Investigating the Effects of Low-Level Chlorpyrifos Exposure on Neuromuscular Function and Locomotory Behaviour in *C. elegans* Wild Type and *gar-3* Mutants, to Identify Potential Regulatory Genes That Could be Associated With Depression.

A report submitted as the examined component of the Project Module SXL390 (B route)

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(4993 words)

Abstract

The use of organophosphates as pesticides are having disruptive effects on the environment and non-target organisms including humans, due to their often-improper application and high neurotoxicity levels. Organophosphates inhibit acetylcholinesterase which breaks down the neurotransmitter acetylcholine. This causes a build up between neuronal synapses causing paralysis in target organisms however, links have been found between exposure and neurological disorders in humans such as depression, though the mechanisms behind this are unknown. This study investigated the effects of low-level Chlorpyrifos exposure on neuromuscular function and locomotory behaviour of wild-type and mutant *C. elegans*, to determine whether any observed effects could be attributed to a potential gene of interest, *gar-3.* An online video database, tracker tool and imaging software were used to obtain measurements for turning frequency, Euclidean and total distance travelled for both wild-type and *gar-3* mutants treated with 0.05 mg of Chlorpyrifos over a 24-hour period, as well as controls for each group. 15 worms were measured in triplicate for each group giving a total of 60 independent samples. A two-way ANOVA followed by post-hoc and Bonferroni correction was used to determine whether results were statistically significant. Exposure to Chlorpyrifos significantly affected locomotory behaviour in all three end points in wild-type ($P = <0.001$) when compared to controls, whereas *gar-3* mutants were unaffected (distance; $p = 0.203$, Euclidean and turn frequency; $p = 1.000$). Wild-type had significantly higher turning frequency and shorter Euclidean distance compared to *gar-3* mutants, implicating Gar-3 receptors in the build-up of acetylcholine at neuromuscular junctions. Further research is needed however observations from this study highlight *gar-3* as a potential gene of interest and could be used a model for future research of *gar-3* homologs in mammalian trials, which could aid further understanding of neurotoxic effects of organophosphates in humans and associated links to depression.

(299 words)

List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
BBF	Body bend frequency
CPF	Chlorpyrifos
ED	Euclidean distance
OPs	Organophosphates
GAR-3	G-protein-linked acetylcholine receptor
Gar-3	Gar-3 mutant phenotype
gar-3	Gene encoding G-protein-linked acetylcholine receptor
G3 +/-	gar-3 treated / untreated (shorthand)
HTF	Head thrash frequency
mAChR	Muscarinic acetylcholine receptors
Mn	Mean
nAChR	Nicotinic acetylcholine receptor
TDT	Total distance travelled

TF	Turning frequency
WT +/-	Wild type treated / untreated (shorthand)

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1 Introduction

1.1 Background

Organophosphates (OPs) are chemical substances commonly used as pesticides in agricultural practices against parasitic nematodes. OPs act as an acetylcholinesterase (AChE) inhibitor preventing break down of the neurotransmitter acetylcholine (ACh) within the synaptic cleft, leading to a build up at neuromuscular junctions and ultimately paralysis of nematodes (figure 1.1) (Holden-Dye and Walker, 2014). ACh is the primary neurotransmitter at neuromuscular junctions of nematodes and so a primary target for anthelmintic drugs (Rand, 2007), (Spensley, 2018). However, extensive use of these highly toxic pesticides are having a huge negative and toxicological impact on the local environment and non-target organisms inhabiting these niches, including humans (Govindarajan et al, 2019) (Ruan et al, 2009), (Wang et al, 2021). Previous studies have indeed found links between pesticide exposure and mood disorders such as depression and other neuropsychological disorders, however the mechanisms behind OP exposure and depression are still unknown (Govindarajan et al, 2019) (Mackenzie Ross and Harrison, 2016), (Siqueira et al, 2019), (Wang et al, 2021).

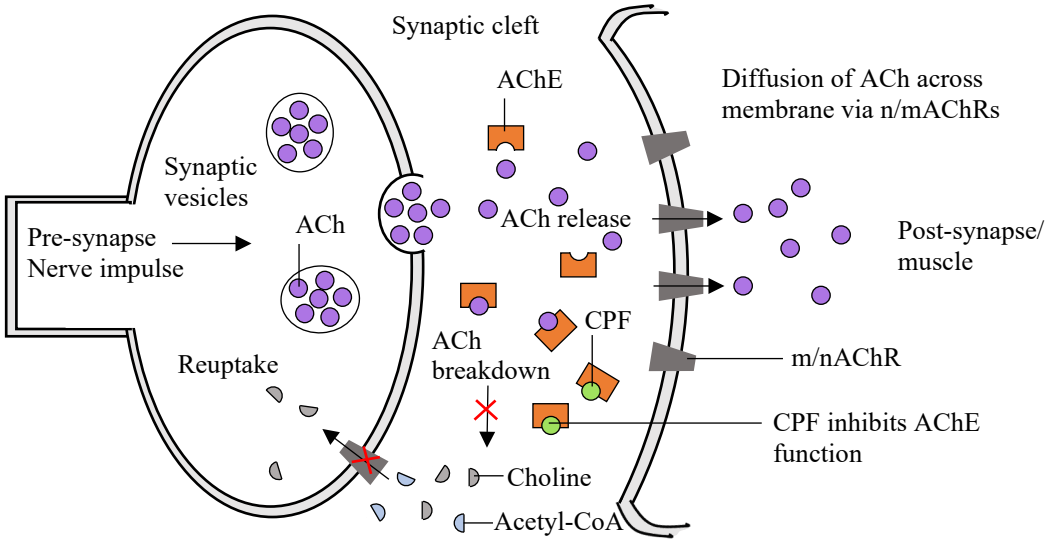

Figure 1.1 Neurotransmission of acetylcholine at neuromuscular junction and inhibition of AChE by CPF (adapted from Rand, 2007, figure 1 and Muppidi, 2012, pp. 1150, figure 91-1).

The nematode *Caenorhabditis elegans* is a well-known model organism, sharing its physiology and pharmacology with the phylum Nematoda and has conserved orthologs; homologous genes, and signalling pathways with humans (Hunt, 2017) (Holden-Dye and Walker, 2014).With 302 neurons and a fully mapped and sequenced genome, any mutations that may give rise to phenotypic traits in *C. elegans* are easily identifiable. Additionally, the entire organism can be assayed unlike cell cultures, in order to monitor its habitual locomotory and neuromuscular systems (Hunt, 2017). This makes *C. elegans* an excellent model to explore effects of OP exposure on neuronal function and a cheap alternative to other mammalian laboratory animals for investigation into human pathways of toxicity and pesticide exposure (Hunt, 2017).

C. elegans move in an undulatory motion. Release of ACh into musculature causes contraction (figure 1.2) followed by a breakdown of ACh by AChE causing relaxation of body wall muscles, creating sigmoidal forwards and backwards motions, as well as turning or pivoting and thrashing, depending on presence of chemical stimulus or medium (Wakabayashi et al, 2004), (Gjorgjieva, 2014).

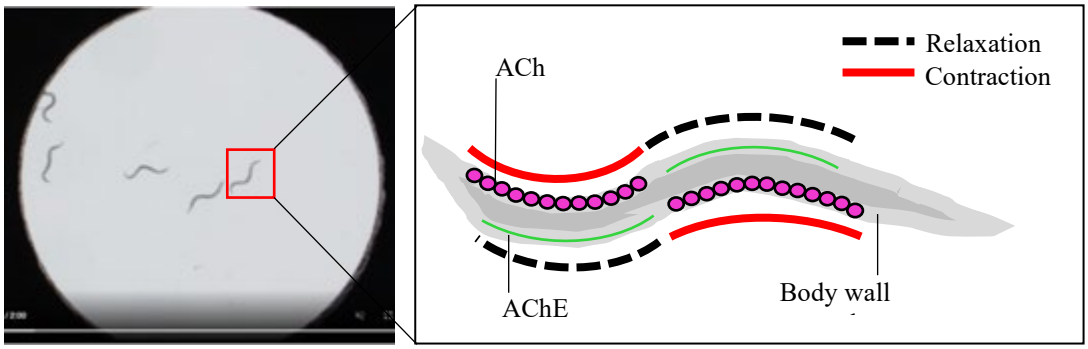

Figure 1.2 Contraction and relaxation of body wall musculature in *C. elegans* (a) Screenshot from video database (b) Blown up representation of *C. elegans* locomotion (adapted from Han et al, 2015. pp. 1160, Fig 1).

