Abstract

Toxic Effects of Chlorpyrifos Exposure on Development of *Caenorhabditis Elegans*

By Kevin Adams

November, 2016

Director: Baohong Zhang

Department of Biology

Chlorpyrifos is a commonly utilized organophosphate pesticide, and as recently as 2007, was the most commonly used insecticide within the United States. Its ubiquity is concerning given its potential to cause developmental issues among younger organisms and its association with neurodegenerative conditions like Parkinson's Disease. Pathways by which these issues arise are unclear since these effects can occur in absence of acetylcholinesterase inhibition. To this end, experiments utilizing *Caenorhabditis elegans* as a model were performed. The organism is useful as a model for several reasons, of which are its rapid maturation, its ease of maintenance, and its transparent body that allows examination of intact bodily systems. *C. elegans* also possesses a fully characterized neuronal lineage with a complete neural wiring diagram, and several conserved neurotransmitter systems.

The primary purpose of this research was to determine if chlorpyrifos would have an impact on the behavior, morphology, or nervous system of *C. elegans* at selected concentrations, ranging from low levels considered generally safe to high levels with noted lethal impacts on *C. elegans*. Populations were grown on media containing chlorpyrifos during larval stages and then transferred to media without chlorpyrifos for 24 hours to separate developmental impacts of chlorpyrifos from its active effects. Analysis of chlorpyrifos-exposed *C. elegans* produced some notable results. Video analysis revealed that worms exposed to high chlorpyrifos concentrations had decrease in body length and width. Exposure to high concentrations of chlorpyrifos showed increasing germline apoptosis over control groups. Pharyngeal pumping assays showed paralysis at high concentrations, and no paralysis at low concentrations. Finally, an assessment of *C. elegans* dopaminergic neurons indicated neuron loss at both low and high developmental exposure. These results show a deleterious impact at high concentrations, with worms showing developmental delays and stunted growth. The compound seems capable of impacting bodily systems outside of the nervous system, as seen in the germline apoptosis assay. Concentrations tested have the desired impacts, with high chlorpyrifos exposure groups showing paralysis and low dose groups showing no paralysis. Most interestingly, the pesticide appears to cause dopaminergic neuron degradation at low concentrations, providing a link between chlorpyrifos and neurodegenerative conditions.

Toxic Effects of Chlorpyrifos Exposure on Development of *Caenorhabditis Elegans*

A Thesis Presented to the Faculty of the Dept. of Biology, East Carolina University

In Partial Fulfillment of the Requirements for the Degree
Masters of Science in Molecular Biology and Biotechnology

By

Kevin Adams

November, 2016

Toxic Effects of Developmental Chlorpyrifos Exposure on *Caenorhabditis Elegans*

By

Kevin Adams

APPROVED BY:

DIRECTOR OF
THESIS: _____
Baohong Zhang, Ph.D.

COMMITTEE MEMBER: _____
Xiaoping Pan, Ph.D.

COMMITTEE MEMBER: _____
Fadi Issa, Ph.D.

COMMITTEE MEMBER: _____
Myon-Hee Lee, Ph.D.

CHAIR OF THE DEPARTMENT
OF BIOLOGY: _____
Jeff McKinnon, Ph.D.

DEAN OF THE
GRADUATE SCHOOL: _____
Paul J. Gemperline, PhD

Contents

List of Tables

List of Figures

I. Introduction

Chlorpyrifos

Chlorpyrifos, trade names Dursban and Lorsban, is an organophosphate compound utilized as a broad-spectrum pesticide, capable of killing a variety of insects and arachnids including pest larva, grubs, cockroaches, flea beetles, flies, termites, ants, and lice.[1] It was introduced for use by Dow Chemical in 1965, and since then has gone on to become one of the most commonly utilized pesticides within the United States and the world.[2] It is registered for use in over 100 different countries and as recently as 2007, was the most commonly utilized insecticide within the United States with between 7 and 9 million pounds of the substance being used.[2,3] It's most commonly used as an agricultural insecticide, and it is mostly used on cotton, corn, almond trees, and various fruit trees including apples, bananas, and oranges.[4] Chlorpyrifos also finds non-agricultural use as a pesticide on golf courses, lawns, sporting fields, and in green houses. Finally, it often finds use as a non-structural wood treatment to prevent pest damage.[5]

Environmental Persistence

In the 1970s, the United States shifted away from organochlorine insecticides, of which there were concerns of toxicity to fish and wildlife along with a potential for bioaccumulation, and towards organophosphate insecticide compounds.[6] Organophosphate compounds tend to degrade more readily; chlorpyrifos in particular has a soil half-life between 10 and 120 days depending on soil conditions.[7] Furthermore, the compound binds tightly to soil, making contamination of water supplies surrounding application sites difficult without significant soil erosion.[8]

The primary ways in which chlorpyrifos finds its way into water sources are through spray drift during application of the pesticide or through instances of significant soil erosion.[9]

Depending on the form of chlorpyrifos used (powder or emulsifiable concentrate versus controlled release formations), the concentrations within water can achieve a rapid high or a low-level persistence.[9] Once in water, chlorpyrifos will normally bind to sediment and organic matter along the bottom of a body of water. From here, slow desorption from sediment can lead to a low (ppb) concentration of chlorpyrifos in water for long periods of time. At a neutral pH at 25ºC, chlorpyrifos has a half-life between 35 and 78 days.[10] Chlorpyrifos that doesn't bind to sediment will usually leave water supplies through volatilization. Within an outdoor atmosphere, chlorpyrifos has a half-life of approximately three days; indoors it can persist for months.[11]

Once chlorpyrifos is in the environment, it tends to degrade relatively quickly compared to other compounds. However, residues of chlorpyrifos can remain on surfaces for much longer periods of time. These residues can be found on fruits and vegetables consumed by people, leading to exposure to the pesticide.[11]

Exposure Routes and Exposed Populations

Populations can come into contact with and absorb chlorpyrifos in a few different ways. The chemical can be absorbed through dermal contact, through compound ingestion, or through inhalation of evaporated chlorpyrifos. Among these routes, dermal contact absorption is the most minor. Over a 24-hour period, only 1-2% of an applied dose appears to be absorbed from the skin, though this absorption can be as high as 11-20% depending on the formulation in which chlorpyrifos is found.[12,13] Outside of groups directly applying chlorpyrifos to surfaces or crops or those coming into contact with recently treated surfaces however, it is unlikely for a person to absorb a significant quantity of chlorpyrifos in this way.

While direct studies on absorption of inhaled chlorpyrifos have yet to be performed, it's generally assumed that the compound is well absorbed through the lungs.[14] Studies examining

workers involved in chlorpyrifos production, where airborne concentrations of the compound ranged from 10 to 1100μg/m^3, showed reductions in butyrlcholinesterase levels 19-32% lower than unexposed matched controls.[15] It should however be noted that these levels of exposure are not expected for a general population. Even among counties with heavy agricultural use of chlorpyrifos, outdoor air concentrations of chlorpyrifos were on average 0.033μg/m^3.[16]

Presently, the most common way people are exposed to chlorpyrifos is through consumption of the compound present as a residue on fruits, vegetables, and nuts. When consumed, chlorpyrifos is thought to be at least 70% bioavailable.[12] It's estimated that the average adult, toddler, and infant in the United States consumes roughly 0.005, 0.014, and 0.009μg/kg-bw per day, respectively.[11] These estimates are expected to be higher among individuals that consume a greater amount of fruits and vegetables. Aside from residues, chlorpyrifos can also be consumed as a component of dust within the environment, though restrictions on indoor use of the chemical likely have limited this.[11]

Overall, it's expected that the general population is exposed to chlorpyrifos predominantly through food consumption, while populations that work directly with chlorpyrifos (processors, applicators, etc.) along with those associated with these populations will absorb the compound through all exposure routes, leading to potential cumulative effect. The most heavily exposed populations are likely to be those working or living in rural or agricultural areas. There is a correlation between agricultural use of an area and increasing airborne concentrations of chlorpyrifos along with evidence that those working with the compound can carry it into their homes, potentially exposing their family members to chlorpyrifos.[16,17]

Primary Toxicity

The primary use of chlorpyrifos, and indeed all organophosphates, is as an agricultural pesticide. The compound has a negative impact on multiple bodily systems.[18] However, it's predominant, and most fully understood, toxic activity is the inhibition of acetylcholinesterase. Chlorpyrifos is a phosphorothioate, meaning it possesses a P=S bond. These phosphorothioate compounds are activated within the body by cytochrome P450 (CYP)-mediated monooxygenases, leading to either a desulfuration reaction or a dearylation reaction (see figure 1).[19]

While dearylation results in conversion of chlorpyrifos to less toxic substances, desulfuration produces a much more acutely toxic compound, chlorpyrifos-oxon. The oxygenated form of chlorpyrifos is capable of permanently inhibiting acetylcholinesterase.[20] This inhibition results in an accumulation of acetylcholine between neurons, which results in stronger, longer lasting signals to receiving neurons until new acetylcholinesterase can be produced by the body. Short term exposure to low concentrations can cause dizziness, fatigue, runny nose or eyes, salivation, nausea, intestinal discomfort, sweating, and changes in heart rate. Exposure to higher concentrations can cause seizures, paralysis, loss of consciousness, and death.[21]

Unknown Toxicology

While the above mentioned mechanism is the primary toxicological pathway for chlorpyrifos, there is evidence to suggest it may have other developmental neurotoxic effects not directly attributable to this action. Introduction of chlorpyrifos directly into the brains of neonatal rodents, bypassing desulfuration metabolism, has resulted in disruption of normal brain cell replication.[22] In fact, chlorpyrifos, along with other organophosphates, can disrupt developmental neuronal proliferation, differentiation, axon, synapse and glial cell genesis, and neural

apoptosis.[23,24] In vitro studies have also provided evidence that chlorpyrifos-oxon may impact neurodevelopment without inhibition of acetylcholinesterase, with potential targets being cell signaling molecules or cytoskeletal proteins.[20]

Chlorpyrifos and Parkinson's Disease

Parkinson's Disease is a neurodegenerative disorder resulting primarily from the loss of dopaminergic neurons in the substantia nigra of the brain.[25] The disease is characterized by shaking or trembling along limbs and the face, stiffness of limbs and torso, slowing of movement, and gradually deteriorating balance and coordination.[26] These symptoms make general movement difficult, and the progressive nature of the disease means the effects of the disease become worse over time. Additional disease symptoms include difficulty in chewing or swallowing, difficulty with speech, urinary and gastrointestinal difficulties, skin problems, and sleep disruption. Depression and other emotional changes are also common among those affected by PD.[27]

To date, Parkinson's Disease is one of the most common neurodegenerative disorders in the world, second only to Alzheimer's Disease; it affects roughly 7 million people across the world and 1 million within the United States.[28] It is more common among the aging and elderly, occurring in 1% of those over 60 and 4% of those over 80, though 5-10% of cases are considered "young-onset" and begin between the ages of 20-50.[29] Roughly 0.3% of the population within industrialized countries is estimated to be afflicted by PD, and it's expected that between 8 to 18 people out of every 100,000 will develop the disease each year.[27]

With the exception of known genetic factors leading to direct disease manifestation, the exact causes of Parkinson's Disease are unknown. However, there is a positive correlation between pesticide exposure and risk of Parkinson's Disease development.[30] Particular interest

has been given to neurotoxic pesticides, including organophosphate pesticides like Chlorpyrifos.[31] In fact, several epidemiological population studies have found an increased risk of Parkinson's Disease associated with exposure to chlorpyrifos.[30] Current studies however do not elucidate a definitive link between exposure and disease development, leaving open the possibility of confounding elements falsely correlating the two phenomena.

Since dopaminergic neuron loss is a characterizing feature of Parkinson's disease, and chlorpyrifos exposure can increase risk of disease development, a portion of this study is dedicated to determining if chlorpyrifos can trigger or enhance degradation of dopaminergic neurons within *C. elegans*. Dopaminergic neurons in *C. elegans* respond to mechanical stimulation from bacteria within their environments, facilitating a basal slowing response that decreases their movement speed in the presence of bacterial food sources.[32,33] Utilizing UA44-strain *C. elegans*, it is possible to directly observe the impact of chlorpyrifos on these neurons and potentially directly link exposure to disease development and progression.

Cause for Concern

Currently, non-occupational exposure to chlorpyrifos should be low enough to avoid adverse developmental effects due to acetylcholinesterase inhibition. However, there is evidence that the compound may impact development through different routes at lower concentrations, which may be cause for alarm given the compound's ubiquity.[34] Studies with rats have shown that exposure to chlorpyrifos during neonatal and postnatal life stages can cause developmental delays in coordination and reflexes as well as locomotion.[35,36] Evidence of developmental issues can also be found in humans. A study evaluating the relationship between chlorpyrifos levels in umbilical cord plasma and behavioral development noted that behavioral disorders were

significantly more likely to occur in toddlers whose umbilical plasma chlorpyrifos concentrations were above 6.17pg/g than in those toddlers who were below this threshold.[37]

New animal models may help elucidate secondary toxicological routes and actions for chlorpyrifos and establish concentrations at which neurodevelopmental or neurodegenerative impairments may occur. Preferable models should possess a well-documented nervous system comparable to the human nervous system.

