Neurotoxic Effects of Low-level Organophosphate Exposure in *C. elegans* and UK Agricultural Workers

Alexander Steven Dawson

PhD Thesis

School of Life Health and Chemical Sciences

July 2023

Declaration

I, Alexander Steven Dawson, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature. . . . . ..

Abstract

Exposure to organophosphate (OPs), pesticides, has been associated with poor mental health among agricultural workers. Although the impacts of acute exposure, such as poisoning due to AChE inhibition and cholinergic hyperexcitation, are well known, the mechanisms by which low-level exposure may impact mental health are not fully understood. Such investigations are complicated by confounding variables like physical health, life stress, and lifestyle factors.

This thesis sought to bridge this gap using an interdisciplinary approach, focusing on both the biological effects of low-level OP exposure, and its impact on human wellbeing. A model was developed using the invertebrate *C. elegans* to examine effects at a molecular level. Results demonstrated that exposure below the threshold for significant AChE inhibition led to behavioural changes in *C. elegans* linked to ACE-2 acetylcholinesterase and GAR-3 muscarinic receptor, suggesting that the cholinergic system mediates some effects of low-level OP exposure, even at very low-levels.

In addition, a survey among UK agricultural workers was conducted to understand the factors affecting mental health in OP-exposed populations. Data suggested that although depression symptoms were higher among agricultural workers compared to a control group of construction workers, lifestyle factors and stress appeared to be more important contributors to poor mental health than pesticide exposure.

In conclusion, the research provides evidence for potential neurotoxic effects of low-level OP exposure from the *C. elegans* model. However, its implication in human mental health is more nuanced, with stress and lifestyle factors playing significant roles, highlighting the complexity of real-world interactions between chemical exposure and human health.

Acknowledgements

I would like to thank my supervisory team for all of their support and assistance with this project. Specifically, to Dr Rachel McMullan for introducing me to *C. elegans*, and for many engaging and enjoyable discussions throughout the project. To Dr Virginia Harrison for guidance on the human aspects, for providing the previously published work that inspired this project, and for the lakeside walks to get me away from my desk during the write up. Thanks also to Dr Claire Rostron for general support throughout the project and to all of the team for their invaluable knowledge, experience, and especially for their patience.

I would also like to thank the School of Life Health and Chemical Sciences for funding the project, and to the staff there for providing an enjoyable academic learning environment. My thanks go to Dr Katja Rietdorf for her additional support, and Dr Robert Saunders for same, and for coffee room discussions, scientific and otherwise. I am grateful to the London *C. elegans* Group, particularly Prof Jonathan Hodgkin, Prof Lindy Holden Dye, Dr Richard Poole, Dr Arantza Barrios, and Dr Queelim Ch'ng, for their interest in this project, and their invaluable insight and feedback. Likewise, the OU Forensic Cognition Research group for valuable feedback, and Professor Sarah Mackenzie Ross for providing insight into her previous organophosphate work. Others to whom I am grateful for their guidance and inspiration during my academic journey, are Prof George Kemenes, Prof John Krebs, and especially Dr Peter McGregor, whose enthusiasm, encouragement, and guidance were positively transformative.

I am grateful to new friends and fellow students for making my journey interesting and enjoyable. To my parents, Ronald, and Janette Dawson, for supporting my return to study, and for their support and belief in me throughout my academic journey.

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ABBREVIATIONS

5-HT, Serotonin

ACh, Acetylcholine

AChE, Acetylcholinesterase

AD, Alzheimer's disease

ADE, Anterior deirid neurons

ALS, Amyotrophic lateral sclerosis

ANOVA, Analysis of variance

BDNF, Brain-derived neurotrophic factor

BChE, Butyrylcholinesterase

BSR, Basal slowing response

CEP, Cephalic neurons

cGMP, Cyclic guanosine monophosphate

CNS, Central nervous system

CPF, Chlorpyrifos

CYP, Cytochrome P-450

DA, Dopamine

DASS, Depression Anxiety and Stress Scale

DAT, Dopamine transporter

DDT, Dichlorodiphenyltrichloroethane

DEP, Diethylphosphate

DETP, Diethylthiophosphate

DFP, Diisopropylfluorophosphate

DSM, Diagnostic and Statistical Manual of Mental Disorders

DTNB, 5,5-dithio-bis-(2-nitrobenzoic acid) – Ellman's reagent

EEG, Electroencephalogram

Egl, Egg-laying deficient

ESR, Enhanced slowing response

EPA, Environmental Protection Agency

EU, European Union

FAO, Food and Agriculture Organization

FDA, Food and Drug Administration

FDR, False discovery rate

GABA, Gamma-aminobutyric acid

GAD, Generalised anxiety disorder

GFP, Green fluorescent protein

GWAS, Genome-wide association studies

HREC, Human Research Ethics Committee

HSE, Health and Safety Executive

IP, Intraperitoneal

IV, Intravenous

LOEL, Lowest observable effect level

mAChRs, Muscarinic receptors

MANOVA, Multivariate analysis of variance

MAO, Monoamine oxidase

MAOI, Monoamine oxidase inhibitor

MAPK, Mitogen-activated protein kinase

MDD, Major depressive disorder

MTL, Medial temporal lobe

mTOR, Mammalian target of rapamycin

NA, Noradrenaline

nAChRs, Nicotinic receptors

NGM, Nematode growth media

NMDA, N-methyl-D-aspartate

NMJ, Neuromuscular junction

NTE, Neuropathy target esterase

OP, Organophosphate

OPIDN, Organophosphorus compound-induced delayed neuropathy

PCR, Polymerase chain reaction

PDD, Persistent depressive disorder

PDE, Posterior deirid neurons

PON1, Paraoxonase

PPE, Personal protective equipment

Rmax, Maximum Euclidean distance

SRRS, Social Readjustment Rating Scale

SSRI, Selective serotonin reuptake inhibitors

SWIP, Swimming induced paralysis

TCA, Tricyclic antidepressants

TRD, Treatment-resistant depression

Unc, Uncoordinated

VTA, Ventral tegmental area

WHO, World Health Organization

WT, Wild type

1. Introduction

1.1. An introduction to neurotoxicology

Over the last 100 years, the use of different chemicals in industrial, commercial, and domestic use has grown exponentially. Modern societies are now heavily reliant on such products with the United Nations Environment Assembly predicting that worldwide production and consumption will double by the year 2030 (UNEP, 2019). Well over two thousand potentially toxic chemicals are estimated to be entered for registration each year, and over 350,000 chemicals and mixtures are already registered (Wang *et al.*, 2020). However, despite their benefits and economic importance, many of these chemicals carry risks to human health and the environment. The field of toxicology aims to understand and mitigate these risks by combining expertise from a wide range of scientific disciplines. Within this broad field, neurotoxicology integrates disciplines such as experimental psychology, toxicology, and behavioural pharmacology, in order to investigate toxic effects on the nervous system (Moser, 2011).

1.1.1. Limitations of toxicity testing

While pre-market toxicology testing is required as part of the regulatory process, a limited range of toxic effects are tested, and the process cannot guarantee complete safety. The complete and comprehensive testing of every possible endpoint for each chemical would be prohibitive in terms of cost and time, and because testing is carried out using animals there are also ethical and animal welfare considerations (Klaassen, 2018). Some insight can be gained from known effects of previously registered compounds, of similar chemical structure. These insights are used to guide the selection of toxic endpoints which are investigated; however, this approach may not always detect

novel effects of new compounds. This is particularly true for neurobehavioral effects, which have not been tested to the same extent as mortality, or histopathological changes (Moser, 2011). However, in comparison to those lethal or physically damaging phenotypes, changes in behaviour can indicate impaired neuronal function, that could be detrimental to human health, but difficult or impossible to detect by physical examination (Moser, 2011).

1.1.2. The need for both animal and human data

Since pre-market toxicology testing is carried out in animals, it also carries other limitations. Animal models are invaluable tools in medical and toxicology research, sharing many conserved characteristics with humans (Mangipudy, Burkhardt and Kadambi, 2014). However, differences in physiology and behaviour, mean that results from animal testing and human health outcomes cannot always be reliably compared. It is therefore essential that animal testing data is supported by human studies where possible. This can take the form of clinical trials, in the case of new medicines. However, human toxicology data is usually limited to retrospective investigation, such as epidemiological studies, and case studies (McGeer and McGeer, 2007).

1.1.3. Misdiagnosis in healthcare settings

Despite efforts to minimise human exposure to harm, the ubiquity of chemical products in the modern world means that some level of exposure is not always avoidable. People who work in laboratories, chemical plants, agriculture, and other industries are at increased risk of occupational exposure. Acute poisoning is a particular risk and is historically not uncommon in some settings, such as in agricultural work with pesticides (Calvert *et al.*, 2008). However, although acute poisoning presents more immediately serious problems for those affected, lower-level and longer-term exposures may also be

harmful, but more difficult to identify. Acute poisoning can be fatal, but usually the circumstances and symptoms are relatively simple to recognise and diagnose, because overtly-toxic effects are more likely to occur following an acute, high-level dose. Occasionally however, people develop toxic symptoms without being aware that chemical exposure is the cause (Hartman, 1998). Symptoms may be especially difficult to recognise in cases where they are a result of chronic, sub-acute, or longer-term exposures at levels that are insufficient to induce classic poisoning symptoms. This highlights the importance of proper product information, workplace education, and full understanding of chemical toxicology profiles at multiple dose levels. Unless the effects of low-level exposure are known to science, medical professionals cannot be expected to correctly diagnose them, risking failure to give appropriate treatment (Li *et al.*, 2014; Harrison and Mackenzie Ross, 2016; Oliverio and Varlet, 2019).

1.1.4. Inter-individual variation

The recognition and diagnosis of low-level toxic effects is complicated further by numerous factors that can alter how individuals respond to different chemicals. For example, children or geriatric patients may be more vulnerable to poisoning than healthy young adults (Bruckner, 2000). Men and women may also respond differently to each other, in terms of how toxins are metabolised, in the manifestations of reproductive toxicity, or in their susceptibility to associated conditions (Miller, 2001; Vahter *et al.*, 2007; Parker and Brotchie, 2010). Lifestyle and occupation can introduce several environmental factors, such as physical health and fitness, and whether any other toxins are present that might interact with the chemical in question (Karalliedde, Edwards and Marrs, 2003). Finally, all of these factors will interact in some way with an individual's genome, which can dramatically alter their predisposition to toxic response, and any

associated health outcomes. For example, individual variation in paraoxonase and cytochrome P450 genes can influence a person's ability to metabolise OPs, and consequently, individual polymorphisms can alter their susceptibility to OP toxicity (Tafet and Nemeroff, 2015; Teodoro *et al.*, 2019).

1.1.5. Organophosphate metabolism

As mentioned, one of the reasons for inter-individual variation in the susceptibility to OP toxicity is a difference in the ability to metabolise the compounds. The first phase of metabolism of OP compounds such as chlorpyrifos is carried out by cytochrome P450 enzymes (CYP450) produced by the liver. Chlorpyrifos contains a sulphur atom which is attached to the phosphorous, this is removed and replaced by an oxygen atom by CYP450s. This highly reactive sulphur atom binds to CYP and reduces the enzymatic activity. The major resulting metabolite of this process is an oxon-version of the chlorpyrifos parent compound; chlorpyrifos-oxon. Such transformations are required for the strong inhibition of AChE, and therefore some 'active' OP metabolites can be more toxic than their 'inactive' parent compounds. After desulphurization, oxons are deactivated via hydrolysis by paraoxonase-1 (PON1) and eventually excreted in urine.

Genomic variation, such as single nucleotide polymorphisms in CYP450s and/or PON1, that can contribute to individual differences susceptibility to OP toxicity.

1.1.6. Concluding summary

Regulatory safety testing of chemical products is of critical importance, yet further work is necessary to improve safety. Neurotoxicology research seeks to address gaps in current knowledge, which in turn can guide best practice in the safe use of toxic substances and inform diagnosis and therapeutic treatment strategies in cases where poisoning has

occurred. Collection of human data is essential in order to maintain clinical relevance, and for the moment at least, experimental animal work is invaluable for investigating the mechanisms that underly toxic effects. Furthermore, neurobehavioral experiments present a set of sensitive tools with which to investigate subtle effects on neuronal function that may occur in the absence of acute toxic injury.

1.2. Organophosphates

1.2.1. A brief history of pesticide use

In 2018 more than 4 million tonnes of pesticides were used worldwide, and overall use has nearly doubled since 1990 (FAO Statistics, 2018). These products are undoubtedly important to maintain agricultural productivity, but the risks to human health must also be considered.