This makes observation of locomotory changes in *C. elegans* relatively easy via microscopy, which reflect neuromuscular function (Qu, 2020). Numerous studies have investigated changes in *C. elegans* neuromuscular function and locomotion following exposure to OPs, as well as methodologies and techniques to interpret such data (Hunt, 2017). Some studies found evidence to suggest chlorpyrifos reduced locomotion as well as body bend frequency (BBF) and head thrash frequency (HTF) in *C. elegans* after 24

hours exposure (Ruan et al, 2009). Similar deleterious effects to locomotion have been found using other OPs, which correlated to similar effects in higher organisms. *C. elegans* have therefore been suggested as a good model for initial neurotoxicity screening given their similarity in toxicity order (Govindarajan, 2019), (Cole, 2004).

Understanding the genes affected by CPF exposure causing certain phenotypic affects is key to determining molecular mechanisms involved and any associations to behaviour in higher organisms. Indeed, studies have found behaviour in rats exposed to CPF resemble models for depression given CPF affects multiple neuronal signalling pathways such as the serotonergic system, which is known to cause depression in humans. Interestingly, these behavioural effects have been caused by exposure levels lower than those causing AChE inhibition, highlighting the requirement to understand specific processes and genes involved which mediate response to CPF exposure (Aldridge, 2005), (Siqueira et al, 2019).

Research into potential genes of interest such as *Ace1-4,* which encode AChE have been conducted using mutants to determine effects of CPF exposure on *C. elegans.* However, two key receptors which facilitate transport of ACh across synapses are located on post synaptic cells; muscarinic acetylcholine receptors (mAChRs) and nicotinic AChRs (nAChRs) (Rand, 2007). Encoded by *gar-3* and *unc-29* respectively, these receptors are expressed in cholinergic motor neurons located in the body wall musculature of *C. elegans* and are Orthologs of mammalian mAChRs (M1/M3/M5 in humans) and nAChRs which mediate ACh functioning within the central nervous system (CNS) (Chan et al, 2013). Previous connections have been made between cholinergic transmission and depression as well as studies into nAChRs and their use as potential drug targets (Philip, 2010), (Bertrand, 2022). Research into mAChRs and depression found agonists can induce pro-depressive behaviour in adult rats following neonatal CPF exposure (Small, 2016). Previous studies into the effects of *gar-3* on locomotion found increased ACh levels in ectopically expressed GAR-3 worms caused accelerated paralysis when compared to other mAChRs, evidenced through increased turning rate, exaggerated body bends and shorter Euclidean distance (ED) (Dittman and Kaplan, 2008). *gar-3* is also found more specifically in the pharyngeal muscles which facilitate pharyngeal pumping. Studies into peristalsis and pumping contractions of the pharynx stimulated by ACh found Gar-3 mutants were insensitive to effects of muscarinic agonist arecoline,

suggesting *gar-3* acts as a mediator in some form in wild type strains (Kozlova, 2019). Indeed, feeding and locomotion are the primary behaviours in survival of *C. elegans* and are both controlled in part by *gar-3* encoded mAChRs (Izquierdo. 2022). Despite this, relatively little is known about *gar-3* and its effect or impact on neuromuscular behaviour when exposed to drugs or OPs such as CPF.

1.2 Scope

Using an online video database, this study investigated the effects of low-level CPF exposure on the neuromuscular function and locomotory behaviour of *C. elegans* wild type and Gar-3 mutants, to determine whether any differences observed in wild type could be attributed to and therefore regulated by *gar-3* using the following hypotheses:

1. There will be a difference in locomotory behaviour in wild type *C. elegans* treated with Chlorpyrifos versus untreated measured by the following endpoints:
    a. Turning frequency (TF)
    b. Total distance travelled (TDT)
    c. Euclidean distance (ED)

2. There will be a difference in locomotory behaviour between Wild type *C. elegans* treated with Chlorpyrifos and treated Gar-3 mutants in measured end points [a] to [c].

gar-3 will be discussed to evaluate whether it can be associated with models for depression through further research.

1.3 Objectives

The objectives of the project as are follows:

1. Determine whether Chlorpyrifos exposure has any effect on neuromuscular function and locomotory behaviour of wild-type *C. elegans.*
2. Analysis of *gar-3* mutant strains of *C. elegans* to determine whether any observed differences in neuromuscular function and locomotory behaviour in wild-type are apparent or absent following Chlorpyrifos exposure.

3. Observe and analyse any differences in assayed end points between groups (Wild type and *gar-3* mutant treated and untreated) over a period of time using a video database, measuring software and two-way ANOVA statistical test with Bonferroni correction.
4. Analysis of quantitative (turning frequency, Euclidean and total distance) and qualitative data (locomotory behaviour) to compare findings to previous studies in the field to assess scientific relevance.
5. Discuss whether findings from this model of work could be applied to behaviour in vertebrates following organophosphate exposure.
6. Discuss whether potential gene of interest (*gar-3*) in *C. elegans* can be associated with depression.

2 Methods

2.1 Database analysis

This was a database investigation. A video database of Wild type (WT) treated and control as well as mutant strains of *C. elegans* were available via the SXL390 online object tracker tool and was used to observe *C. elegans* to record and obtain the data. Treated *C. elegans* were exposed to 0.05 mg of CPF for 24 hours. A lower dose was chosen to allow visual observation of any locomotory changes and behaviours via microscopy, given previous work shows worms exhibit reduced locomotion following treatment in a concentration-dependent manner, significantly higher doses of CPF leading to full paralysis preventing observation of effects (Ruan et al, 2009), (Izquierdo, 2021). Control groups were exposed to acetone only, given Chlorpyrifos is made up in acetone. Well-fed *C. elegans* were loaded onto plates without food for observation. All worms were already rested on the plate following exposure and so already acclimatized at the time of recording.

2.2 Pilot studies

Initial pilot study data collection (supplementary file 1) began with use of *Unc-29* as the chosen mutation to observe however, it became apparent there were insufficient numbers of videos within the database to collect enough samples for viable statistical analysis. This limitation led to further exploration and selection of *gar3* as the chosen mutation for observation. During this pilot study (supplementary file 1) there was a noticeable difference in the total distance travelled (TDT) between treated and untreated WT groups as well as Gar-3 (G3) mutants, which led to the observation and measurement of ED. Observation and tracing of movement via the tracker showed untreated WT travelled in their usual undulatory pattern and mostly in a forwards direction, creating simplistic and smooth traces. Traces from treated worms showed much less uniformity and more erratic, angular movements (figure 2.1 a, b). These observations seemed to coincide with a change in direction, which correlate with a shorter total and ED, setting turning frequency (TF) as the third dependent variable and end point in the assay. Little difference was observed between treated and untreated G3 subjects (figure 2.1 c, d).

2.3 Study Methodology

15 worms from each group (WT untreated: WT-, treated: WT+, G3 untreated: G3-, treated: G3+) were measured for TDT , ED and TF by placing the centre of the cursor on the tip of the head and tracing movement of each subject at 2 second intervals by setting the video to 10 frames per step for a period of one minute using the object tracker (Wang et al, 2021). Fiji image processing software was used to measure ED and TF (figure 2.2 b and c). To adequately measure distance the scale was set to 1 mm per 67.43 pixels on Fiji, which was based on measurements of 20 wild-type control worms given their known size is approximately 1 mm in length (supplementary file 2). Three technical replicates were carried out for each test subject to obtain mean values for each group and end point. Given the plates contained a random sampling of same age worms, each worm was classed as biologically distinct and therefore one worm was equal to one biological replicate in this investigation. An individual external to this investigation selected videos at random in order to remove any bias from previously collected data and pilot studies.