II. Significance

Unknowns of Chlorpyrifos

While the primary route of chlorpyrifos toxicity is known and accounted for when establishing limits on exposure, potential secondary routes of toxicity aren't well characterized, with only a few hypothetical routes identified in vitro. Exposure of neuronal cells to chlorpyrifos concentrations 0.1-1nM showed decreases in axon length, and in vitro treatment of rat cortical neurons to chlorpyrifos and chlorpyrifos-oxon caused increased phosphorylation of CREB, a gene that plays a major role in gene expression.[34] These effects are shown at concentrations below those at which chlorpyrifos inhibits acetylcholinesterase. Perhaps more troubling, analysis of effects resulting from these concentrations are limited. With some evidence linking neural disorders in young children with fetal and early infancy chlorpyrifos exposure,[37] coupled with the wide spread use of chlorpyrifos as an insecticide,[38,3] it is a matter of urgency that the unknown toxicology of chlorpyrifos be described as fully as possible.

Validation of C. elegans Model

C. elegans provides several advantages as an animal model, particularly in regards to analyzing neurodevelopmental impacts of toxicant exposure. With a fully characterized and simple nervous possessing several neurotransmitter systems shared in common with vertebrates,[39] *C. elegans* could potentially allow one to examine how chlorpyrifos affects the developing nervous system in full and in detail. Given its small transparent body, these effects can even be viewed within living organisms. Its rapid life cycle is also ideal for examining developmental effects in general, given the relatively short time frame between larval exposure and analysis of adults.

The greatest challenge to *C. elegans* as a model however is its taxonomic distance from humans when compared to other mammalian model organisms. As of yet, there is not enough certainty that *C. elegans* will react to chlorpyrifos in ways comparable to a higher eukaryote. Without evidence of comparability, the total effects of chlorpyrifos on *C. elegans* may be less applicable when considering its effects on humans. The necessity of this work thus lies in supporting or refuting *C. elegans* as a model chlorpyrifos toxicity.

Thus, analysis of behavior, and the search for signs of developmental impairment, is critical for establishing similarity between *C. elegans* and other model organisms that have helped elucidate secondary toxic activities for chlorpyrifos thus far. Assuming this point of similarity is established, more detailed research can be conducted to examine the effects of chlorpyrifos on *C. elegans*, taking full advantage of all the aspects of the *C. elegans* model.

Elucidation of Chlorpyrifos-Parkinson's Disease Connection

One primary focus of this research is determining if chlorpyrifos exposure can cause symptoms associated with Parkinson's Disease in absence of confounding factors seen in larger population-based studies. The primary symptoms being examined are abnormalities in *C. elegans* movement and coordination, though the more direct symptom being observed is degradation of dopaminergic neurons, which is the cause of most primary symptoms of Parkinson's Disease.

A related focus is determining at what concentrations chlorpyrifos causes or enhances dopaminergic neuron loss. Will dopaminergic neurons only show damage at the highest levels of exposure, levels which should already be mitigated by existing law and regulation, or can damage occur at lower concentrations that might otherwise be considered innocuous? Assuming lower concentrations are found harmful, guidelines for chronic exposure may need review.

III. Hypothesis and Specific Objectives

Hypothesis

It was expected at the start of this experiment that chlorpyrifos would have a range of negative effects on *C. elegans* due to its neurotoxic nature. At high chlorpyrifos exposure, it was hypothesized that body size would be reduced, movement speed would decrease, and that germline cell apoptosis would increase. Since chlorpyrifos is known to cause paralysis at high doses, it was expected that active exposure to the compound would inhibit *C. elegans* pharyngeal pumping. Finally, exposure to chlorpyrifos was expected to cause an increase in head dopaminergic neuron degeneration within *C. elegans*. Each of these outcomes was expected to a far lesser degree within low chlorpyrifos exposure groups, with the exception of pharyngeal pumping since chlorpyrifos levels in low dose groups weren't expected to reach a threshold where muscle paralysis would occur.

At the outset of this experiment, it was expected that *C. elegans* would display behavioral patterns comparable to reactions shown by rodents exposed to chlorpyrifos during early development. Exposure to chlorpyrifos was expected to inhibit *C. elegans* movement and coordination, as visualized through movement speed decrease along with changes in body wavelength and amplitude. It was assumed that decreases in worm speed would be dependent on dose, with greater decreases observed with increasing dose.

Assuming that movement and feeding are impaired due to developmental chlorpyrifos exposure, it was also assumed that increased chlorpyrifos exposure might result in stunted growth. It was hypothesized that worm body length and width would decrease upon exposure to chlorpyrifos and that the decrease would be greater in heavily exposed groups.

Though chlorpyrifos is not a known genotoxin, environmental stressors have been noted to induce germline apoptosis within *C. elegans*.[40] It was thus expected that chlorpyrifos would cause some degree of stress and result in a greater count of apoptotic germline cells, identifying the chemical as a stressing factor. The count of apoptotic cells was expected to increase with exposure to greater chlorpyrifos concentrations.

To see if *C. elegans* feeding was inhibited in any way due to exposure, which would possibly be a contributing stress factor resulting in previous stress assays like the germline apoptosis assay, a pharyngeal pumping assay was performed, with the assumption that pumping rate would be mildly inhibited at low doses and noticeably decreased with high doses.

Given the links between organophosphate pesticide exposure and Parkinson's disease development, and knowing that a key sign of Parkinson's disease is dopaminergic neuron loss, it was expected that exposure to chlorpyrifos would result in loss of *C. elegans* dopaminergic head neurons. This loss was expected to be minor in low dose groups and more pronounced in high dose groups. There was no expectation that exposure would show trends among a single class of head dopaminergic neuron, for example causing more loss among ADE neurons than CEP neurons or vice versa.

Research Objectives

One of the primary objectives of this research is to identify what, if any, morphological and locomotive changes occur when *C. elegans* is exposed to chlorpyrifos during development. Lethal concentrations of chlorpyrifos in *C. elegans* have been determined previously. The compound has a 24hr LC_{10} of 0.298mg/L and a 24hr LC_{90} of 3.135mg/L within liquid media.[41] However, behavioral and physiological effects of chlorpyrifos exposure haven't been reported in great detail. WormLab analysis of behavioral videos should reveal and quantify what, if any,

changes occur in *C. elegans* behavior, movement, and body size. If changes can be observed, especially at lower test concentrations, this will provide evidence of chlorpyrifos toxicology beyond its acetylcholinesterase inhibition.

A secondary goal of research is to see if chlorpyrifos is capable of eliciting a deleterious response outside of the nervous system; apoptosis assays can answer this and may provide some evidence as to the cause of any observed changes among exposed worms in appearance, movement, size, or development. Apoptosis pathways in *C. elegans* encompass several genes, with groups of genes associated with apoptosis caused by genetic damage, starvation, and oxidative, osmotic, and heat stress.[40] Positive identification of germline cell apoptosis is thus an important step in identifying the effects chlorpyrifos has *C. elegans*. It is also important to see what chlorpyrifos concentrations can induce apoptosis.

Two important goals of this research are to begin identifying what factors may contribute to any observed increase in apoptosis and under what conditions chlorpyrifos exhibits its typical paralytic action. Examining pharyngeal pumping rate allows observation of how efficiently a worm feed, and a decreased rate might indicate starvation. However, if no decrease in pharyngeal pumping rate is observed at concentrations where other instances of stress were recorded, that might indicate multiple potential stressors arising from chlorpyrifos exposure.

A final goal of this research is to further elucidate a link between Parkinson's Disease and chlorpyrifos exposure by seeing if the compound can contribute to dopaminergic neuron loss in the *C. elegans* model. Within rat models, neonatal exposure to chlorpyrifos resulted in reductions in dopaminergic neurons in the substantia nigra.[42] Within *C. elegans*, could chlorpyrifos trigger similar neuron loss? This experiment attempts to answer this question and attempts to the link the neurodegenerative disorder and compound more definitively.

IV. Experimental Design

Model Organism-C. Elegans

As a model for toxicity, *Caenorhabditis elegans* has several advantageous qualities. It possesses a rapid rate of maturation, growing from fertilized egg to reproducing adult in three days. The small, 1mm round worm can also reproduce prolifically, with a single hermaphrodite individual being capable of laying 300 to 350 eggs all while being grown and sustained on a petri dish seeded with *E. coli*.[43] These factors mean that large populations of the round worm are not difficult to produce and subject to environmental treatments, and utilizing a larger number of test subjects adds statistical power to results obtained. Additionally, the rapid maturation of *C. elegans* means that the effects of developmental exposure can be observed within a relatively short time frame; within three days, the effect of larval chlorpyrifos exposure can be measured within an adult worm population.

C. elegans also has multiple features that make it a useful model for in vivo neurotoxicity studies. It possesses 302 neurons with 118 characterized neuronal subtypes.[44] So far, it is the only organism to have the entire structure of its nervous system mapped out.[45] This means it is theoretically possible to examine the exact effects of a toxicant at a complete anatomical level within *C. elegans*. Additionally, it also shares cholinergic, γ-aminobutyric acid, (GABA)ergic, glutamergic, dopaminergic, and serotonergic neurotransmitter systems with vertebrates.[39] As a model for Parkinson's disease in humans, *C. elegans* is remarkably useful; all major proteins with mutations linked to Parkinson's disease are conserved in *C. elegans*, with the exception of α-synuclein.[46]

However, if *C. elegans* is to be a useful model for human toxicology, there must be evidence that it reacts to toxic compounds in ways comparable to mammals. In regards to

organophosphate pesticides at least, there are indications that *C. elegans* responds to the chemicals similarly to higher order eukaryotes. Research found strong correlations in the rank order of organophosphate toxicity between *C. elegans* and rodents. Behavioral toxicity tests with *C. elegans* ordered a group of 15 organophosphate pesticides by toxic effect and found these rankings statistically consistent with the rankings of the compounds in rats and mice.[47] Given these similarities, it is not implausible to use *C. elegans* as a model for developmental toxicity concerning Chlorpyrifos.

In summary, *C. elegans* offers easily maintained, proliferative, and manageable populations upon which we can test the effects of chlorpyrifos. The round worm avoids the costs and time investments associated with higher eukaryotes while allowing the observation of a greater number of organisms. The simplistic nervous system of *C. elegans* provides a compromise between in vitro studies on neural cells, which cannot observe the responses of cells within living systems, and in vivo studies on more complex eukaryotes, whose nervous systems cannot be analyzed in full detail. Furthermore, several components of the *C. elegans* nervous system are conserved among higher eukaryotes, including humans, making observations based on them applicable to human medical research. Finally, there is evidence that *C. elegans* reacts to organophosphate compounds in a manner comparable to mammals, which potentially gives direction to future experimentation involving chlorpyrifos in other animal models.

Strains Utilized

Three different strains were used within the proposed experiments. For the majority of experiments, N2 strain *C. elegans* were the model organism of choice. This is a common laboratory strain of wild type *C. elegans*; most protocols for culturing and analyzing *C. elegans*

are designed with the N2 strain in mind. Video analysis and pharyngeal pumping assays were performed utilizing this strain.

The MD701 (Plim-7::ced-1::gfp; lin-15(+)) transgenic strain of *C. elegans* was utilized for studies of adult worm germline cell apoptosis. The strain possesses a CED-1:GFP marker which identifies cell corpses within the worm germline that are being engulfed by neighboring cells. This allows for the identification and quantification of apoptotic cells in still living *C. elegans*.

The *C. elegans* strain UA44 (baIn11; $P_{dat\text{-}1}$::α-syn, $P_{dat\text{-}1}$::gfp) is used as a model for Parkinson's Disease.[48] Under control of the *dat-1* (sodium-dependent dopamine transporter) promoter, the strain produces human α-synuclein along with GFP. Transgenic α-synuclein production supplements worm neurons with the single Parkinson's Disease associated protein not present in *C. elegans* and GFP production allows for direct visualization of C. elegans head dopaminergic neurons. This strain was utilized for dopaminergic neuron assessment.

Chlorpyrifos Treatment

C. elegans were exposed to chlorpyrifos through the media upon which they are grown and sustained. Nematode growth media was prepared following the methods covered in *Maintenance of C. elegans*.[49] There were slight modifications however to ensure that chlorpyrifos adequately dissolved within the media. Solutions of chlorpyrifos were prepared within DMSO to create a stock, which was then mixed with the media mixture (replacing added water) in order to create medias with varying 'environmental concentrations' of chlorpyrifos. To minimize effects of DMSO on the study organisms, concentrations of the substance in media did

not exceed 0.1%. To control for these effects, media containing 0.1% DMSO without dissolved chlorpyrifos was used to grow control group *C. elegans.*

Medias were prepared for two test concentrations along with the 0µg/L control media. The lowest concentration tested will be 1µg/L, which is an established environmentally acceptable concentration.[50] The second observed concentration was 3mg/L which is the LC90 for *C. elegans* in liquid media.[41] Current LC values within solid media are unknown. These concentrations range from those that are theorized to have no effect or negligible effects on humans, to those of which there is experimental evidence for lethality in *C. elegans*. Testing at lower concentrations has the potential to either validate or challenge current measures of guidance concerning chlorpyrifos, while testing at the higher concentration should produce a measureable effect on *C. elegans*, allowing a starting point for future studies of chlorpyrifos action in this and other model organisms.

Exposure Period

C. elegans populations were grown on treatment or control media during their L1 to L4 larval stages, a period that should simulate persistent exposure to chlorpyrifos during a childhood developmental period in other organisms. To ensure all organisms in treatment groups are within the same life stage, a synchronization procedure was performed. The procedure used for synchronization was taken from Porta-de-la-Riva et al. (2012) with minor modifications.[51] Briefly, *C. elegans* worms at varying points of development are washed off of their media and into a 15mL tube using M9 buffer. The solution in the tube is centrifuged at 2000RPM for 2 minutes to produce a worm pellet at the bottom of the tube. The supernatant of the tube is discarded and the worm pellet is washed with 5mLs of M9 buffer. It then undergoes repeated cycles of centrifugation and washing as stated until the observed discarded supernatant is clear.