Since the early domestication of plants and animals around 10,000 years ago (Smith, 1997) the protection of agricultural produce from pests and other disease has been important to human survival. The first recorded use of pesticide was around 4,500 years ago, when sulphur compounds were believed to repel mites and insects because of their foul smell. Since then, numerous compounds derived from plants or animals, and inorganic substances have been used to control pests, including arsenic, mercury, sulphuric acid, and lead (Matthews, 2018). Unfortunately, the non-specific toxicity of many of these substances would lead to undesirable, non-target effects.

From the 1940s onward, modern insecticides as we now know them started to be commercialised, following the synthesis of dichlorodiphenyltrichloroethane (DDT) by Dr. Paul Hermann Müller (Bynum, 2002). DDT is an organochlorine (OC) compound with broad spectrum insecticidal action, the discovery of which Müller would go on to win the Nobel Prize in Physiology or Medicine in 1948 (Bynum, 2002). Until the 1960s, DDT saw widespread use as an insecticide, and was used in an attempt by the World Health Organisation (WHO) to eradicate mosquito borne diseases such as malaria (Nájera, González-Silva and Alonso, 2011). DDT was seen as a safe, effective, and economical insect control solution until the 1960s when concerns about its environmental impact

were brought to public attention by Rachel Carson in her book, Silent Spring (Carson, 2002). At the time, Silent Spring raised much controversy, but has since been described as one of the most important books of the of the twentieth century (Lutts, 1985; Dunn, 2012). Despite suggestions that Carson had made some claims that were not fully supported by scientific evidence, the US government acknowledged that her claims of ecological damage being caused by the use of large quantities of pesticides were valid, and that DDT had been widely detected, in fish, birds, mammals and invertebrates in several countries spanning the globe (Greenberg, 1963).

With the ecological impacts of DDT already in question, its initially celebrated efficacy as an insecticide was also reduced, as over time insects were found to have developed resistance. This resistance can occur due to two primary mechanisms; firstly, mosquitos with mutations in the voltage-gated sodium channel (*Vgsc*), which is the target site of DDT, showed resistance to DDT and pyrethroids, and were consequently selected for, producing resistant populations such as has been noted with the malaria carrying mosquito *Anopheles gambiae* (Jones *et al.*, 2012). Secondly, DDT-resistant *A. gambiae* have also shown 5-times elevated expression of glutathione-S-transferase (GSTE2), which confers additional metabolic resistance to DDT. Wild-caught *A. gambiae* that exhibit both increased GSTE2 expression, and specific *Vgsc*-mutations together are extremely resistant to DDT, and such resistance has been replicated through expression in *Drosophila* (Mitchell *et al.*, 2014).

As a result, use of DDT was banned in many countries in the 1970s, although its importance to malaria control remained a hotly debated topic (Taverne, 1999). The widespread attention brought by Carson was instrumental in the creation of environmental regulatory agencies, including the US Environmental Protection Agency

(EPA) and European Environment Agency (EEA), and the landscape of agricultural chemical use was changed completely.

1.2.2. Organophosphates

As the use of DDT and other OC pesticides began to be phased out, the need for a range of alternative options to control insect populations, and to combat the development of resistance to new and existing agents became increasingly urgent.

This need focused the attention of the agricultural industry onto a large group of compounds, the organophosphates (OPs). OPs had already been synthesised following their discovery in the 19th century by Lange and Krueger (Petroianu, 2010), but were recognised for both their insecticidal, and chemical warfare potential, by Dr Gerhard Schrader in 1937.

Following WWII, advances in OP research enabled the development of around 2000 OP compounds by Schrader and his colleagues (Ballantyne and Marrs, 2017), and the use of DDT was largely replaced by the OP chlorpyrifos (CPF) following its introduction in 1965 (Ballantyne and Marrs, 2017). Although OPs were more expensive to use, and less persistent than DTT, they helped to address the problem of developed resistance in target organisms because OPs exert their effects through different molecular targets than DDT. Nevertheless, some cross-resistance to pesticides of different classes can occur, even when the mechanisms of action differ. For example, if enzymes involved in detoxification have broad substrate specificity then resistance to DDT and OPs, as well as other classes, could be possible (Denholm et al. 2002).

1.2.3. What are organophosphates?

OPs are a large class of organic compounds that are most commonly used as pesticides, but they have some other industrial uses such as flame retardants, and they are used as nerve agents in chemical warfare (Gupta, 2006). There are thousands of different OP compounds with varying different effects and potencies, many of which remain unknown, however they all have at least two things in common: firstly, their general structure includes a phosphorous atom, and either a defining phosphoryl (P=O) or thiophosphoryl bond (P=S) (Figure 1).

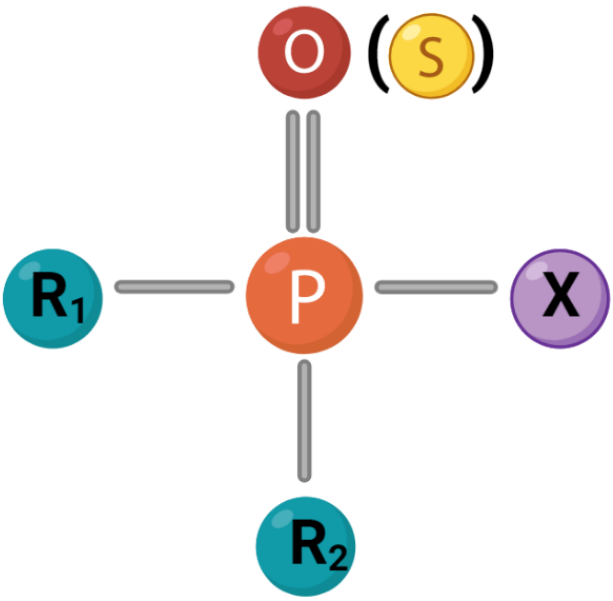

Figure 1. The general chemical structure of organophosphates. All OP compounds have a phosphorous which is attached by a double bond to either an oxygen, or sulphur atom. R1 and R2 are most often alkoxy groups but other substitutes are possible. When acetylcholinesterase is phosphorylated by the OP, the acyl residue (X) is displaced (Ballantyne and Marrs, 2017).

1.2.4. Organophosphates inhibit acetylcholinesterase.

The other main characteristic that different OP compounds have in common is a shared mechanism of action, whereby they all inhibit the enzyme acetylcholinesterase (AChE). Inhibition of AChE prevents degradation of the excitatory neurotransmitter acetylcholine (ACh), leading to a build of ACh in the synaptic cleft, and consequent hyper-excitation at cholinergic synapses and the neuromuscular junction (**Figure 2**).

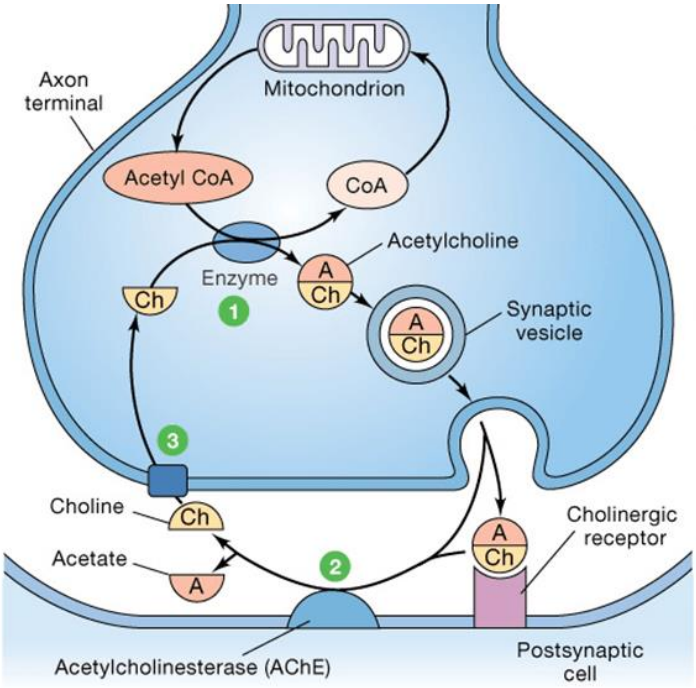

Figure 2. The cholinergic pathway. 1) ACh is synthesised inside the presynaptic terminal before being packed into vesicles for transport. 2) AChE rapidly breaks down ACh, effectively terminating the signal. 3) Choline is taken back up into the synapse (Adapted from: www.pasadena.edu).

1.2.5. Organophosphates irreversibly inhibit acetylcholinesterase through covalent modification of the enzyme.

AChE inhibitors generally feature different modes of inhibition, including competitive, un-competitive, and non-competitive inhibition (Čolović *et al.*, 2013). In competitive inhibition, the inhibitor competes with the ACh substrate and both cannot bind simultaneously to AChE, and availability of AChE to hydrolyse ACh is reduced.

Uncompetitive inhibitors alter the AChE enzyme and ACh substrate by binding together with both, thus creating an enzyme-inhibitor-substrate complex and preventing both the release of the ACh degradation product and any further degradation.

During non-competitive inhibition, the inhibitor binds to an allosteric AChE site, which is different from the orthosteric, or active site. This inhibits the activity of the AChE enzyme regardless of any further binding of ACh with AChE (Čolović *et al.*, 2013).

However, OPs cannot be strictly defined as competitive, uncompetitive, or non-competitive inhibitors of AChE. This is because they permanently inhibit AChE by covalently binding to its active site, forming a stable enzyme-inhibitor complex. This means that the activity of AChE can only be restored through the synthesis of new AChE (Čolović *et al.*, 2013; Ballantyne and Marrs, 2017).

1.2.6. Organophosphates as pesticides

ACh is the most abundant excitatory neurotransmitter in insect nervous systems (Gauthier, 2010) and as a result, insects are particularly vulnerable to the cholinergic hyperexcitation caused by OPs. This qualifies OPs as effective insecticides, inducing paralysis and death in target organisms, with high rates of efficacy. This combined with relatively low persistence within the environment in comparison with DDT and the OCs,

meant that OPs have become some of the most widely used pesticides worldwide (FAO Statistics, 2018). There are many compounds currently in use, including CPF, diazinon, dichlorvos, malathion, parathion, and many more. Some of the most common use cases are the spraying of crops for protection against herbivorous pests, and the treatment of livestock against ectoparasitic infestation, such as the dipping of sheep.

1.2.7. Risks to human health from organophosphates

While the reliance on cholinergic signalling by insects means that OPs are particularly effective against them, ACh and AChE are also important to many other organisms, including mammals, which suggests that some off-target effects are still possible. However, in addition to differences in neuronal signalling, the relative body mass of humans combined with an advanced ability to detoxify OPs, means that they can more effectively tolerate the levels of OPs present in most cases (Timchalk *et al.*, 2002). Despite this, acute OP poisoning is not uncommon in humans, particularly in cases where health and safety measures are suboptimal such as in developing countries (Razwiedani and Rautenbach, 2017).

However, even in developed countries it is likely that people will encounter OPs to some extent, and although acute poisoning is relatively less-common, negative impacts on human health remain a concern. Agricultural workers may be at risk through occupational exposure to higher concentrations, or larger volumes of pesticides as they are prepared and applied. Over recent years, and since the implementation of the Health and Safety at Work Act (1974), increased efforts have been made in the UK to ensure the safety of workers and to enforce the use and development of best practice, including the use of personal protective equipment (PPE). However, this has not always been the case, and even now best practice may be enforced with varying degrees of success. Education

of employers and employees is important to help them recognise risks, which has been shown in turn to increase uptake of PPE and good safety behaviours (Remoundou *et al.*, 2015; Okoffo, Mensah and Fosu-Mensah, 2016). Moreover, even with best practice accidents can happen, and in some cases inappropriate items of PPE, or items used incorrectly can actually increase the risk of exposure (Beránková, Hojerová and Peráčková, 2017). The next section will give an overview of the symptoms associated with OP exposure.

1.2.8. Organophosphate Toxicity

OP toxicity can take different forms depending on the duration or severity of exposure. These can be associated with several different outcomes and sets of symptoms, which will now be discussed. Some common symptoms of organophosphate poisoning separated by nicotinic and muscarinic mode of action are listed in **Table 1.**

1.2.8.1. Acute OP toxicity

Cholinergic syndrome - The initial clinical manifestation of OP poisoning follows an acute exposure incident and presents in a range of symptoms which can each be attributed to excessive ACh acting on nicotinic and/or muscarinic receptors (nAChRs and mAChRs respectively), at specific areas within the nervous system (Eddleston *et al.*, 2008). These symptoms are well known and although each compound carries its own individual toxicological profile, the treatment protocols are the same, with respiratory failure being the most common cause of death in fatal OP poisonings.

