ABSTRACT

CHARACTERIZATION OF ORGANIC CHALCONE 17 RESISTANT *CAENORHABDITIS ELEGANS* MUTANT

Plant Parasitic Nematodes (PPNs) are a challenging problem in today's agriculture causing an annual loss of about $157 billion (Li et al., 2015). Until recently, methyl bromide was the most used agent for controlling PPNs. However, methyl bromide is now not preferred because of its high toxicity (Li et al., 2015). This establishes a problem by losing a way to control these PPNs. In order to effectively kill these PPNs, Dr. Calderón-Urrea's laboratory used *Caenorhabditis elegans* (*C. elegans*) as a model organism and identified certain chalcones as effective nematicidal agents (Attar, 2011). Chalcones are organic chemical compounds found to be effective in killing the reference strain used in the ACU laboratory (PD4251) of *C. elegans* (Attar, 2011). For the purposes of this thesis the PD4251 strain, which contains the green fluorescence protein (GFP), is noted as the reference strain. Currently the lab has found three chalcones, **17**, **25**, and **30**, that are effective in killing the reference strain, and although the mechanism behind the chalcones' mode of action is unknown, research in the lab suggests these three chalcones may target different biochemical pathways in the nematode.

Previously, Dr. Calderón-Urrea's laboratory identified a mutant strain, 17.1.2, of *C. elegans* that is resistant to chalcone **17** (Tamayo, 2016). The current research aims to further characterize this mutation using fluorescence staining techniques, coordination ability by using the WormTracker software analyses, genetic mapping, and further genetic screenings to identify additional chalcone mutants. The data shows the mutant having a significant difference in movement, speed, and distance traveled when compared to the reference strain

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CHARACTERIZATION OF ORGANIC CHALCONE 17 RESISTANT *CAENORHABDITIS ELEGANS* MUTANT

by

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INTRODUCTION

Background

Agriculture faces many problems while trying to produce quality product; one of the main challenges is management of plant parasitic nematodes (PPNs). PPNs cause monetary loss worldwide by damaging living plant cells. This has been a significant issue due to the presence of a common parasite known as *Meloidogyne incognita,* which is known to cause infection in more than 2,000 plant species. *M. incognita* is a root knot nematode and is a parasite that causes major concern in agriculture (Li *et al*., 2015). These worms feed on the cytoplasm of living plant root cells and ultimately make plants incapable of producing fruit (Abad *et al*., 2008). Until recently, methyl bromide was the most commonly used agent for controlling PPNs. However, methyl bromide is now not preferred because of its high toxicity. It has been widely demonstrated as being harmful to the environment (Earth's ozone layer) and to people (Li *et al*., 2015). This establishes a problem by losing an effective way to control these plant parasitic nematodes. As farmers, and those in the food industry, try to grow and sell efficient product they are constantly facing economic loss because of the damage that these nematodes are causing. The infection begins in the soil and seeps its way to the crops and eventually to the human population. Now, an easy solution might be to spray the crops with nematicides (pesticides for the purpose of killing nematodes). However, it has been proven through previous studies that these nematodes are growing resistant to many of the common nematicides used around the world. It was found that not only are they resistant for each class of compound used in these nematicides, but some species are even multi-compound resistant (Burns, 2015). These nematicides that are currently available on the market are

found in different forms including fumigants, organophosphates, and carbamates (Gowen, 1995). These chemicals are depicted in Table 1 below. Although these have been commonly used around the world, each class of chemicals has a huge disadvantage linked to their use. Fumigants perform best but are the most toxic class of chemicals (Gowen, 1995). These chemicals are extremely volatile and expose toxicity to the soil and to the environment. Many fumigants are already banned in multiple countries due to the danger they introduce to society. Non-volatile nematicides like organophosphates and carbamates are applied on the surface of the soil. For this reason, they are not as effective as fumigants because they can't reach the root of the crops, where the nematodes are found. Although they are not as effective these compounds are still just as toxic. Its toxic degradation products seeps into the soil and is absorbed by organic matter (Gowen, 1995). Since these are less effective, increased concentrations and prolonged treatment is essential in trying to successfully eradicate the nematodes presence within the soil. However, as farmers increase exposure to these types of nematicides, they not only increase risk to the environment, but they also increase the risk of resistance as the PPNs are exposed to only the residual amounts that make it deep within the soil (Gowen, 1995). Resistance to nematicides is not well characterized and data is insignificant in trying to pinpoint a broad mechanism (Chitwood, 2003). Studies observing *Meloidogyne naasi, G. rostochiensis,* and *Pratylenchus crenatus* exposed to aldicarb for 15 years found species specific differences among the nematodes (Moens & Hendrickx, 1998). In another study, *Rhabditis oxycerca* was bred for 400 generations and two mutant strains resistant to aldicarb and oxamyl were characterized compared to the wild type. The mutants were characterized by their decreased size, tolerance of warm temperatures, and their ability to reproduce when exposed to the chemicals (Kampfe & Schutz,

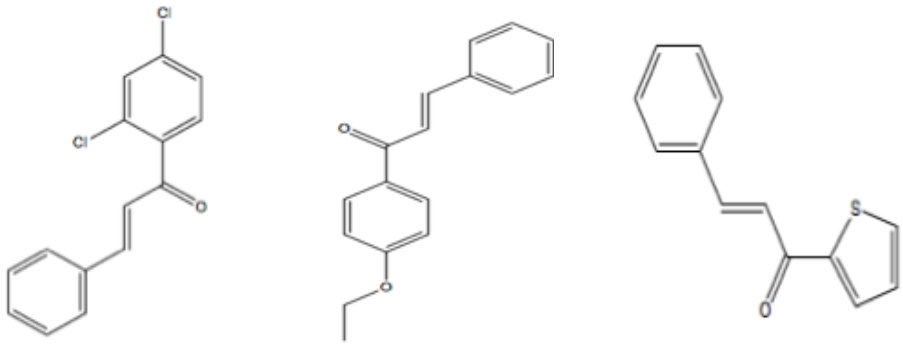
1995). In a third study, *Heterorhabditis bacteriophora* was seen to have resistance towards fenamiphos, avermectin, and oxamyl (Glazer, Salame, & Segal, 1997). Although extensive data has been collected towards resistance among PPNs, the mechanism of how these nematodes are building resistance is still unclear, especially because each species reacts differently. This growing resistance and the risks associated with using nematicides puts farmers and agriculture in a tough position as they keep losing methods of control. Pesticides are commonly used incorrectly and with poor management, resulting in poisoning and increased mortality in many countries (Kottegoda, 1985). 1,3-Dibromochloropropane was officially banned in the United States when one third of the male workers in a manufacturing plant became sterile due to exposure to this chemical (Chitwood, 2003). Most of these chemicals listed, dichloropropene, methyl bromide, chloropicrin, dazomet, metam sodium, methyl isothiocyanate, have all proven to be human carcinogens (Chitwood, 2003). However, even with all these risks, the remaining unbanned chemicals (Table 1) are the current way of controlling PPNs. Future developments to regulate these PPNs is extremely dependent on research. Nematologists are focused on understanding how the nematodes life cycles may be interrupted, how the resistance to these compounds is being manifested, and if there is a way to control these PPNs without introducing a high risk of toxicity to the environment and its surrounding organisms.

In order to find a new way to effectively kill these PPNs, Dr. Calderón-Urrea's laboratory used *Caenorhabditis elegans* (*C. elegan*s) as a model organism to identify certain organic chalcones as effective nematicidal agents (Attar, 2011). *C. elegans* is a model organism to test in these experiments due to its similarity to *M. incognita*, its size, reproduction rate, well known genetics, and life cycle characteristics. *C. elegans*, although not a PPN, is crucial to this experiment

Table 1: Currently available chemicals, their corresponding trade names and formulation (Chitwood, 2003).

Chemical name	**Trade name**	**Formulation**
Fumigants		
Methyl bromide	Dowfume	Gas
1,3 dichloropropene	Telone/DD-95	Liquid
Ethylene dibromide[1]	Dowfume W-85	Liquid
Metam-sodium	Vapam	Liquid
Dazomet	Basamid	Dust (prill)
Methyl isothiocyanate	Di-Trapex	Liquid
Chloropicrin[1]	Larvacide	Liquid
Organophosphates		
Thionazin	Nemafos	Granular or emulsifiable liquid
Ethoprophos	Mocap	Granular or emulsifiable liquid
Fenamiphos	Nemacur	Granular or emulsifiable liquid
Fensulfothion	Dasanit	Granular
Terbufos	Counter	Granular
Isazofos	Miral	Granular or emulsifiable liquid
Ebufos	Rugby	Granular or emulsifiable liquid
Carbamates		
Aldicarb	Temik	Granular
Aldoxycarb	Standak	Flowable
OxamyI	Vydate	Granular or emulsifiable liquid
Carbofuran	Furadan/Curaterr	Granular or flowable
Cleothocarb	Lance	Granular

and research as it lays a foundation for our methods. Two previous experiments in Dr. Alejandro Calderón-Urrea's laboratory found promising results in similarly killing both *C. elegans* and *M. incognita* (Singh, 2013). Three organic chalcones **17**, **25**, and **30** represented in Figure 1, have been proven to effectively kill the reference strain used in this laboratory. Chalcones are formed by aromatic ketones and an enone that makes the basis of this molecule. There are many derivatives of this molecule, and that family is collectively known as chalcones. Their molecular structures can be observed in Figure 1.



Chalcone **17**	Chalcone **25**	Chalcone **30**
$C_{15}H_{10}Cl_2O$	$C_{17}H_{16}O_2$	$C_{13}H_{10}SO$
MW= 277.15 g/mol	MW= 252.33 g/mol	MW= 214.29 g/mol

Figure 1: Structures of the organic chalcones used in the laboratory.