At this point, 5mLs of synchronization solution (a mixture of 3.5mL water, 1mL bleach and 0.5 mL NaOH solution) is added to the tube and the tube is shaken to disturb the worm pellet. The synchronization solution, after 5 to 8 minutes of vigorous mixing, kills all worms and worm larvae while leaving worm eggs unharmed. The solution is then centrifuged at 2000RPM for 2 minutes to create an egg pellet, and the synchronization solution supernatant is removed and replaced with 5mLs of M9 buffer. The dispersed eggs undergo repeated cycles of centrifugation, supernatant removal and replacement at least four times in total. These wash cycles ensure removal of any remaining synchronization solution that may kill or impact eggs.

At this point, there are *C. elegans* eggs suspended within M9 buffer containing no food supply for any hatching larva. The solution is placed on a shaker in a 20ºC incubator for 20 hours, which is sufficient time for eggs in solution to develop into L1 larva. Because there is no food for the larva to feed on within the solution, they are arrested at this state. The worm larva are then be transferred onto appropriate treatment plates seeded with OP50 *E. coli*, where they are grown until worms are in late L4 state in a 20ºC incubator. At this point, they are transferred to non-treatment media to grow until they are at the appropriate life stage for experimentation.

The procedure for transfer to treatment-free media is as follows. Worms are washed from treatment plates and deposited into microcentrifuge tubes for rounds of centrifugation, supernatant removal, and washing with M9 buffer to remove chlorpyrifos or other potential contaminants from the worms. The worms are deposited on treatment-free nematode growth media seeded with OP50 strain *E. coli*. Worms are allowed to grow to full egg-laying adulthood from the young adult stage in absence of chlorpyrifos. The treatment free growth period is an effort to separate active effects of the chlorpyrifos within organisms from developmental effects the experiments are attempting to note and quantify. The exception to this free growth period are

the pharyngeal pumping assays, which had the goal of observing the active effects of chlorpyrifos on pharyngeal pumping rate.

WormLab

After completion of the above detailed exposure procedure, worms were transferred to nematode growth media plates seeded with OP50 *E. coli.* After a 30-minute acclimation period on the new media, videos were taken of each treatment group and the control group. Videos focused on individual worms and were roughly 30 seconds in length. These videos were analyzed via Wormlab software, which tracks all worms within a treatment group and measures track lengths covered by worms, worm wavelength, worm amplitude and bending, angle, speed of movement, and instances of omega bend behaviors. From data provided by WormLab video analysis, average forward and reverse movement speeds and distances covered were determined. Data provided was also used to track instances of reversals and proportion of time spent in reversal. Differences in behavior and morphology between treatment groups and the control group were interpreted for signs of effects from chlorpyrifos.

WormLab software was also utilized to quantify morphological differences between worm populations exposed to varying levels of chlorpyrifos during development. Worm length, width, and area were accounted for to determine physical effects of exposure.

Pharyngeal Pumping Assay

Synchronized N2-strain *C. elegans* were plated in triplicate onto 0.05% DMSO control NGM, 1ug/L Chlorpyrifos NGM, and 3mg/L Chlorpyrifos NGM, all seeded with OP50 *E. coli* as a food source. Populations of *C. elegans* were left on treatment or control media for 48 hours, allowing the control group to mature to the L4/Young Adult stage before egg laying had begun. At this point, worms were transferred to fresh treatment media of the type on which worm

populations were raised with fresh OP50 *E. coli.* After a two-hour acclimation period, 30 second videos of individual worms were recorded. For each video, pharyngeal pump rate for the recorded worms was determined by counting the total number of pumps and dividing this by the length of the video in seconds.

Apoptosis Assay

Apoptosis assays were done utilizing the MD701 *C. elegans* strain. Apoptotic germ cells are visualized through a CED-1::GFP fusion protein that marks somatic cells clustering around the apoptotic cells during engulfment.[52] Samples of adult worms were taken after the previously outlined exposure period, suspended on agarose on slides, and paralyzed with a 5mM solution of Levamisole.

Once suspended on slides, 40 to 50 worm gonads per treatment were examined utilizing fluorescent microscopy. GFP positive cells within the gonad were quantified, with particular interest paid to apoptotic cells residing within the gonad loop. Average numbers of apoptotic cells per treatment group were taken and compared.

Dopaminergic Neuron Assessment

Synchronized UA44-strain *C. elegans* were plated as outlined in the exposure period procedure. Worm populations were allowed to grow on treatment or control media for 48 hours, leaving the majority of worms in the L4/Young Adult life stage. At this point, *C. elegans* populations were washed and transferred onto fresh non-treatment NGM seeded with OP50 *E. coli*, where they were allowed to grow for at least 24 hours. At this point, worms were fully mature adults, producing observable eggs.

Exposed and control worms were then mounted onto slides and paralyzed with exposure to 5mM Levamisole solution. For each treatment, 100 individual worms were observed with a

GFP fluorescent microscope, specifically examining the head dopaminergic neurons. These neurons are the set of four CEP neurons and the pair of ADE neurons. Counts of fluorescent neurons were taken for each individual worm and differences between these counts among treatment groups were analyzed.

V. Results

Impact of Chlorpyrifos Treatment on Worm Body Size

Analysis of videos through Wormlab shows nonsignificant changes between the control and low dose treatment groups among all tested parameters. At low doses of environmental chlorpyrifos, worms showed a small increase in both length and width that was not significant at the 95% confidence level. *C. elegans* grown for 48 hours on 1ug/L Chlorpyrifos Nematode Growth Media showed an average body size slightly above the control group, with greater length (840.69±6.69μm) and greater width (102.8±2.2μm). In comparison, control *C. elegans* that weren't exposed to chlorpyrifos had an average body length of 813.4±9.5μm and an average body width of 98.44±1.77μm. Worm area, as a derivative endpoint of length and width, was likewise higher among the low dose group than among the control. An average low dose group worm had a body area of 87915.9±2057.8μm^2 while a control worm had an average body area of 81452.21±1809.01μm^2. The difference in body size is small, roughly a 3% increase in length a 4% increase in width, and an 8% increase in body area; future testing would be necessary to determine if such an increase is significant or due to treatment.

Worms exposed to chlorpyrifos at the 3mg/L level during development did show significant ($P<0.05$) decreases in body size when compared either to control groups or low dose groups. Worm bodies were shorter (601.23±14.18μm), thinner (86.01±2.21μm), and overall smaller in total area covered (53033.89±2389.87μm). It is theorized that these decreases in body size were at least partially attributable to starvation during development, along with some developmental delay within heavily exposed worms. Results of body length and width analysis are presented in fig. 5 and table 1.

Impact of Chlorpyrifos Treatment on Body Wavelength and Amplitude

When corrected for body size, differences in wavelength were minor and statistically indistinguishable among treatment groups. Control group worms had an average wavelength to body length ratio of 0.515±0.007, low dose worms had a ratio of 0.504±0.009, and high dose worms had a ratio 0.508±0.011. Differences within the amplitude to body length ratios among groups were likewise statistically indistinguishable. The average ratio was 0.0488±0.0029 among the control group, 0.04955±0.0051 within the low dose group, and 0.0502±0.0050 among the high dose group. Based on this data, it seems unlikely that developmental exposure to chlorpyrifos influences worm body posture in adulthood. Results for wavelength and amplitude are shown in fig.6 and fig.7. Results are listed in table 2.

Impact of Chlorpyrifos Treatment on Movement Speed

As with body size, lightly exposed worms showed non-significant increases in speed over the control group. An average control worm had a forward speed of 28.08±4.59μm/s and an average reverse speed of 31.48±5.05μm/s. Worms exposed to low levels of chlorpyrifos during development had an average forward speed of 36.87±4.77μm/s and an average reverse speed of 40.32±6.91μm/s. On average, low dose worms were roughly 31% faster than control worms moving forward and 28% faster moving in reverse. The differences in speed shown between the control and low group was more drastic than other measured endpoints and the observed increase in movement speed goes opposite of expectations. Assuming future studies on movement speed show significance, it is possible that chlorpyrifos may be acting as a stimulant at very low concentrations.

Heavily exposed worms responded to developmental treatment as expected, with decreases in both forward and reverse movement speeds compared both to control and low dose

worms. An average high dose worm had a forward speed of 18.92±2.72μm/s and a reverse speed of 19.57±3.02μm/s. The observed difference between control and high dose worms was not significant at the $p<0.05$ level, though there was significance ($p<0.05$) when comparing the high dose and low dose groups. All groups showed greater reverse speeds than forward speeds. Results are shown in fig. 8 and measurements are listed in table 3.

Impact of Chlorpyrifos Treatment on Reversal Ratios

Except for a significant ($p<0.05$) difference between high and low dose worms, there was no determinable difference in reversal ratios between the three groups. Disregarding significance, *C. elegans* exposed to low doses of chlorpyrifos during development had the lowest reversal ratios, spending 22% of recorded time moving in reverse. The control group had the second highest reversal ratio, with 27% of recorded time spent moving backwards. Finally, the heavily exposed group spent the greatest amount of time in reversal, with 34% of recorded time spent moving in reverse. With the only statistically distinguishable difference between groups being between the low and high dose treatment groups, it's difficult to draw conclusions from these results. However, it is possible that high dose worms are more sensitive to adverse stimuli that would trigger a reversal response. Conversely, low dose worms may lose a degree of this sensitivity. Results are shown in fig. 9 and listed in table 3.

Chlorpyrifos Exposure Induced Germline Apoptosis

Looking specifically at numbers of apoptotic germ cells within the gonad loop, control and low dose chlorpyrifos groups showed comparable levels of apoptotic cells, with the low dose group showing an observable but not significant increase compared to control. An average control gonad contained 0.77±0.11 observed apoptotic cells, while the gonad of a worm exposed to 1μg/L chlorpyrifos during development contained 1.25±0.19 apoptotic cells. There is an

increase observed between the two groups, though the difference between the two groups doesn't meet a $p<0.05$ significance threshold. Further testing with a greater number of worm gonads may show a statistically supportable difference that potentially indicates stress beyond that which is caused by bodily paralysis. The degree of difference between the two groups is substantial, if not statistically significant, indicating an increase in the occurrence of apoptosis by 60% over control with just mild exposure to chlorpyrifos.

C. elegans exposed to high doses of chlorpyrifos showed a notable and significant ($p<0.05$) increase in the number of apoptotic loop germline cells, with a 64% increase in apoptotic cells compared to the low dose group and a 166% increase in apoptotic cells compared to control groups. An average worm exposed to 3mg/L chlorpyrifos during development showed 2.05±0.21 observed apoptotic cells per gonad. As of yet, it is unclear if this increase is due to direct stress caused by chlorpyrifos or an indirect result due to physical impairment during larval stages. Representative images of germlines are shown in fig.10. Apoptotic cell counts are shown in fig.11 and counts are recorded in table 4.

Chlorpyrifos Exposure induced aberrant Pharyngeal Pumping

As with other experiments utilizing the N2-strain of *C. elegans*, the pharyngeal pumping assay attempted did not indicate any significant differences between the control treatment group and the 1ug/L Chlorpyrifos treatment group. Both groups showed pumping rates slightly above 2.5 pumps per second. Specifically, the control group showed a rate of 2.66±0.06pumps/sec, just slightly above the 2.59±0.06pumps/sec rate for worms exposed to 1μg/L chlorpyrifos. Differences in pharyngeal pumping were nigh indistinguishable between the two groups, and it seems that chlorpyrifos does not have a paralytic effect on *C. elegans* at such a low concentration.

The 3mg/L Chlorpyrifos treatment group however, showed a significant decrease ($p<0.001$) when compared to the other two groups. The average pharyngeal pumping rate of a worm under heavy chlorpyrifos exposure was 1.697±0.062pumps/sec. This represents a roughly 36% drop compared to the control pumping rate. This suggests chlorpyrifos, at higher doses, has the expected paralytic effect on *C. elegans*. Assuming that this paralysis is inhibiting food consumption, it is possible that the impact chlorpyrifos has on worm body size and development may be partially due to induced starvation. Results are shown in fig.12 and recorded in table 5.

Chlorpyrifos Exposure induced aberrance in Dopaminergic Neurons

Both the 1ug/L Chlorpyrifos treatment group and 3mg/L Chlorpyrifos treatment group showed a significant ($p<0.05$) increase in the proportion of showing damage or degradation to dopaminergic neurons, as measured by loss of GFP visibility. This is significant within low dose groups, given previous experiments' inability to assess any differences between control worms and lightly exposed worms.

The ratio of control worms showing any signs of dopaminergic neuron degradation was 0.10±0.03. One in ten worms examined showed loss of GFP in one or more neurons. Among worms exposed to 1μg/L chlorpyrifos during development, the ratio of worms showing GFP loss within neurons was 0.25±0.04, meaning one in four worms showed neural degradation. This ratio was similar within heavily exposed worm populations; a ratio of 0.26±0.04 for GFP loss was observed among the 3mg/L chlorpyrifos treatment group. There was no statistically distinguishable difference between the ratios of GFP loss among the low and high treatment groups.

Breaking down GFP loss by neuron type, ADE neurons show more dramatic differences among groups. The control group had GFP loss within ADE neurons in only 4±2% of

individuals. The percentage of ADE-GFP loss among the low dose treatment group was 11±3% and was 14±4% among the high dose group. While differences in damaged proportion could not be statistically distinguished between the 1ug/L Chlorpyrifos exposed group and either the control or 3mg/L treatment group, the 3mg/L treatment group showed a significant difference ($p<0.05$) in proportion showing ADE damage when compared against the control.