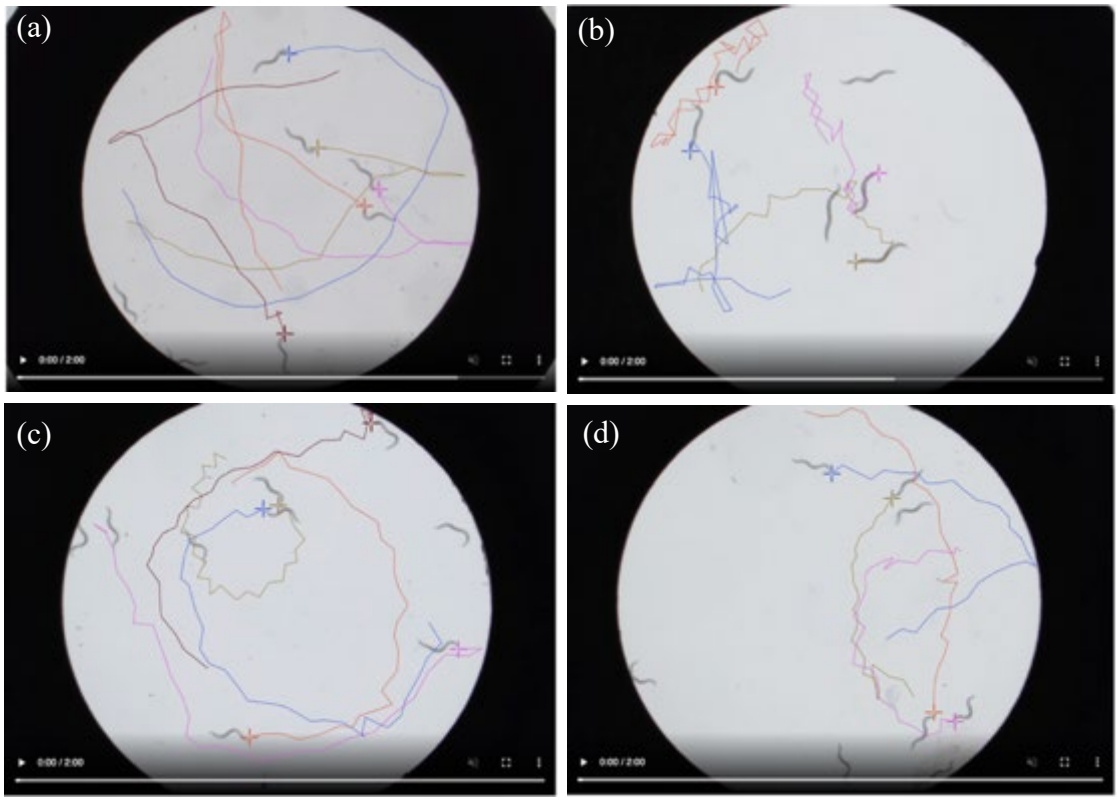

Figure 2.1 Screenshots from the video database object tracker and trace lines from test subjects after 1 minute for **(a)** WT- **(b)** WT + **(c)** G3 - **(d)** G3+.

2.3.1 Turning Frequency (TF)

Locomotory behaviour in *C. elegans* has been mapped to find a series of movements and behavioural states including pivoting or turning, a forward movement governed by sensory structures in the nose which cause the bending of the head during foraging (Wakabayashi, 2004), (Dittman and Kaplan, 2008), (Ouellette et al, 2018). The head region contains body wall and pharyngeal muscles in which *gar-3* is expressed (Kozlova et al, 2019). Additionally, AChE's such as OPs have been found to cause inhibition of pharyngeal pumping, therefore TF was chosen as an end point (Izquierdo, 2021). A pilot study confirmed measuring from the tip of the head was the most effective way to observe changes in direction. Given the functionality of the object tracker, measuring body bends and turns through analysis of curvature and wavelength proved too difficult with much room for inaccuracy and error. Therefore, worm movements were traced using the object tracker and angles from each trace were measured using Fiji processing software. A 'turn' was defined as an angle of 100ºC or less. TF was defined as the number of times worms changed direction (forward) over one minute from the start of the video. The number of turns for each subject were measured and counted, followed by number of reversals. Any reversals were subtracted from the total number of turns to give the final TF in that test.

2.3.2 Total distance travelled (TDT)

Exposure to pesticides such as CPF are known to cause paralysis in nematodes (Holden-Dye and Walker, 2014). The distance travelled by such organisms is therefore one of the most effective ways to study whether there are any changes in movement and is often used in this area of research (Cole, 2004), (Zhang, 2022). Distance was an easy and identifiable behavioural end point to observe and record using the object tracker. Once each eligible subject was traced, the data coded to the trace could be exported and analysed (Supplementary file 2).

2.3.3 Euclidean distance (ED)

ED is a common measurement of distance when studying *C. elegans* and remains a useful end point to work out the distance between two points (Dittman and Kaplan, 2008), (Zhang, 2022). In this case, ED refers to the furthest distance travelled by each subject from their starting point. ED was calculated using Fiji by measuring from the centre of the tracker cursor at the starting point of the test subject to the furthest part of the trace (figure 2.2c).

2.4 Exclusion criteria

During pilot studies it was found that the cursor disappears from view if subjects moved below the time stamp of the video screen. This is due to a technical error in the object tracker and a limitation of the data. To counter this limitation, if the head of the subject fell below the time stamp, the subject was excluded from the investigation. In addition, subjects proved difficult to observe if they migrated and remained close to the edge of the plate and fell out of range of view. Therefore, any subjects that remained at edge of plate for longer than 4 seconds (2 frames per step) were discounted from the trial (figure 2.2a).

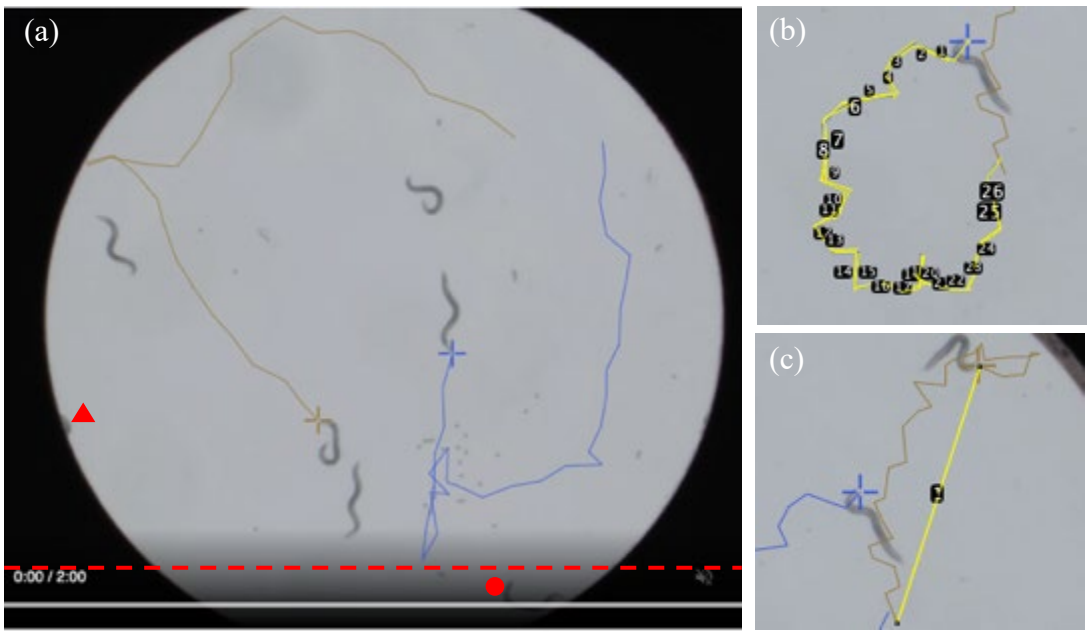

Figure 2.2 (a) Exclusion criteria; subjects fall below the boundaries of the tracking device (ineligible subject denoted by red dot denotes), subjects that spend >4 seconds at the plate boundary (ineligible subject denoted by red triangle) **(b)** Number of turns and **(c)** Euclidean distance, both measured using Fiji imagine software tool.

2.5 Statistical analysis

Given most of the data from this two factorial design experiment was normally distributed and therefore parametric (supplementary file 2, 3) a two-way ANOVA test was conducted for all results given robustness of the test, followed Bonferroni correction to account for multiple pairwise comparisons within and between groups. The alpha value for all tests was set at 0.05. Statistical analysis was performed to determine whether results observed were significant.

3 Results

3.1 Turning Frequency (TF)

The box plot for WT shows that the WT- group had a lower number of turns (*Mn* = 1.27) than WT+ (*Mn* = 7.84). In Gar-3 mutants, the box plot shows G3- had a higher number of turns (*Mn* = 5.27) than G3+ (*Mn* = 4.09).