The mainly physiological features of acute poisoning are relatively simple to measure and identify. Furthermore, the events leading to such poisoning are likely to have been recognised by the patient or witnesses, particularly as the symptoms appear within a few

minutes of inhalation or within 30-90 minutes of ingestion. Dermal absorption may lead to a later onset of symptoms of up to 48 hours (King and Aaron, 2015). In addition to the compound involved, and the quantity or concentration consumed, the route of exposure can also dictate the order and presentation of specific symptoms. For example, vomiting and other gastrointestinal responses might be expected following ingestion, whereas tremors and sweating may more closely follow dermal exposure. Standard treatment for OP poisoning involves administration of atropine, which blocks some of the effects of OPs by competitively antagonising mAChRs (Eddleston and Chowdhury, 2016), and oximes, which reverse the inhibition of AChE itself (Eddleston *et al.*, 2002). However, even after improvement of this initial 'cholinergic syndrome', other forms of neurotoxicity can follow.

Intermediate syndrome - In some cases, a secondary phase involving weakness or paralysis of skeletal - including respiratory - muscles occurs, at around 24-96 hours after cholinergic syndrome. This is thought to occur due to dysfunction at the neuromuscular junction and may persist for weeks (Coulson, 2015).

Extrapyramidal syndrome - A relatively rare, but nonetheless recurrent and potentially relevant occurrence is the presentation of transient Parkinsonism following OP exposure (Müller-Vahl, Kolbe and Dengler, 1999; Brahmi *et al.*, 2004; Shahar *et al.*, 2005; Kalyanam, Narayana and Kamarthy, 2013; Panda, Bala and Bhirud, 2014; Reji *et al.*, 2016). This has been reported to manifest either alongside initial cholinergic syndrome or with delayed onset, and it has been shown to be reversible and responsive to the Parkinson's drug amantadine (Shahar *et al.*, 2005; Kalyanam, Narayana and Kamarthy, 2013). In fact, the transient nature of this phenomenon is likely to have prevented it from having been more often detected or reported (Müller-Vahl, Kolbe and Dengler, 1999). Nonetheless, it is

perhaps unsurprising given the necessary balance between cholinergic and dopaminergic neurotransmission (Calabresi *et al.*, 2006; Pádua-Reis *et al.*, 2017). A link between OP exposure and altered dopamine (DA) signalling might support a suggested mechanism for OP-related affective disorders, since DA is implicated in the pathophysiology of depression (described further in 1.10).

Furthermore, there is growing evidence of an important role for OPs in the onset of Parkinson's Disease (PD) itself (Baltazar *et al.*, 2014; Chuang *et al.*, 2016; Paul *et al.*, 2016, 2017). Levodopa, a precursor to DA, can be administered as a drug to relieve the symptoms of Parkinsonism (Tomlinson *et al.*, 2010). While depression in particular is often reported in PD patients, the motor related symptoms that are treated with levodopa are distinct from the symptoms of depression, and a combination of various quality of life factors likely contribute to depression experienced within those groups (Zis *et al.*, 2015). Nevertheless, potential interactions between OP exposure and DA function may be worth consideration in investigation concerning OP exposure and its possible role in affective disorders.

Table 1. Organophosphate poisoning symptoms separated by nicotinic and muscarinic mode of action. (King and Aaron, 2015).

Nicotinic Poisoning	Muscarinic Poisoning
Increased heart rate	Slowed heart rate
High blood pressure	Low blood pressure
Tremors, twitching	Muscle weakness
Seizures	Excessive sweating
Restlessness	Excessive salivation
Anxiety, agitation	Abdominal cramps
Headache	Nausea, vomiting
Dizziness	Diarrhea
Confusion, delirium	Blurred vision
Respiratory distress	Constricted pupils
	Urination difficulties
	Lacrimation (tearing)
	Bronchoconstriction
	Excessive mucus production

1.2.8.2. Chronic OP toxicity

With proper education, cases of acute poisoning in the workplace should be simple to recognise allowing any deficiencies or departures from proper safety protocols to be addressed. However, in cases where lower levels of exposure occur, the absence of acute poisoning symptoms may not necessarily indicate complete safety. Symptoms might be different and therefore unrecognised, or they could be absent altogether in the short term but emerge as a chronic illness later. The symptoms and effects of low-level, long-term, chronic OP toxicity are still not well-understood.

Delayed neuropathy - Organophosphorus compound-induced delayed neuropathy (OPIDN) can present weeks after the initial exposure and can occur in the absence of overt cholinergic syndrome. Indeed, OPIDN leads to paralysis via axonal degeneration

caused by inhibition of neuropathy target esterase (NTE), and thus has a completely separate pathophysiology (Richardson *et al.*, 2013).

1.2.8.3. Links to other diseases

Since the turn of this century, concerns have emerged in response to evidence that longer term exposure to OPs is associated with several other serious diseases, including diabetes (Rathish *et al.*, 2016; Leonel Javeres *et al.*, 2020), deep vein thrombosis and pulmonary thromboembolism (Lim *et al.*, 2015), breast cancer, ovarian, thyroid, colon, and lung cancers, glioma and non-Hodgkin lymphoma (Lerro *et al.*, 2015; Yang, Lee and Park, 2020). Furthermore, chronic, and acute OP exposure have been implicated in heart disease (Hung *et al.*, 2015; Kuo *et al.*, 2017), as well as neurodegenerative diseases, including Parkinson's Disease (Baltazar *et al.*, 2014; Chuang *et al.*, 2016; Paul *et al.*, 2017), Alzheimer's Disease (Sánchez-Santed, Colomina and Herrero Hernández, 2016; Voorhees *et al.*, 2019), and amyotrophic lateral sclerosis (ALS) (Merwin *et al.*, 2017; Yu and Pamphlett, 2017).

Lastly, there is mounting evidence that alterations in neuronal function caused by chronic and acute OP exposure, more specifically the occupational pesticide exposure experienced by agricultural workers, could be a causal factor in neuropsychological and neuropsychiatric diseases, including anxiety, depression, and suicide risk (London *et al.*, 2005, 2012; Harrison and Mackenzie Ross, 2016; Stallones and Beseler, 2016).

1.3. Organophosphates and mental health

Reports of psychiatric effects following OP exposure emerged as early as the 1940s, including some early studies which would be considered unethical by modern standards. One early example reported increased dreaming, sleep disruptions, emotional lability, changes in libido, and visual hallucinations, tremor, and paraesthesia after injecting healthy volunteers continuously with low concentrations of the OP diisopropylfluorophosphate (DFP) (Harvey and Lilienthal, 1947). A few years later, direct links between DFP and psychiatric symptoms were investigated in patients with pre-existing conditions, including 17 with psychotic disorders, 10 with bipolar disorder and 10 healthy controls (Rowntree, Nevin and Wilson, 1950). Depression, unhappiness, dejection, apathy, irritability, and general changes in emotional affect were reported by healthy volunteers injected with DFP. Depression was also increased in bipolar patients along with increased dreaming and disturbed sleep, although episodes of mania were reduced. Of the 17 schizophrenic patients, 6 experienced psychotic symptoms which persisted for 6 months after cessation of DFP administration (Rowntree, Nevin and Wilson, 1950).

Over the next couple of decades, evidence began to mount from case studies involving varying levels of OP poisoning which strengthened the links to mental health. Severe cases of OP poisoning were accompanied by noticeable changes in behaviour, including difficult speech, mental confusion, disorientation, difficulty concentrating, drowsiness, tremor, and ataxia (Grob, Garlick and Harvey, 1950), and later, withdrawal, apathy, depression, and schizophrenia (Gershon and Shaw, 1961; Durham, Wolfe and Quinby, 1965). Where physical poisoning symptoms were more moderate, headaches and sleep disturbances were common, including increased dreaming, nightmares, and insomnia

(Grob, Garlick and Harvey, 1950; Durham, Wolfe and Quinby, 1965). Furthermore, feelings of uneasiness, restlessness, trembling, and anxiety were noted, even after lower exposures (Grob, Garlick and Harvey, 1950).

By the middle of the 1970s, it was generally accepted that OP poisoning was linked to several psychological and psychiatric issues. Some of these include impaired vigilance and reduced concentration, slowing of information processing and psychomotor speed, memory impairments, linguistic disturbances, depression, or anxiety (Stallones and Beseler, 2016).

Having accepted the association with these effects, researchers began to investigate the wider circumstances surrounding behavioural impairments and OP exposure. Importantly, whether acute poisoning is necessary for the development of such behavioural impacts, or whether regular exposure in the absence of acute incident or symptomology might be sufficient, is a question that remains difficult to answer. Early survey studies, in which agricultural workers with history of acute OP poisoning were included alongside their asymptomatic counterparts produced mixed results regarding behavioural outcomes. However, the specific type or frequency of exposure may be of equal importance, as specialised sprayers exhibited higher levels of anxiety and lower plasma AChE than general farmers, in the absence of other toxic symptoms (Levin, Rodnitzky and Mick, 1976). Another unknown factor that could potentially influence results, was length of time that symptoms persist after exposure. Tabershaw and Cooper (1966) monitored acutely poisoned workers for three years after their respective incidents and found that around 38% suffered symptoms of acute poisoning for at least six months. Forty three of the 114 people tested continued to experience symptoms

including weight loss, digestive problems, headaches, blurred vision, weakness, nervousness, and chemical hypersensitivity (Tabershaw and Cooper, 1966).

One follow-up study which started in 1952 (Metcalf and Holmes, 1969), took a multidisciplinary approach to assess the psychological, neurological, and neurophysiological effects of OP poisoning in workers involved in the manufacture of OP compounds. A battery of psychological tests showed no evidence of brain damage within 72 hours of any poisoning incident, however psychological functioning was always erratic and slow in workers that showed any other toxic symptoms. Complaints of drowsiness were matched with associated electroencephalogram (EEG) changes, and general confusion and weakness were confirmed by neurological examinations (Metcalf and Holmes, 1969). Further, psychiatric interviews were conducted which found thinking problems, impaired vision, forgetfulness, and physical aches and pains to be more prevalent in exposed workers compared with controls. Exposed workers were also 40% more likely to complain of drowsiness, fatigue, and reduced interest in their work. Those results were then followed up with several cognitive and sleep tests, which demonstrated clear problems with memory, attentional focus, and difficulty remaining alert, as well as excessive drowsiness following exposure in workers that needed treatment for acute toxicity (Metcalf and Holmes, 1969).

Since these early findings, a series of other studies have reported cognitive abnormalities in people following OP pesticide exposure. Common cognitive symptoms across studies are difficulty concentrating (Metcalf and Holmes, 1969; Savage *et al.*, 1988; Stallones and Beseler, 2002a), confusion or disorientation (Metcalf and Holmes, 1969; Rosenstock *et al.*, 1991; Stallones and Beseler, 2002a), memory impairment (Metcalf and Holmes, 1969; Levin, Rodnitzky and Mick, 1976; Savage *et al.*, 1988; McConnell, Keifer and Rosenstock,

1994; Stallones and Beseler, 2002a) and impaired ability to read or process written information (Metcalf and Holmes, 1969; Steenland *et al.*, 1994; Stallones and Beseler, 2002a). However, all of these cognitive abnormalities were reported following acute poisoning, and studies which did test for effects of low-level exposure, despite mixed results generally, did not specifically report any cognitive effects in the absence of acute poisoning symptoms (Levin, Rodnitzky and Mick, 1976). Therefore, the safety implications and health risks associated with the low-level use of OP pesticides has yet to be properly addressed.

1.4. Separating the effects of acute poisoning from low-level exposure

Despite increased interest in the specific effects of low-level exposure in recent years, the majority of epidemiological studies concerning OP use by agricultural workers have failed to properly address the question of whether long-term, low-level exposure is sufficient to cause ill health in the absence of acute poisoning incidents (Mackenzie Ross *et al.*, 2013). However, investigating the effects of low-level exposure is now arguably more important, for several reasons. Firstly, the effects of acute OP toxicity are already relatively well-understood, partly because they have been studied over a longer period, they are easier to recognise in epidemiological and clinical settings, and acute exposures are more amenable for testing in pre-clinical models (Munro, 1977; Rhomberg *et al.*, 2007). Secondly, the proportion of people who work with OPs that experience acute poisoning in most workplaces should be a relatively small minority, and those unlucky enough to experience this should be identified for medical treatment. However, any health risks associated with low-level exposure could apply to all who work with OPs, but suffering might go unnoticed or untreated in a potentially larger group of people. Importantly, while the known effects of acute poisoning are undoubtedly serious, the potential implications of low-level exposure should not be downplayed. For example, increased risk of depression and suicide are both suspected in relation to low-level OP exposure (London *et al.*, 2005; Harrison and Mackenzie Ross, 2016). Depression has been described as the leading cause of disability worldwide (Friedrich, 2017), and suicide is one of the leading causes of death, being responsible for 27.1% of deaths in UK men, and 16.7% in UK women aged 20 to 34, between 2001 and 2018 (ONS, 2020).