The percentage of the control group with CEP neuron degradation was 8±3%. Among the low and high treatment groups, this percentage was elevated to 17±4% and 14±4% respectively. Damage to CEP neurons seems elevated among the two treatment groups, but the observed differences cannot be statistically distinguished from control and damage does not seem to increase with increasing dose.

Images of C. elegans dopaminergic neurons post treatment are shown in fig.13. Results of Dopaminergic Neuron Assay are presented in fig.14 and fig.15. Results of assays are listed in table 6.

Statistical Analysis

Statistical significance among body size measurements, posture/coordination measurements, movement speeds, reversal ratios, apoptotic cell counts, and pharyngeal pumping rates was assessed by way of one-way ANOVA analysis, utilizing a Bonferroni Correction post-hoc test for comparisons between groups. To determine significant differences in proportions of worms showing GFP loss in dopaminergic neurons among different treatments, a chi-square test was utilized at the 0.05 significance level with a post-hoc Bonferroni Correction.

VI. Discussion

Morphological Impact

Among all bodily measurements, *C. elegans* exposed to low levels of chlorpyrifos during development showed no significant difference from control worms at the adult stage, though there was a nonsignificant increase in length and width. From this, along with data from other experiments, it is possible to conclude that chlorpyrifos at the 1ug/L level does not have a strong toxicological impact on *C. elegans* when excluding impacts on the nervous system. The chemical at this level does not appear to cause overwhelming bodily stress to the point where development is impacted. However, further experiments may need to be done to see if any delayed effects can be observed later in the *C. elegans* lifespan.

In contrast to low dose chlorpyrifos treatment, the 3mg/L chlorpyrifos treatment led to an observed developmental delay and decrease in body size. The developmental delay is consistent with previous reports on the effects of chlorpyrifos exposure on *C. elegans.*[24] However, the decrease in body size seems to go beyond a simple developmental delay during video recording and extend to a genuine decrease in adult body size, an indicator of stress. Without further experimentation, the exact cause of this decrease is unknown, though other performed trials indicate starvation due to paralysis may be a possible contributing factor.

Of note, significant changes in morphology seem limited to a decrease in body size among heavily exposed *C. elegans*. Body wavelength and amplitude are comparable to control and low dose treatment groups. These two measurements can be thought of as partially analogous to coordination, or how *C. elegans* tend to propagate their bodies along a substrate. The similarity among the groups indicates no lingering paralysis within muscles in the heavily

exposed group, indicating that any lingering chlorpyrifos exposure was minimal to nonexistent once exposed worms were transferred to non-treatment agar.

Mobility Impact

The lasting impact of chlorpyrifos on wild-type *C. elegans* mobility seems difficult to distinguish. Except for the differences in forward and reverse speeds observed between the low and high dose treatment groups, no significant difference in speed could be observed. Looking at the whole of the data, changes in speed do not follow an expected trend line, with speed decreasing slightly under low doses and decreasing noticeably at high exposure. Rather speed seems to increase slightly with light exposure during development and then decrease with heavy exposure. At low doses, chlorpyrifos does not seem to induce a Parkinson-like phenotype at the chosen time point, with exposed worms showing no evidence of movement impairment.

In the very least, the results obtained may be indicative of differing effects of chlorpyrifos, and potentially different toxicologies, when exposure occurs at different doses. The observed nonsignificant movement increase is perhaps too great to fully disregard and may need further trials to determine if changes are truly significant. What would cause such an increase in speed also requires investigation. It is known that CEP and ADE neurons within *C. elegans* help to produce a basal slowing response when food is present. Damage in these neurons could potentially produce a worm that doesn't readily slow its movement on food sources, which could explain the observed increases.

As of now however, it cannot be said that developmental exposure to chlorpyrifos at the 1μg/L concentration has any effect on adult *C. elegans* movement speed. There is some evidence that heavier exposure, at the 3mg/L concentration, could have a lasting impact on movement

speed. However, without significant difference from an unexposed control group, this cannot be stated definitively.

Chlorpyrifos as a Stressor

At high concentrations, chlorpyrifos seems to possess the ability, either directly or indirectly, to increase apoptosis within *C. elegans* germline cells. Worms exposed to high doses of the compound during development, high enough doses to cause developmental delays and smaller adult sizes, showed apoptotic cell counts over twice that of unexposed MD701-strain worms. While it wasn't assessed in current experiments, this might indicate an impact on later reproductive success within exposed worms along with multigenerational toxic effects. A portion of this increase in apoptotic cell count might be attributable to partial paralysis during development, resulting in the observed decrease in pharyngeal pumping rate among heavily exposed worms. This decrease could inhibit a worm's ability to feed, causing starvation which is a known cause of increased germline cell apoptosis.[23]

Though low doses of chlorpyrifos (1μg/L) during development did not produce significant increases in apoptotic cell count over unexposed worms, there was a nonsignificant increase which may deserve further investigation. As expected, the average number of apoptotic cells per worm gonad seems to fall along a trend line, with increasing numbers occurring with increasing dose. If this increase is supported in later studies, this might indicate action of chlorpyrifos as a cellular stressor beyond nutrient deprivation via muscle paralysis. At this concentration, chlorpyrifos does not seem to significantly inhibit acetylcholinesterase but may have other impacts on cells.

Effects on Dopaminergic Neurons

Initial results seem to indicate that chlorpyrifos, whether directly or indirectly, is causing some degree of damage to dopaminergic neurons. Given previous results showing that high exposure to chlorpyrifos caused significant bodily stress within worms, at least partially these results are not particularly surprising. Within high dose groups, a portion of neuron degradation might be attributable to poor nutrition and starvation, in addition to any direct stress the compound may have had upon the cells.

What is more surprising however, is the degree to which *C. elegans* exposed to a low dose of chlorpyrifos showed dopaminergic neuron loss. The experiment showed a rate of abnormality significantly greater than unexposed worms and statistically indistinguishable from worms receiving a high dose. This is particularly startling since *C. elegans* in previous experiments did not show either significant signs of stress or impairment at this chemical dose. It should be noted that behavioral studies were not performed with the transgenic UA44 strain, which may show greater morphological impairment or locomotion alterations with developmental chemical exposure.

As of yet, it is unknown how chlorpyrifos might be impacting dopaminergic neurons within *C. elegans.* The compound can be considered either a direct cause or an enhancer, which may exacerbate the disease progression to a point in which it is notable. Since UA44-strain of *C. elegans* experiences age-related dopamine neuron degradation due to α-synuclein expression, the latter explanation may be considered more probable at this point.[53] Further studies will need to be performed to see if the increase in apparently absent neurons occurs in absence of α-synuclein.

Lifespan Studies

The vast majority of research detailed above focused on endpoints found within a very narrow age range. Observed *C. elegans* were usually adults right at the onset of egg-laying. It's possible that developmental exposure could cause damage observable at later life stages and might potentially accelerate age-related deterioration. This is of particular interest in the study of Parkinson's Disease, which tends to emerge later in life. Behavioral observations of older worms might produce more pronounced differences between treatment groups than did observation of younger worms. Conversely, it is possible that some of the morphological changes observed in heavily exposed *C. elegans* (shorter body length and narrower width) might dissipate with age; worms could continue to grow over their lifespan and differences between the treatment groups might vanish.

Concentration Variances

These studies were limited to two distinct and separate chlorpyrifos doses: a low dose (1μg/L) which was hypothesized to have limited effect and cause little disruption of acetylcholinesterase, and a high dose (3mg/L) that would follow expected toxicological mechanisms for chlorpyrifos. In this dose selecting, middling concentrations were ignored. Seeing *C. elegans* reactions to a wider variety of doses might help establish trend lines among measured endpoints and better characterize the exact reactions of the organism to the compound. This will be especially pertinent if future research finds significant differences in *C. elegans* locomotion and morphology between no exposure and 1μg/L developmental chlorpyrifos groups. Assuming such research agrees with the data presented above, which showed nonsignificant increases in locomotion and body size among the low exposure group, a greater number of concentration treatments both below and above the low exposure group will be necessary to determine the presence of a potential hormetic effect.

Determining Apoptotic Action

While developmental chlorpyrifos exposure has seemingly increased the incidence of *C. elegans* apoptotic germline cells during adulthood, it is unknown by what mechanism it causes this phenomenon. Fortunately there are several proteins known within *C. elegans* apoptotic pathways that can be examined for their role in chlorpyrifos-triggered apoptosis. For example, it is known that oxidative, osmotic, and heat stress induced apoptosis in *C. elegans* depend on the MAPK kinases MEK-1 and SEK-1 along with ABL-1.[54] Observing the effects of chlorpyrifos in absence of any one of these proteins would help elucidate the exact contribution of starvation towards germline apoptosis in previously mentioned experiments. It would also potentially implicate chlorpyrifos as an oxidative stressor. Though chlorpyrifos is not a known genotoxin, further experiments could also be performed on *C. elegans* that lack CEP-1, which is required for DNA damage induced germline apoptosis.[55] No change in rates of germline apoptosis would further specify the action of the compound; a decrease in apoptosis however might indicate previously unaddressed toxicological facets of chlorpyrifos.

Elucidating Effects on Dopaminergic Neurons

The above experiments indicated the potential for developmental chlorpyrifos exposure to directly cause or enhance dopaminergic neuron degeneration, but there are still several questions to be answered. The UA44 strain of *C. elegans* is partially humanized due to production of human α-synuclein, allowing its dopaminergic neurons to function as a better simulacrum for human dopaminergic neurons. With the addition of α-synuclein, *C. elegans* possesses all major proteins whose dysfunction is associated with Parkinson's Disease. However, as a result of α-synuclein expression, *C. elegans* naturally experiences dopaminergic neuron loss over its lifespan. An important question is whether an increase in neuron loss would occur in

absence of α-synuclein, or are the observed negative effects of chlorpyrifos dependent on action upon α-synuclein. To this end, similar trials should be performed on other strains of *C. elegans*, possibly with dopaminergic neurons visualized in a different manner. It has also yet to be confirmed that UA44-strain *C. elegans* has the same vulnerability to chlorpyrifos that wild-type *C. elegans* possesses. Behavioral studies with WormLab on the UA44-strain could provide evidence that the two react similarly to the compound. Alternatively, signs of poor coordination like slower movement or irregular body wavelength or motions could be related to the symptoms of Parkinson's disease and support the use of the strain further as a PD model for other compounds.

VII. References

1. U.S. Environmental Protection Agency. 1986 (Sept.). Ambient water quality criteria for chlorpyrifos-1986. Office of Water Regulations and Standards. Criteria and Standards Division. Washington, DC.
2. http://npic.orst.edu/factsheets/chlorptech.html
3. http://www.epa.gov/opp00001/pestsales/07pestsales/market_estimates2007.pdf
4. http://www.pestmanagement.info/nass/app_usage.cfm
5. https://www.epa.gov/ingredients-used-pesticide-products/chlorpyrifos#basic
6. Barron, Mace G., and Kent B. Woodburn. "Ecotoxicology of chlorpyrifos." *Reviews of environmental contamination and toxicology*. Springer New York, 1995. 1-93.
7. Racke, Kenneth D., Dennis A. Laskowski, and Mark R. Schultz. "Resistance of chlorpyrifos to enhanced biodegradation in soil." *Journal of Agricultural and Food Chemistry* 38.6 (1990): 1430-1436.
8. Poletika, N. N., et al. "Chlorpyrifos and atrazine removal from runoff by vegetated filter strips: Experiments and predictive modeling." *Journal of environmental quality* 38.3 (2009): 1042-1052.
9. U.S. Environmental Protection Agency. 1986 (Sept.). Ambient water quality criteria for chlorpyrifos-1986. Office of Water Regulations and Standards. Criteria and Standards Division. Washington, DC.
10. Howard, P.H. (ed.). 1989. Handbook of Environmental Fate and Exposure Data for Organic Chemicals, Vol. III: Pesticides. Lewis Publishers, Chelsea, MI.
11. Eaton, David L., et al. "Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment." *Critical reviews in toxicology* 38.sup2 (2008): 1-125.
12. Nolan, R. J., et al. "Chlorpyrifos: pharmacokinetics in human volunteers." *Toxicology and applied pharmacology* 73.1 (1984): 8-15.
13. Griffin, P., et al. "The in vitro percutaneous penetration of chiorpyrifos." *Human & experimental toxicology* 19.2 (2000): 104-107.
14. Geer, L. A., et al. "Comparative analysis of passive dosimetry and biomonitoring for assessing chlorpyrifos exposure in pesticide workers." *Annals of Occupational Hygiene* 48.8 (2004): 683-695.
15. Brenner, F. E., et al. "Morbidity among employees engaged in the manufacture or formulation of chlorpyrifos." *British journal of industrial medicine* 46.2 (1989): 133-137.
16. Harnly, Martha, et al. "Correlating agricultural use of organophosphates with outdoor air concentrations: a particular concern for children." *Environmental health perspectives* (2005): 1184-1189.
17. Bradman, Asa, et al. "Pesticides and their metabolites in the homes and urine of farmworker children living in the Salinas Valley, CA." *Journal of Exposure Science and Environmental Epidemiology* 17.4 (2007): 331-349.
18. Gupta, R. C. (Ed.). (2011). Toxicology of organophosphate & carbamate compounds. Academic Press.
19. Chambers, J. E., & Levi, P. E. (1992). Organophosphates: chemistry, fate, and effects. pp 3–17
20. Flaskos, J. (2012). The developmental neurotoxicity of organophosphorus insecticides: a direct role for the oxon metabolites. *Toxicology letters*, 209(1), 86-93.