Additional work in Dr. Alejandro Calderón-Urrea's laboratory generated and identified mutant strains of *C. elegans* that were resistant to organic chalcones (Tamayo, 2016). Although chalcones are organic chemical compounds that are found to be effective in killing the reference strain of *C. elegans* in the lab (PD4251), the mechanism behind this resistance is unknown. This reference strain is important because it contains a green fluorescent protein (GFP) gene that can be

seen in all body wall and vulval muscles. This is useful to us because it helps us identify when a worm has died because it would also lose its ability to fluoresce. *C. elegans* were synchronized (performed to ensure that all nematodes are at the same life stage when mutagenesis is performed) and then mutated using the mutagen EMS [0.2 M EMS (ethyl methane sulphonate)], which induces random heritable nucleotide mutations (such as point mutations, missense, nonsense, insertions, or frame shift mutations) (Milligan, *et al.*, 1998). This is how the 17.1.2 (chalcone **17** resistant mutant) was obtained. Out of the fifteen strains that were found to survive against these chalcones, only this one strain remained resistant to organic chalcone **17**. This was kept on Nematode Growth Media (NGM) plates and kept alive by seeding these plates with OP50 an *Escherichia coli s*train. This mutant is said to be resistant because when they are plated on the chalcone **17** containing plates instead of dying like the reference strain, they survive and reproduce.

We now have this mutant and are keeping the population growing in order to characterize the mutation that has been observed. Dominance/recessive tests were also done on these strains against the reference strain of *C. elegans* used in our lab. It was concluded that the mutant strain for chalcone **17** (17.1.2) was due to a recessive gene (Tamayo, 2016).

Project Rationale

In order to characterize the recessive mutant strain that the lab generated, we used fluorescent staining techniques on these nematodes using DAPI and Hoechst 33258 dyes. DAPI has a molecular weight of 277.33 g/mol, which is very close to chalcone **17** (277.15 g/mol). Hoechst 33258 is larger with a molecular weight of 533.88 g/mol which sparked curiosity on whether this molecule with

greater mass could make it into the worm. Hoechst 33258 also has an ethyl group, which is important because it makes the molecule lipophilic, which helps cross membranes that are still intact (Larsson, Akerman, & Jonsson, 1995). Both dyes are soluble in water and work similarly in attaching to DNA at the minor groove of the DNA double helix (Larsson, Akerman, & Jonsson, 1995). DAPI passes through disturbed cell membranes and stains the nucleus, whereas Hoecsht is used to demonstrate the ability to stain the nucleus even when the membrane is not disrupted. Chalcones, on the other hand, are not soluble in water. They are soluble in DMSO, chloroform, ether, benzene, and ethanol. This is why when first obtaining the chalcone we dissolve it in dimethyl sulfoxide (DMSO) and ethanol. If DAPI and Hoecsht are able to stain the nuclei of the nematodes, and make it into the body of the worm, then it might be possible that the organic chalcones are also making their way into these mutants. This suggests that the mutation could possibly not be a problem of uptake of the molecule, but rather a resistance to the chemical even when it is present inside the nematode.

We observed and studied the movement of this mutant by measuring the number of body bends per second, and also recording the rates of these movements through speed and distance traveled using the WormTracker plugin (Nussbaum-Krammer et al., 2015) through the ImageJ 1.x software (Schneider et al., 2012). This helped distinguish differences and similarities seen between the mutant strain and the reference strain used in the lab. If movement differences are statistically significant between these two strains, then we can possibly infer that there is a motor neuron effect caused by exposure to these chalcones. We also tested whether this mutant is resistant to chalcone **25** or **30**. This test is essential in showing that although these organic chalcones are similar in effectively killing the reference strain of *C. elegans,* we believe and prove their pathways are different. If

our hypothesis is true, then the three chalcones act independently of each other, and the mutants that are resistant to chalcone **17,** will not be able to resist exposure to **25** or **30**. We also tried to genetically map this mutation by crossing our mutant strain with a strain that contains three different known phenotypic mutations (KK1) obtained from the University California of Davis in collaboration with Dr. Lesilee Rose. This strain contains dumpy, long, and blistered worms which serve as visible markers for our genetic crosses. We were also given an additional strain (KK75) which contains the dumpy, long, and uncoordinated mutations. We were able to cross with all the markers included in KK1 which include chromosomes 1-3 but were not able to run any crosses with KK75 yet. Crosses done with the KK75 strain can be included in future work for this experiment so that chromosomes 4-6 can be tested as well. These strains are essential because these mutations cause distinct phenotypic changes that makes crossing easier for us to observe and track. Before the crosses were started, we needed to obtain males through a series of heat shocks. Naturally, *C. elegans* have two sexes: males and hermaphrodites. Males occur naturally with a ratio of 1/1000, while hermaphrodites make up the majority of each generation (Horvitz, 1997). These hermaphrodites can self-fertilize by making identical copies of themselves. Heat shock procedures increase the rate of nondisjunction during gamete formation and increase the chances of producing males. This procedure was crucial to our experiment, since mapping and genetic crosses cannot be done without males. Once these crosses were conducted, the F_2 generations were tested on chalcone plates containing chalcone **17**. Later, tests and analyses of lethality in the offspring were supposed to depict a certain probability of survivors.

In addition to characterization of this recessive mutant (17.1.2), we expanded the amount of mutagenesis screens done so that we can identify more

mutants. We followed previous protocol used in the lab with ethyl methanesulfonate (EMS) as the mutagenizing agent (Tamayo, 2016). By running more screens, we increased the chances of obtaining more mutants against each organic chemical and ultimately more characterization of mutations, which would support the hypothetical pathways introduced. Since genetic screens often examine tens of thousands of individual nematodes, optimizing the genetic screen is important for maximizing the number of mutagenized gametes examined. A general algorithm for optimizing genetic screens in *C. elegans* has been developed and can be used for large-scale screens (Shaham, 2007). This algorithm is based on a few parameters: the desired certainty of obtaining one mutant, the mutagenizing agent, and whether or not balancers are used. Sequential screens look at phenotypes over multiple generations since there might be difficulty in observing the phenotype in the first generation. To make genetic screens more applicable, it is often possible to score a more easily detected phenotype, such as lethality or uncoordinated movement (primary screen), which allows isolation of mutations. The specific mutations are then identified through a secondary screen (Huang and Sternberg, 2006). Since we are looking for a specific phenotype (resistance to chalcones), we tested the mutagenized worms on chalcone containing plates and used a selection screen in our experiment to pick those worms that survive when exposed to the chemicals.

Mapping and identification of the implicated DNA sequences will eventually be conducted in a single step by using Whole Genome Sequencing (WGS) and single nucleotide polymorphism (SNP) mapping strategies (Doitsidou *et al.*, 2010). We hope to eventually locate the single mutation, which causes this chalcone resistance.

Gaps in Current Knowledge

The main gap that exists for this research topic is identifying the pathway of resistance in these nematodes. Our hypothesis seeks to test the possible molecular mechanism of these chalcones. However, there could also be a complete different molecular pathway that targets a specific enzyme which we are not testing in this experiment. The source of this resistance can vary on multiple possible explanations. Through this first step in identifying the mechanism behind this resistance, we hoped to get closer for finding the reason, even if it is in the form of eliminating a possibility. The literature and previous research suggests that the best way to control plant parasitic nematodes is actually to couple different chemicals (Huang et al., 2014). Current research in the ACU laboratory aims to demonstrate this as well. Different combinations of all three of these chalcones (**17**, **25**, and **30**) are being tested in order to find the most effective combination for killing the reference strain of *C. elegans*. If an effective treatment is found, this can be a harmless alternative for replacing methyl bromide in the control of PPNs. However, because nematodes have the ability to adapt and become resistant to chemicals, it is still essential to find out the pathway of this resistance. This would ultimately lead to a much more efficient control method.

The research done in the ACU lab seeks to identify the molecular target(s) of these chalcones. This research project is specifically focused on organic chalcone **17**, since that is what the obtained mutant is resistant to. Once this pathway is found, more mutagenesis protocols and screens need to be done in order to generate mutants for organic chalcones **25** and **30**. The importance of these screens is paramount in order to build a solid foundation of data to back up our proposed mechanism of resistance once it is found. It would be very beneficial to generate more mutants resistant to chalcone **17**, in order to gather data in

support of the already generated mutant (17.1.2). Ideally, we would love to determine the molecular pathways for chalcones **17**, **25**, and **30**.

Importance of This Study

The problem of PPNs suggests that agriculturalists need a new method of control, especially as the monetary loss is becoming more prominent because methyl bromide has become completely useless (Luken and Grof, 2006). When agriculturalists purchase controls, they need to know as much background information as possible, so they can decide if it is a product their crops can use. The type of crop grown is specific to the PPNs that infect them. Some main areas that need to be known are: how the control works, how effective it is, whether or not it will harm any other soil microorganisms, and particularly if it has the potential to affect human or environmental health. This project could address this need by extending our knowledge of the mechanism of action of organic chalcones. This is why we hope to get closer to identifying the molecular targets of chalcones. By characterizing mutant 17.1.2 and generating more mutants to the rest of the chalcones used in the laboratory we believe we can achieve this goal. By understanding the chalcone pathway in *C. elegans*, future work can discover an optimal cocktail of organic chalcones for PPN control. This work is very unique because no other researchers are studying these novel organic molecules for PPN control. The importance of this research can be seen in the long-term goal, which is to develop an effective and efficient form of PPN control and thereby significantly lower the monetary loss that agriculturalists experience. Achieving the proposed research objectives will further our knowledge of how *C. elegans*, and likely also PPNs, are affected by chalcones, by running our characteristic analyses through the WormTracker plugin (Nussbaum-Krammer et al., 2015). This

project will help us become one step closer to identifying the molecular mechanism of the organic chalcones by first focusing on chalcone **17**.

