom across all four species. The degree to which these gene families affect resistance in each species is described in Figures 24 and 25. Figure 24 looks at the effect of a loss of GST function across all four species, and Figure 25 looks at the effect of a loss of CYP450 function across all four species. Figure 24 shows D. sechellia to experience
the most extreme decrease in survival rate when GSTs are knocked down, followed
by *D. mauritiana*, *D. melanogaster* and finally *D. simulans*. The extremity of *D.
sechellia* in this patterning may support the fact that detoxification mechanisms in *D.
sechellia* are different than its sister species. As described previously, this may be due
to geographic isolation, bottleneck effect, and evolved noni fruit detoxification
processes (Linz et al. 2013 and Stensmyr 2009).

If one looks at the significant differences between species in Figure 24, the
phylogeny proposed above with *D. sechellia* and *D. simulans* as the least diverged of
the four species (Figure 8b in Introduction) is further supported. The difference in
decrease in survival between these two species is not significant. The difference
between all three species in the *simulans* clade from *D. melanogaster*, as well as the
difference between *D. sechellia* from *D. mauritiana*, and *D. mauritiana* from *D.
simulans* are significant. This suggests the divergence of both *D. melanogaster* away
from the *simulans* clade, as well as the divergence of *D. mauritiana* away from *D.
simulans* and *D. sechellia*.

In Figure 25 (Dibrom+PBO) there is a far less conclusive pattern of decrease
in survival between the four species. There is no dramatic difference between them,
indicating that all four species are similarly dependent on *CYP450s* for Dibrom
resistance. While there are significant differences between species, such as between
*D. melanogaster* and *D. simulans* as well as between *D. melanogaster* and *D.
mauritiana*, other comparisons such as that between *D. melanogaster* and *D. sechellia*
are not significant. Let’s consider the lack of significance between *D. melanogaster*
and *D. sechellia*. Although it is possible that similar patterns of resistance in species that exist in geographically and evolutionarily distinct regions may arise, one must also investigate the intricacies of gene expression before solidifying any sameness in resistance mechanisms. Even with less conclusive results for Dibrom, I use the same phylogeny as that investigated for Deltamethrin to visualize my data. A summary figure of this phylogeny and the effects that both gene families have on each species with regard to Dibrom resistance is included in Figure 27.

![Dibrom phylogeny](image)

**Figure 27: A phylogeny to consider the gene families associated with a decrease in resistance to Dibrom**

Figure 27 considers the phylogeny proposed by Garrigan et al. 2012 (See Figure 8b in Introduction). It then assigns the significance of both CYP450s and GSTs in Dibrom resistance for each species. Like for the Deltamethrin summary figure, I will define the magnitude of the $\beta$ values as indicators for how detrimental each gene family is to resistance. * = the gene family has a moderate effect on resistance ($0.00 < \beta < | -1 |$). ** = the gene family has an intermediate effect on resistance ($| -1 | < \beta < | -2.5 |$). *** = the gene family has a large effect on resistance ($| -2.5 | < \beta \leq | -4.5 |$).

### 8.2. Targeting Specific Genes within CYP450 and GST Gene Families

I will now consider the different ways that specific genes within a family may be targeted to specify how resistance to a pesticide arises. Three different techniques
can be implemented. One could inhibit gene expression, overexpress genes, or mutate the genes themselves, deleting them and considering the repercussions. Inhibition of specific genes via RNAi can be performed on the *D. melanogaster* transgenic RNAi line W\(^{1118}\). For this reason I have made this the *Drosophila* line used for all experimentation described thus far. It can be used as both the control line for all pesticide testing, as seen in experimentation for this thesis, and can also be used as the driver line for subsequent gene knock-down lines in future experimentation.

Using overexpression or transgenic techniques, we could determine how increases in specific gene expression may affect resistance. Lastly, mutant *Drosophila* that lack a gene associated with resistance altogether could be bred through techniques such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas9).

CRISPR-Cas9 is capable of mutating a gene in vivo, which could actively verify overexpression or knockdown techniques described. These three techniques are used in experimentation suggested below.

Within the *CYP450* and *GST* families, a number of specific genes have already been determined to be essential to detoxification. Also, it has been observed that the same target gene may be implicated in detoxification of a variety of different pesticides. Because the families are so large, it would be beneficial to begin research of specific genes using loci that have already been identified for pesticide resistance. One can see a few examples of this is past literature. Within the *CYP450* family, *Cyp6g1* has been overexpressed in *Drosophila* resistance testing, causing DDT resistance, and the neonicotinoid, nitenpyram resistance (Ffrench-Constant et al.
2004, Daborn et al. 2007, Dworkin and Jones 2009). Therefore, Cyp6g1 could also be overexpressed to test for resistance capacities in relation to Deltamethrin and Dibrom. Another gene to test is Cyp450 CM6. Cyp450 CM6, has been shown to be downregulated in both Deltamethrin and Permethrin-resistant mosquitoes. Using RNAi, Cyp450 CM6 could also be investigated for its resistance role in relation to Dibrom and Deltamethrin. A third example lies within the GST family. One might consider GSTE2, found to be upregulated or overexpressed in Permethrin-resistant mosquitoes (Bonizzoni et al. 2012). Using over-expression techniques, GSTE2 could also be tested for Deltamethrin and Dibrom resistance.