21. Christensen, K.; Harper, B.; Luukinen, B.; Buhl, K.; Stone, D. (2009). *"Chlorpyrifos Technical Fact Sheet"*. National Pesticide Information Center. *Retrieved 2014-07-03*.
22. Whitney, K. D., Seidler, F. J., & Slotkin, T. A. (1995). Developmental neurotoxicity of chlorpyrifos: cellular mechanisms. *Toxicology and applied pharmacology*, 134(1), 53-62.
23. Slotkin, T. A. (2005). Developmental neurotoxicity of organophosphates: a case study of chlorpyrifos.
24. Flaskos, J., & SAcHANA, M. A. G. D. A. L. I. N. I. (2010). Developmental neurotoxicity of anticholinesterase pesticides. Anticholinesterase Pesticides: Metabolism, Neurotoxicity, and Epidemiology, 203-223
25. Dauer, William, and Serge Przedborski. "Parkinson's disease: mechanisms and models." *Neuron* 39.6 (2003): 889-909.
26. Jankovic, Joseph. "Parkinson's disease: clinical features and diagnosis." *Journal of Neurology, Neurosurgery & Psychiatry* 79.4 (2008): 368-376.
27. http://www.ninds.nih.gov/disorders/parkinsons_disease/parkinsons_disease.htm
28. de Lau, Lonneke ML, and Monique MB Breteler. "Epidemiology of Parkinson's disease." *The Lancet Neurology* 5.6 (2006): 525-535.
29. Samii, Ali et al. (2004). Parkinson's Disease. The Lancet, Volume 363, Issue 9423, 1783-1793
30. Freire, Carmen, and Sergio Koifman. Pesticide exposure and Parkinson's disease: epidemiological evidence of association. *Neurotoxicology* 33.5 (2012): 947-971.
31. Costa, L.G., Giordano, G., Guizzetti, M., Vitalone, A., 2008. Neurotoxicity of pesticides: a brief review. Front. Biosci. 13, 1240–1249.
32. Kang, Lijun, et al. "C. elegans TRP family protein TRP-4 is a pore-forming subunit of a native mechanotransduction channel." *Neuron* 67.3 (2010): 381-391.
33. Sawin, Elizabeth R., Rajesh Ranganathan, and H. Robert Horvitz. "C. elegans locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway." *Neuron* 26.3 (2000): 619-631.
34. Eaton, D. L., Daroff, R. B., Autrup, H., Bridges, J., Buffler, P., Costa, L. G., ... & Spencer, P. S. (2008). Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. *Critical reviews in toxicology*, *38*(S2), 1-125.
35. Dam, K., Seidler, F. J., & Slotkin, T. A. (2000). Chlorpyrifos exposure during a critical neonatal period elicits gender-selective deficits in the development of coordination skills and locomotor activity. *Developmental Brain Research*, 121(2), 179-187.
36. Carr, R. L., Chambers, H. W., Guarisco, J. A., Richardson, J. R., Tang, J., & Chambers, J. E. (2001). Effects of repeated oral postnatal exposure to chlorpyrifos on open-field behavior in juvenile rats. *Toxicological sciences*, 59(2), 260-267.
37. Rauh, Virginia A., Robin Garfinkel, Frederica P. Perera, Howard F. Andrews, Lori Hoepner, Dana B. Barr, Ralph Whitehead, Deliang Tang, and Robin W. Whyatt. (2006) Impact of prenatal chlorpyrifos exposure on neurodevelopment in the first 3 years of life among inner-city children. *Pediatrics* 118(6), e1845-e1859.
38. http://www.epa.gov/oppsrrd1/REDs/chlorpyrifos_ired.pdf
39. Leung, M. C., Williams, P. L., Benedetto, A., Au, C., Helmcke, K. J., Aschner, M., & Meyer, J. N. (2008). Caenorhabditis elegans: an emerging model in biomedical and environmental toxicology. *Toxicological Sciences*, 106(1), 5-28.

40. Lakso, Merja, et al. "Dopaminergic neuronal loss and motor deficits in Caenorhabditis elegans overexpressing human α-synuclein." *Journal of neurochemistry* 86.1 (2003): 165-172.
41. Roh, J. Y., & Choi, J. (2008). Ecotoxicological evaluation of chlorpyrifos exposure on the nematode *Caenorhabditis elegans*. *Ecotoxicology and environmental safety*, 71(2), 483-489.
42. Zhang, Jie, et al. "Neonatal chlorpyrifos exposure induces loss of dopaminergic neurons in young adult rats." *Toxicology* 336 (2015): 17-25.
43. C. Elegans II, 2nd Edition Section I The Biological Model
44. Hobert, O. (2005). Specification of the nervous system. In WormBook, pp.1–19.
45. White, J. G., Southgate, E., Thomson, J. N., & Brenner, S. (1986). The structure of the nervous system of the nematode Caenorhabditis elegans. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, 314(1165), 1-340.
46. Benedetto, Alexandre, Catherine Au, and Michael Aschner. "Manganese-induced dopaminergic neurodegeneration: insights into mechanisms and genetics shared with Parkinson's disease." *Chemical reviews* 109.10 (2009): 4862-4884.
47. Cole, R. D., Anderson, G. L., & Williams, P. L. (2004). The nematode *Caenorhabditis elegans* as a model of organophosphate-induced mammalian neurotoxicity. Toxicology and applied pharmacology, 194(3), 248-256.
48. http://wormshack.ua.edu/Harrington-Knight-Methods.pdf
49. Brenner, F. E., et al. "Morbidity among employees engaged in the manufacture or formulation of chlorpyrifos." *British journal of industrial medicine* 46.2 (1989): 133-137.
50. Harnly, Martha, et al. "Correlating agricultural use of organophosphates with outdoor air concentrations: a particular concern for children." *Environmental health perspectives* (2005): 1184-1189.
51. Porta-de-la-Riva M, Fontrodona L, Villanueva A, Cerón J. Basic *Caenorhabditis elegans* Methods: Synchronization and Observation. *Journal of Visualized Experiments : JoVE*. 2012;(64):4019. doi:10.3791/4019.
52. Schumacher, B., Hanazawa, M., Lee, M.H., Nayak, S., Volkmann, K., Hofmann, E.R., Hengartner, M., Schedl, T., and Gartner, A. (2005). Translational repression of *C. elegans* p53 by GLD-1 regulates DNA damage-induced apoptosis. Cell *120*, 357–368.
53. Lakso, Merja, et al. "Dopaminergic neuronal loss and motor deficits in Caenorhabditis elegans overexpressing human α-synuclein." *Journal of neurochemistry* 86.1 (2003): 165-172.
54. Salinas, L. S., E. Maldonado, and R. E. Navarro. "Stress-induced germ cell apoptosis by a p53 independent pathway in Caenorhabditis elegans." *Cell Death & Differentiation* 13.12 (2006): 2129-2139.
55. Schumacher, Björn, et al. "The C. elegans homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis." *Current biology* 11.21 (2001): 1722-1727.
56. Tang, J., Cao, Y., Rose, R. L., Brimfield, A. A., Dai, D., Goldstein, J. A., & Hodgson, E. (2001). Metabolism of chlorpyrifos by human cytochrome P450 isoforms and human, mouse, and rat liver microsomes. *Drug metabolism and disposition*, 29(9), 1201-1204.
57. Taki, Faten A., Xiaoping Pan, and Baohong Zhang. "Nicotine exposure caused significant transgenerational heritable behavioral changes in Caenorhabditis elegans." *EXCLI journal* 12 (2013): 793.

58. Gumienny, Tina L., et al. "Genetic control of programmed cell death in the Caenorhabditis elegans hermaphrodite germline." *Development* 126.5 (1999): 1011-1022.

VII. Tables and Figures

Endpoint	Treatment	N	Mean (µm)	Std. Deviation	Std. Error
Length	**DMSO-Control**	30	813.40	52.03	9.50
	1ug/L Chlorpyrifos	25	840.69	33.47	6.69
	3mg/L Chlorpyrifos	23	601.23	68.00	14.18
Width	**DMSO-Control**	30	98.44	9.68	1.77
	1ug/L Chlorpyrifos	25	102.80	11.07	2.21
	3mg/L Chlorpyrifos	23	86.01	10.58	2.21
Area	**DMSO-Control**	30	81452.21	9908.33	1809.01
	1ug/L Chlorpyrifos	25	87915.86	10289.00	2057.80
	3mg/L Chlorpyrifos	23	53033.89	11461.44	2389.87

Table 1. Worm Body Measurements

Endpoint	Treatment	N	Mean	Std. Deviation	Std. Error
Wavelength:Body Length Ratio	**DMSO-Control**	30	0.5150692	0.03682317	0.006723
	1ug/L Chlorpyrifos	25	0.5035531	0.04636112	0.009272
	3mg/L Chlorpyrifos	23	0.5079139	0.05385663	0.01123
Amplitude:Body Length Ratio	**DMSO-Control**	30	0.0487664	0.01576667	0.002879
	1ug/L Chlorpyrifos	25	0.0495531	0.02565579	0.005131
	3mg/L Chlorpyrifos	23	0.0502066	0.02402462	0.005009

Table 2. Worm Posture Data.

Endpoint	Treatment	N	Mean	Std. Deviation	Std. Error
Forward Speed	**DMSO-Control**	28	28.0753 µm/s	24.28366	4.58918
	1ug/L Chlorpyrifos	24	36.8651 µm/s	23.38231	4.77289
	3mg/L Chlorpyrifos	23	18.924 µm/s	13.0344	2.71786
Reverse Speed	**DMSO-Control**	28	-31.4809 µm/s	26.74447	5.05423
	1ug/L Chlorpyrifos	24	-40.317 µm/s	33.8729	6.91428
	3mg/L Chlorpyrifos	23	-19.5694 µm/s	14.50439	3.02437
Reversal Ratio	**DMSO-Control**	28	0.2718	0.1011	0.01911
	1ug/L Chlorpyrifos	24	0.2167	0.13526	0.02761
	3mg/L Chlorpyrifos	23	0.3358	0.11609	0.02421

Table 3. Worm Movement Data

Treatment Group	N	Mean Apoptotic Cells	Std. Error
DMSO Control	61	0.7705	0.11062
1ug/L Chlorpyrifos	60	1.25	0.1881
3mg/L Chlorpyrifos	60	2.05	0.21448

Table 4. Apoptosis Data

Treatment	N	Pumps/sec	Std. Error
DMSO Control	25	2.6638	0.06191
1ug/L Chlorpyrifos	20	2.5882	0.06322
3mg/L Chlorpyrifos	20	1.6975	0.0619

Table 5. Pharyngeal Pumping Rate Data

Treatment Group	N	Proportion LG	Proportion LG (ADE)	Proportion LG (CEP)
Control	100	0.1±0.030	0.04±0.020	0.08±0.027
1ug/L Chlorpyrifos	100	0.25±0.043	0.11±0.031	0.17±0.038
3mg/L Chlorpyrifos	100	0.26±0.044	0.14±0.035	0.14±0.035

Table 6. Dopaminergic Neuron Assessment Data

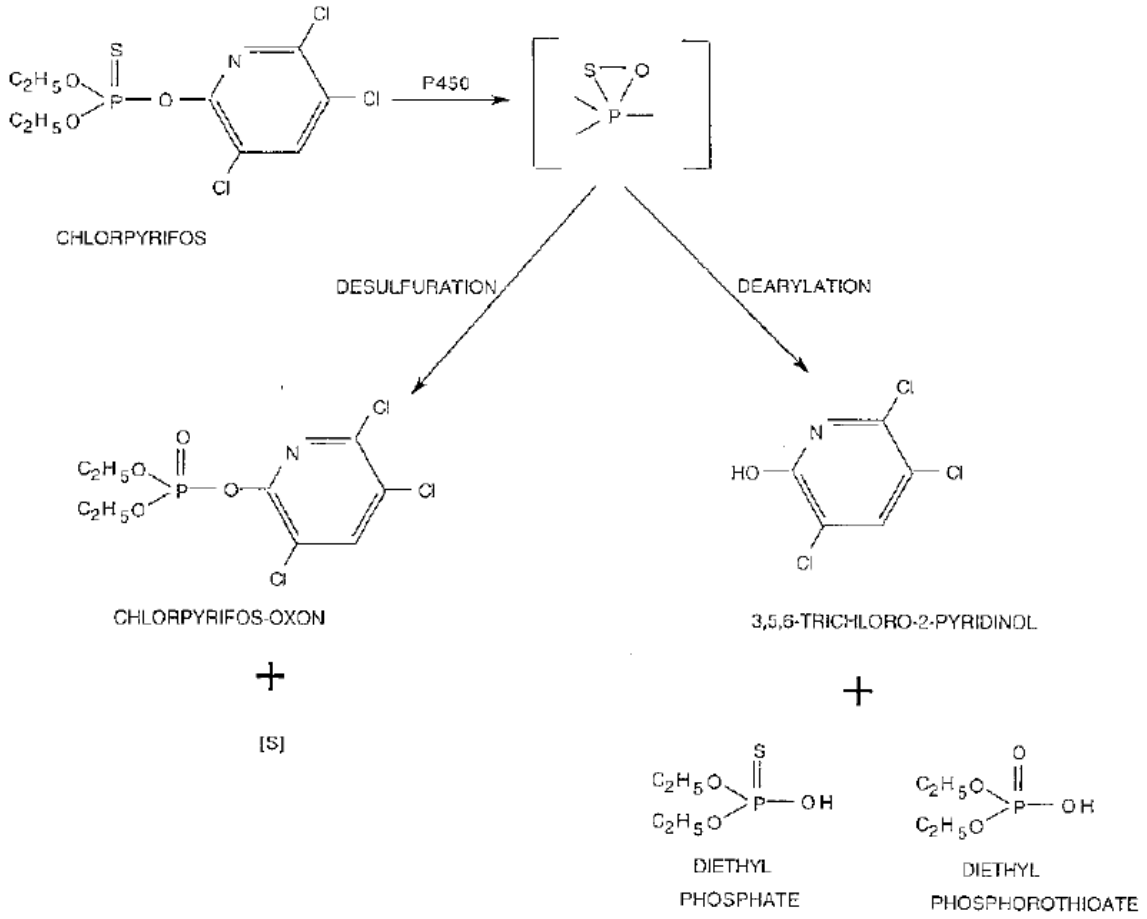


Fig 1. Cytochrome P450 dependent Chlorpyrifos metabolism.[56]