HYPOTHESIS AND OBJECTIVES

Previous work from the Calderón-Urrea laboratory suggests that chalcones **17** and **25** may work to affect different biochemical pathways in the nematode. First, the effects of each chalcone are different in either *C. elegans* or the plant parasitic nematode *M. incognita* (Singh, 2013). Second, they show synergistic effects when used in combination to kill *C. elegans* and *M. incognita*. Third, chalcone **25** is more efficient in killing the worms at a later stage of development when compared to chalcone **17** (Singh, 2013). Furthermore, results from this early work also suggests that both chalcone **17** and **25** induce death in *C. elegans* by a different pathway other than the apoptotic programmed cell death since *ced-3* and *ced-4* mutant nematodes die at the same rate as wild-type nematodes (Singh, 2013). With the addition of chalcone **30**, we have found that this chemical is even more efficient at killing the reference strain of *C. elegans* at even lower concentrations, when compared to the other two chalcones. However, these possible different and unique pathways are still not known. For these reasons, our working hypothesis is that "chalcone **17, 25,** and **30** affect independent pathways in *C. elegans,* and the effect of these chalcones on the mutant strain cause significant changes in behavior when compared to the reference strain (PD4251)". Before we can address this hypothesis, there are outstanding questions that we must answer first. These include: do chalcones kill the worm after they enter the body, or is it a problem of uptake of the chalcone molecule? Is this pathway linked to a genetic mutation? Is it possible to map where this mutation takes place? Are the resistant mutants affected by exposure to these organic chalcones, and if so, how? We propose the following aims in order to get closer to test our hypothesis and the outstanding questions:

1) Stain mutants with dyes of similar molecular size to chalcone **17**.

2) Determine whether chalcone **17** resistant nematodes are also resistant to chalcone **25,** and **30.**
3) Run WormTracker characteristic analyses to compare movement, speed, and distance traveled between the resistant mutant strain (17.1.2) and the reference strain.
4) Genetically map this mutation by making males through heat shock procedure, and then crossing with the supplied mapping strains KK1 obtained by Dr. Lesilee Rose.

METHODOLOGY

Preparation of Chalcones and Plates

Two organic chalcones **17** and **25** were originally used as the primary chalcones until the addition of the synthesis of chalcone **30**. These compounds were synthesized by our collaborator, Dr. Saeed Attar, and his laboratory. The chalcones were proven to kill *C. elegans* with 100% lethality at the 10^{-4} M concentration (Tamayo, 2016). This is the concentration that was used to dilute the chalcones and ultimately make chalcone containing plates, in order to test both the mutant and reference strain for our experiment. The reference strain used in this experiment was PD4251, which contains a GFP protein in all body wall and vulval muscles. This strain was obtained by purchase from Caenorhabditis Genetic Center (Tamayo, 2016). Previous work in the laboratory has outlined the proper protocol for how to properly dilute these compounds using chalcone **25** as an example (Tamayo, 2016). A quick description of the process is first weighing out the molecular weight of the desired chalcone, then adding 1mL of DMSO (dimethyl sulfoxide) in order to achieve 10^{-1} molar concentration. The following step dissolves that solution in 900 μl of 100% ethanol since the chalcones are soluble in these solutions. Then, that solution is dissolved in 900 μl of 50% ethanol, and finally placed in the nematode growth media (NGM) agar after it has already been autoclaved and the reagents have been added. The chalcone solution is added before the agar has solidified and been poured into plates, in order to achieve the desired chalcone concentration of 10^{-4} M. The NGM agar was made with the following protocol before being autoclaved for an 800 mL solution: 2.4 g of NaCl, 13.6 g of difco agar, and 10 g of peptone. The reagents added after the solution is autoclaved were: 20 mL of 1M KPO_4, 800 μl of 1M $CaCl_2$, 800 μl of

1M $MgSO_4$, 800 μl of 5 mg/mL concentration of cholesterol, and 800 μl of 2 mg/mL concentration of uracil. This NGM agar was poured in 100 mm sterile plates in order to grow and maintain the reference strain (PD4251) and the 17.1.2 (mutant) strain separately. These NGM plates were seeded with the *Escherichia coli* strain OP50, which served as the food source for the worms. Two spots of 100 μl of OP50 were placed on each of the 100 mm NGM plates. These plates were kept in a 25° C incubator, and the worms were then chunked (moving a small piece of agar with a populated number of worms onto a new 100 mm seeded plate) using sterile spatulas in order to keep the population from starving or becoming contaminated. Chunking or picking worms from a populated, starved plate onto a new plate was done every week, never exceeding seven days. When the experiments called for chalcone plates for tests of resistance or lethality, the dilution protocol was used as the reference, and the NGM agar (with all reagents previously added) was used as the final 10^{-4} M solution. This means that the 10^{-3} solution prepared with 50% ethanol, was then added to the NGM agar in order to obtain the needed concentration.

DAPI and Hoechst 33258

In order to successfully stain the isolated mutant in addition to the reference strain as our control, we followed established staining protocols (Shaham, 2006). Before introducing the dyes to the worms, the NGM populated plates that were maintained weekly were washed. The plates were washed with M9 buffer, which for a 1L stock consists of the following reagents: 3 g of KH_2PO_4, 6.0 g of Na_2HPO_4, 5.0 g of NaCl, and 1mL 1M $MgSO_4$ (Stiernagle, 1999). After this solution is autoclaved, 2 mL of M9 was used to wash off separate plates of 17.1.2 and the reference strain and then placed in 1.5 mL microcentrifuge tubes. The

tubes were left in room temperature until a pellet had been formed through gravity, the supernatant was removed and 200 µl of M9 was placed back in each of the tubes. After this was done the tubes were introduced to the specific dyes. 17.1.2 was stained with DAPI and Hoechst, and another tube was just left unstained. Same procedure was done with the reference strain as well. The final concentration of the fluorescent dyes that was added to the tubes was the following: Hoechst 33258 at 1 µg/1000 µl, and for DAPI a concentration of 100 ng/1mL. In order to obtain this concentration, proper procedure and staining protocols were followed (Shaham, 2006). For the Hoechst 33258 (Sigma) dye, a concentration of 1 µg/1000 µl was made from the frozen stock solution. In order to do this, the frozen stock solution was diluted. 1µg of dye into 1 mL of dimethyl sulfoxide (DMSO). For the DAPI (4',6-diamidino-2-phenylindole) dye, a concentration of 100 ng/1 mL was made in phosphate buffered saline (PBS). PBS is made with the following: 80 g of NaCl, 2 g KCl, 6.1 g anhydrous Na_2HPO_4, 2 g KH_2PO_4. 1 L of H_2O. This was then autoclaved and sterilized in order to use it for diluting the stock. To make the final concentration, the DAPI stock powder was dissolved in 70% ethanol to a concentration of 100 µg/mL. From that, it was diluted further in PBS to obtain the final concentration of 100 ng/mL.

After the desired concentrations for both fluorescent dyes were made and observed that the worms had settled and formed a pellet, the next step in staining was prepared. Prior to the staining, the worms were anesthetized with Levamisole. This presents the worms in a resting state after the staining is complete, so that it is easier to take pictures using fluorescence microscopy. The tubes were then stained with the desired concentration of each type of dye. The tubes then incubated in the dark for fifteen minutes with very gentle agitation, in order to complete the staining process. While these incubated, 1g of agarose was diluted in 50 mL H2O,

to make a 2% solution for agarose pads used to prepare the microscope slides. After the fifteen minutes of staining, the worms were washed with M9 three times to ensure the removal of any excess dye on the outside of the worm. Since the pellet is so small, 200 µl of M9 was used for each of the washes. Then same procedure was conducted, waiting for the pellet to settle then removing the supernatant, and then repeating the wash two more times for each of the tubes. When this was done, 40 µl of the stained worm pellet was transferred to the agarose pads (70 µl placed on each slide) that were dried on the microscope slide. A slide cover with a bit of Vaseline on the edges was then placed on the pads with the worms so that it would keep the worms and solution in place. The worms were then observed under a Leica fluorescent microscope, using the designated filters (FITC filter sets) to visualize both DAPI and Hoechst. Controls did not have any stains in their designated tubes. This was shown with their expression of their green fluorescent protein (GFP) which is incorporated in the reference strain. If the fluorescence is seen inside the worm (could be staining nerves or nuclei), then the conclusion can be made that they are not uptake mutants. The reference strain was also stained to see if the dyes were making their way into those worms as well. This served as the control, so that if the mutants were unable to uptake the dye, then perhaps the chalcone resistance mutation had changed this ability. This possibility can be seen by the fact that the organic chalcone **17** is of the same size as the fluorescent dye DAPI. DAPI has a molecular weight of 277.33 g/mol, which is very close to chalcone **17** (277.15 g/mol). Hoechst 33258 is a bit larger because of its ethyl group with a molecular weight of 533.88 g/mol. Hoecsht is significant because it can be used on live and fixed samples, as if often used as a substitute for DAPI as well. This is also very useful to our project because it shows that it is possible that these worms are actually taking up and ingesting the chalcone, which

suggests that they could indeed be resistant to the chemical when they are able to survive against it.

Testing for Connections in Resistance

Since the mutant 17.1.2 is known to be able to survive against exposure to chalcone **17**, we wanted to test to see if chalcones **25** and **30** had any similar effect on the worms and their resistance. In order to achieve this, we wanted to see if these worms could also survive exposure to chalcones **25** and **30** at the same concentration of 10^{-4} M. This was done by testing the chalcone **17** resistant strain on chalcone **25,** and **30** plates. In order to do this, we made sure to have negative controls during each of our test runs. The negative control has the reference strain (non-mutants) plated on chalcone **17, 25,** and **30** plates. These controls were to show the standard being unable to survive once exposed to the organic chemicals. We also had another set of controls where the chalcone **17** mutant was plated on chalcone **17** plates, so that we could ensure that the mutant is able to survive on its own designated compound. Along with these controls, worms were picked off of a chalcone **17** resistant strain NGM populated plate (without chalcone) and placed on a plate that is inoculated with chalcone **25,** and then another plate with chalcone **30**.

The experiment was setup as five different replicate tests with three plates per chalcone. This means that the dilutions of 10^{-4} (as explained in dilution procedure earlier) were made for each of the chalcones five times, and worms were picked on three different small (50 mm) chalcone plates for each of the compounds. Thirty worms were picked on each of these plates, and then checked every day for a week to record lethality. This was done through the use of a dissecting scope, observing whether the worms were still alive, mobile, or

expressing the GFP protein seen in our reference (PD4251) strain. This records a total of 90 worms per compound in addition to the negative and positive controls. The time frame of one week leaves substantial time for the adults to reproduce and for their eggs to hatch if they are able to resist and withstand exposure. Results were taken by keeping record of eggs hatched or not and observing the probability of deaths overall the total sample size placed on the plate. Scoring of the viability of these worms was done using a dissecting microscope. Worms that immediately died were evident due to their straight carcasses counted on the plate.