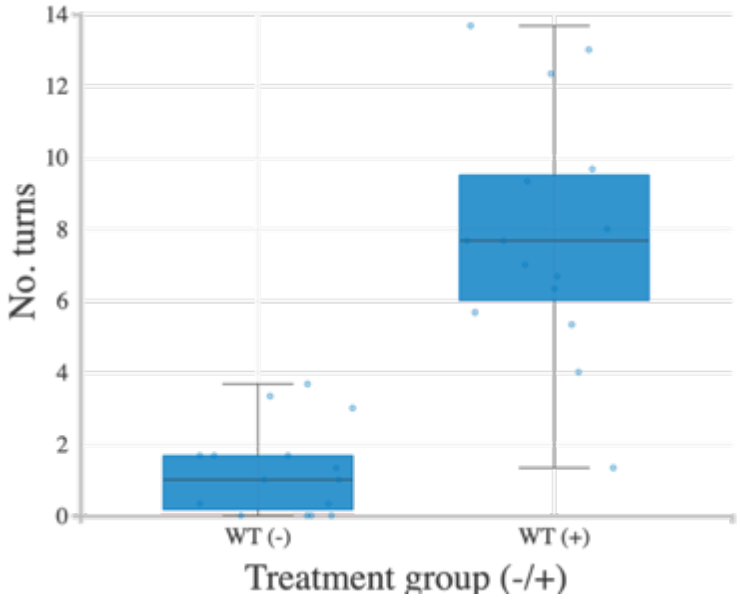

Figure 3.1 Box plot showing mean values for turn frequency in WT+ (*Mn* = 7.84) and WT- (*Mn* = 1.27).

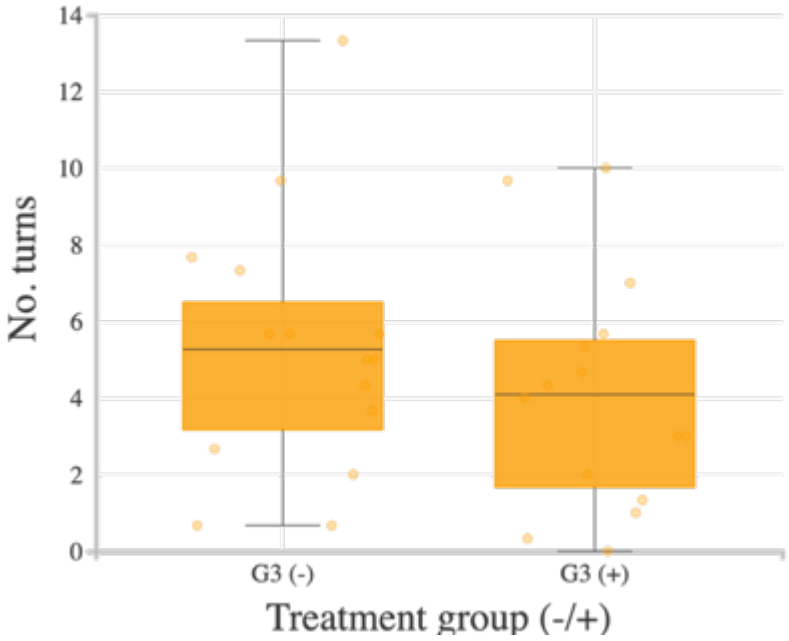

Figure 3.2 Box plot showing mean values for turn frequency in G3+ (*Mn* = 4.09) and G3- (*Mn* = 5.27).

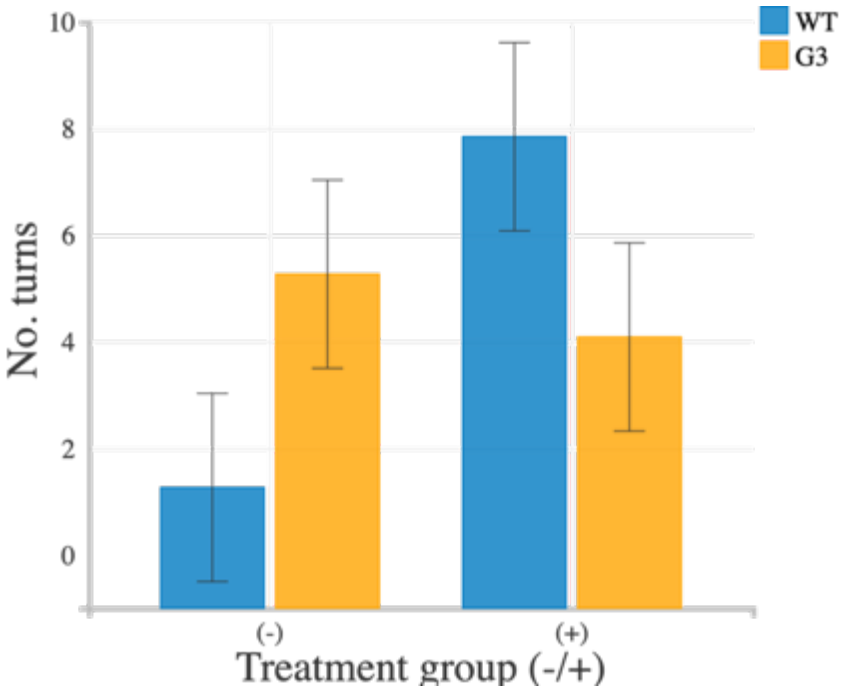

Figure 3.3 Bar chart showing mean values for turn frequency across all 4 groups (WT+/- & G3 +/-).

A two-way ANOVA to compare variance between groups was conducted. Results showed there to be a significant interaction between phenotypes and treatment groups (p = < 0.001). A post hoc test revealed a significant difference in turn frequency between WT+/- (p = <0.001), WT+/G3+ (p = 0.005) and G3-/WT- (p = 0.002), however no significant difference between G3+/G3- (p = 1.000), G3+/WT- (p=0.061) and G3-/WT+ (p = 0.110).

ANOVA - No. Turns

Cases	Sum of Squares	df	Mean Square	F	p	η^2_p
Treatment Group (+ / -)	109.296	1	109.296	12.949	< .001	0.188
Phenotype (WT / G3)	0.226	1	0.226	0.027	0.871	4.773e-4
Treatment Group (+ / -) ✻ Phenotype (WT / G3)	225.661	1	225.661	26.735	< .001	0.323
Residuals	472.674	56	8.441			

Note. Type III Sum of Squares

Table 3.1 Two-way ANOVA summary of results for turn frequency (treatment groups: p =< 0.001, phenotypes: p = 0.871, interaction between treatment groups and phenotypes: p = < 0.001).

Post Hoc Comparisons - Treatment Group (+ / -) ✻ Phenotype (WT / G3)

		Mean Difference	SE	t	p_{bonf}
(+ G3)	(- G3)	-1.179	1.061	-1.112	1.000
	(+ WT)	-3.756	1.061	-3.541	0.005
	(- WT)	2.822	1.061	2.660	0.061
(- G3)	(+ WT)	-2.577	1.061	-2.429	0.110
	(- WT)	4.001	1.061	3.772	0.002
(+ WT)	(- WT)	6.578	1.061	6.201	< .001

Note. P-value adjusted for comparing a family of 4

Table 3.2 Two-way ANOVA for turn frequency: post hoc comparison showing pairwise comparisons for each group.

Results confirm that the null hypotheses for TF in *C. elegans* can be rejected:

1.

   a. There will be no difference in Turning frequency in wild type *C. elegans* treated with Chlorpyrifos versus untreated.

2.

   a. There will be no difference in Turning frequency between wild type *C. elegans* treated with Chlorpyrifos and Gar-3 mutants treated with Chlorpyrifos.

Therefore, the alternative hypotheses were supported.

3.2 Total distance travelled (TDT)

The WT- group had higher scores for Total distance travelled (Mn = 13.06) than WT+ (Mn = 8.71). The G3- group had higher value for Total distance travelled (Mn = 12.25) than G3+ group (Mn = 10.52).