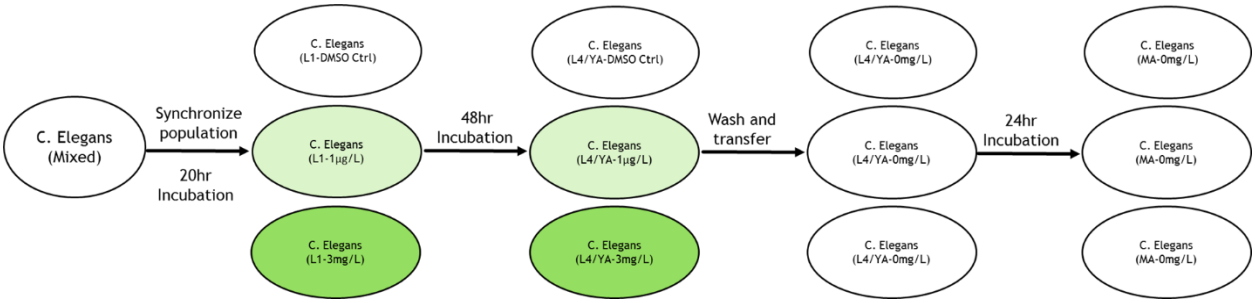


Fig 2. Overview of *C. elegans* treatment timeline Organisms to be synchronized to produce an initial population consisting entirely of L1 larvae. Organisms are exposed to chlorpyrifos through larval stages up to young adulthood (~48hr), allowed to grow in absence of chlorpyrifos for 24hr, then observed as mature egg-laying adults.

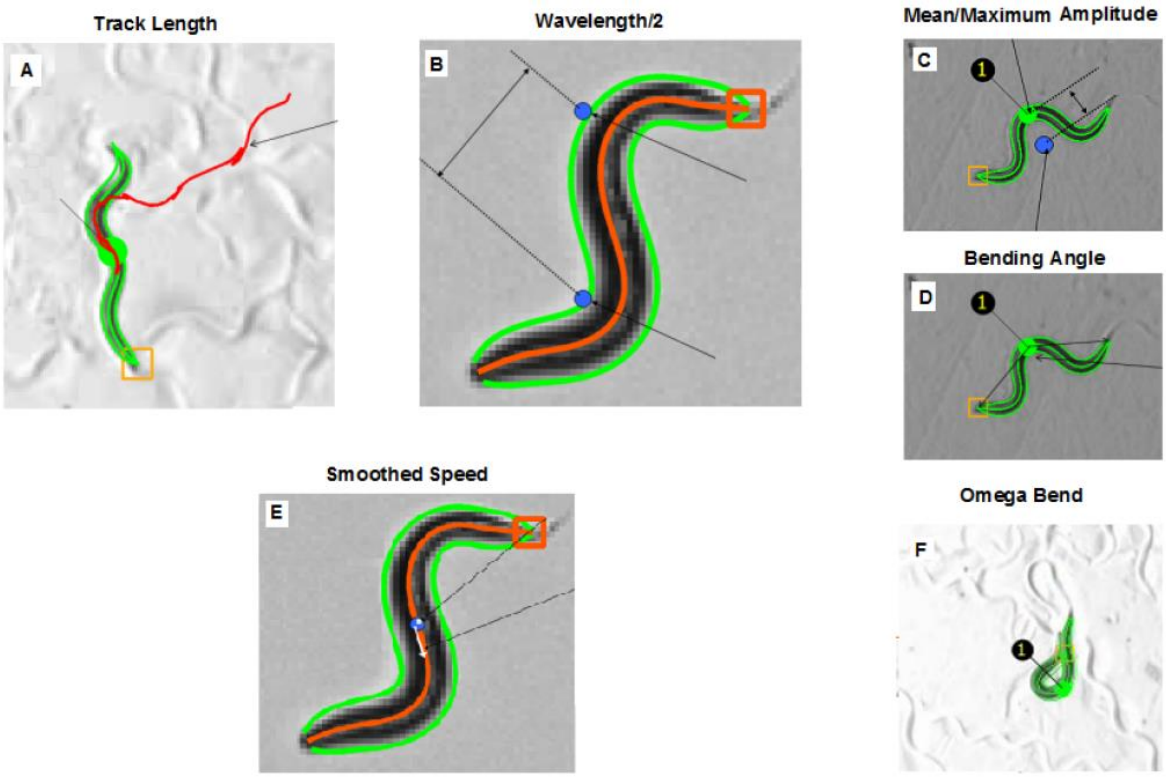


Fig 3. Overview of factors recorded by Wormlab software.[57]

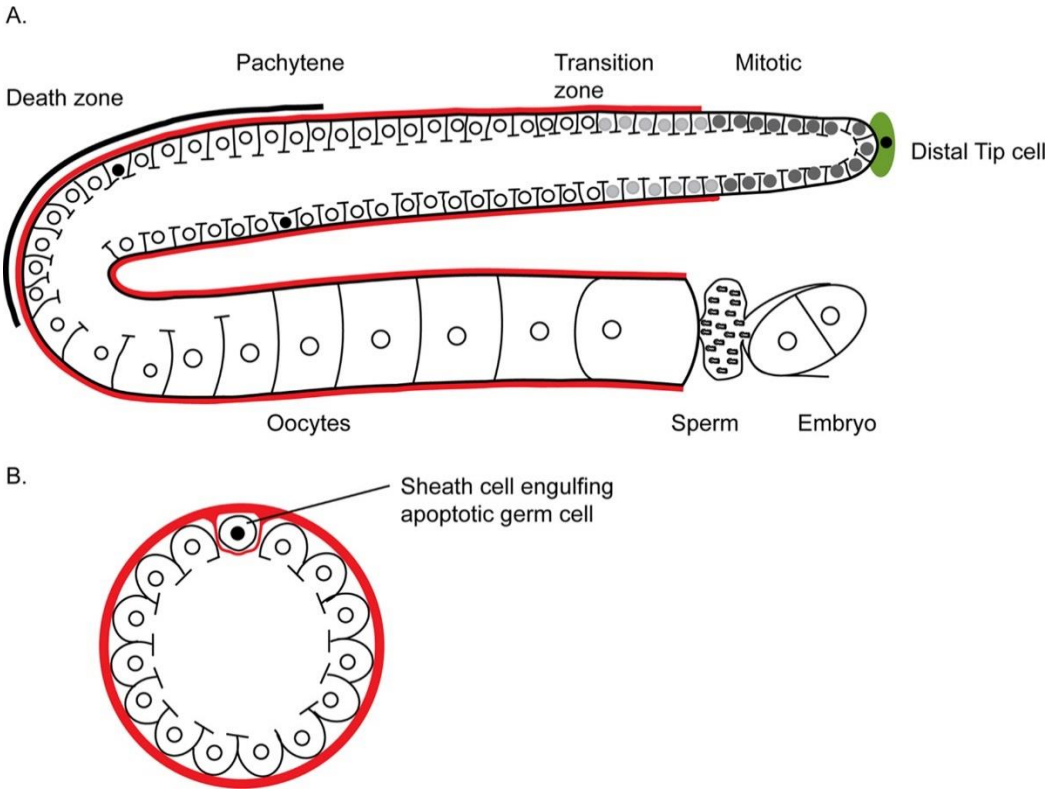
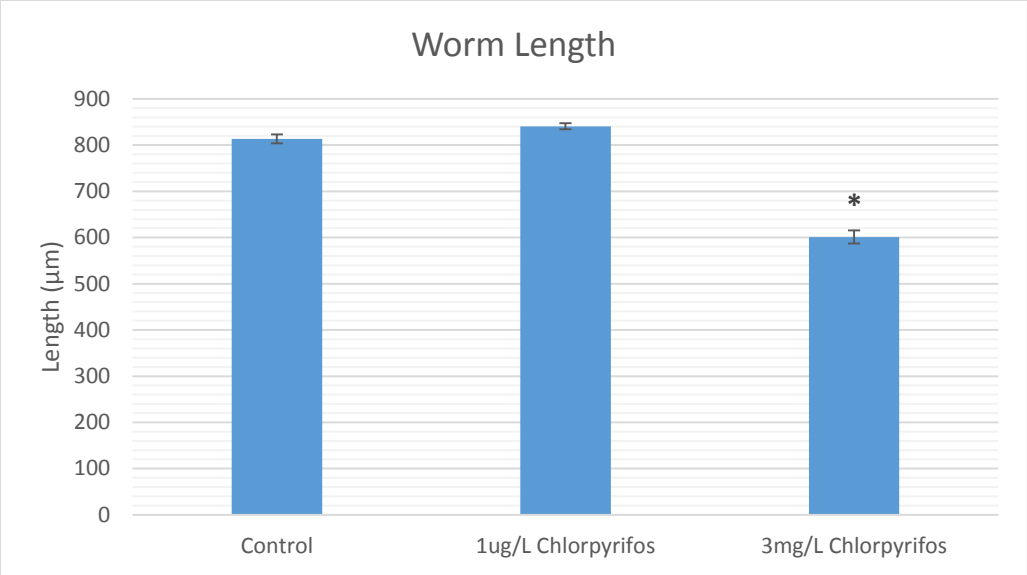


Fig 4. *C. elegans* gonad overview, taken from Gumienny et al. 1999 .[58] All GFP positive cells along gonad are counted, with particular attention paid to cells residing within the gonad loop or "Death Zone."



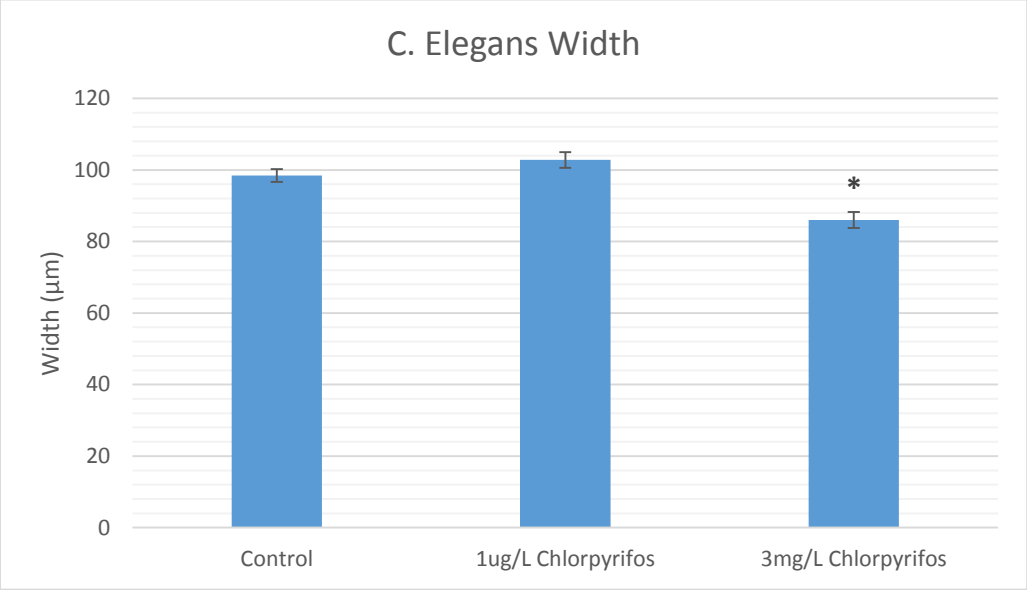


Fig 5. Average *C. elegans* length and width among low and high chlorpyrifos treatment groups. Hermaphroditic worms exposed for 48 hours from larval stage 1 to stage 4. Videos taken of young adult worms 9 to 11 hours after treatment. High treatment group experienced developmental delay during treatment. Measurements from WormLab analysis of videos. * indicates significant difference from control ($p<0.05$). Error bars indicate standard error.