WormTracker Characteristic Analyses

In order to characterize the mutant that is resistant to chalcone **17**, we wanted to use a method that could clearly depict the effect these chalcones have on not only the way these worms look, but also on their behavior and even perhaps their effect on their muscular system. In order to do this, videos were recorded at the L4 stage of the worm's life cycle (young adults) using a microscope and the DP Controller software and manager. Both the reference strain and the mutant strain were recorded at each of these times in their designated plates. The easiest method in achieving this was running a synchronization which leaves us with the embryos of the nematodes. The embryos then all hatch at the same time, and the stages of the worm's life cycle occur consecutively throughout each of the following days. The Leica microscope was used for the recordings, and then imported to the ImageJ software obtained through imagej.net (Schneider et al., 2012). The settings for the videos were adjusted so that they could be imported and compatible with ImageJ 1.x (Schneider et al., 2012). The settings were recorded under 15 second timeframes for each of the videos, high contrast so that the WormTracker plugin (Nussbaum-Krammer et al., 2015) could easily recognize

each of the individual worms and track their movement. The video file format was saved as an AVI so that it could be read by the ImageJ 1.x software (Schneider et al., 2012). Once the WormTracker plugin (Nussbaum-Krammer et al., 2015) had been installed into ImageJ 1.x, the high contrast of the videos was crucial in order for the software to generate data by following the movement of these worms. Once this was done, the 15 second time frame of each of the videos was run through the software and the data was exported. The values were then imported into an excel spreadsheet where the graphs of individual and averages were taken. The sample size for the mutant was n=37 and the reference was an n=43. The parameters recorded with this software covered distance traveled in pixels, speed in pixels per second, and also body bends per second. The reference strain and wild type of *C. elegans* typically move in a sinusoidal fashion which consists of body bends as their method of movement. The software was able to also record this value and export it into excel where we were able to generate our graphs, and our statistical analyses. The statistical analysis done for these were grouped in averages and in individual tracks. We first ran an f-test to see if the variances were equal or not, then when we saw the obvious unequal variances we ran a t-test for two samples of unequal variances for each of the parameters recorded (distance traveled, speed, and body bends per second). This helped us clearly represent the differences seen between the reference strain and the mutant, clearly depicting the effect that these chalcones have on the nematodes.

Genetically Map the Cha17.1.2 Mutation with strain KK1

Two strains (KK1 and KK75) were provided by Dr. Lesilee Rose from University of California, Davis. Both strains have three unique phenotypic mutations with very distinct and observable features. The list and location of these

mutations on their designated chromosomes are shown in Tables 2 and 3. This experiment solely focused on crosses done with the KK1 strain. The KK75 strain still needs to be crossed with the mutant in future work. By crossing our mutant (17.1.2) males with the specific phenotypes in KK1, we can test the next generations (F_2) by exposing them to chalcone **17** and looking for the phenotype of interest in addition to recording the probability of lethality caused. This is done on the same concentration as the other chalcone tests at 10^{-4} M **17** plates.

Table 2: Depicts three different genetic mutations that cause strong phenotypic differences in the KK1 strain, along with their chromosome locations.

Chromosome	1	2	3
Gene	dpy-05	bli-02	unc-32
Characteristic	Strong dumpy	Blisters (L4)	Uncoordinated (coiler)

Table 3: Depicts three different genetic mutations that cause strong phenotypic changes in the KK75 strain, along with their chromosome locations.

Chromosome	4	5	X
Gene	unc-5	dpy-11	lon-2
Characteristic	Uncoordinated; severe coiler	Medium dumpy	1.5X longer

The three visible markers in the KK1 strain are recessive mutations represented as follows: dpy/dpy, bli/bli, unc/unc. The dumpy marker phenotype is very clear as the worms appear to be shorter, fatter, and less mobile when compared to the heterozygotes in the strain. This mutation is a marker located on chromosome 1. The bli/bli contain blisters on the adult worms and this serves as a visible marker for chromosome 2. Lastly, the unc/unc gene causes the worms to be

very uncoordinated in their movement, and they typically tend to coil on the plate. This gene is the marker for chromosome 3. With this information, we hoped to cross each of these visible markers (which are recessive mutations) with our own 17.1.2 mutant. Resistance to chalcones in the 17.1.2 mutant is also a recessive mutation. This is important to note because it affects the expected ratio of survivors in the F_2 generation. By testing these phenotypic markers through genetic crosses, we tried to locate or eliminate possible locations of the chromosome that could be responsible for this resistance.

This portion of the experiment was highly dependent on the availability of males. As mentioned earlier, males occur naturally in *C. elegans* at a rate of 1/1000 worms (Horvitz, 1997). To be able to run all our crosses a stock of males needed to be available.

In order to do this, males needed to be generated every week. Dr. Ross's heat shock protocol was followed, which consists of the steps listed in Figure 2. A starved populated plate of 17.1.2 mutant worms was taken, which meant these worms were in their dauer stage, where they do not have enough food to grow or reproduce. The worms were chunked off of these plates and onto new plates, and by the next day they were in the L4 stage, which is the desired phase to run a heat shock. This proved to be essential throughout our experiment. These plates were then placed in a 30°C incubator for 4.5 hours. This was done every Monday of every week until sufficient males were produced for the crosses. The temperature was set back to 25° C after the 4.5 exposure time, and the plates were left to grow and reproduce for two more days. These heat-shocked plates were then chunked onto 6-8 small 50 mm NGM plates so that it would be easier to locate males in a population of more than 1000 worms. This allowed us to generate males in order to run genetic crosses with the KK1 strain.

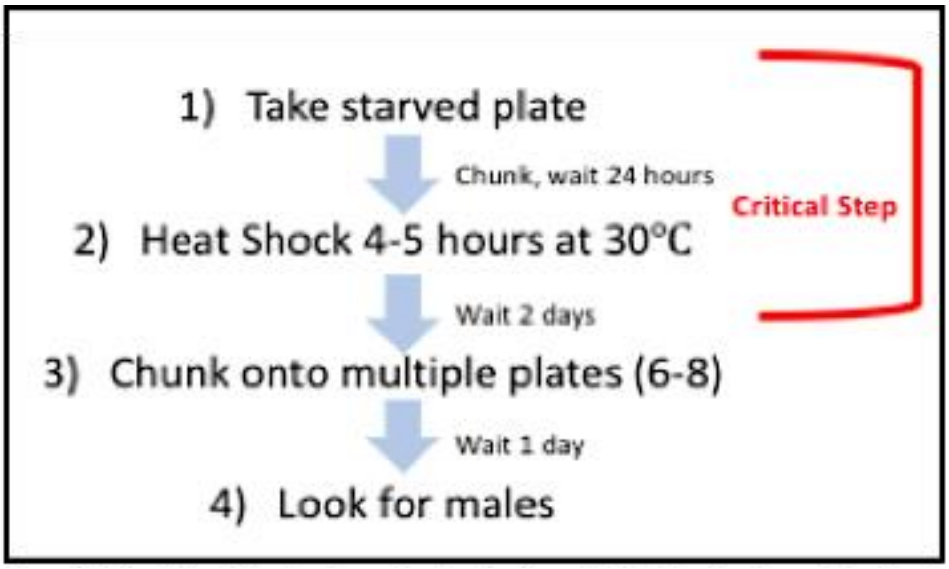


Figure 2: Heat Shock Protocol provided by Dr. Ross. Critical step is shown to be the first step, as that is the stage of increased nondisjunction during meiosis and gamete formation.

The genetic crosses were set up with the following procedure. Each of the designated markers present in the KK1 strain were separately crossed with 17.1.2 males. Two to three male 17.1.2 worms were picked onto an NGM plate along with three to four sperm depleted hermaphrodites. Sperm depletion helps to ensure that the worms are not self-reproducing and that the offspring generations are indeed produced from the cross that was set up. Since both the markers and the resistance mutations are recessive, we wanted to test the F_2 generation. After the males were crossed with the pseudo-females from KK1, we allowed the F_1 generation to self-reproduce and left these untouched until the F_2 embryos had been produced. Once these eggs were visible, the plates were washed, and the embryos were collected through centrifugation. These embryos were then plated on chalcone **17** plates. Because both these mutations are recessive, there is a specific phenotypic ratio that should occur if the mutations are linked to the mapping strain. For example, if looking at the dumpy trait located on chromosome 1, then the expected outcome of the F_2 generation is 25% non-dumpy but resistant

worms, 50% non-dumpy and not resistant worms, and 25% dumpy and not resistant. This is what we expect to see as results for each of the recessive mutations included in the KK1 strain.

Screens

Prior to mutagenesis, and to guarantee that our nematode population is synchronized (they should reach adulthood and reproduce together), begin by chunking ten starved plates of worms onto ten new NGM plates, where they are left to grow to gravid adults. Then by using the adults, a synchronous population of nematodes is obtained by bleaching the adults, so that only the embryos remain. After this is done, hatching of these eggs are observed and cultured on plates until they reach the L4 stage (Jorgensen, E. M. and S. E. Mango, 2002). To begin mutagenesis, the L4 nematodes are washed with M9 from these plates. M9 is the buffer medium that is used throughout this experiment. They are then centrifuged in a 15mL conical tube, and the pellet is collected (20-30 seconds at 150-200 x g). Then, the worms are washed with M9 to remove any contamination and the pellet of worms (approximately 500-1000 worms, depending on the scale of the screen) are resuspended in 5 mL of M9. Next, the EMS solution (41.2 μL of EMS to 1.958 mL of M9 solution; mix well to suspend EMS) which is the mutagenizing agent, is added to the 5 mL of worm suspension. The tube is then rocked, in the hood, for four hours. After the four hours, another run of centrifuging allows us to collect a pellet of embryos. The supernatant was removed, and the worms were washed twice with 8 mL of M9 to remove EMS thoroughly. The worms are then resuspended in 0.5 mL of M9 and plated on seeded NGM plates (~100- 200 worms per plate, using 2 plates) where the mutated worms (the P_0 generation) were left to grow to adulthood. We are interested in the F_1's that will be L4 in

three days (Tamayo, 2016). The F_2 progeny are then inspected for the phenotype of interest (worms that are alive after exposure to the organic chalcones). Along with the F_2 progeny, controls of the reference strain are plated to make sure that the standard worms are responding to exposure of the chalcone plates. If F_2 plated on the same chalcone plates are surviving exposure to the chemical, then we compare that reaction with our controls that were not able to survive exposure. If F_2 are alive then we can confirm the mutagenesis protocol as being successful (Tamayo, 2016). These mutagenesis protocols were conducted for both organic chalcone **17**, **25**, and **30**.