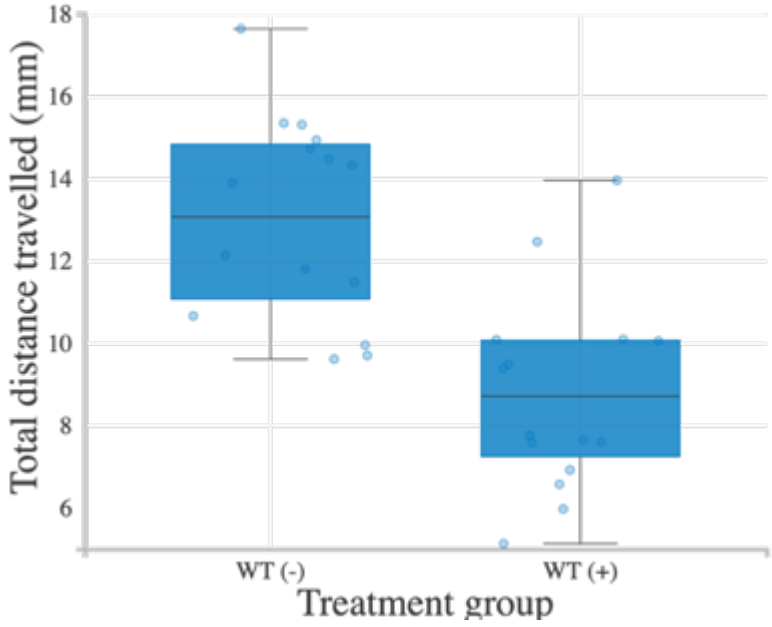

Figure 3.4 Box plot showing mean values for total distance travelled in WT+ (*Mn* = 8.71) and WT- Mn = 13.06).

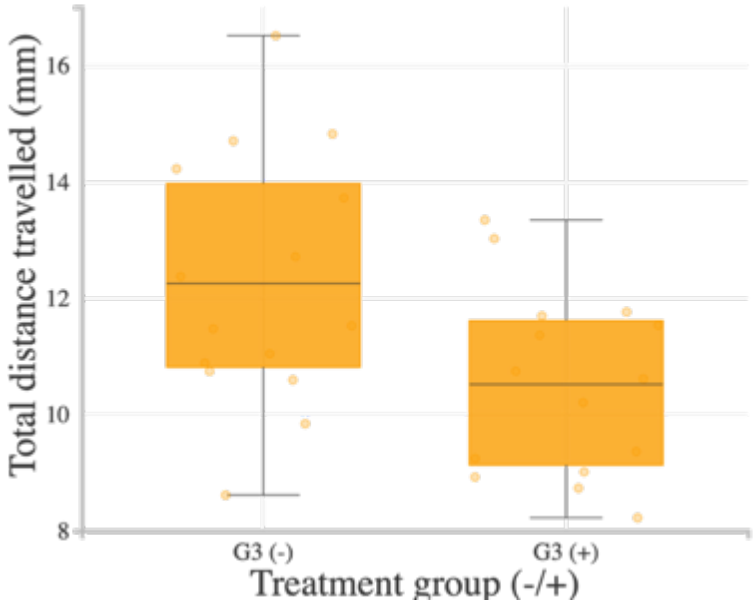

Figure 3.5 Box plot showing mean values for total distance travelled in G3+ (*Mn* = 10.52) and G3- *(Mn* = 12.25)

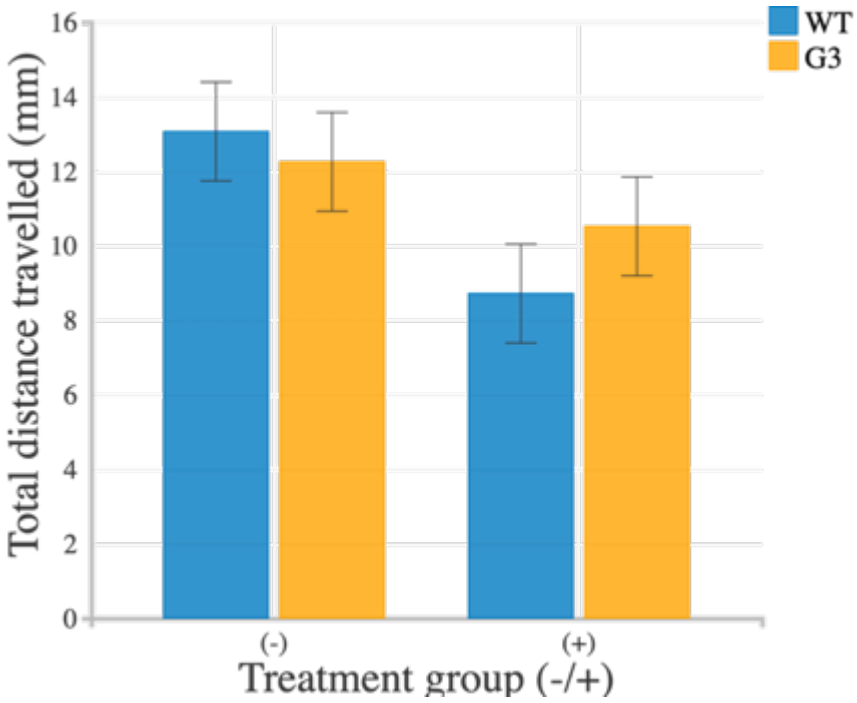

Figure 3.6 Bar chart showing mean values for total distance travelled across all 4 groups (WT+/- & G3+/-).

Results from the two-way ANOVA showed there to be a significant interaction between phenotypes and treatment groups. A post hoc test revealed a significant difference in TDT between WT+/- (p = < 0.001), G3+/WT- (p = 0.014) and G3-/WT+ (p= < 0.001), however no significant difference between G3+/- (p = 0.203) G3 +/WT+ (p= 0.165) and G3-/WT– (p = 1.000).

ANOVA – Total distance travelled (mm)

Cases	Sum of Squares	df	Mean Square	F	p
Treatment Group (+ / -)	138.867	1	138.867	29.136	< .001
Phenotype (WT / G3)	3.690	1	3.690	0.774	0.383
Treatment Group (+ / -) ✻ Phenotype (WT / G3)	25.689	1	25.689	5.390	0.024
Residuals	266.904	56	4.766		

Note. Type III Sum of Squares

Table 3.3 Two-way ANOVA summary of results for total distance travelled (treatment groups: p =< 0.001, phenotypes: p = 0.383, interaction between treatment groups and phenotypes: p = 0.024).

Post Hoc Comparisons - Treatment Group (+ / -) ✻ Phenotype (WT / G3)

		Mean Difference	SE	t	p_{bonf}
(+ G3)	(- G3)	-1.734	0.797	-2.175	0.203
	(+ WT)	1.805	0.797	2.264	0.165
	(- WT)	-2.547	0.797	-3.195	0.014
(- G3)	(+ WT)	3.539	0.797	4.439	< .001
	(- WT)	-0.813	0.797	-1.019	1.000
(+ WT)	(- WT)	-4.351	0.797	-5.458	< .001

Note. P-value adjusted for comparing a family of 4

Table 3.4 Two-way ANOVA for total distance travelled: post hoc comparison showing pairwise comparisons for each group.

The results confirm the null hypothesis for TDT in wild type *C. elegans* can be rejected:

1.

b. There will be no difference in the total distance travelled in wild-type *C. elegans* treated with Chlorpyrifos versus untreated.

The alternative hypothesis was supported.

Results confirm that the null hypothesis for total distance travelled between wild type and gar-3 mutant strains must be accepted:

2.

b. There will be no difference in Total distance travelled between wild type *C. elegans* treated with Chlorpyrifos and Gar-3 mutants treated with Chlorpyrifos

The alternative hypothesis was therefore rejected.

3.3 Euclidean Distance (ED)

The WT- group had higher scores for ED *(Mn = 4.46)* than WT+ group (*Mn* = 2.40). The G3- group had higher scores for Euclidean distance (*M* = 4.59) than G3+ group *(M = 4.54)*.

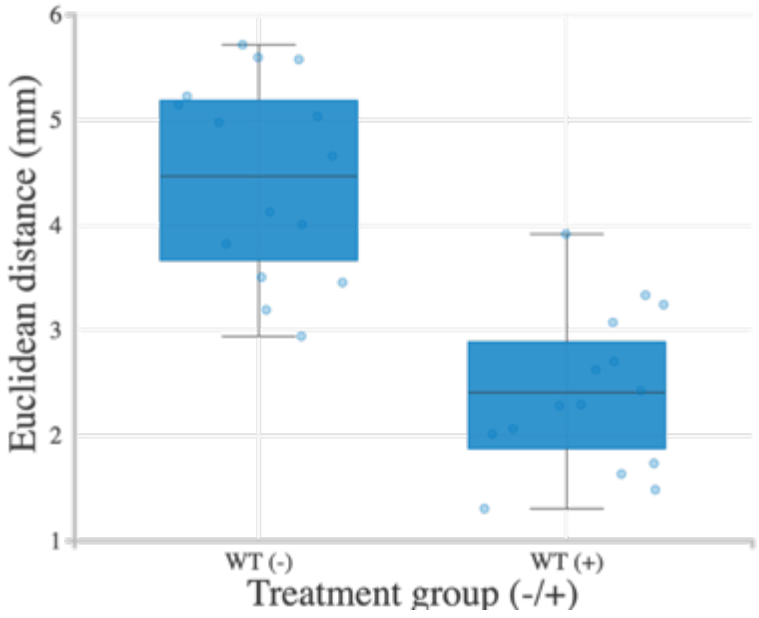

Figure 3.7 Box plot showing mean values for Euclidean distance in WT+ (*Mn* = 2.40) and WT- (*Mn* = 4.46).