After these experiments, CRISPR-Cas9 could be used to mutate and or delete the gene in question to confirm the role of inhibition or over-expression of the genes. In cases of over-expression like GSTE2, deleting the gene and observing the effects on resistance could confirm or reject the up-regulatory role this gene has in detoxification. If deletion decreased resistance, overexpression of GSTE2 would be supported. Then we could look at a case where a gene is inhibited, such as CYP450 CM6. If after deletion by CRISPR-Cas9 resistance remained high, the role of downregulation of the gene could be confirmed. After considering candidate genes like these, one could move on to test other specific genes, further specifying the genetic basis for resistance to the pesticides Dibrom and Deltamethrin.

8.3. Considering Other Gene Families

There is no doubt that resistance as a trait is complex. In order to explore the full range of genes and families associated with resistance, one must look to other
gene families besides \textit{CYP450s} and \textit{GSTs}. There has been substantial research done on the gene family of \textit{esterases} on its own and in conjunction with both \textit{GSTs} and \textit{CYP450s} in relation to pesticide resistance (Feyereisen et al. 1995, Ffrench-Constant et al. 2004, Willoughby et al. 2006, Daborn et al. 2007). The synergist tribufos directly targets this gene family (National Institute of Health 2018). This would be a logical and tangible next step in this project in order to determine the role of the last major gene family identified as being involved in pesticide resistance.

Outside of families directly associated with pesticide resistance, chemoreceptive gene families such as \textit{Odorant Receptors (Ors)} or \textit{Gustatory Receptors (Grs)}, would also be reasonable gene families to investigate. Given their role in noni detoxification in \textit{D. sechellia}, it would be beneficial to see if they also had a function in detoxifying human-produced xenobiotics such as pesticides. One could first target \textit{Ors} and \textit{Grs} as entire gene families. By performing similar experiments to the ones already done on \textit{CYP450s} and \textit{GSTs}, it would be possible to observe if these gene families affect resistance to pesticides. Because these genes are so essential for food recognition in insects, inhibiting their function may be too detrimental. Perhaps it would be more viable to use the RNAi system discussed above to target specific \textit{Ors} and \textit{Grs} needed for detoxification. For example, one could target genes within subfamily E of the \textit{Grs} to look into how a family that is involved in putative bitter tasting in \textit{D. sechellia} (McBride and Arguello 2007) may constitute some of the genes associated with sensing toxins in pesticides.
8.4. Considering Other Pesticides

In addition to looking at other gene families associated with resistance in these two pesticides, it would also be helpful to look at the way *Drosophila* respond to *CYP450* and *GST* inhibition when exposed to other pesticides. This may help to answer the questions about the globality of the role of these gene families in pesticide resistance. In the future, I hope that this project continues to look at pesticides ranging throughout all pesticide classes. The lab plans to look at the neonicotinoid Imidacloprid, which has proved to be so harmful to insect species such as the honeybee (Lu et al. 2014), and Permethrin, another pyrethroid, which would be useful to see how it compares to results found in Deltamethrin, a pesticide of the same class. It should not go without mention that the use of DDT testing in this thesis was attempted, but it was impossible to reach high enough concentrations of the pesticide in the *Drosophila* food medium for any death in *Drosophila* species to be induced. The species proved to be too resistant. These examples are just the start of extensive pesticide testing. From this point, one could also look at pesticides in classes such as ivermectins, carbamates and insect growth regulators.

8.5. A Goal for Pesticide Use in the Future

The goal of this research was to establish a way to elaborate on our knowledge of resistance mechanisms in the model species *Drosophila*. Using this model species we can then strive to describe phenomena that may represent a more general mechanism seen across many other insects and vertebrate species. In understanding the molecular basis of pesticide resistance, we can thus grow to be far more precise
with our approaches to pest management. Instead of using broad-spectrum chemicals, a pesticide could be engineered to be far more specific to the pest it intends to target. If pointed and precise, the amount of pesticide used could hypothetically be reduced, also diminishing the effects on other species as well as the emission of toxins into the environment. A model such as this one would favor farmers and environmentalists alike.

By looking into the gene families that are in part responsible for pesticide resistance, we can consider ways to broaden our knowledge of these resistance mechanisms. We look to future experimentation with the hopes of being able to to expand our consideration of gene families and genes in particular associated with pesticide resistance. Simultaneously, our understanding of the field of integrative genetics and environmentalism can only stand to grow. A more complete, inclusive and considerate picture of how to control our agricultural system is on the rise.
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