RESULTS

Similar Size Molecules Suggest Chalcone Uptake

To shed some light onto whether strain 17.1.2 is an uptake mutant, worms were stained with DAPI (277.324 g/mol) and Hoechst 33258 (533.88 g/mol). These dyes were chosen because of their similar molecular weight and size to chalcone **17** which has a molecular weight of 277.15 g/mol. We reasoned that if the fluorescent dyes successfully made it into the worm and stained the inside of their body (whether whatever cells they may stain), then it was possible that the chalcones could also make it into the worms due to the similarity in the molecular size to the dyes. Both DAPI and Hoechst 33258 dyes were successful in staining the mutant worms as seen in Figures 3-6. Based on their similarity in structure and in molecular weight, especially that of the DAPI dye, to the chalcone, this experiment was fundamental in possibly getting one step closer in answering whether these organic molecules could make it into the worm. The fluorescent dyes successfully made it into the worm and stain the inside of their body (whether it stains nerves or nuclei), which suggests that it is possible that the chalcones are also making it into the worms (Figures 3-7). Although we do not know whether the chalcone is being ingested or absorbed, it still shows that molecules of similar size are able to be taken up by these worms which means that they are not uptake mutants. At first, we were not sure if the chalcones were even able to make it into the worm. Now we know that both the reference and the mutant strain are able to uptake molecules with similar size to both DAPI and Hoechst dyes as seen in Figures 3-7.

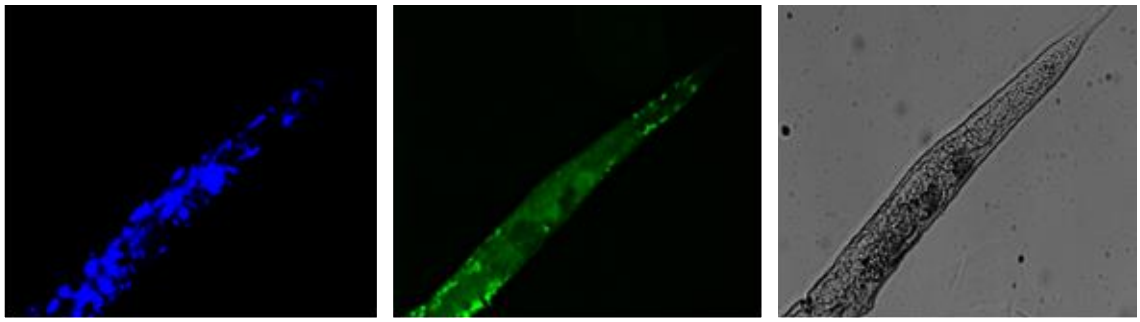


Figure 3: (From left to right) 17.1.2 stained with Hoechst 33258 dye, same worm expressing GFP protein with different filter, and last white light filter of same mutant worm.

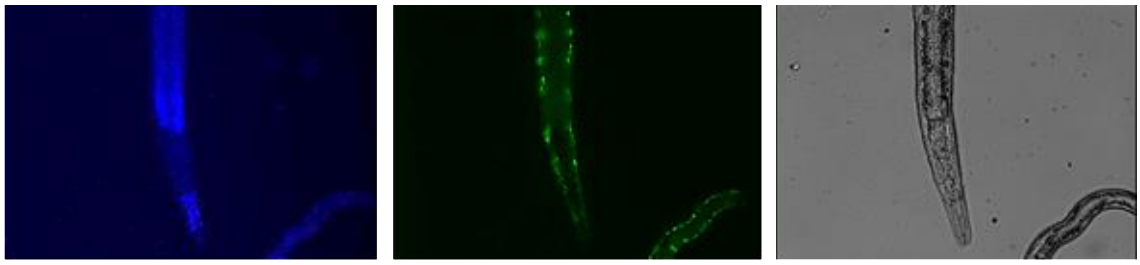


Figure 4: (From left to right) Different worm of same mutant strain with Hoechst 33258 dye, same worm depicting GFP, and far right in white light.

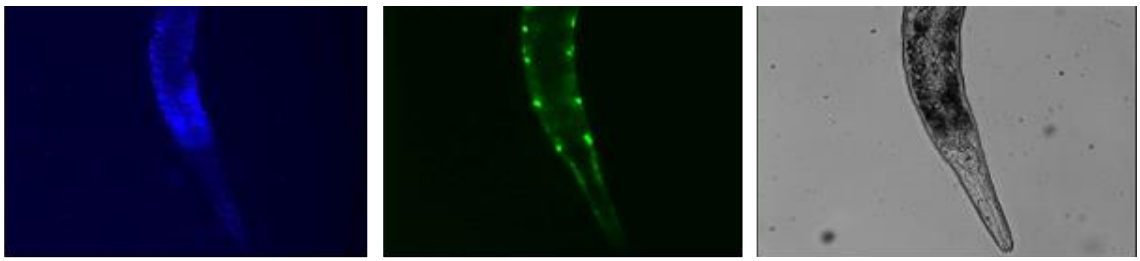


Figure 5: (From left to right) Mutant strain stained with now DAPI dye, then same worm pictured expressing GFP, lastly in white light.

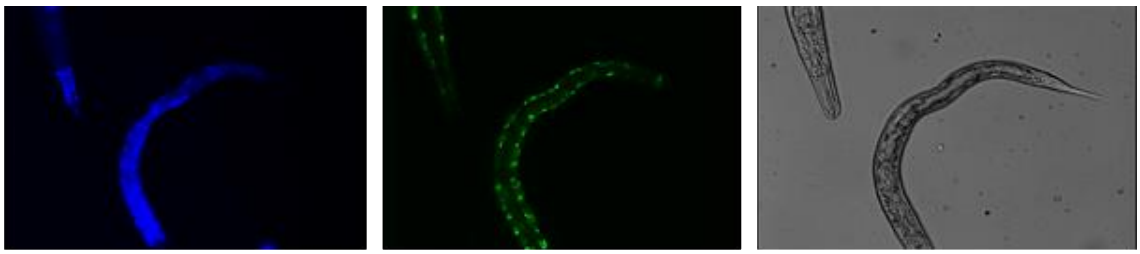


Figure 6: Another mutant worm stained with DAPI dye in same order of filters.

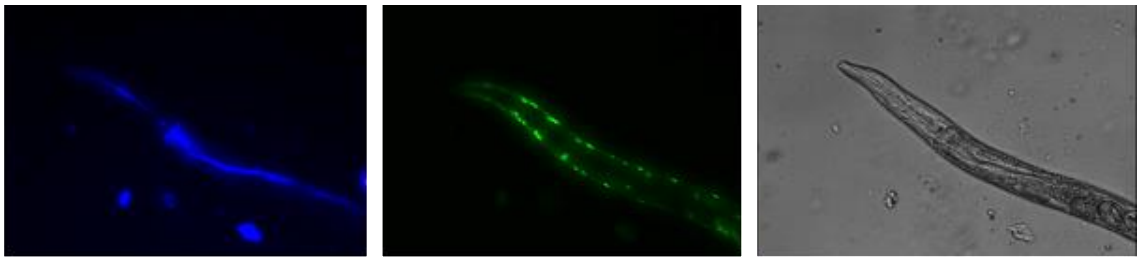


Figure 7: PD4251 strain depicted with Hoechst 33258 dye, then GFP filter and lastly white light.

Chalcone **17** Resistant Nematodes are Not Resistant to Chalcone **25** and **30**

Our expected results matched our observed and the designated mutants were not resistant to the other chalcones (chalcone **17** mutants did not survive on chalcone **25** and **30** plates). The 17.1.2 mutant had 100% lethality in all three of the plates for both chalcone **25** and **30** during all five trials. This means that for all three plates for each of the five replicates, all thirty of the worms died after exposure to the chalcones. Death was recorded using a dissecting microscope and after exposure to the chalcones within a three-day span. Worms were flat and straight observed by their carcasses and then checked for any GFP fluorescence. No fluorescence and no motility along with the carcass count on each of the plates was how the 100% lethality was recorded. The reference strain (non-mutants) died on all chalcone plates and survived on our NGM (no exposure to chalcone) control plates. The 17.1.2 mutant was only able to withstand exposure to chalcone **17** plates. The results of this aim prove that although these three organic chalcones are similar in effectively killing the wild type of *C. elegans*, their pathways are different. Our hypothesis was true, and the three chalcones acted independently and the 17.1.2 mutants were not able to resist exposure to all chalcones simultaneously. Our expected results were that the designated mutants would not be resistant to the other chalcones (chalcone **17** mutants will not be resistant to

chalcone **25**, **30** and any other mutant obtained in the future will be specific to only one compound). This expectation was confirmed as all the mutants on chalcone **25** and **30** plates did not survive. Our positive control of the 17.1.2 mutants on chalcone **17** plates remained the foundation of this experiment as those mutants were able to withstand chalcone **17** exposure exclusively.

Behavioral Characterization of 17.1.2 Shows Significant Differences

The purpose of this aim was to characterize the differences and to record how chalcone exposure affects worms when compared to the reference/standard strain. The WormTracker plugin (Nussbaum-Krammer et al., 2015) is installed through the ImageJ 1.x software (Schneider et al., 2012) and allows us to track worms individually and in groups. This records their speed, distance traveled, and body bends per second. This is great information for us to have, because we are able to record and track these worms.