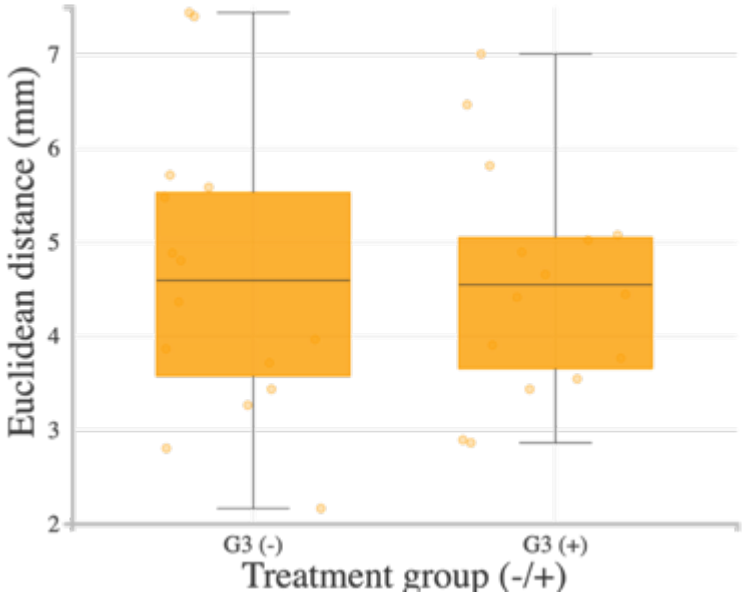

Figure 3.8 Box plot showing mean values for Euclidean distance in G3+ (*Mn* = 4.54) and G3- (*Mn* = 4.59).

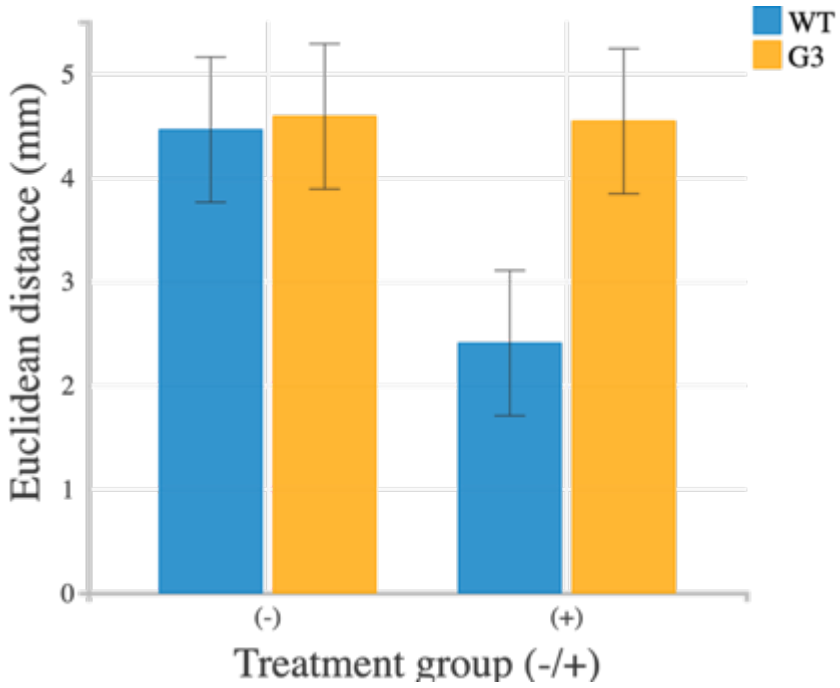

Figure 3.9 Bar chart showing mean values for Euclidean distance across all 4 groups (WT+/- & G3+/-).

A two-way ANOVA showed there to be a significant interaction between phenotypes and treatment groups. A post hoc test revealed a significant difference in Euclidian distance between WT+/- ($p = < 0.001$), WT+/G3+ ($p = < 0.001$) and G3-/WT+ ($p = < 0.001$), however no significant difference between G3+/- ($p = 1.000$), G3-/WT- ($p = 1.000$) and G3+/WT- ($p = 1.000$).

ANOVA - Euclidian distance (mm)

Cases	Sum of Squares	df	Mean Square	F	p	η^2
Treatment Group (+ / -)	16.559	1	16.559	12.511	< .001	0.132
Phenotype (WT / G3)	19.244	1	19.244	14.540	< .001	0.154
Treatment Group (+ / -) ✻ Phenotype (WT / G3)	15.140	1	15.140	11.439	0.001	0.121
Residuals	74.120	56	1.324			

Note. Type III Sum of Squares

Table 3.5 Two-way ANOVA summary of results for Euclidean distance (treatment groups and phenotypes: $p =< 0.001$, interaction between treatment groups and phenotypes: $p = 0.001$).

Post Hoc Comparisons - Treatment Group (+ / -) ✻ Phenotype (WT / G3)

		Mean Difference	SE	t	p_{bonf}
(+ G3)	(- G3)	-0.046	0.420	-0.110	1.000
	(+ WT)	2.137	0.420	5.088	< .001
	(- WT)	0.082	0.420	0.195	1.000
(- G3)	(+ WT)	2.183	0.420	5.197	< .001
	(- WT)	0.128	0.420	0.305	1.000
(+ WT)	(- WT)	-2.055	0.420	-4.893	< .001

Note. P-value adjusted for comparing a family of 4

Table 3.6 Two-way ANOVA for Euclidean distance: post hoc comparison showing pairwise comparisons for each group.

Results confirm that the null hypotheses for ED in *C. elegans* can be rejected:

1.

c. There will be no difference in Euclidean distance in wild-type *C. elegans* treated with Chlorpyrifos versus untreated.

2.

c. There will be no difference in Euclidean distance between wild type *C. elegans* treated with Chlorpyrifos and Gar-3 mutants treated with Chlorpyrifos.

Therefore, the alternative hypotheses was supported.

4 Discussion

4.1 Wild-type key findings

The results from this investigation support the hypotheses that low-level exposure to chlorpyrifos had a significant effect on locomotory behaviour of WT *C. elegans* in all three end points assayed ($p = < 0.001$). Treated WT showed an increase in TF following chlorpyrifos exposure, coupled with a shorter ED and TDT, suggesting there is a change in neuromuscular function following exposure. Findings for Euclidean and TDT support previous work which also reported a reduction in locomotion after exposure to CPF for 24 hours (Ruan et al, 2009), (Govindarajan et al, 2019). However, HTF and BBF have been shown to decrease in these studies, whereas TF increased in this investigation. Such end points to determine HTF and BBF could not be easily assayed in this investigation due to limitations and simplicity of the object tracker. Given worms were small, there was no zoom function or rotation functionality and the tracking cursor was moderately large in size, accurate readings for statistical analysis and comparison was out of scope for this investigation. However, an increase in the number of turns following CPF exposure coincides with a shorter distance travelled and ED, causing an overall reduction in locomotion, which correlates with previous work. Additionally, although BBF has been shown to decrease, some studies have actually shown an initial increase in BBF from control following lose dose CPF exposure for 24 hours, before decreasing in a concentration dependent manner (Ruan et al, 2009). An increase in TF following ectopic expression of GAR-3 in worms has also been found, therefore supporting these findings (Dittman and Kaplan, 2008).