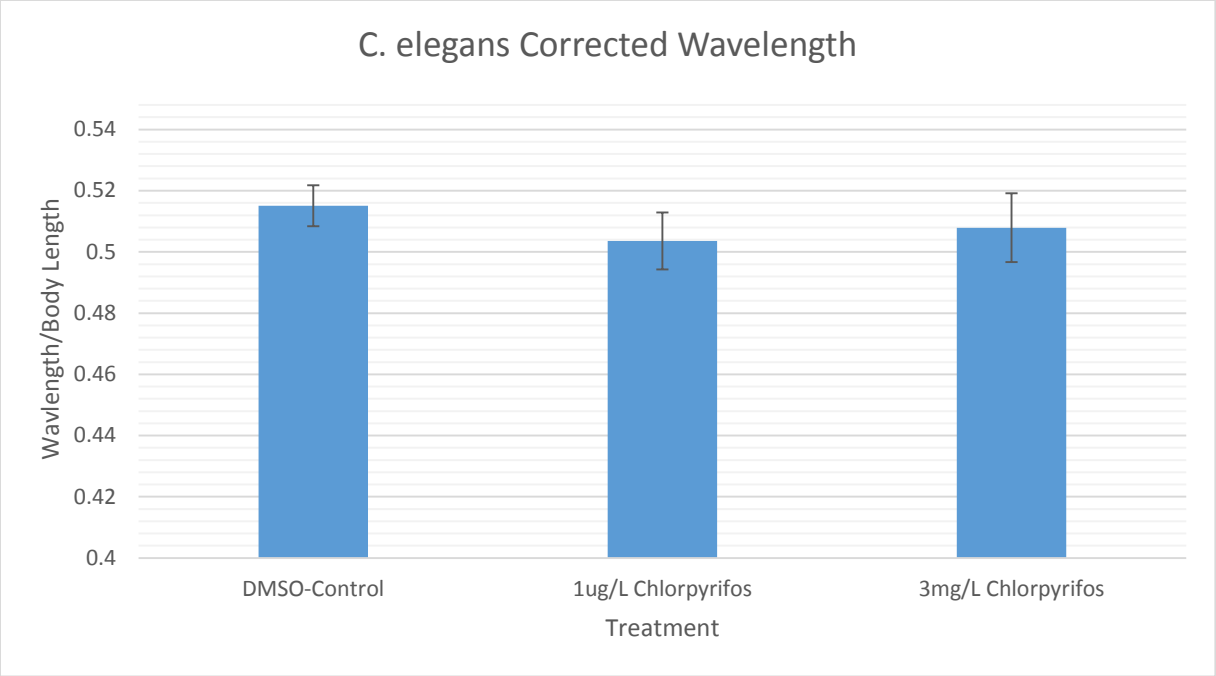


Fig 6. Average corrected wavelength (wavelength/body length) of *C. elegans* body after developmental chlorpyrifos treatment. Worms exposed for 48 hours from larval stage 1 to stage 4. Videos taken of young adult worms 9 to 11 hours after treatment. Measurements from WormLab analysis of videos. Error bars indicate standard error.

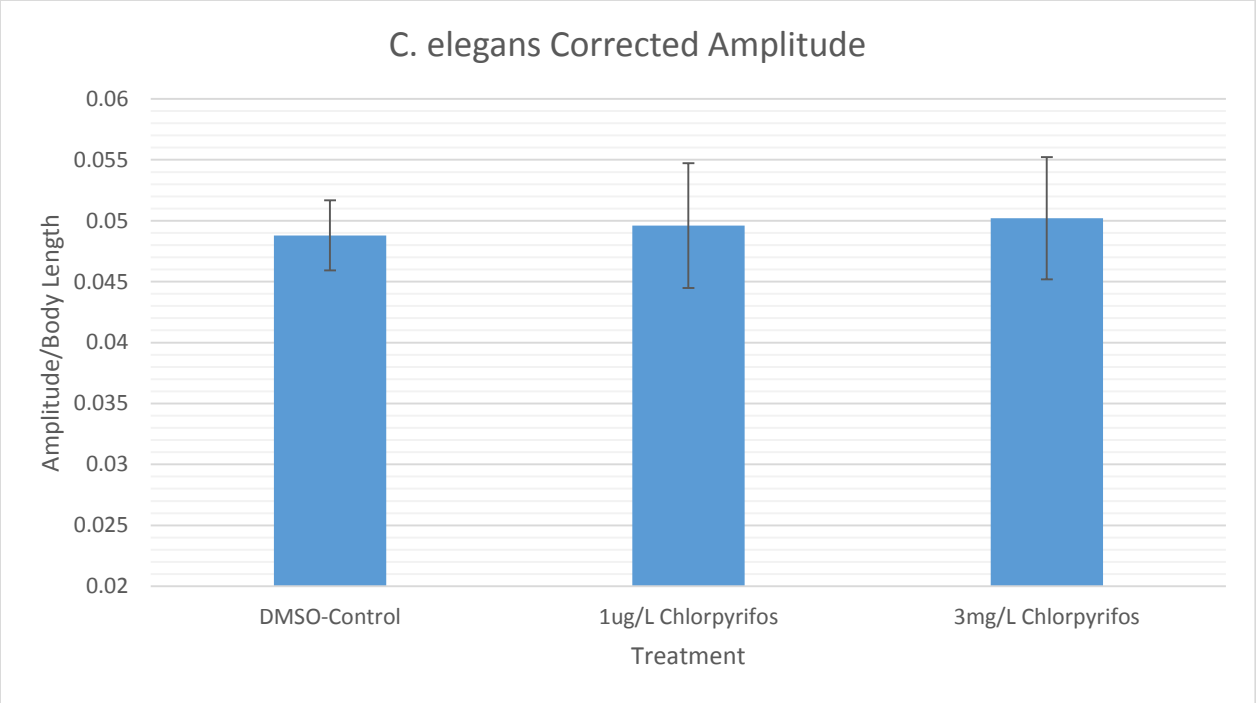


Fig 7. Average corrected amplitude (amplitude/body length) of *C. elegans* body after developmental chlorpyrifos treatment. Worms exposed for 48 hours from larval stage 1 to stage 4. Videos taken of young adult worms 9 to 11 hours after treatment. Measurements from WormLab analysis of videos. Mean amplitude of a single organism an average of amplitude measurements over course of video. Error bard indicate standard error.

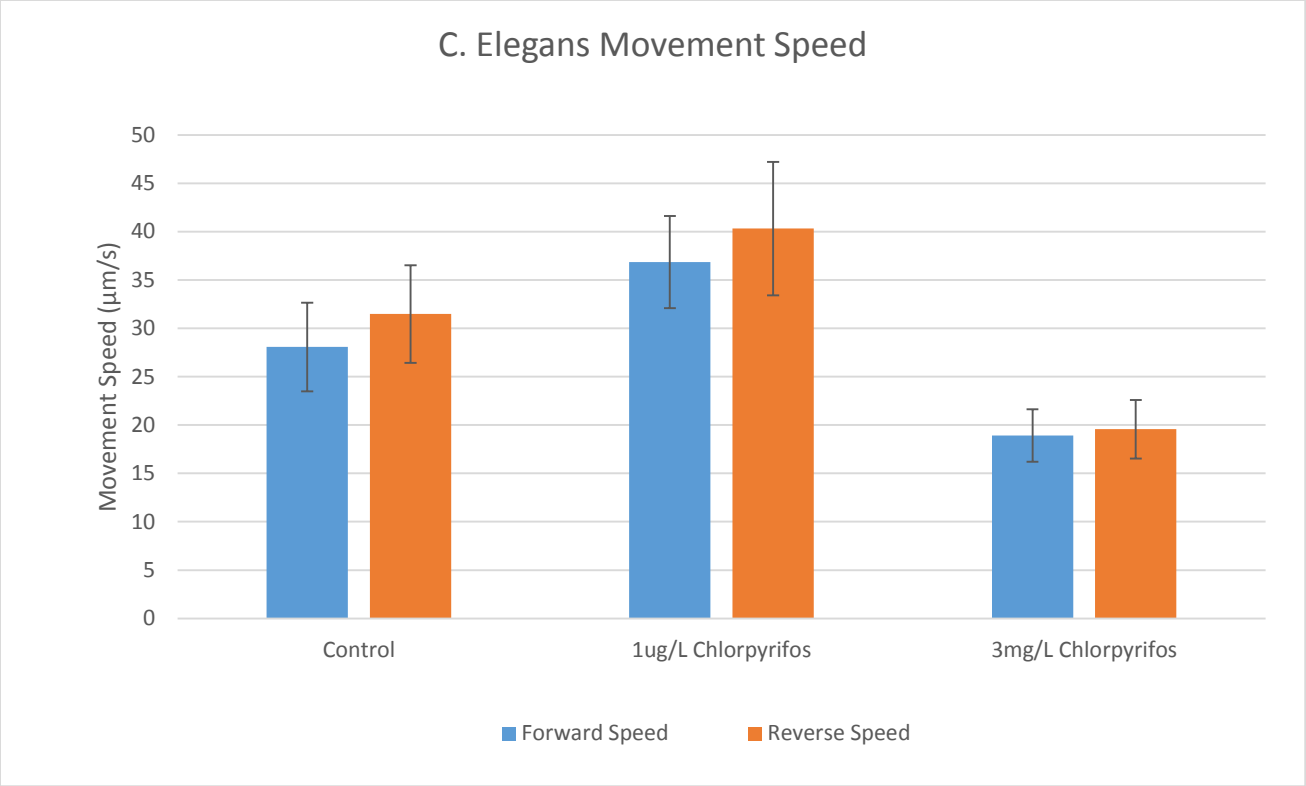


Fig 8. Average *C. elegans* forward and reverse speed among low and high chlorpyrifos treatment groups. Worms exposed for 48 hours from larval stage 1 to stage 4. Videos taken of young adult worms 9 to 11 hours after treatment. Measurements from WormLab analysis of videos. Error bars indicate standard error.

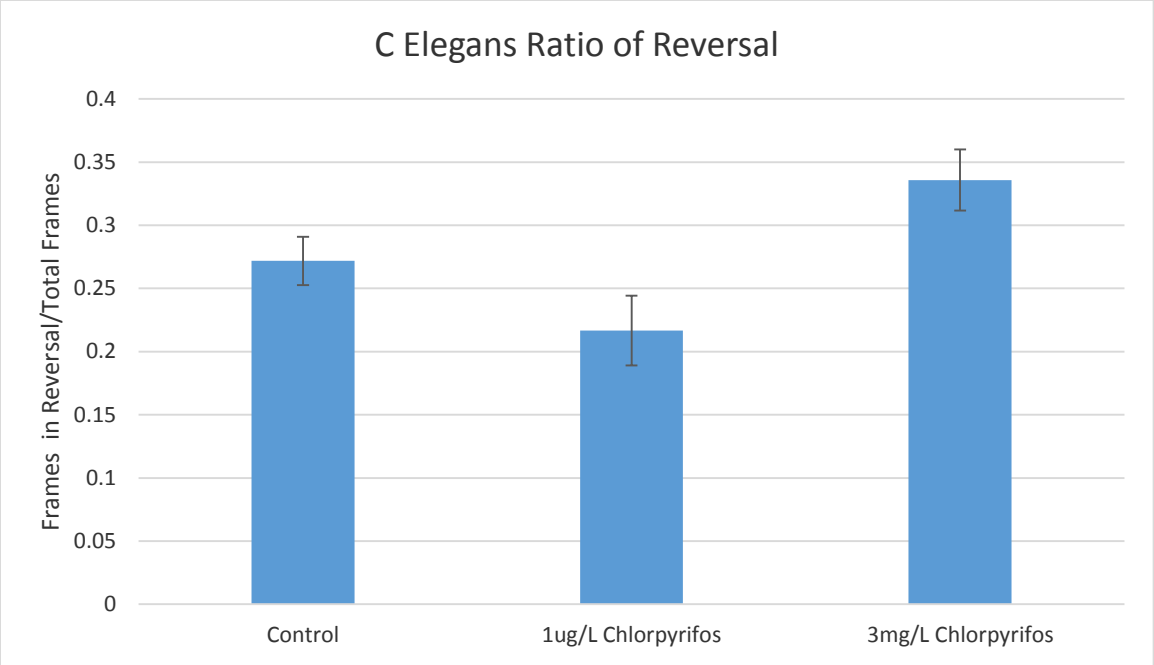


Fig 9. Average portion of time spent in reversal for low and high treatment groups. Ratio calculated by taking number of frames in video where worms were in reversal and dividing by total number of frames for the video. Measurements from WormLab analysis of videos. Error bars indicate standard error.

Apoptosis Assay

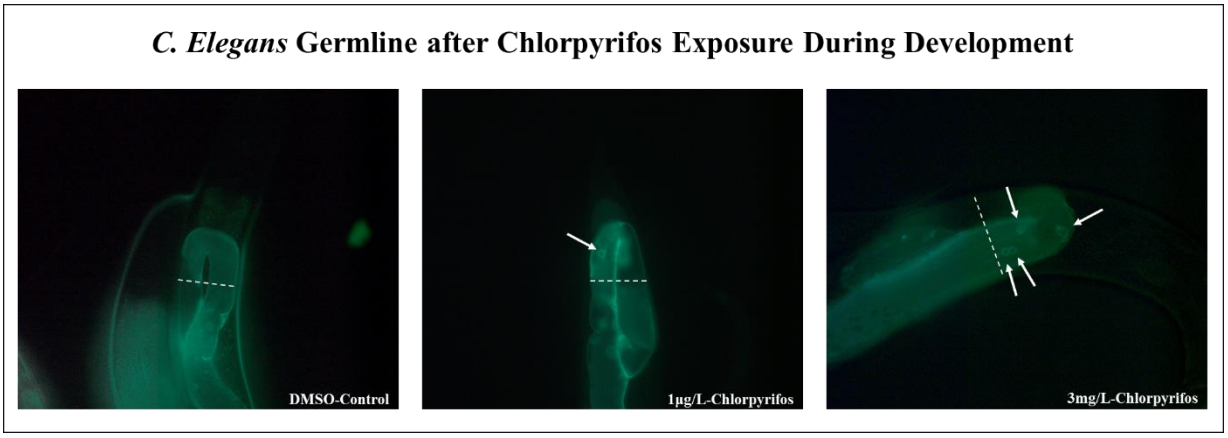


Fig 10. Images of MD701-strain *C. elegans* germlines after exposure to chlorpyrifos during development. Line indicates demarcation of gonadal loop, wherein apoptotic cells were counted. Rings of GFP fluorescence identify apoptotic cells undergoing engulfment, identified by white arrows. Heavily exposed worms showed an increase in observable apoptotic cells.