Our results show a statistically significant difference when running a two-sample unequal variance t-test using an alpha level of 0.05. This aim characterized and measured the mutants and reference strain for three parameters. These three were: speed, distance traveled, and body bends per second. For distance traveled, our P-value (two tail test) was 0.0046, and for body bends per second our P-value (two tail test) was 0.0026. These are both significant. The reference strain does five times more body bends and travels twice as fast and covers twice the distance than the mutant strain. Figures 8-13 are graphs representing each of the strains. Figures 8, 10, and 12 show graphs of individual tracked worms and figures 9, 11, and 13 show the averages of the two strains.

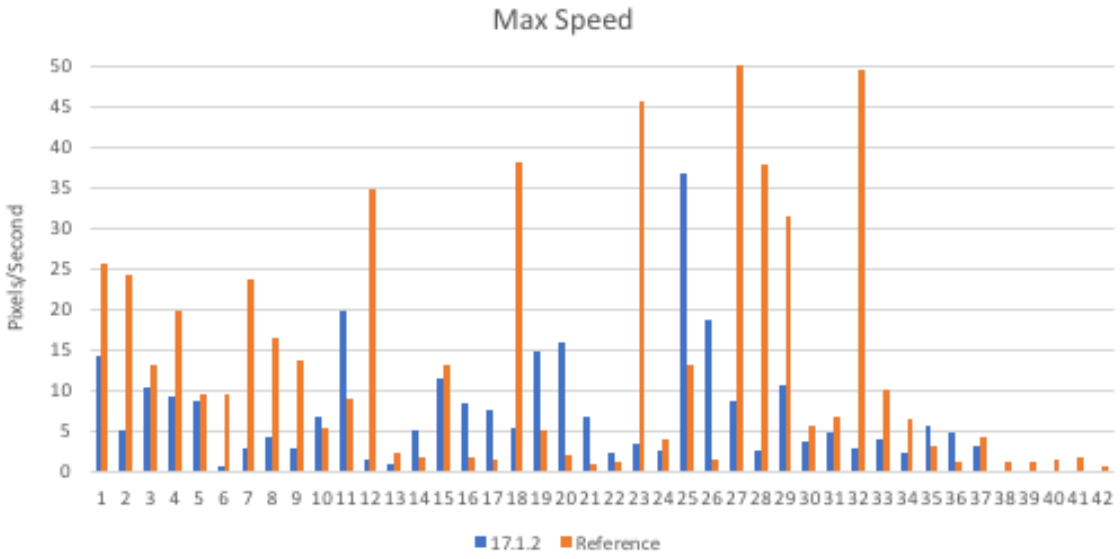


Figure 8: Max speed calculated individually for each of the worms in the mutant strain (depicted in blue) in comparison to the reference strain (shown in orange).

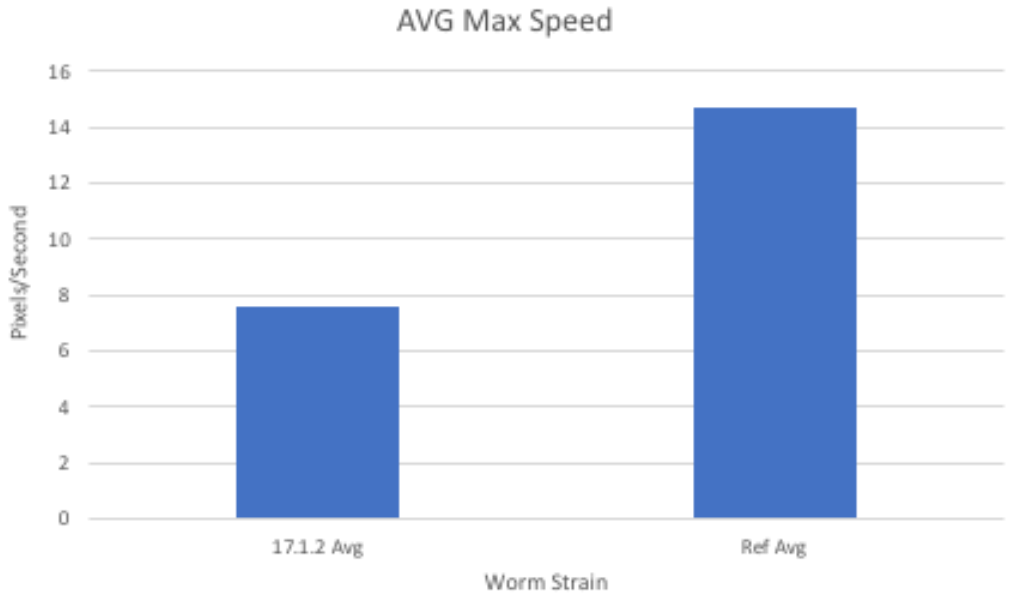


Figure 9: The averages of the total sample of both the mutant and the reference strain.

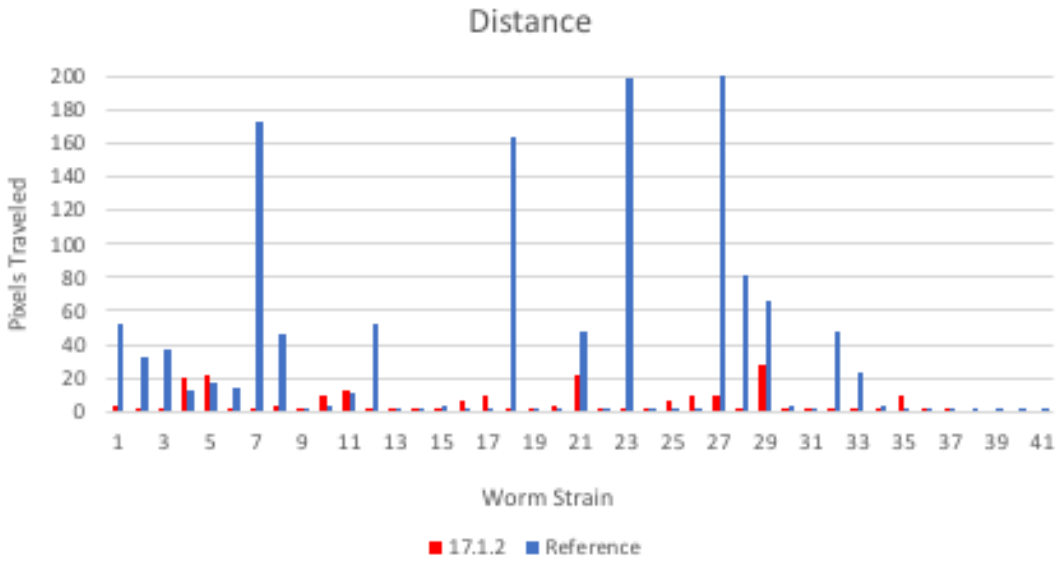


Figure 10: Distance tracked for each worm in both the mutant strain (red) and the reference strain (blue). Shows how the distance traveled differs greatly as mutants are unable to move across the plates even in a 15 second time interval.

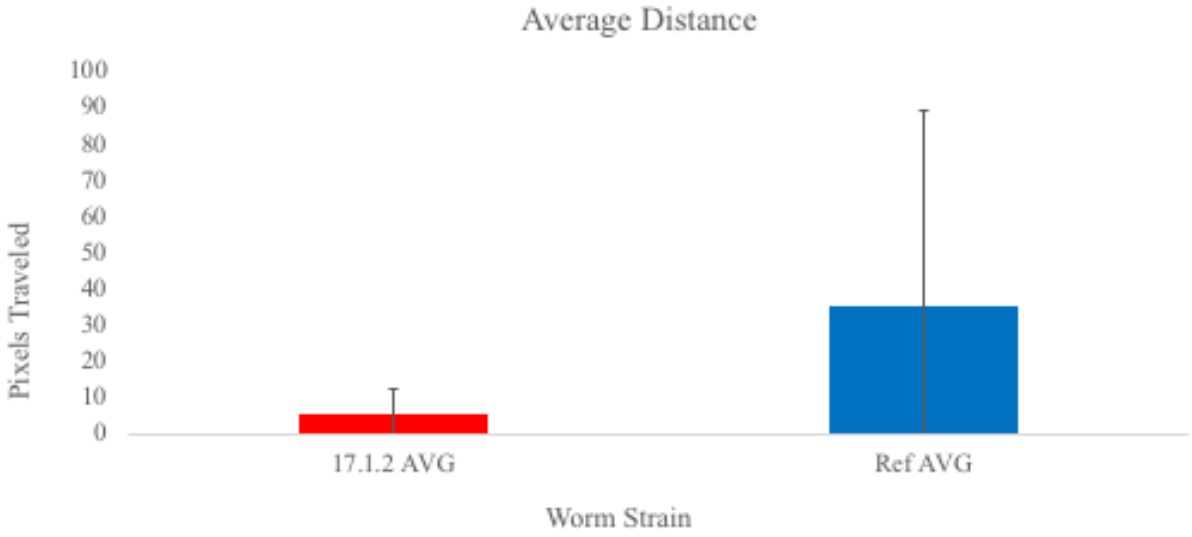


Figure 11: Average distance calculated by pixels per second. Both averages shown for mutant and reference strain.

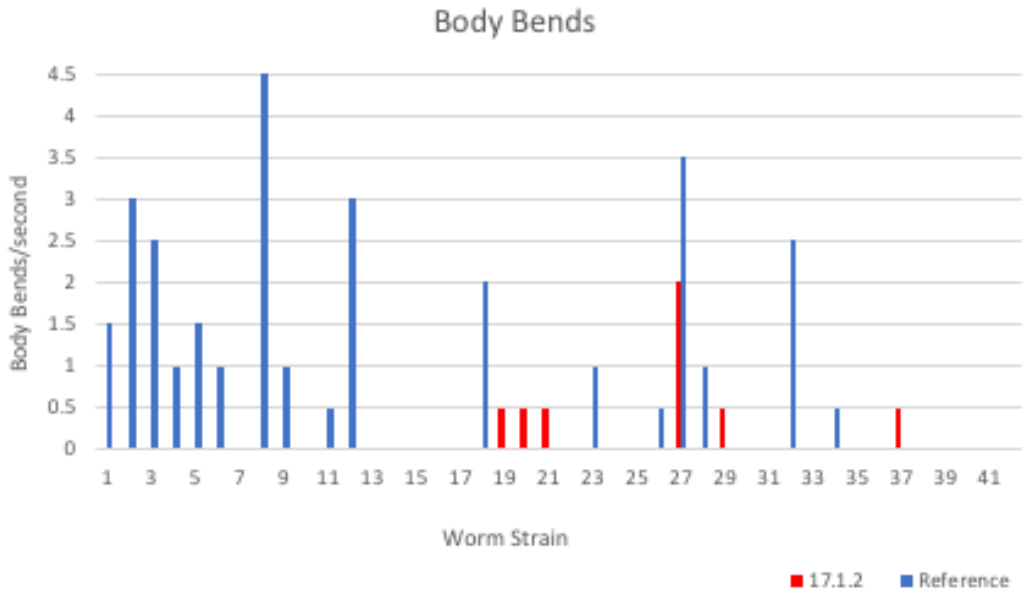


Figure 12: Body bends per second were so also analyzed using the WormTracker plugin. Great differences as the mutant strain (red) hardly follows the normal pattern behavior when compared to reference strain (blue).