4.2 Gar-3 mutant key findings

Whilst results showed that untreated G3 mutants had a slightly higher turn frequency than the treated group as well as a higher ED, statistical analysis confirmed these differences were not significant ($p = 1.000$). Results for TDT were also not significant ($p = 0.203$). These results suggest chlorpyrifos exposure had no significant effect on these end points of locomotory behaviour in Gar-3 mutants, suggesting Gar-3 mutants confer some degree of resistance to low level CPF exposure, coinciding with previous findings of OP resistance in mutant strains (Rand, 2007), (Lee et al, 2002). These findings correlate somewhat to previous work into peristalsis and pumping contractions of the pharynx in feeding behaviour which are stimulated by ACh and found that Gar-3 mutants were

insensitive to the effects of exogenous muscarinic agonist arecoline (Kozlova, 2019). Other studies reported the inhibition of AChE by organophosphates was linked to the inhibition of pharyngeal pumping as well as paralysis of the body wall muscles, the sites in which we know GAR-3 mAChRs are located (Izquierdo, 2021). This work therefore supports *gar-3* as a potential target for further investigation.

4.3 Wild-type and Gar-3 mutant interaction

Given there was a significant difference in locomotory behaviour between treated and untreated WT but no significant difference in G3 mutants, suggests the *gar-3* gene could be implicated in the response to increased levels of ACh causing the neuromuscular effects observed in WT strains and therefore a potential gene of interest for pesticide exposure and toxicity. Two-way ANOVA post hoc comparisons were conducted to determine whether the changes to locomotory behaviour observed in treated WT could be attributed to *gar-3.*

Although untreated G3 had a significantly higher TF when compared to untreated WT ($p = 0.002$), statistical analysis confirmed WT+ were shown to have a significantly higher TF when compared to G3+ mutants ($p = 0.005$). Indeed, statistical analysis for TF confirmed a significant difference between treatment groups ($p = < 0.001$) but not between phenotypes ($p = 0.871$) indicating the significant differences observed between treated phenotypes are not due to any differences in baseline. This result correlates with previous research which found increased levels of ACh in ectopically expressed Gar-3 worms caused accelerated paralysis when compared to other mAChRs (GAR1 and 2), evidenced through increased TF (Dittman and Kaplan, 2008).

Additionally, WT+ exhibited a significantly lower ED compared to G3, ($P = < 0.001$) which is further supported by an insignificant difference in ED between untreated groups ($p = 1.000$) and that ED in G3 mutants was not significantly affected following exposure to CPF. This supports the work of Dittman and Kaplan (2008) to some degree who found WT had a smaller ED when compared to gar-2 mutants and that GAR-3 receptors mediate areas of exploration as well as change of direction.

Although G3 treated travelled a further total distance than WT treated, the results were not significant as were those between G3 and WT controls. These findings contradict the work of Dittman and Kaplan (2008) who found a significant difference in distance of travel between mutant and wild type strains whereby mutants travelled 25 % further. This disparity could be due to difference in measuring parameters and methodology which should be considered in future investigations.

4.4 Considerations, strengths and limitations

Results clearly show a marked deleterious impact on locomotory behaviour and neuromuscular function of WT *C. elegans* following CPF exposure when compared to Gar-3 mutant phenotypes, exhibiting a supressed response in two of the three end points. However, further research is needed to determine whether *gar-3* is indeed responsible for observed changes to neuromuscular function, to ascertain whether these findings can be conveyed in mammalian orthologs M1,2 and 3.

Although ample data was available for *gar-3* mutants and WT, time constraints limited sample size to 15 subjects per group therefore, a repeat of the investigation using a larger sample size would increase reliability and validity of these results and avoid any potential sampling errors. This repeated test would also confirm validity of chosen statistical analysis given ANOVA was chosen based on most of the data being parametric, the outliers remaining close to normal distribution, however an increased sample size would rule out any potential sampling errors from this investigation. Additionally, although data for TDT was computer-generated, data points were selected manually as well as data for TF and ED which required a considerable amount of time and lends itself to some subjectivity and therefore user error. These limitations arose due to capability and usability of the object tracker and remote working conditions. Any follow-on research should make use of computer-generated data where possible as well as multiple researchers for manual counts to increase validity and reduce bias. The limited number of recently published results for *gar-3* in this area must also be noted as a limitation of the data available from the tracker following a change of mutant from the originally selected *unc-29.*

However, it must also be emphasised that the methods, sample size and statistical analysis used emulate that of previous studies in this area and were relatively robust to yield

significant results for most end points, the reliability of which maintained through use of Bonferroni correction to provide a sufficient response to the research question (Govindarajan et al, 2019), (Izquierdo et al, 2021).

4.5 Where next

Whilst there are previous studies to corroborate findings from this investigation (Dittman and Kaplan, 2008) (Izquierdo, 2021), (Kozlova, 2019), (Lee et al, 2002), (Ruan et al, 2009), other wider research suggests there's a level of cross talk between mAChRs and nAChRs following pesticide exposure in *C. elegans* and more specifically that GAR-3 receptors may in fact play a part in recovery from nAChR-induced paralysis, rather than facilitating these deleterious effects (Spensley et al, 2018). There are a number of variables for this disparity such as use of carbamate Aldicarb as primary pesticide rather than CPF, and so although a noteworthy distinction, can't be used to discount findings from this investigation. However, this does suggest further research is needed into the relationship between nicotinic and muscarinic receptors and any cross talk following CPF exposure in *C. elegans*, given research into *unc-29* was considered and discounted due to limitations of the database, through lack of adequate sample sizes for analysis and inability to adjust for this cofounding variable. A comparative study with *unc-29* would further strengthen the case for *gar-3* or refute such evidence. Such research could be advantageous given the disproportionate number of recent published findings for mAChR regulation in *C. elegans* compared with nAChRs as well as CPF compared to other OPs such as aldicarb, when clearly there is a link to CPF exposure and human health (Kori et al, 2020).

Findings from this investigation for *gar-3* as a potential gene of interest in *C. elegans* could to help mark an important scientific advance in the understanding of molecular mechanisms behind CPF neurotoxicity in higher organisms. Indeed, one study speculates OPs affect cholinergic as well as serotonergic and glutaminergic neurotransmission in brain areas such as the hippocampus, which are known to be involved in depression (Siqueira et al, 2019). Low level exposure to CPF in rats caused depressive-like behaviour which correlated with a reduction in AChE in the brain and subsequent increase in ACh neurotransmission via muscarinic receptors, such as in *C. elegans* neuromuscular junctions (Holden-Dye and Walker, 2014), (Rand, 2007), (Spensley, 2018). Given the orthologous nature of mammalian mAChRs with *gar-3*, the suggestion

that CPF activates mAChRs in brain areas involved in depression in rats, corelates on some level with these findings suggesting *gar-3* encoded mAChRs could facilitate deleterious changes to locomotory behaviour in *C. elegans*. This suggests *gar-3* could be associated to models for depression when studying CPF exposure and neurotoxicity to identify effective treatments and antidepressants through further research in higher organisms and vertebrates.

5 Conclusion

It's clear that further research is needed into the effects of *gar-3* on neuromuscular function in response to CPF, to identify if in fact *gar-3* can be implicated as a cause of the differences observed in treated wild type *C. elegans*. However, findings from this investigation clearly meet the objectives set out and answer the research question to confirm low-level CPF exposure does affect the neuromuscular function and locomotory behaviour of *C. elegans* wild-type with results to suggest *gar-3* involvement in the regulation and mediation of the observed behavioural effects. These findings could provide preliminary information for future research of *gar-3* homologs in mammalian trials, given *C. elegans* AChRs are orthologous to human and other vertebrate isoforms. Such research could eventually lead to a better understanding of the effects of OP neurotoxicity and depression in humans (Izquierdo, 2021).

References

Aldridge, J. E., Levin, E. D., Seidler, F. J., & Slotkin, T. A. (2005) 'Developmental exposure of rats to chlorpyrifos leads to behavioral alterations in adulthood, involving serotonergic mechanisms and resembling animal models of depression', *Environmental health perspectives*, 113(5), pp. 527–531. https://doi.org/10.1289/ehp.7867

Bertrand, D. (2005) 'The possible contribution of neuronal nicotinic acetylcholine receptors in depression', *Dialogues in Clinical Neuroscience*, 7(3), pp. 207-216. DOI: 10.31887/DCNS.2005.7.3/dbertrand

Chan, J, P., Staab, T, A.,Wang, H., Mazzasette, C., Butte, Z., Sieburth, D. (2013) 'Extrasynaptic Muscarinic Acetylcholine Receptors on Neuronal Cell Bodies Regulate Presynaptic Function in Caenorhabditis elegans', *Journal of Neuroscience* 33 (35), pp. 14146-14159. DOI: 10.1523/JNEUROSCI.1359-13.2013

Cole, R. D., Anderson, G. L., Phillip, W. L. (2004) 'The nematode Caenorhabditis elegans as a model of organophosphate-induced mammalian neurotoxicity', *Toxicology and Applied Pharmacology,* 194(3), pp. 248-256. https://doi.org/10.1016/j.taap.2003.09.013.