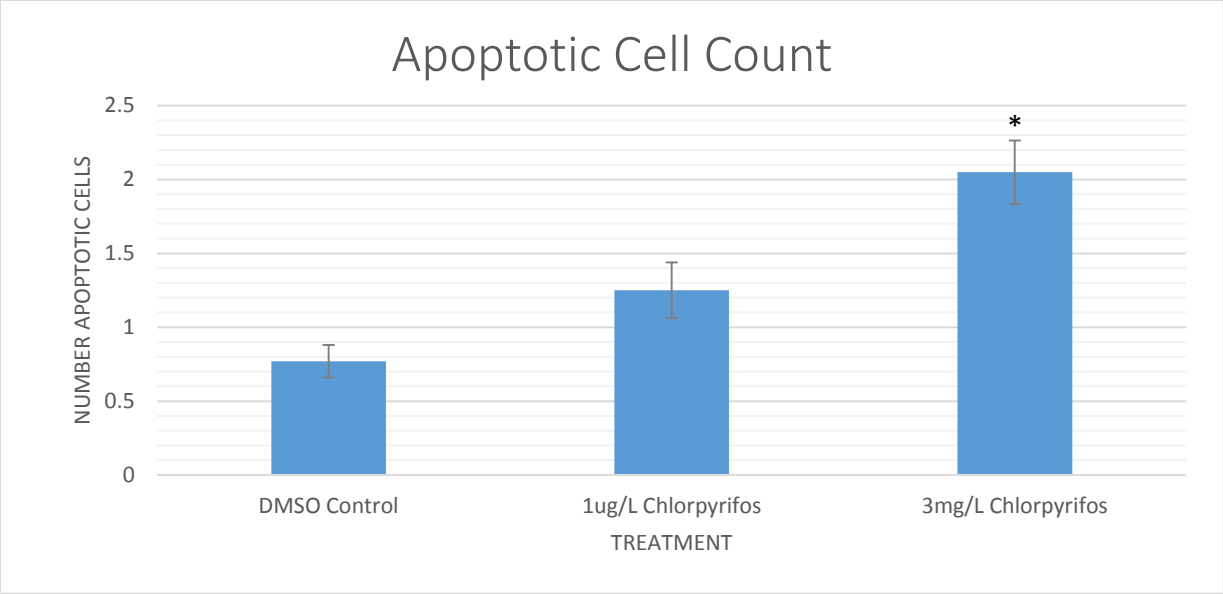


Fig 11. Observed number of apoptotic germline cells in MD701 strain *C. elegans* after 48 hours chlorpyrifos exposure and 1 day free of exposure. Adult, egg-laying worms were examined and apoptotic cells within the gonad loop were counted. * indicates significant difference from control ($p<0.05$). Error bars indicate standard error.

Pharyngeal Pumping Assay

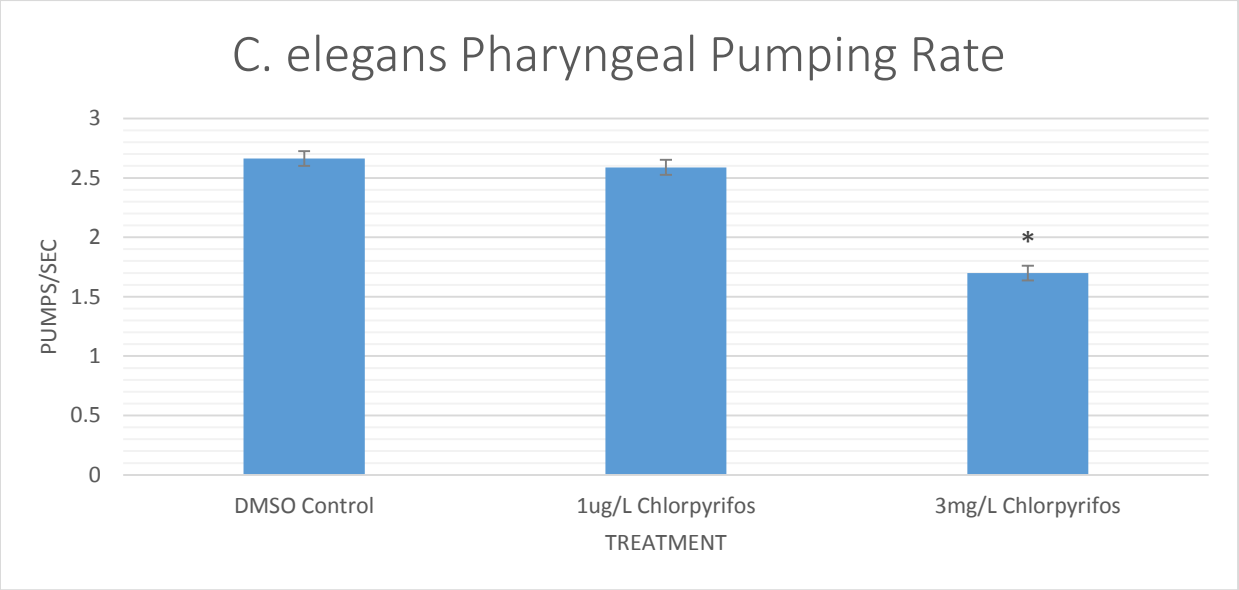


Fig 12. Pharyngeal pumping rates of *C. elegans* exposed to low dose and high dose chlorpyrifos treatments. Videos were taken of 20-25 worms per treatment group and the average pumping rate for the group was calculated by counting the number of pharyngeal movements and dividing by video time. Young adult worms recorded on treatment. * indicates significant difference from control ($p<0.05$). Error bars indicate standard error.

Dopaminergic Neuron Assay

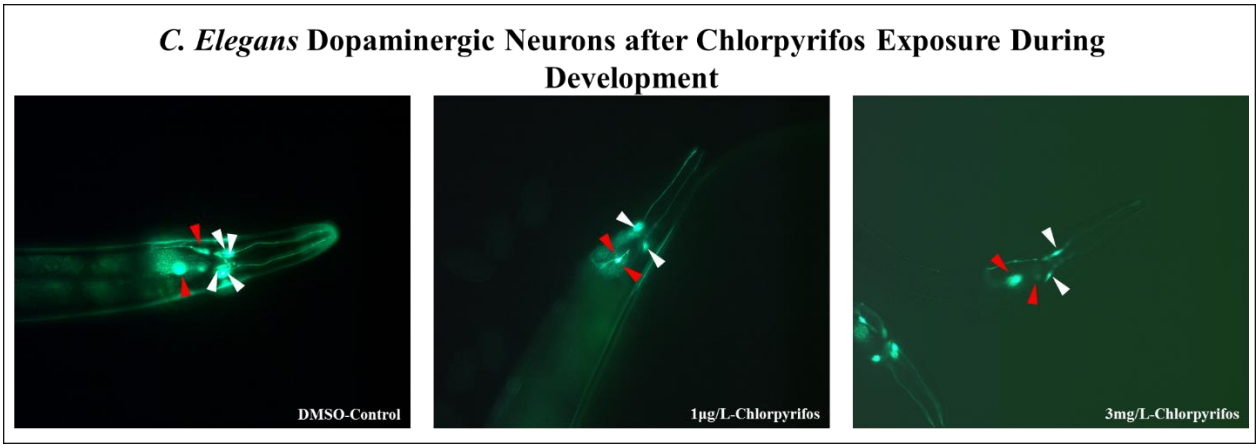


Fig 13. Images of Head Dopaminergic Neurons in UA44 *C. elegans* exposed to chlorpyrifos during development. White arrows identify CEP neurons (4 present in DMSO-Control, 2 in both 1µg/L and 3mg/L Chlorpyrifos exposed individuals) and red arrows identify ADE neurons (2 present in all individuals). Treatment groups showed loss of GFP in more neurons than control.

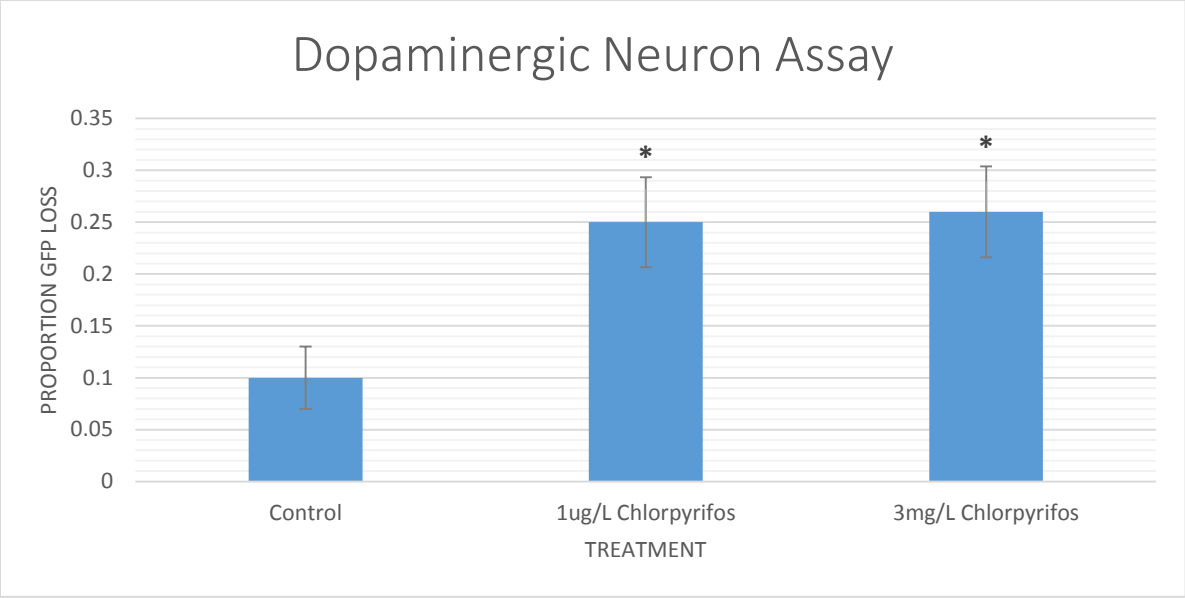


Fig 14. Proportion of UA44-strain *C. elegans* displaying loss of GFP in head dopaminergic neurons after 48hr exposure to Chlorpyrifos and 1 day free of exposure. Per group, 100 organisms were examined. An organism was considered positive for GFP loss if one or more of its two ADE neurons or four CEP neurons could not be found. * indicates significant difference from control ($p<0.05$). Error bars indicate standard error.

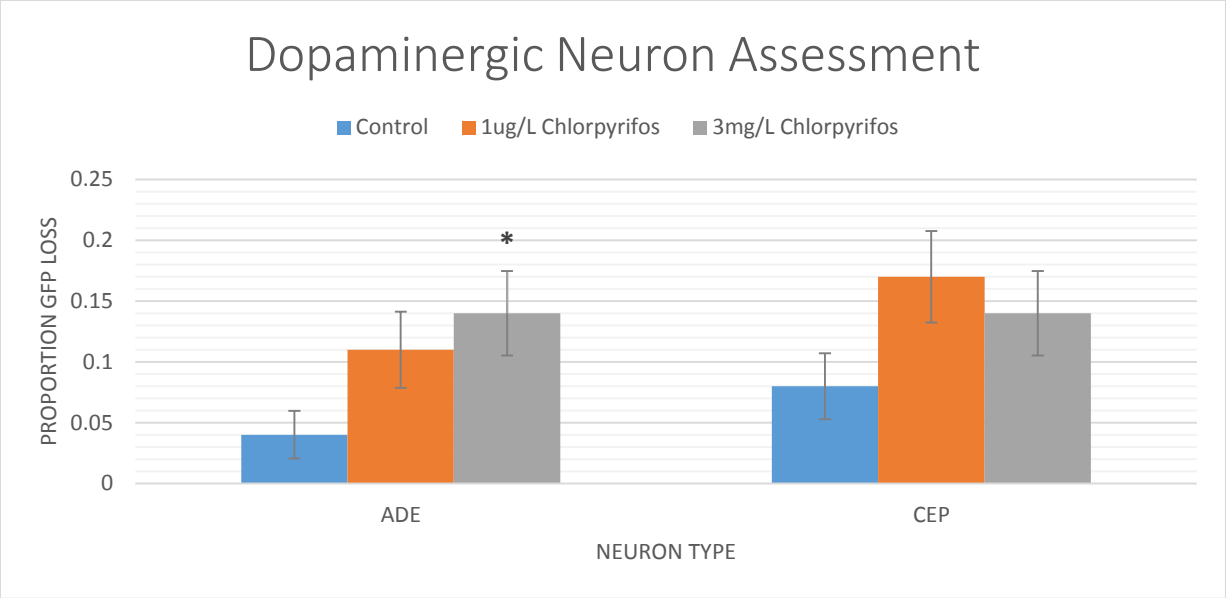


Fig 15. Breakdown of GFP loss within UA44-strain *C. elegans* dopaminergic neurons by neuron type. Shown is the proportion of each treatment group showing loss of GFP in either ADE or CEP neurons. * indicates significant difference from control ($p<0.05$). Error bars indicate standard error.