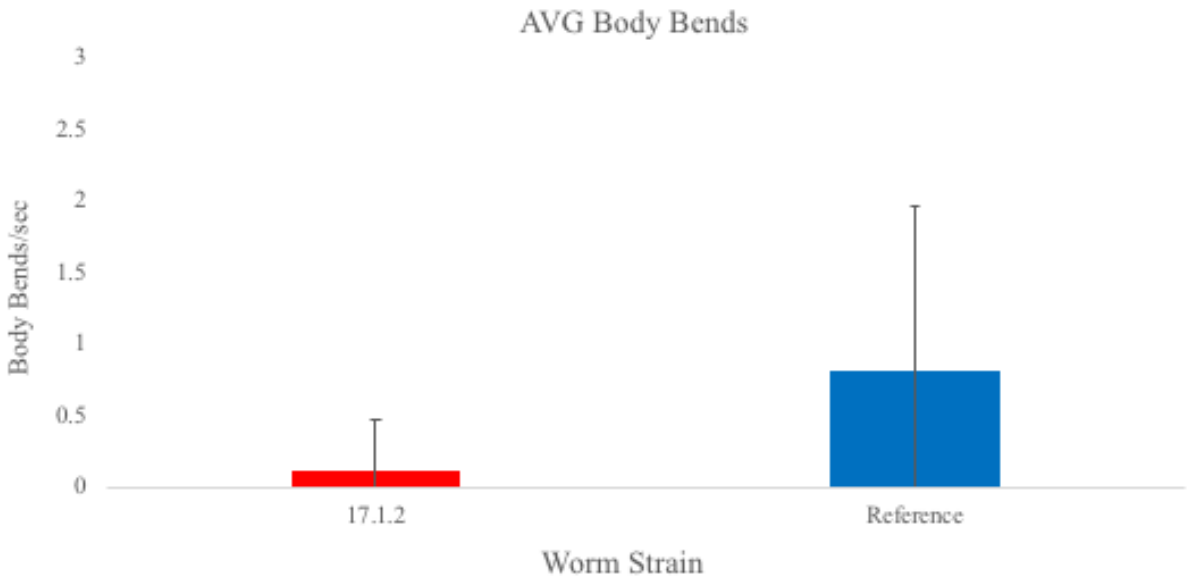


Figure 13: Averages for both the mutant and reference strain depicted for body bends per second. Great difference in how the chemically exposed mutants have altered behavioral functions.

Table 4: Body bends per second statistical analysis using a t-test assuming unequal variances due to first running an F-test to determine whether or not the variances were different. Shows a statistically significant difference between the

t-Test: Two-Sample Assuming Unequal Variances		
	17.1.2	REF
Mean	0.121621622	0.709302326
Variance	0.130630631	1.324197121
Observations	37	43
Hypothesized Mean Difference	0	
df	51	
t Stat	-3.171982975	
P(T<=t) one-tail	0.001281827	
t Critical one-tail	1.67528495	
P(T<=t) two-tail	0.002563653	
t Critical two-tail	2.00758377	

Table 5: Statistical Analysis for distance traveled of the two strains of worms in pixels per second. Two sample t-test was done assuming unequal variances, due to first running an F-test to determine the differences in variances.

t-Test: Two-Sample Assuming Unequal Variances		
	17.1.2	REF
Mean	5.468891892	30.41209302
Variance	52.95836177	2939.397295
Observations	37	43
Hypothesized Mean Difference	0	
df	44	
t Stat	-2.985777954	
P(T<=t) one-tail	0.002303658	
t Critical one-tail	1.680229977	
P(T<=t) two-tail	0.004607317	
t Critical two-tail	2.015367574	

Resistance Mutation and Chromosomes 1, 2, and 3

Results for this section of the genetic mapping with KK1 did not produce expected outcomes as described in methods. Heat shock protocol listed in Figure 2 was followed to obtain the males needed for the genetic crosses. Males looked a bit different from the hermaphrodites that are seen in the majority of the population. They have a tapered tail that looks similar to an arrowhead and is shown in Figure 14. These males were then crossed with the genetic mapping strains listed in Table 2 which shows the specific chromosomes linked with each observable mutation. To visualize the different phenotypes, present in KK1 and KK75, refer to Figures 15 and 16.

Figure 14: Male 17.1.2 mutant.

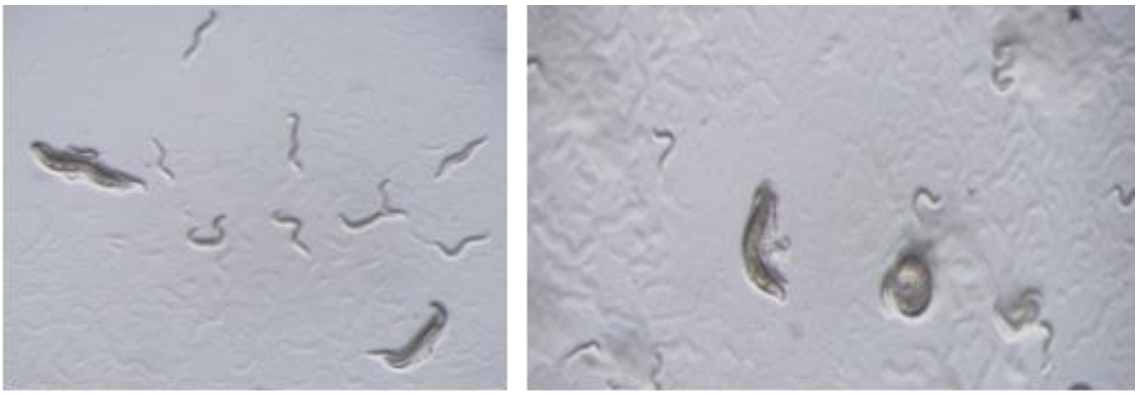


Figure 15: KK1 strain showing the different mutations present.

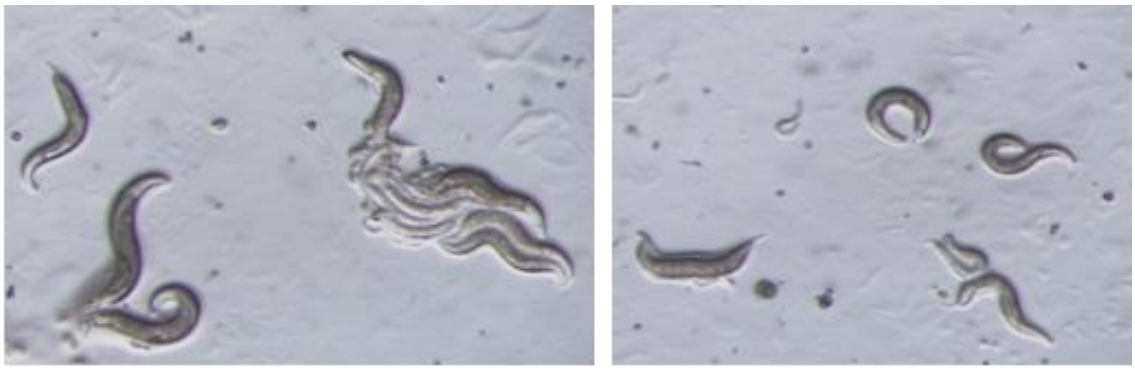


Figure 16: KK75 strain showing the different phenotypes present.

The crosses for each of the recessive mutations seen in KK1 were conducted with two to three 17.1.2 males as mentioned above. The results, however, were not what should be expected. The expected phenotypic ratio that should have been observed in these crosses, for example with the dumpy gene, should have been 25% resistant to chalcone **17** but non-dumpy, 50% non-dumpy and non-resistant, and 25% dumpy and non-resistant. However, we did not get any survivors in any of the F_2 offspring produced from any of the mapping crosses. The 100% lethality of all of the F_2 offspring when plated on chalcone **17** 10^{-4} M concentration plates leads us to not be able to conclude a location for this mutation.

Additional Screens

Each of the chalcone compounds had five mutagenesis screens conducted. After EMS exposure about 100-200 mutagenized nematodes were used and then suspended and plated on two seeded 100 mm NGM plates. This was the P_0 generation which were left to grow and generate the F_1 generation. After this generation had laid embryos, the worms were individually picked off (all the P_0 generation adults). The goal here was to let the F_1 generation reproduce and populate enough where there was plenty of F_2 embryos ready to test on chalcone

plates. We estimate that there were about 500-700 F_1 nematodes used on every screen performed. Once the F_2 embryos were isolated by performing a synchronization protocol, consisting of exposure to: 3.75 mL of ddH_2O, 0.25 mL of 5 M NaOH, and 1 mL of 5% bleach, they were transferred to chalcone plates to their designated compound. With 100-200 nematodes being mutagenized that is a pool of 600-1200 chromosomes being exposed to a mutagen. Once the F_1 were taken and washed about 100 eggs were placed on each of the ten chalcone plates used along with the control plate. Mutagenesis screens were continuously being run for chalcone **30** and **17**, unfortunately no new mutants were generated.

Further support of my hypothesis relies on the availability of more mutants. The purpose of this aim was to conduct more screens, so that more mutants can be generated and used for mapping as well. When more mutants are generated, comparisons and observations can be recorded in order to have more support to this experiment's findings.