1.5. Psychiatric problems in farmers

In general, farmers are reported to suffer from lower rates of psychiatric morbidity than the rest of the general population (Thomas *et al.*, 2003). However, the same study found that farmers were more likely than the average UK household to feel that their lives were not worth living (Thomas *et al.*, 2003). It is known that certain groups of people within society carry higher burdens of depression and anxiety than others, including for example, people suffering with poor physical health, or working in particular professions (NICE, 2010). Contrary to the initial finding by Thomas *et al.*, (2003), agricultural professions, and farming more specifically, are among the most likely to experience anxiety and depression of any occupational group (Roberts and Lee, 1993; Eisner, Neal and Scaife, 1999; Sanne *et al.*, 2003). Suicide is also more common in farmers than other occupations in the UK, with suicide being the second most common cause of death, other than accidents (Gregoire, 2002).

1.5.1. Suicide in farmers

The reasons for elevated depression and suicide rates in farmers are unclear. One theory supposes that farmers are more likely to have access to the means to carry out successful suicide attempts, which is based on the fact that firearms, hanging or self-poisoning with pesticides account for the majority of suicide verdicts (Hawton *et al.*, 1998). However, there is little to support this theory other than circumstantial evidence, and others suggest that lifestyle factors, life-stress, poor health, or even pesticide exposure could be to blame (Monk, 2000; London *et al.*, 2005; Judd *et al.*, 2006; Freire and Koifman, 2013).

1.5.2. Common mental disorder in farmers: links to organophosphates?

Common mental disorders encompass a range of conditions such as depression and anxiety, which can significantly impact an individual's emotional well-being and disrupt their daily functioning. While they are less debilitating than major psychiatric disorders, the high prevalence of common mental disorders results in substantial costs to society. Over the last few decades several epidemiological studies have sought to address the relationship between common mental disorders and farming, yet results are rarely consistent (Freire and Koifman, 2013a; Mackenzie Ross et al., 2013). The reasons for this could be many, but methodological inconsistencies are common and therefore worth considering when carrying out research in this area (Summarised in **Table 2**). While these effects are mostly noted to occur following cases of acute OP poisoning (Beseler *et al.*, 2006; Beseler and Stallones, 2008), there is mixed, and contradictory evidence for a role for low-level OP exposure in poor health outcomes, including cognitive deficit and common mental disorder (Freire and Koifman, 2013a; Mackenzie Ross *et al.*, 2013; Harrison and Mackenzie Ross, 2016). Several studies have reported no association between low-level exposure, cognitive impairments, and changes in mood (Daniell *et al.*, 1992; Fiedler *et al.*, 1997; Roldán-Tapia, Parrón and Sánchez-Santed, 2005; Solomon, Poole, Palmer, Peveler, *et al.*, 2007).

One study conducted with Egyptian pesticide formulators compared a group of 172 pesticide applicators with a control group of 233 non-exposed participants (Amr, Halim, and Moussa, 1997). The authors used DSM-III criteria and found that greater numbers of the exposed group suffered with psychiatric disorder than controls, reporting irritability and erectile dysfunction as common, and higher frequencies of individuals meeting the criteria for diagnosis of depression. However, there was insufficient information

regarding exposure history reported in this study to exclude the possibility of acute poisoning events, which therefore could have contributed to the symptoms reported. While this was still useful, the value to our understanding of low-level exposure risks is limited.

Another study which assessed Brazilian 37 tobacco workers using DSM IV criteria found almost half of participants suffered with psychiatric disorders, including anxiety (35%) and major depression (21%), when they were assessed shortly after a 3-month period of pesticide use. This was despite all participants showing normal plasma AChE levels throughout the study. The same group were assessed again after 3 months without coming into contact with pesticides and the number of diagnoses for depression and anxiety almost halved, respectively. Lifetime exposure history was recorded from this group, and although none of the participants reported having suffered acute cholinergic symptoms within year prior to the study, at least one had previously required pharmacological treatment and hospitalisation for acute poisoning. A further 52% of the participants had a history of "at least mild cholinergic syndrome" (Salvi *et al.*, 2003). The authors reported that, for cultural and economic reasons, these tobacco workers do not use effective protective equipment. Furthermore, they are regularly exposed to combinations of chemicals, including OPs, herbicides, fungicides, and neonicotinoid insecticides. It is difficult to determine whether the results of this study could be strictly attributed to low-level exposure. It is also difficult to pick apart the contribution of OPs from other individual, or mixed, effects of the other pesticides to which these groups are exposed. Several pesticides are often used on the same crop or area within agriculture, and so the confounding effects of different substances may present a limitation for most retrospective studies including agricultural workers. Nevertheless, the finding that AChE

activity was within normal levels throughout this group, and that psychiatric disorders were fairly common, together suggest that biological measures other than AChE inhibition should be considered in future studies.

Harrison and Mackenzie Ross (2016) studied UK sheep farmers using both self-report measures and structured clinical interviews, which are considered to be the gold standard of diagnostic measures used in such studies, according to DSM-IV criteria. These authors collected detailed exposure history and excluded participants with history of acute exposure from the study. The sheep farmers as the exposed group were compared against a control group of rural police officers with no history of exposure. Based on self-report screening questionnaires, the exposed group showed greater evidence of anxiety and depression than controls. However, results from the more stringent, structured diagnostic interviews did not show greater evidence for depression, and only anxiety was more common in the exposed group. In this study, other factors known to contribute to mental health problems, such as physical health, and stress were controlled for. However, the scale used to measure life factors that contribute to stress only considers acutely stressful events, such as losing a partner, losing one's job, getting married, or other events that cause a marked change in the subject's lifestyle (Holmes and Rahe, 1967). Stress can be an important contributor to depression (Tafet and Nemeroff, 2015). However, there are many aspects of day-to-day life that could cause stress that do not necessarily involve a major single event, or acute lifestyle change of lifestyle (Chamberlain and Zika, 1990). For example, long-term health conditions, ongoing financial issues, or even an abusive domestic relationship, would not have been acknowledged in the scale used by Harrison and Mackenzie Ross (2016), unless those things had started or increased in severity during the previous 12 months. Such day-to-day stressors could be important to mental

health in farmers, since they may be geographically or socially isolated and their livelihoods may be vulnerable to weather and other uncertainties (Eisner, Neal and Scaife, 1999; Matthews *et al.*, 2016; BBC, 2019). Despite this, day-to-day stressors do not seem to have been controlled for in studies investigating OPs and psychiatric health.

Several other studies have found positive relationships between low-level OP exposure, and poor psychiatric health outcomes, with depression and anxiety being commonly reported in exposed groups (summarised in **Table 2**). Conversely, a few studies have found no such relationships (Cole *et al.*, 1997; Fiedler *et al.*, 1997; Berent *et al.*, 2014).

Cole *et al.* (1997) compared 144 farm workers against 72 non-farming rural inhabitants that were matched for age and time spent in education. Despite this matching, age and education were reported to be the most important factors influencing the results, and farmers showed similar mood scores to the non-farming controls. Notably, the farming group used as the exposed group in this study had a mixed exposure history, ranging from those who worked directly spraying OP insecticides and carbamate fungicides, to farm-based workers with no direct exposure to pesticides. On the other hand, three percent of the control group reported having suffered one or more incidents of having been poisoned by pesticides, which suggests that the rural non-farming group could not be considered as strictly non-exposed.

Similarly, Fiedler *et al*. (1997) compared 57 tree fruit farmers, with known exposure to OPs, but with no reported history of acute poisoning, against 42 age-matched controls. The control group in this study was made up from cranberry and blueberry growers with "minimal" exposure history, and from hardware store owners. The groups were tested for depression as part of a psychiatric assessment but no difference between groups was

found. A limitation perhaps, was there could presumably have been some exposure among the control group. The exact details of how exposure history was assessed in the control group are not supplied. Another limitation was that the control group had generally higher level of education and reading performance than the tree fruit farmers used as the exposed group. The authors concluded that in the absence of acute poisoning, any effect of low-level exposure on neurobehavioral or neuropsychiatric function must be minimal, and at most, rarely associated with symptoms.

Why this evidence is mixed is likely to be the result of the methodological challenges involved with the study of low-level OP exposure, and also with measuring common mental disorders, which complicate interpretation of the evidence. Firstly, most studies have used different methodologies to explore common mental disorders in farmers, especially in relation to measuring exposure levels, measuring mood symptomology, demographic information, and accounting for other specific and potentially confounding factors. The lack of standardised exposure metrics means that comparisons are difficult, or impossible between studies, and dose-response modelling is lacking for human exposure. Discrepancies may also be due to methodological differences and limitations, such as a lack of reliable exposure data. Biological measurements such as serum AChE levels can be used but are unreliable at lower exposure levels. They rarely include a baseline measurement and have very limited temporal sensitivity because OPs are metabolised too quickly for detection in retrospective studies (Cocker *et al.*, 2002; Garfitt *et al.*, 2002). The detection of metabolites in urine have been used with some success, although the problem with temporal sensitivity remains (Hardy *et al.*, 2021). Recent pilot studies have shown promise in the use of hair samples, which have more sensitive detection levels, and have a much wider time window for analysis because the

metabolites found in hair and nail samples, and the tissue samples themselves are more stable. They are also more easily transported and stored, and sample collection is only minimally invasive (Hardy *et al.*, 2021).

Measures for estimating or quantifying pesticide exposure could influence study results. Another important factor is the reliability of detecting common mental disorder in these populations. These methods vary greatly and range from asking participants to report whether they have ever received a medical diagnosis for specific disorders (Beard *et al.*, 2014), as a very simple measure, to conducting structured clinical diagnostic interviews with participants directly, which is considered the 'gold standard' of diagnostic option (Harrison and Mackenzie Ross, 2016). Another common and convenient tool for assessing mood related symptoms, is the use of self-report questionnaires (Henry and Crawford, 2005; Golden, Conroy and O'Dwyer, 2007; Calvert *et al.*, 2008; Ronk *et al.*, 2013). These are useful for screening participants for possible symptoms of common mental disorder and exist in a range of forms and levels of detail, however differences in the reliability of these scales in comparison to clinical interviews has been demonstrated in a study that specifically addressed low-level OP exposure in relation to mood (Harrison and Mackenzie Ross, 2016). These authors had previously reported that clinically significant depression and anxiety levels occurred in 40% of UK sheep farmers that had been exposed to OPs compared to 23% in the control group (Mackenzie Ross *et al.*, 2010).

Author	Research Question	Design	Participants (Exposed/Referent)	Job Title	Developed/ Developing	Exposure Measures	Mood Measures
Stephens et al 1995	LTLL exposure to OPs & NB function	Group comparisons	146/143	Sheep Dippers	Developed (UK)	EHQ	Self-Report: GHQ
Fiedler et al 1997	Effect of LTLL exposure to OPs on NB function	Group comparisons	27/42	Fruit Tree Sprayers	Developed (USA)	EHQ	Self-Report: MMPI-2
Cole et al 1997	Compared NB performance of farm and non-farm members	Group comparisons	Farm members: 23 consumers, 28 exposed, 123 applicators/72	Farm Members	Developing (Ecuador)	EHQ, AChE	Self-Report: POMS
Bazylewicz-Walczak et al 1999	Behavioural effects of chronic exposure to OPs	Group comparisons & Pre/Post	26/25	Greenhouse Workers	Developed (Poland)	Air and clothing concentrations	Self-Report: POMS
Steenland et al 2000	Chronic neurological effects of OP exposure	Group comparisons	191/189	Pest Control	Developed (USA)	EHQ, PON1, Urinary metabolites	Self-Report: POMS
Salvi et al 2003	NB outcomes after 3 months with and without OP exposure	Pre/Post	37/25	Tobacco Workers	Developing (Brazil)	EHQ, AChE	Structured Clinical Interview
Roldan-Tapia et al 2005	Continuous exposure to OPs (subsymtomatic) and NB effects	Group comparisons	40/26	Greenhouse Workers	Developed (Spain)	BuChE, EHQ	Self-Report: POMS
Roldan-Tapia et al 2006	Association between different levels of exposure to OPs & NB function	Group comparisons	24 acute/40 chronic/26 controls	Greenhouse Workers	Developed (Spain)	BuChE, EHQ	Self-Report: POMS
Mackenzie Ross et al 2007	Nature & extent of NB problems in farmers who report chronic ill health	Group comparisons	25/22	Sheep Dippers	Developed (UK)	EHQ	Self-Report: HADS

Mackenzie Ross et al 2010	Does LTLL exposure to OPs cause ill health (NB problems) in sheep farmers	Group comparisons	127/78	Sheep Dippers	Developed (UK)	EHQ, PON1	Self-Report: HADS
Malekirad et al 2013	OP effects on neurocognitive impairment and health	Group comparisons	187/187	Horticultural workers	Developing (Iran)	EHQ	Self-Report: GHQ
Berent et al 2014	Compare NB function of exposed and non-exposed workers over 1 year period	Group comparisons & Longitudinal	53/60	Chemical factory workers	Developed (USA)	BuChE, AChE and Urinary	Self-Report: BSI

Table 2. Summary of studies investigating mood and psychiatric functioning following low-level exposure to organophosphates. The information in the table was compiled as part of a systematic review in progress at the time of writing, which follows on from previous work on which this project was based. Included here with permission from the authors(Mackenzie Ross and Harrison, Personal communication).