Dittman, J, S., Kaplan, J, M. (2008) 'Behavioral Impact of Neurotransmitter-Activated G-Protein-Coupled Receptors: Muscarinic and GABAB Receptors Regulate Caenorhabditis elegans Locomotion' *Journal of Neuroscience,* 28 (28), pp.7104-7112. DOI: 10.1523/JNEUROSCI.0378-08.2008

Gjorgjieva, J., Biron, D., Haspel, G. (2014) 'Neurobiology of *Caenorhabditis elegans* Locomotion: Where Do We Stand?', *Bioscience*, *64*(6), pp. 476–486. DOI: https://doi.org/10.1093/biosci/biu058

Govindarajan, D., Chatterjee, C., Shakambari, G., Varalakshmi, P., Jayakumar, K., Balasubramaniem, A. (2019) 'Oxidative stress response, epigenetic and behavioral alterations in Caenorhabditis elegans exposed to organophosphorus pesticide quinalphos', *Biocatalysis and Agricultural Biotechnology,* 17, pp. 702-709. https://doi.org/10.1016/j.bcab.2019.01.031

Han, B., Bellemer, A., Koelle, M. (2015) 'An Evolutionarily-Conserved Switch in Response to GABA Affects Development and Behavior of the Locomotor Circuit of Caenorhabditis elegans', *Genetics*, 199, pp. 1160. DOI: 10.1534/genetics.114.173963

Holden-Dye, L., & Walker, R. J. (2014). 'Anthelmintic drugs and nematicides: studies in Caenorhabditis elegans', *WormBook : the online review of C. elegans biology*, 1–29. https://doi.org/10.1895/wormbook.1.143.2

Hunt P. R. (2017) 'The *C. elegans* model in toxicity testing', *Journal of applied toxicology : JAT*, *37*(1), pp. 50–59. https://doi.org/10.1002/jat.3357

Izquierdo, P, G., O'Connor, V., Green, C., Holden-Dye, L., Tattersall, J, E, H. (2021) '*C. elegans* pharyngeal pumping provides a whole organism bio-assay to investigate anti-cholinesterase intoxication and antidotes', *NeuroToxicology,* 82, pp. 50-62. https://doi.org/10.1016/j.neuro.2020.11.001

Kozlova, A, A., Lotfi, M., Okkema, P. G. (2019) 'Cross Talk with the GAR-3 Receptor Contributes to Feeding Defects in *Caenorhabditis elegans eat-2* Mutants' *Genetics*, *212*(1), pp. 231–243. https://doi.org/10.1534/genetics.119.302053

Lee, Y. S., Park, Y. S., Nam, S., Suh, S., Lee, J., Kaang, B. K., Cho, N. J. (2002) 'Characterization of GAR-2, a Novel G Protein-Linked Acetylcholine Receptor from *Caenorhabditis elegans*.', *Journal of Neurochemistry,* 75, pp. 1800-1809. https://doi-org.libezproxy.open.ac.uk/10.1046/j.1471-4159.2000.0751800.x

Mackenzie Ross, S.J. & Harrison, V. (2016) 'What's your poison? Neurobehavioural consequences of exposure to industrial, agricultural and environmental chemicals', *Cortex*, 74, pp.353-357. https://doi.org/10.1016/j.cortex.2015.12.002

Muppidi, S., Wolfe, G. I., Barohn, R. (2012) 'Diseases of the neuromuscular junction', *Pediatric Neurology: Principles and Practice,* pp. 1549-1569. https://www.researchgate.net/publication/284287403_Diseases_of_the_neuromuscular_junction

Ouellette M. H., Desrochers M. J., Gheta I., Ramos R., Hendricks M. (2018) 'A Gate-and-Switch Model for Head Orientation Behaviors in *Caenorhabditis elegans*', *eNeuro,* 5(6). DOI: 10.1523/ENEURO.0121-18.2018.

Philip, N.S., Carpenter, L.L., Tyrka, A.R. (2010) 'Nicotinic acetylcholine receptors and depression: a review of the preclinical and clinical literature', *Psychopharmacology*, 212, pp. 1–12. https://doi.org/10.1007/s00213-010-1932-6

Rand, J.B. (2007) 'Acetylcholine' *WormBook, ed. The C. elegans Research Community,* doi: /10.1895/wormbook.1.131.1

Ruan, Q., Ju, J., Li, Y., Liu, R., Pu, Y., Yin, L., Wang, D. (2009) 'Evaluation of Pesticide Toxicities with Differing Mechanisms Using *Caenorhabditis elegans', Journal of Toxicology and Environmental Health,* 72(11-12), pp. 746-751. DOI: 10.1080/15287390902841532

Siqueira, A. A., Cunha, A. F., Marques. G. L. M., Felippe. I. S. A., Minassa. V. S., Gramelich. T. C. S., Cicilini. M. A., Alarcon. T. A., Pires. R. G. W., Sampaio. K. N., Beijamini. V. (2019) 'Atropine counteracts the depressive-like behaviour elicited by acute exposure to commercial chlorpyrifos in rats', *Neurotoxicology and Teratology*, 71, pp 6-15. https://doi.org/10.1016/j.ntt.2018.11.002

Small, K. M., Nunes, E., Hughley, S., Addy, N.A. (2016) 'Ventral tegmental area muscarinic receptors modulate depression and anxiety-related behaviors in rats', *Neuroscience Letters,* 616, pp. 80-85. https://doi.org/10.1016/j.neulet.2016.01.057

Spensley, M., Del Borrello, S., Pajkic, D., Fraser, A. G. (2018) 'Acute Effects of Drugs on *Caenorhabditis elegans* Movement Reveal Complex Responses and Plasticity', *G3 Genes|Genomes|Genetics*, 8(9), pp. 2941–2952. https://doi.org/10.1534/g3.118.200374

Qu, M., Wang, D. (2020) 'Toxicity comparison between pristine and sulfonate modified nanopolystyrene particles in affecting locomotion behavior, sensory perception, and neuronal development in Caenorhabditis elegans', *Science of The Total Environment,* 703, DOI: https://doi.org/10.1016/j.scitotenv.2019.134817.

Wakabayashi, T., Kitagawa, I., Shingai, R. (2004) 'Neurons regulating the duration of forward locomotion in Caenorhabditis elegans', *Neuroscience Research,* 50 (1), pp. 103-111. https://doi.org/10.1016/j.neures.2004.06.005

Wang, Y., Liu, S., Huang, P., Wang, Z., Xu, Y. (2021) 'Assessing the combined toxicity of carbamate mixtures as well as organophosphorus mixtures to Caenorhabditis elegans using the locomotion behaviors as endpoints', *Science of The Total Environment,* 760. https://doi.org/10.1016/j.scitotenv.2020.143378

Zhang, G., Roberto, N.M., Lee, D. (2022) 'The impact of species-wide gene expression variation on *Caenorhabditis elegans* complex traits', *Nat Commun*, 13 (3462). https://doi.org/10.1038/s41467-022-31208-4

Glossary

Acetylcholine	A major neurotransmitter in cholinergic neurons made up of acetic acid and choline, which sends signals Causes muscle contraction
Acetylcholinesterase	An enzyme that breaks down the neurotransmitter acetylcholine at neuromuscular junctions
Agonist	A chemical or drug that binds to receptors to elicit a response
Chlorpyrifos	A type of pesticide typically used to kill pests by inhibiting the enzyme acetylcholinesterase thereby affecting neuromuscular function
Euclidean distance	The distance or length between two points; the first and furthest point
gar-3	The G-protein-linked Acetylcholine receptor gene encodes the acetylcholine receptor which is involved in a number of processes, such as the transmission of acetylcholine
Organophosphate	A compound formed from phosphoric acid and alcohol that is typically used in pesticides
Ortholog	Homolog genes that are conserved across different species via a common ancestor and retain the same function across species.
Pharyngeal pumping	Movement of the pharyngeal muscle to facilitate feeding in nematodes
unc-29	A gene that encodes the a-subunit of the nicotinic acetylcholine receptor and is involved in multiple processes such as acetylcholine transmission

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Appendices

Supplementary file 1 – Pilot Study

Supplementary file 2 – Full Investigation - raw data collection and analysis

Supplementary file 3 – Results and statistical analysis