DISCUSSION

Support for a Molecular Mechanism of Resistance

The first aim of this research focused on finding out whether the fluorescent dyes (DAPI and Hoechst) were taken up by both the reference strain and the mutant strain. The reason we were so interested in finding this information out was because of their similar size in molecular weight to the organic chalcone used in the laboratory. DAPI's molecular weight is 277.324 g/mol which closely resembles chalcone **17** which is what our mutant is resistant to. Hoechst 33258 weighs 533.88 g/mol and is the molecule with the greatest mass, so we were interested in seeing whether this molecule would make it into the worm. The results and pictures taken with the fluorescence microscope showed us that both these dyes were making it into the body of these worms, whether they be mutant or the reference strain. This demonstrates that it is possible that the organic chalcones are also able to make it into the worm. Although we do not know whether these chemicals are being ingested or not, it is still clearly shown through our data that the inside of the worms are efficiently stained. This is key because literature supports the fact that the molecular mechanism of the effect of these chalcones on organisms is unclear (Diaz-Tielas et al., 2016). Although the use of chalcones is clear in fungicides, antivirals, and effects of phytotoxicity on plant metabolism is shown, literature still make it evident that more investigations must be done in order to fully understand the mechanism behind how these chalcones work (Diaz-Tielas et al., 2016). Before this aim was conducted, it was a very likely possibility that the chalcones were not making it into the worm, and that the mutants had just developed a way to prevent its uptake. However, the results from this aim help us in suggesting that this resistance does indeed have a molecular mechanism behind it. This supports literature and links effects of these chalcones

back to a mechanism that is still unfortunately unclear. Hence the purpose of the following steps of this research in order to answer the question of whether this mechanism could rely on a genetic mutation on a specific chromosome.

Chalcone Exclusivity

The second aim of this research was to determine whether or not the chalcone **17** mutant (17.1.2) is also resistant to chalcone **25** and **30**. Multiple tests on chalcone plates determined that the mutants were exclusively resistant to only chalcone **17**. They were not able to withstand exposure to the other two compounds. This is notable because it supports our hypothesis of each of these organic chalcones having independent pathways. It is also crucial because it agrees with the literature that certain chalcones have independent effects and functions. Although we know all these chalcones are effective in killing the wild-type of *C. elegans*, the three chalcones still act independently because the mutant is not resistant to all three (Attar, 2011). If the worms that were initially from the chalcone **17** plates survived in the midst of exposure to the other two chalcones, then that could conclude that the mutation would be sufficient to protect worms from two different chalcones. However, because the chalcone **17** resistant mutants were not resistant to chalcone **25** and **30,** then this indicates that each chalcone affects a different pathway in the nematode.

Although chalcones are all flavonoids, their derivatives all have different functions and uses as literature states. For example, 2',3',4',4-tetrahydroxy-chalcone serves as an antiviral (Onyilagha et al., 1997) while 2',3,4,4'-tetrahydroxy-3'-geranyl-chalcone serves as a fungicide (Jayasinghe et al., 2004). Although similar in compound formation, just like our compounds, their functions and pathways can be completely different. This suggests that although

compounds' structure can be similar, their effects can be entirely independent from each other.

Effects of Chalcone Resistance: Closer to the Molecular Mechanism

The third aim was to characterize the behavior of the mutant, and therefore to determine if there was any tradeoff for their ability to grow in the presence of chalcones. Our goal was to analyze their movements based on body bends per second, distance traveled, and their speed or rate of movement. This is important to us because although they are able to survive against the chemical, there are great differences in behavior between these mutants and the reference strain used in the laboratory. It shows how the chalcone is greatly affecting not only the neuronal track of these worms but even possibly their muscular system. Chalcone exposure, although does not kill these resistant mutants, it clearly slows them down, and distorts the normal sinusoidal movement shown through body bends. Our graphs and statistical analysis, through the use of a t-test of two samples with unequal variances, showed us that there is a significant difference between the two strains. The reference strain does five times more body bends per second than the mutant strain. We observed that the mutants do not move as fast or in the normal sinusoidal curves that the wild-type and reference strains of *C. elegans* do. The reference strain also moved twice as fast as the mutant strain, leading us to conclude that their speed is much faster than the mutant. In addition to this, the distance traveled of the mutant strain was six times less than the reference strain. This provides quantitative data that supports the fact that these chalcones greatly affect these nematodes, even when they are able to withstand them.

With this data, we can say that the mutant worms are greatly affected by exposure to this organic chemical. Although it does not kill the resistant strain, it

greatly hinders not only their neural reactions but also their muscular system and activity. This could be due to a motor neuron effect in the body where the muscles of the worms are being compromised. *C. elegans* works with a TRPV channel protein that is involved in both OSM-9 and OCR-2 proteins which are key in facilitating mechanosensation, chemosensation, sensory transduction, and osmosensation (Tobin et al., 2002). OSM-9 is a *C. elegans* gene that is directly involved in expressing sensory neuron function and mechanosensory responses (Colbert et al., 1997). These channels and genes could be affected by the mutation that is causing the resistance in these mutants, and perhaps these distorted responses are one of the effects that chalcone **17** exposure causes. This could be an explanation to why our data shows such a great difference in motility seen in our mutants when compared to the reference strain.

The literature also shows different effects due to chalcone exposure. A study done on oxidatively stressed *C. elegans* showed that chalcone exposure actually increases thermal tolerance in these nematodes (Duong et al., 2012). Although their target gene is unclear, similar to our experiment, they were able to find the significance of antioxidant chalcones in being able to greatly reduce harmful effects of reactive oxygen species *using C. elegans* as their model (Duong et al., 2012).

Genetic Mapping

The fourth aim was to genetically map the location of this mutation that causes resistance to the chalcone. By conducting the genetic crosses between the male mutants, and the mapping strain (KK1), we hoped to get closer to the location of this mutation. Although we were able to run all the crosses with the three markers in KK1 with our 17.1.2 mutant, we are unable to claim any kind of

location for this resistance due to the fact that none of the F_2 generations (from the three crosses) were able to withstand chalcone **17**. There are six chromosomes in *C. elegans* and we were hoping to have pinpointed a possible location for the resistance mutation. All the crosses done collectively tested for chromosome 1, 2, and 3 as the possible location of this mutation. However, in order for any of these markers to be linked to the resistance mutation, we needed to have recorded and observed at least a 25% resistance (survival on the chalcone plates) in order to claim a linked mutation. Although unexpected, there can be an explanation as to why none of the F_2 embryos were able to survive and therefore have to be left with an unassigned location. This can be due to the F_2 generation and the double mutants having variable expression (Brenner, 1974). Possible explanations can include certain traits (like resistance in this case) to be obscured due to the introduction of a new mutation and its background (Brenner, 1974). For example, a dumpy resistant double mutant can look to be the same as just a dumpy tester, which can make scoring in crosses and in the F_1 generation more difficult. Linkage also makes it a bit more difficult as the pattern of the expected outcomes depends on the recombination frequencies occurring in the F_1 generation (Brenner, 1974). Experiments done with *C. elegans* and the location of mutants on linkage groups is stated to depend on how these mutations segregate by recombination, and the exact pattern depends on the each of the strains in the hermaphrodites. Perhaps during the process of our crosses, either the expression of resistant was obscured by the introduction of the new genome and its background, or the pattern of the expected outcome was affected in the F_1 selfing step in the cross.

Additional work is definitely needed in order to claim a location for this resistance. Right now, it remains unassigned based on the results that we observed. Concurrently in the lab, another graduate student is trying to use a Whole Genome

Sequencing approach to fine map this mutation. Additional work can also include mapping with the KK75 strain which includes markers for chromosomes 4-6 of *C. elegans*. Results must be closely observed and noted in order to compare our results done with the KK1 strain.

Role of EMS in Mutagenesis

The fifth aim was to run more screens and mutagenesis protocols. Although we ran multiple screens, we were unable to generate more mutants that were resistant to any of the chalcones. Some might argue that the role of EMS might be the problem as it was not able to generate more mutants. This is proven to be incorrect throughout the literature. Other types of mutagens such as nitrosoguanidine and diethyl sulfate (DES) have been said to be very effective, however they also possess a great danger to the organisms being tested (Bessereau and Boulin, 2002). These mutagens are carcinogenic to animals and therefore would be toxic and even might contribute to the death of *C. elegans* in this experiment (Hoffmann, 1980). EMS is a very effective mutagen that has been successful in the laboratory before (Tamayo, 2016). However, due to the other aims that took up a majority of our efforts, the screens are always ongoing in the laboratory. Our hopes are to generate much more mutants so that if a molecular mechanism is proposed, we can have more than one mutant backing up our idea. This would help us have a foundational basis to our arguments in the future.

CONCLUSION AND FUTURE WORK

The results of this project provide strong evidence in strengthening our hypothesis: the susceptibility to chalcones is due to different and independent pathways for each of the chalcones. In addition to this, characterization of the recessive chalcone 17 mutant, shows how much the exposure of this chemical has an effect on these worms. The action of the chalcone could perhaps be on a protein product, and the mutation on the gene that the protein codes for renders the chalcone action ineffective (Tamayo, 2016).

The future work of this project relies on collecting more data to support our proposed pathway of this resistance. In order to this, more mutants must be obtained in order to have multiple comparisons for each of the chalcones that we have obtained in our lab. Once those steps have been completed, DNA for each mutant can be prepared and sent out for Whole Genome Sequencing (WGS) and SNP mapping. These would help officially sequence the mutant's genetic composition and allow us to compare it to the reference strain. All the research done in this project is mapping by hand. Once companies and the mutants actually get sent out, we will have results that can back up this experiment and the findings of my research. Although we have not found the chromosome that the mutation belongs to, we hope that the data we have obtained will be confirmed by further studies done in the lab. Future work can also incorporate MAQGene, which can help us identify the SNP distribution in comparison with our reference strain and give us the ability to discover if there is a genetic mutation responsible for causing this resistance in our mutant. If more mutants are generated, WGS can help us pinpoint the location of not only this mutation, but ideally for all the mutants generated for each of the organic chalcones we have.

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