AChE–acetylcholinesterase; BSI-Brief Symptom Inventory; BuChE–butyrylcholinesterase; LTLL–long-term low-level; EHQ–Exposure History Questionnaire; GHQ – General Health Questionnaire; HADS – Hospital Anxiety and Depression Scales; NB–neurobehavioural; MMPI-2-Minnesota Multiphasic Personality Inventory-2; MMSE-Mini Mental State Examination; POMS – Profile of Mood States; PON1–serum paraoxonase/arylesterase; For further information about the psychometric tests in this table see Lezak et al, 2012 and Strauss et al, 2006.

1.6. Gaps and limitations of existing studies

1.6.1.1. Exposure assessment

One of the most important, but also most difficult measures to account for in the investigation of OP exposure and any health outcome, is the measure of exposure itself. While OP exposure can be measured indirectly using metabolites in blood or urine sample analyses, such measures can only provide a 'snapshot' of very recent exposure history. OPs are metabolised relatively quickly within the body. Enzymes facilitate their breakdown into various metabolites, which undergo subsequent processing and elimination via urine or faeces (Costa *et al.*, 2003). Although the specific rates of metabolism and elimination may vary, OPs typically exhibit a half-life ranging from a few hours to a few days in the body. Therefore, it is very unlikely that even a recently collected sample would be of much use when assessing the effects of longer-term, or historic pesticide use in retrospect. This is a particular problem in studies of low-level exposure, where medical treatment is unlikely to have been sought. More often in low-level studies, there is a reliance on self-reported history of exposure, or an assigned estimate which may be calculated based on proxies such as occupational roles and activities. For example, sheep farmers are more likely to have been exposed to OPs through their work than police officers (Mackenzie Ross *et al.*, 2010). These distinctions may justify the qualitative assignment of 'exposed' and 'non-exposed' category groups for between group comparisons and can be supported by simply asking participants to confirm whether or not they have ever been exposed, as a yes/no exclusion criterion.

1.6.1.2. Exposure metrics

However, measuring exposure within groups poses a greater challenge. Exposure metrics are sometimes created, but their value is difficult to determine, because it is unclear what the most important aspects of exposure are. For example, whether it is the dose, frequency, intensity, or duration of exposure, the specific OP compounds an individual is exposed to, or the route of administration. Crucial factors may therefore be overlooked in some studies. Consensus in the literature is also lacking, regarding whether these aspects of exposure should be evaluated independently or merged into a single value that captures numerous characteristics of exposure. Where exposure measurements have been employed, they vary greatly and are unlikely to be directly comparable. Although metrics are generally preferred over simple measurements of exposure, such as exposed/non-exposed, or measures of time/duration of exposure, they may be overly complex and misleading if the values used to calculate them are misjudged or chosen arbitrarily (Mackenzie Ross *et al.*, 2013).

1.6.1.3. A definition of low-level exposure

The absence of precise and agreed-upon definitions complicates the identification of low-level effects. In addition, the onset of adverse symptoms from AChE inhibition may be more closely related to the rate of inhibition than the final level of inhibition, and detection limits may be well below those required for the onset of crisis (Darvesh et al., 2003; Freire and Koifman, 2013; Mackenzie Ross et al., 2013; Harrison and Mackenzie Ross, 2016). It seems most simply classified as 'any exposure that does not induce acute poisoning symptoms'. This term is likely to cover a wide range of exposures, with some groups reporting daily exposure for extended periods of time, such as pesticide formulators, and others experiencing less-frequent exposure, for a few days per year as

may be the case with dipping sheep on an annual basis. This definition also implies that individuals can self-diagnose pesticide poisoning; it does not account for the reality that some people may be more likely than others to seek medical help, regardless of the severity of their illness (Phillips and Pitts, 2002).

1.6.1.4. Additional and synergistic effects of multiple substances

Furthermore, potential additional, or synergistic effects of exposure to more than one toxicant can occur when treating crops or working in industrial environments, but these are rarely considered in retrospective investigations. This means not only that it is difficult to assign any observed effect to any specific substance but could offer a theoretical explanation for why an effect is seen in one population, but not in another where different combinations of substances may have been present during exposure.

1.6.1.5. Other confounding factors

Several other factors also need to be controlled for, which are not always considered, such as age, gender, years of education, alcohol and drug consumption, major stressful life events, and day-to-day stressors. Some studies overlook the potential confounding effects between common mental disorders or other factors such as general physical health. Even when accounting for these effects, some reported evidence of poor mental health (Mackenzie Ross et al., 2010). Another potentially misleading practice could be statistically controlling for similarly correlated variables as covariates, as some may be strongly linked to exposure variables, such as age and lifetime exposure (Fiedler *et al.*, 1997).

1.6.2. Mood and anxiety disorders

'Mood disorder' is a term that covers several mental health conditions within depression and bipolar disorders (Pizzagalli, Whitton and Webb, 2018). Mood disorders are strikingly common, with 20 percent of people being likely to suffer one or more at some point during life. They cause some of the greatest burdens of disability, not just in psychiatric disease, but in all diseases including major physical ailments such as heart disease, diabetes, and cancer (WHO, 2017). As a result, the personal, economic, and societal costs associated with mood disorders are substantial. Furthermore, research has suggested that anxiety and depression may be particularly prevalent in certain occupations, with those working in farming and agriculture demonstrating the highest incidence rates (e.g., Roberts and Lee, 1993; Sanne et al, 2003). This thesis will investigate one potential reason for this elevated risk of mood disorder in farming populations: exposure to pesticides. However, to illustrate this fully, definitions and explanations for mood and anxiety disorders will first be explored. The following subsections will review the DSM-5 diagnostic criteria for mood disorders and discuss their impact.

1.6.3. Unipolar depression

The most well described of the unipolar disorders is major depressive disorder (MDD), in which patients suffer at least five depressive symptoms over a period exceeding (often considerably) 2 weeks. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5; American Psychiatric Association (APA), 2013) for MDD to be diagnosed, a person must experience depressed mood for a large part of the day on most days, and/or anhedonia. Anhedonia is characterised by a reduced ability to experience pleasure from previously pleasurable activities. In addition to one or both of these, a person with MDD will have experienced several of the following symptoms: disturbed

sleep, general fatigue, feelings of guilt, feeling worthless, difficulty concentrating or decision making, changes in weight or appetite, psychomotor agitation or slowing, and suicidal thoughts or behaviour. In addition to the presence and 2-week minimum duration of these symptoms, the person must have experienced significant distress and/or functional impairment (APA, 2013).

Dysthymia, more recently described as persistent depressive disorder (PDD) (Murphy and Hallahan, 2016), is another well-characterised form of unipolar depression. A diagnosis of PDD depends on an extended period of symptoms for no less than 2 years, and with no periods of remission lasting more than 2 months. Depressed mood for most of the day, on most days must be accompanied by at least two of the following symptoms: feelings of hopelessness, low self-esteem, low energy, trouble concentrating, or changes in appetite (APA, 2013).

Other subtypes of unipolar depression include melancholic, and atypical depression, although criteria for these as defined conditions are not supported by statistical modelling techniques (Pizzagalli, Whitton and Webb, 2018). However, characteristic features of melancholic depression include reduced appetite and weight loss, anhedonia, and insufficient sleep. Conversely, atypical depression consists of increased appetite and weight gain, excessive daytime sleepiness, and enhanced mood reactivity.

1.6.4. Bipolar disorder

Bipolar disorders may include elements of depression but require specifically a history of manic episodes lasting for at least one week. Mania is characterised by irritable, elevated, or expansive mood, which is accompanied by highly energetic, goal-focused behaviour, despite noticeably impaired functional performance. On most days, and for

most of the day, bipolar patients will experience racing thoughts, reduced need for sleep, or inflated self-esteem. They may also display an increase in risk-taking behaviour, psychomotor agitation, or a high level of distractibility. A selection of four of these symptoms, including altered mood, may result in a diagnosis of 'bipolar I disorder' whether depression is present or not, although some experience of depression is likely in most cases (Pizzagalli, Whitton and Webb, 2018). On the other hand, a diagnosis of bipolar II disorder requires a history of major depression, in addition to at least one episode of hypomania. Hypomania is similar to mania, but with lesser severity and duration (APA, 2013).

1.6.5. Anxiety disorders

Anxiety in itself is a natural part of the human threat response, and so some level of concern, or worry about negative or stressful situations is often perfectly normal and rational. However, when these responses are disproportionate to the given situation, either in terms of severity or longevity, and if they cause regular distress or prevent a person's ability to function or enjoy life, then anxiety becomes a mental health problem.

Several anxiety disorders have been described, many of which are defined by the focus or cause of a person's worries, concerns or behaviours (APA, 2013). For example, certain phobias may be specifically related to social situations, heights, or certain animals (Eaton, Bienvenu and Miloyan, 2018). Panic disorder is a categorised by the experience of multiple episodes of sudden and severe physical anxiety symptoms (panic attacks), and specifically the potentially self-perpetuating fear of experiencing another panic attack (Roy-Byrne, Craske and Stein, 2006). Physical anxiety symptoms include hyperventilation, heart palpitations/pounding heartbeat, trembling or shaking, sweating, chills or hot flushes, chest pain, difficulty breathing, nausea, dizziness and numbness or

tingling. Other specific anxiety disorders may be directed at gaining weight, or separation from a close family member, among other things (Silove *et al.*, 2010; Attia *et al.*, 2013).

In cases where anxiety is related to a multitude of different things (such as different aspects of everyday life) rather than having a single, specific focus, a diagnosis of generalised anxiety disorder (GAD) may be given, provided symptoms do not result from any other illness, drug, or experience of trauma (APA, 2013). For a diagnosis of GAD, excessive anxiety and worry lasting at least six months, must be accompanied by three or more symptoms, including fatigue, irritability, poor sleep, muscle tension, difficulty concentrating, or feeling restless or on edge.

Whilst not technically categorised within mood disorders, anxiety is commonly found comorbidly with depression, with some research suggesting that nearly 80% of patients diagnosed with MDD also meet the criteria for anxious distress (Zimmerman *et al.*, 2019). Symptoms of both disorders commonly present simultaneously and this has raised questions as to whether their separation within diagnostic guidelines is arbitrary and unhelpful (Möller *et al.*, 2016; Mulder *et al.*, 2019). There are also several parallels between anxiety and depression, including some common symptoms such as repetitive negative thinking. The first-line treatment options are also almost identical between the disorders, including behavioural and pharmacological treatments (Carek, Laibstain and Carek, 2011; Gorka *et al.*, 2019). Furthermore, anxiety and depression have been reported to frequently occur together in agricultural workers following organophosphate exposure, suggesting that there may be value in further investigation of both anxiety and depression in that context (Harrison and Mackenzie Ross, 2016).

1.7. What causes mood disorders?

Understanding what causes changes in mood can be critical for diagnosis, treatment, and prevention. In some cases, the cause of the mood change may be obvious, such as a bereavement, or a sudden life change like a person losing their job. In other cases, there might be no clear life circumstance that appears to trigger the episode. This has been a subject of much research, and many theories which attempt to explain the causes and mechanisms of mood disorders have emerged (Schildkraut, 1965; Janowsky *et al.*, 1972; Sanacora, Treccani and Popoli, 2012; van Enkhuizen *et al.*, 2015; Lener *et al.*, 2017). The aetiology of diseases like depression and anxiety, however, has proven to be incredibly complex. As a result, many therapeutic options have been successfully applied in the treatment of mood disorders, yet none are perfect. The precise value and suitability of pharmacological treatments for depressed patients has been hotly debated (Penn and Tracy, 2012; Hengartner, 2020). Antidepressant drugs must show better efficacy than placebo to achieve regulatory authorisation, however how depressive symptoms are reduced by antidepressants with different mechanisms of action, and the possible contribution of placebo effects, can be difficult to disentangle (Kirsch, 2014; McGirr *et al.*, 2015). Onset of therapeutic effect can also be delayed, necessitating extended treatment. Changes in neuronal chemistry and function can lead to developed drug tolerance, sensitisation, and cessation of treatment can lead to withdrawal symptoms (Hengartner, 2020; Khushboo *et al.*, 2022).

Efforts to demystify their causes and find better treatments are ongoing. In the meantime, there are several circumstantial and environmental factors that are thought to increase a person's likelihood of suffering from depression, the majority of which are shared with anxiety disorders (Rodgers *et al.*, 2000). Further to the investigation of pathophysiology,

a combination of biopsychosocial factors is also likely to play important roles, and these provide important context in which to understand such complex conditions (Engel, 1977; Inerney, 2021). Some of the known factors in anxiety and depression aetiology will now be discussed.

1.7.1. Lifestyle factors

Although depressive symptoms can sometimes occur for no obvious reason, it is well known that certain lifestyle factors can affect a person's chances of suffering, and in many cases lifestyle changes alone can be sufficiently therapeutic, and even life-saving (Bohnert *et al.*, 2012; Sarris *et al.*, 2014). While some lifestyle changes may emerge as a result of mood disorder, including falling into categories of diagnostic symptoms, the direction of the cause-and-effect relationship is not always entirely clear. For example, a person experiencing difficulties at work or at home could contribute to, and/or be a symptom of depression (APA, 2013). In terms of treatment, there is a broad range of research to suggest that both lifestyle and medical factors should be considered (Sarris *et al.*, 2014).

1.7.2. Sleep, diet, and exercise

Links between levels of satisfactory sleep and depressive symptoms have long been acknowledged (Burton, 1845). More recently, disturbed sleep is noted as an incredibly common, transdiagnostic symptom in several psychiatric disorders, and most closely linked with depression (Baglioni *et al.*, 2016). While lack of sleep alone may not be a complete cause of depression, there is considerable overlap between factors implicated in insomnia and those for depression. Some examples include stress, lifestyle factors, and physical or mental health conditions (Drake, Roehrs and Roth, 2003). Like depression, the underlying pathophysiology of insomnia is less-well understood, however recent insights from genome-wide association studies (GWAS) indicate possible casual links

between the two conditions (Lane *et al.*, 2019). Moreover, because most depressed patients report at least some sort of sleep disruption, and patients suffering insomnia have increased risk of depression and suicidal behaviour, a bi-directional relationship between the two seems evident (Riemann *et al.*, 2020).

Diet and exercise have also been implicated in the risk of developing mental health disorders, however, whether diet alone is directly responsible for longer-term, or pathological changes in mood such as depression has been difficult to prove (Molendijk *et al.*, 2018). Metanalyses have found that high quality diets, specifically Mediterranean diets, diets low in animal produce, and high in leguminous proteins, fish, and vegetables, were associated with lower depression risk (Khalid, Williams and Reynolds, 2016; Molendijk *et al.*, 2018). However, the evidence for a direct causal link between diet and depression is lacking, despite some interesting hypotheses suggesting that neurotrophic, immune, or anti-inflammatory processes could mediate dietary effects on depression (Sarris *et al.*, 2015). Another possible explanation for the association between diet and exercise, and depression, could be via changes in metabolic health. For example, bodyweight, obesity, and diabetes are notably often comorbid with depression (Katon, 2008; Luppino *et al.*, 2010).

There are many theories as to how exercise itself might protect against depression, and some evidence to support the use of exercise as an effective treatment, however more research into how, and the types of exercise that may be most effective are needed (Carek, Laibstain and Carek, 2011; Cooney *et al.*, 2013; Paolucci *et al.*, 2018).

1.7.3. The endorphin hypothesis

One theory which has claimed to explain the effects of exercise on mood relates to the endogenous opioid system, known as the endorphin hypothesis (Hoffmann, 1997). This hypothesis is based on the fact that levels of endogenous β-endorphins are increased following acute exercise, and it is suggested that these bind to brain opioid receptors, consequently reducing anxiety, and producing a euphoria referred to as 'runners high' (Howlett *et al.*, 1984; Hoffmann, 1997; Dishman and O'Connor, 2009). The mood enhancing effects of exercise are well recognised, as are the analgesic effects of endorphin release following exercise (Dishman and O'Connor, 2009; Carek, Laibstain and Carek, 2011; Cooney *et al.*, 2013). However, the endorphin hypothesis as a direct causal explanation for the effects of exercise on mood remain controversial. While theoretically plausible, the positive effects have been difficult to measure empirically, and it is unknown whether increases in plasma β-endorphins are meaningfully replicated in the brain. Furthermore, treatment with the opioid antagonist naloxone has been shown to prevent the analgesic effect of exercise, but not the positive effect on mood, suggesting that mood may be affected by some other mechanism (Yeung, 1996; Dishman and O'Connor, 2009). Nevertheless, not all opioid receptors are sensitive to naloxone, and the longer-term effects of exercise could work through naloxone-insensitive aspects of the opioid system (Woods, Shahabi and Sharp, 1997; Koppert *et al.*, 2005). This might also occur via interactions of β-endorphins with other neurotransmitter systems (Dishman and O'Connor, 2009).

1.7.4. Exercise and monoamines

The effects of exercise on mood may also occur via monoaminergic signalling, in combination or independently of opioids. The implication of the monoamines, DA,

serotonin (5-HT), and noradrenaline (NA), in depression and anxiety will be discussed in further detail later in this chapter. However, briefly: the mesocorticolimbic DA system includes a circuit adjoining the nucleus accumbens, frontal cortex and the ventral tegmental area (VTA), is important in motivation and hedonic response, and is regulated by endogenous opioids (Werme *et al.*, 2002; Dishman and O'Connor, 2009). It has been suggested that these pathways may interact with motivational control of physical activity such as hedonic drive to exercise (Dishman and O'Connor, 2009). Furthermore, changes in 5-HT and NA levels and turnover are known to occur during and following exercise (Chaouloff, 1997; Dishman, 1997; Dishman and O'Connor, 2009). As will be discussed later in this chapter, these systems are important in the function of several clinically effective antidepressant medicines, and therefore present possible intersections between exercise and mood.

1.7.5. Drug and alcohol use

Another relationship that is frequently observed is that between substance use and depression. While substance use disorders are categorised separately from mood disorders (APA, 2013), it is acknowledged that several mental disorders, including mood disorders, can often develop following the use of some medications or drugs of abuse. These are termed 'substance induced mental disorders' (APA, 2013). This is another bi-directional relationship, in which people who are suffering from a disorder may attempt to self-medicate by using substances to treat their symptoms. Whereas on the other hand, as a depressant, frequent use of alcohol can cause some symptoms of depression, and although some other drugs might appear to have the opposite effect, there is a long-term negative impact of addiction on various aspects of a person's life and neurobiology (Volkow, 2004; Fergusson, Boden and Horwood, 2009).

1.8. Health and wellbeing

1.8.1. Other mental health problems

It is not uncommon for more than one mental health problem to present simultaneously, and often one set of symptoms may precede another set. Comorbidities between sleep disorders, substance use, depression and anxiety have already been discussed in previous sections of this chapter, and bi-directional effects are a common feature in these relationships. The following subsections will explore some other factors which are known to contribute to depression and anxiety, and some factors which may influence inter-individual vulnerability to such disorders.

1.8.2. Physical health issues

Physical health can be a particularly important predictor of mental health and mood. This may be a particularly important factor for consideration because farming is one of the highest risk occupations for work-related injury and illness (Dixon and Welch, 2000; Solomon, Poole, Palmer, and Coggon, 2007). Elements of physical health status can be associated with most of the other factors mentioned so far, such as the metabolic health impacts of a poor diet and a lack of exercise. Similar issues may arise in alcohol abuse, alongside diseases such as liver cirrhosis (Gutteling *et al.*, 2007). Drug addiction, while largely considered to be a mental health issue (APA, 2013), can often present with powerful physical symptoms, particularly during withdrawal (Hughes, Higgins, and Bickel, 1994). Other issues, such as dental problems may also result from taking certain drugs which can also lead to poor mental health outcomes (Anttila *et al.*, 2006; Reece, 2007).

Physical health issues can impact in many ways and while depression is in itself a major cause of disability, other conditions that force significant changes to a person's lifestyle, especially when present over a longer term, can greatly increase the risk of developing depression (Benedetto *et al.*, 2014). Likewise, serious and life-threatening conditions can understandably induce anxiety, and depression (Smith, Gomm and Dickens, 2003). In such serious cases the level of worry may be entirely proportionate to the situation, and many of the symptoms associated with mood disorders might be expected. Significant levels of pain, discomfort, or the effects of medications may also contribute. However, even comparably less serious conditions might lead to changes in mood, particularly when overall health status is poor. In these cases, the development of comorbid depression can lead to a worsening of the severity of other complaints (Moussavi *et al.*, 2007).

While some conditions could foreseeably cause mood related symptoms, some others might not seem as predictable, yet could be directly responsible for depression through more direct biological mechanisms. Some of these include nervous system disorders. A diagnosis of Parkinson's disease for example, might be expected to negatively impact mood, and lifestyle changes forced by the associated debilitating motor symptoms might by themselves increase the risk of depression. However, in many cases anxiety and depression are some of the first symptoms in Parkinson's disease to emerge, and it is thought that the DA signalling deficiency characteristic of Parkinson's may bring on these symptoms before the motor symptoms appear (Belujon and Grace, 2017; Dallé and Mabandla, 2018). Similarly, depression is common in Alzheimer's disease (AD) patients, for which it has been suggested serotonergic signalling, and/or neuroendocrine

signalling is responsible, providing a direct link between the pathophysiology of AD and depression (Sierksma *et al.*, 2010).

1.8.3. Physical pain

Physical pain may be associated with many of the health conditions related to altered mood and may itself be an important factor in the aetiology of mood disorders. Depression and chronic pain are both common complaints, which often occur comorbidly leading to increased suffering (Surah, Baranidharan and Morley, 2014). There has been some debate as to whether depression is a consequence of chronic pain, or if it could precede and influence the subjective experience of pain (Fishbain *et al.*, 1997). Interestingly, neurotransmitter pathways implicated in the regulation of pain are also frequently associated with suggested biological theories for the pathophysiology of depression, the analgesic and mood-altering effect of exercise discussed earlier being just one example (Fishbain *et al.*, 1997; Dishman and O'Connor, 2009; Surah, Baranidharan and Morley, 2014; Naser and Kuner, 2018).

1.9. Life history and environmental stress

1.9.1. Childhood experiences

Experiences from childhood can also greatly influence the risk of developing depression in later life. Finding direct causation for these associations has been difficult, largely due to the various types of childhood experiences and psychopathologies reported, however history of childhood trauma is disproportionately more common in mental disorders in general (Moskvina *et al.*, 2007). Major depression as an adult is commonly reported in childhood victims of emotional abuse, neglect, and physical abuse (Bernet and Stein, 1999; Moskvina *et al.*, 2007). Whereas, physical and sexual abuse are specific predictors of post-traumatic stress, and anxiety disorders (Gibb, Chelminski and Zimmerman, 2007). These sorts of terrible experiences have also been shown to lead to significantly increased risk of suicide (Janiri *et al.*, 2018), and the effects of multiple negative experiences can have a more severe effect than a single major trauma, such as a sudden parental bereavement (Pham *et al.*, 2018).

1.9.2. Major life events

The contributions of stressful life events on major depression have long been recognised, and have been widely studied (Holmes and Rahe, 1967; Lei and Skinner, 1980; Paykel, 1994; Kessler, 1997). Even outside of the developmental vulnerabilities associated with childhood, there are numerous events which can significantly affect our mental health, specifically in cases where risk is already increased by other factors, like poor lifestyle or illness. Events that are decidedly negative, like losing a job, a relationship breakdown, or the death of a close friend or relative, can obviously impact mood. However, some events that might be considered as positive can be highly stressful and increase vulnerability to anxiety or depression. Examples such as moving home, starting a new job, having a child,

or getting married, can all increase risk depending on circumstances and individual susceptibility (Paykel, 1994; Kessler, 1997; Clark, 2005).

1.9.3. Chronic stressors

For the most part, research concerning the effects of life stress on mood has focused on major life events, based on strong associations with depression in particular (Kessler, 1997). However, it is possible that the occurrence of individual, acute life events do not account for a significant proportion of stress experienced by people throughout their lives (Hutchinson and Williams, 2007). For many people, and particularly those who experience low mood over longer periods of time, the effects of chronic, ongoing life issues may be more likely to be a major source of distress. As an illustrative example, a person living in the same place, with the same job, and with a consistently stable family situation, might not experience any notably stressful life events for many years. However, that same person could suffer from a permanent health condition, long-term financial difficulties, and have to look after a relative with a long-term health problem. In this case, the individual could be at risk of developing depression for several reasons that would not be detected by considering stressful events alone. Therefore, ongoing factors that cause stress without changing from day to day may be more important in the maintenance and development of depression (Kanner *et al.*, 1981; DeLongis *et al.*, 1982; Chamberlain and Zika, 1990; Hutchinson and Williams, 2007). This is a factor that may be of particular significance to farming cohorts, as recent research has highlighted a number of chronic stressors that may serve as risk factors for mental health issues, including ongoing financial concerns and persistent physical health issues (Daghagh Yazd, Wheeler and Zuo, 2019).

1.9.4. The effect of psychological resilience

While any combination of the factors described here may increase the likelihood of a person experiencing mood disorders, some individuals could be less prone to suffering than others. A person's resilience is their ability to cope with adverse life events, stress, and trauma (Southwick *et al.*, 2014). Resilience has been shown to correlate positively with mental well-being, positive emotional state, and life satisfaction (Hu, Zhang, and Wang, 2015). Conversely, several studies support the suggestion that symptoms of depression and anxiety are negatively correlated with resilience (Smith, 2009; Hu, Zhang, and Wang, 2015; Shapero *et al.*, 2019). Rather than being a fixed personality trait, resilience can result from experience and can be proactively built upon (Southwick *et al.*, 2014). This holds potential for interventional mitigation of mood related symptoms for individuals, while at the same time adding a level of complexity in terms of understanding the prevalence and causes of mood disorders at a population level, due to added inter-individual variation.

1.9.5. Genetic factors

Additional variation in susceptibility to mood disorders may be caused at a biological level. In recent years, researchers using candidate study approaches have largely failed to identify specific gene variants that can be reliably implicated in depression, despite twin-based studies showing that depressive disorders are around 37% heritable (Sullivan, Neale and Kendler, 2000). Genome-wide association studies (GWASs) had proven similarly fruitless with regard to depression, until very recently when over 80 associated loci were successfully replicated (Howard *et al.*, 2019). These discoveries are exciting and will no doubt lead to important advances in the understanding and treatment of depression pathology in the future. However, before their benefit is fully

realised more work must be done to pick apart and define roles for each variant within potential causal mechanisms, and from associated phenotypes and comorbidities (Ormel, Hartman and Snieder, 2019). In the meantime, it is helpful to understand the various features and pathways which have been implicated in the pathophysiology of mood disorders, and the justifications for each of several theories proposed to date. Therefore, some of the most popular explanations for a biological basis for depression will now be discussed.

1.10. Biological basis for depression

Understanding the potential biological mechanisms underlying mood disorders is important for, and often informed by, successful treatment of existing cases. It also helps to understand whether there is biological plausibility to hypotheses in epidemiological investigations. Here, it will be useful to understand some background on the known mechanisms of mood disorders to address whether exposure to organophosphates might plausibly play a causal role.

1.10.1. The monoamine hypothesis

Perhaps the most pervasive explanation for the pathophysiology of depression over the last century is the monoamine hypothesis, originally proposed by Joseph Schildkraut (1965). The theory proposed that depression resulted from an imbalance of the neurotransmitters 5-HT, DA, and NA, and was based on responses to drugs which appeared to mediate depression.

1.10.2. "Reserpine-induced depression"

Some of the earliest evidence of a biological mechanism for depression was observed following the effects of different drugs on neurotransmitter levels, and associated variations in depressive symptoms (Bunney and Davis, 1965; Purves *et al.*, 2001). A classic example is the antihypertensive drug: reserpine, a natural alkaloid which had been previously used in traditional Indian medicine for the treatment of nervous disorders (Healy and Savage, 1998). Following the use of reserpine in the West in the 1950s, some patients were reported to suffer depression-like, dysphoria and reduced motor activity (Bunney and Davis, 1965). Comparable behavioural responses to reserpine treatment were observed in animals which led to the view that reserpine actually induced clinical

depression (Schildkraut, 1965). It was supported that the depletion of catecholamines (**Figure 3)** and 5-HT at synaptic nerve terminals caused by reserpine, was at least in part responsible for the onset of depressive symptoms (Schildkraut, 1965).

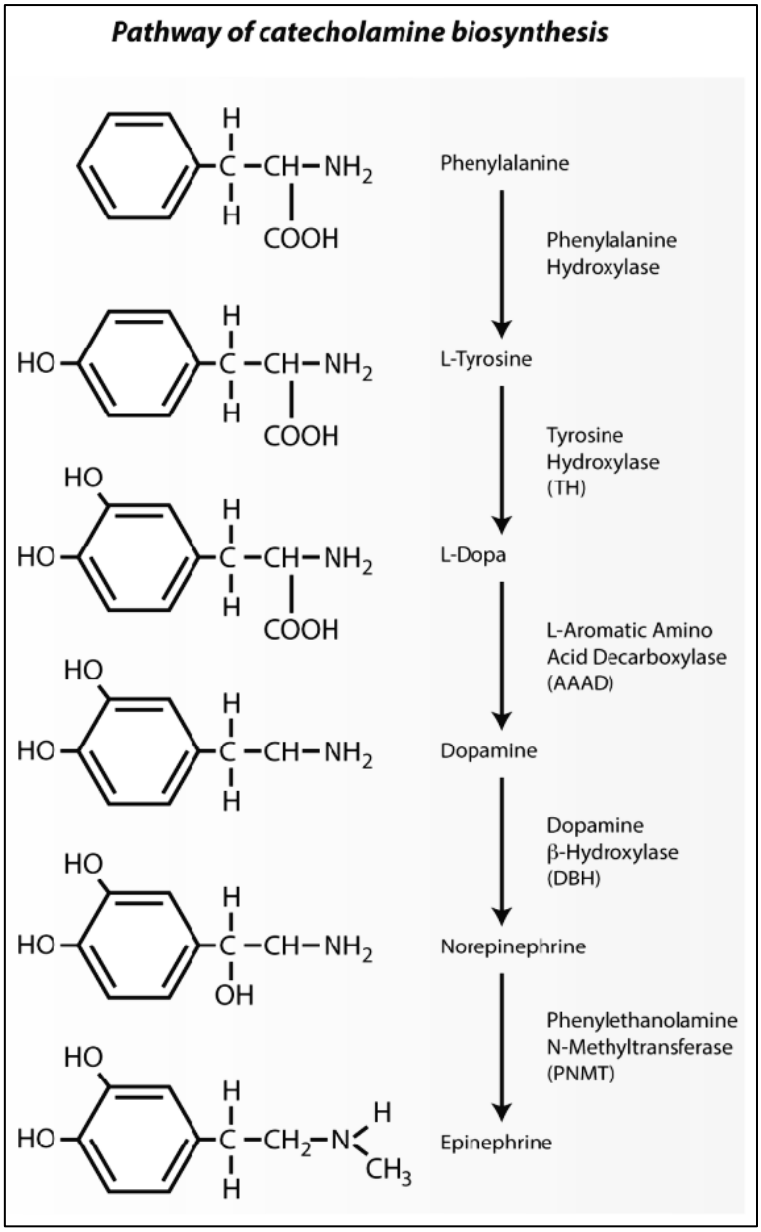

Figure 3. The catecholamine biosynthetic pathway. All three catecholamine neurotransmitters are derived from the precursor tyrosine. Tyrosine hydroxylase is the catalyst for the initial step in the reaction and is a rate-limiting factor in the synthesis of these neurotransmitters (Adapted from: Végh *et al.*, 2016).

1.10.3.Monoamine oxidase inhibitors

The idea of reduced biogenic amine function as causal mechanism for depression, although inconclusive, was supported by the unexpected discovery that tuberculosis patients being treated with the monoamine oxidase (MAO) inhibitor isoniazid, experienced some relief from pre-existing depressive symptoms (Oldham, 1955). Inhibition of MAO prevents the normal degradation of monoamine neurotransmitters and consequently causes elevated levels of 5-HT, DA, and NA, based on which MAO inhibitors (MAOIs) were adopted as the first group of drugs to be marketed specifically as antidepressants (Wax, 2014).

1.10.4.Tricyclic antidepressants

Continued support for the biogenic amine hypothesis emerged around the same time as imipramine, and later: amitriptyline, were adopted as some of the earliest tricyclic antidepressants (TCAs) (Peet, 1994). TCAs also elevate biogenic amine levels but via a different mechanism to MAOIs: primarily, TCAs bind and block NA and 5-HT transporters (NET and SERT, respectively), preventing the reuptake of those neurotransmitters and prolonging the signal between neurons (**Figure** 4).

TCAs can also be potent antagonists of histamine H_1 receptors, and muscarinic acetylcholine receptors (Richelson, 1977, 1978). It has been argued that the blockade of H_1 and muscarinic receptors likely explains some side effects, but not the therapeutic activity of TCAs used in the treatment of depression (Alvarez *et al.*, 1986). Conversely, some more recent studies in rodents have indicated that histamine signalling via H_1 receptors may be important in motivated arousal, and the processing of emotionally salient memories (Valdés *et al.*, 2010; Riveros *et al.*, 2019; Provensi *et al.*, 2020). While emotional and motivational behaviours are important factors in depression, evidence for

decreased histamine H_1 receptor function in depressed human patients is limited. Furthermore, understanding the interactions between depression and histamine H1 receptor function can be made more difficult by confounding factors, such as whether patients have been previously treated with TCAs, or antihistamine medication (Kano *et al.*, 2004).

The role of muscarinic acetylcholine receptors in depression has shown some promise as a potential therapeutic target following some success in the use of the muscarinic antagonist scopolamine (Jaffe, Novakovic and Peselow, 2013; Guo *et al.*, 2018). The potential role of the cholinergic system in depression (and how it might serve as a biologically plausible route to mood disorder following exposure to organophosphates) is discussed in further detail later in this and following chapters.

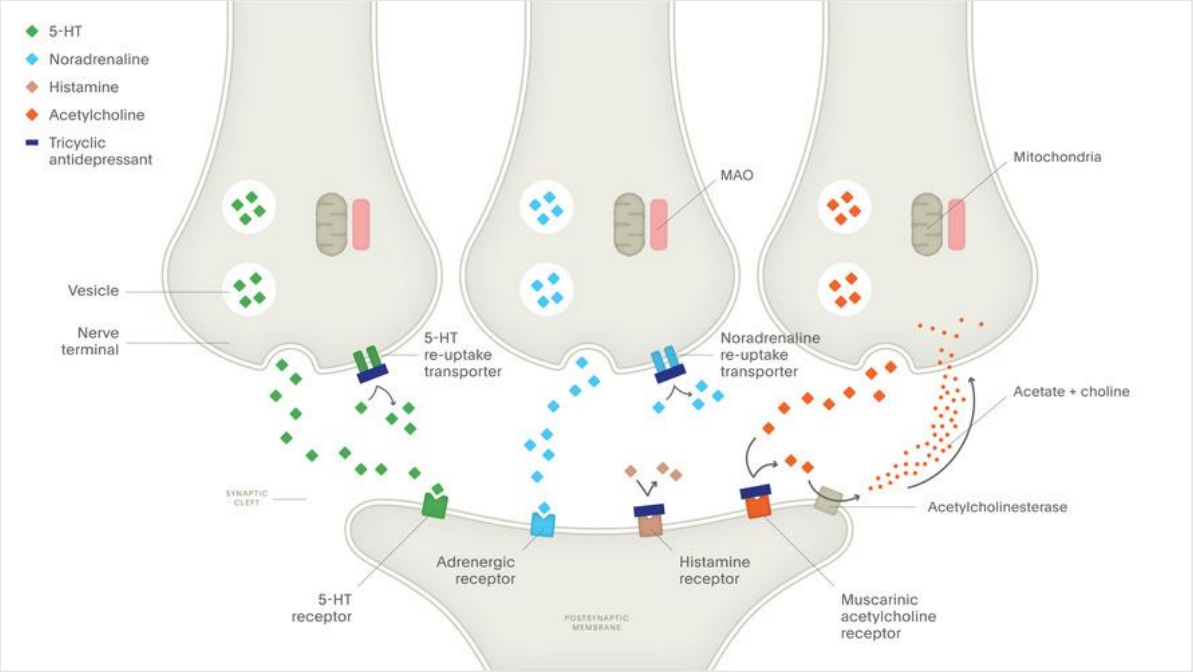

Figure 4. Tricyclic antidepressants mechanism of action. TCAs primarily block reuptake of 5-HT(Green squares - left) and NA (blue squares - centre) by binding to the respective transporter molecule on the presynaptic terminal membrane. TCAs also block histamine receptors and muscarinic acetylcholine receptors (Adapted from: Okafor and Aquino, 2019).

1.10.5.Selective 5-HT reuptake inhibitors (SSRIs)

TCAs and MAOIs have proven efficacy, even when compared to more modern antidepressants, and furthermore, may still be considered important for the treatment of treatment resistant depression (Chockalingam, Gott and Conway, 2019). However, they have relatively broad action and exert their effects via multiple neurotransmitter systems. Consequently, side-effects are not uncommon, and they may not be well tolerated. Tolerability and the range of side-effects were somewhat improved in patients with the emergence of drugs with higher selectivity during the 1980s. Fluoxetine (Prozac®) was one of the first of several antidepressants (SSRIs) that inhibit 5-HT

transporters with 20-1500 fold better selectivity than NA transporters, and with fewer 'off-target' effects, such as histamine H_1 or muscarinic acetylcholine receptor blockade which can occur following treatment with tricyclic antidepressants (Owens *et al.*, 1997; Andersen *et al.*, 2011).

In the UK, current treatments for depression include a range of psychological, psychosocial, and drug treatments, which can be used in combination or in isolation depending on the severity of symptoms (NICE, 2009). In cases of moderate to severe depression, or subthreshold depression where non-drug approaches have been tried unsuccessfully, SSRIs may be prescribed. The selection of SSRIs over other drug options is because of their relatively better safety profile, rather than efficacy (Peretti, Judge and Hindmarch, 2000; Clevenger *et al.*, 2018), however several other medicines with modes of action relating to biogenic amine pathways remain important options for the treatment of depression (**Table 3**).

Table 3. Medicines targeting biogenic amine pathways currently prescribed as antidepressants. (Adapted from: Kupfer, Frank and Phillips, 2012).

Drug Class	Named Examples	Mechanism of action
Selective 5-HT reuptake inhibitors (SSRIs)	Citalopram, Escitalopram, Fluoxetine, Fluvoxamine, Paroxetine, Sertraline	Selectively inhibit the reuptake of 5-HT
Tricyclic antidepressants	Amitriptyline, Desipramine, Doxepin, Imipramine, Maprotiline, Nortriptyline, Protriptyline, Trimipramine	Non-selectively inhibit the reuptake of monoamines, including 5-HT, DA, and NA.
NE/DA reuptake inhibitor	Bupropion	Inhibits the uptake of NA and DA
5-HT modulator	Nefazodone, Trazodone	Primarily antagonises 5-HT2 receptors
5-HT/NE reuptake inhibitors	Desvenlafaxine, Duloxetine, Venlafaxine	Inhibits the reuptake of 5-HT and NA
Noradrenergic and specific serotonergic modulator	Mirtazapine	Primarily antagonises alpha-2 and 5-HT2C receptors
5-HT reuptake inhibitor and 5-HT1A receptor partial agonist	Vilazodone	Potently and selectively inhibits 5-HT reuptake and acts as a partial agonist at 5-HT1A receptors
MAO inhibitors	Isocarboxazid, Phenylzine, Tranylcypromine, Selegiline	Non-selectively inhibit enzymes (MAO-A and MAO-B) involved in the breakdown of monoamines, including 5-HT, DA, and NA

1.10.6. Beyond 5-HT: the catecholamines

Despite a major focus on the importance of 5-HT in depression pathology and treatment, roles for NA and DA have also been acknowledged for the same amount of time (Schildkraut, 1965; Nutt *et al.*, 2007). In attempts to address the 28-55% of cases in which SSRIs did not achieve satisfactory therapeutic effects (Nutt *et al.*, 2007), models were explored that addressed different factorial components of mood, and neurotransmitter systems which appeared to be implicated in each (e.g., Figure 5).

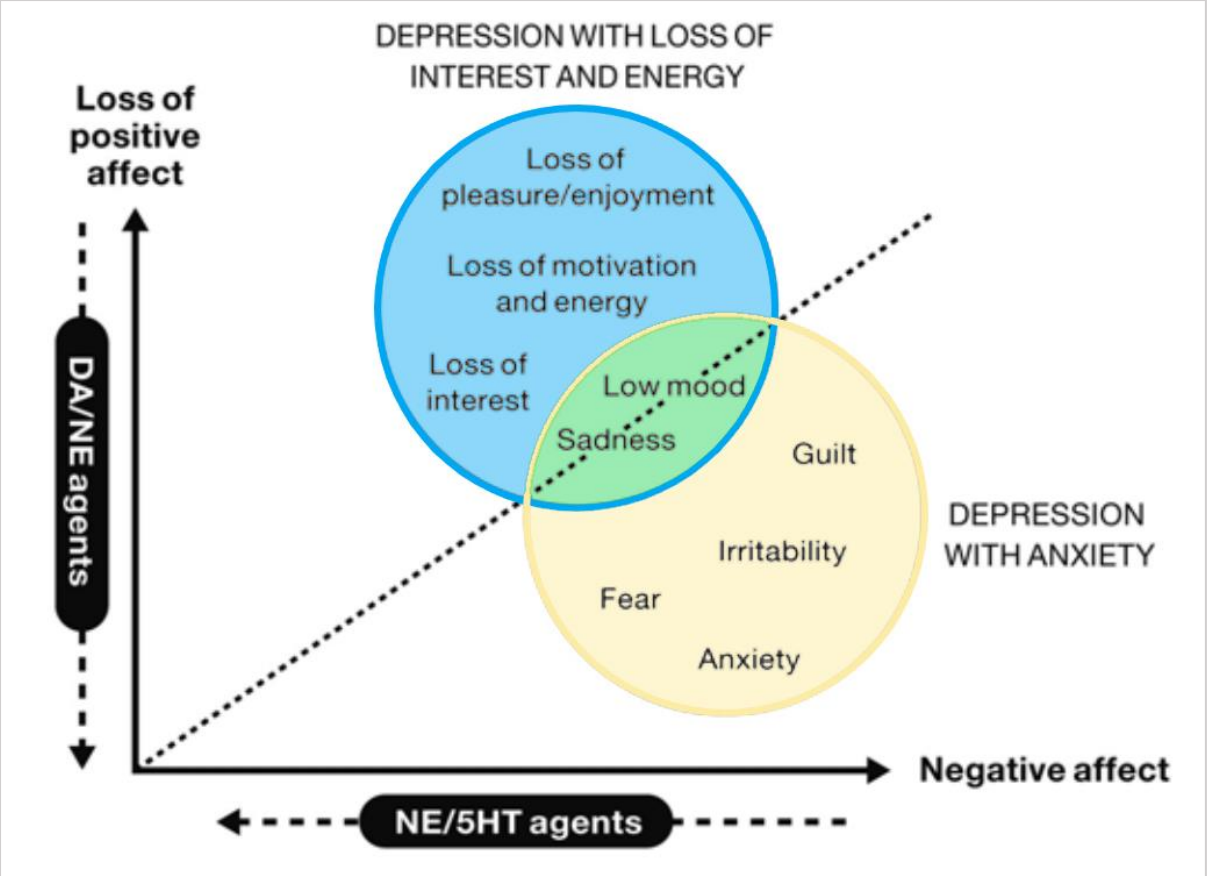

Figure 5. Different classes of antidepressant drugs are used to treat symptoms relating to positive and negative affect. Loss of pleasure, motivation or interest in enjoyable activities are associated with a loss of positive affect and could be treated with agents that act on DA and NE signalling. Negative affect-related symptoms, such as guilt, irritability, fear and anxiety, could be treated using drugs that act on NE and 5-HT signalling. Low mood and sadness are common to both sets of symptoms and could be reduced by using the appropriate drug for the accompanying symptoms (theoretical model adapted from: Nutt et al., 2007).

One example is the separation of mood symptoms into two distinct and non-correlated factors, termed positive and negative affect (Watson *et al.*, 1995; Shelton and Tomarken, 2001). Positive affect includes the more positive mood states such as self-confidence, enthusiasm, alertness, energy, interest, and joy. Conversely, negative affect contains

states which are often associated with anxiety and depression, such as fear, anxiety, loneliness, guilt, disgust, and irritability (Clark and Watson, 1991). The model illustrated in **Figure 5** suggests that drugs which target DA and NA signalling systems are more effective for the treatment of depression where a decrease in positive affect is specifically observed. Whereas depression with comorbid anxiety might be better treated by targeting 5-HT and NA systems (Nutt *et al.*, 2007). Such a model does seem to partly explain the differential efficacy of classes of antidepressants in different cases. The distinction could also serve as a guide to more appropriate selection of treatments in a clinical setting. Nevertheless, the use of SSRIs continues to be the first line antidepressant strategy (Chockalingam, Gott and Conway, 2019).

1.10.7.Monoamines may not tell the whole story.

That SSRIs are the first line drug choice prescribed for depression, and so many other currently prescribed antidepressants are considered to exert their effects through one or more components of monoamine signalling mechanisms, supports the implication of such pathways in a biological explanation for depression. However, despite the range of such antidepressants available, and the clear benefits experienced by many patients, none of these medicines offers a complete solution for the management of depression.

A common problem among different classes of antidepressants has been the delay in the onset of therapeutic effects. At least 14-28 days of continuous treatment are generally recommended for MAOIs, SSRIs, tricyclic antidepressants and selective NA reuptake inhibitors (SNRIs), and satisfactory results regularly take several months (Trivedi *et al.*, 2006). Such delays can extend patient suffering and are particularly problematic in cases where there may be a risk of suicide, as this may be increased by delayed relief of symptoms (Souery *et al.*, 2007). In addition to delayed onset, some depressed patients

failed to show any satisfactory response at all, even following several treatment steps and multiple different classes of drugs (Rush *et al.*, 2006; Trivedi *et al.*, 2006). Furthermore, following successful remission following initial treatment, some patients go on to relapse even when antidepressant treatment is continued (Kennard *et al.*, 2018). Cases where therapeutic response does not occur after two or more consecutive treatments with different classes of antidepressants, are classed as treatment-resistant depression (TRD) (Souery *et al.*, 2007; Mrazek *et al.*, 2014). In addition to prolonged suffering of patients with TRD and the increased risk of suicide, they are also among the most likely to depend on medical services and disability benefits, adding a substantial economic cost (Pearson *et al.*, 1999; Lecrubier, 2000).

Clearly, a full biological explanation for the pathology of depression must be more complex than a simple imbalance of monoamines. In agreement with this, ongoing efforts to address the limitations of common antidepressants and treat TRD have alluded to several additional mechanisms and putative targets on which to base a range of promising and novel therapeutics. These efforts continue to improve our understanding of depression pathology.

1.10.8.A possible role for glutamate signalling in depression.

Glutamate is a primary excitatory neurotransmitter and neuromodulator, and its importance to neuronal function is widely known. It is therefore unsurprising that glutamate signalling has also been a focus for research concerning several neuropsychiatric disorders, including depression. A precise role for glutamate signalling in depression is yet to be determined, however a clearer understanding of the interactions between drugs that alter glutamatergic signalling, and the symptoms of

depression may help to understand its underlying pathophysiology (Sanacora, Treccani and Popoli, 2012).

1.10.9.Ketamine as a novel antidepressant

Following the discovery, adoption and common use of classical, monoamine-based antidepressants, decades passed without any truly novel drug classes being successfully identified for the treatment of depression (Papakostas and Ionescu, 2015). However, at the turn of this century it was demonstrated that intravenous treatment with 0.5 mg/kg ketamine hydrochloride significantly improved symptoms in depressed patients within 72 hours (Berman *et al.*, 2000). Ketamine has been used as an anaesthetic since the 1960s and is an N-methyl-D-aspartate (NMDA) receptor antagonist (Sinner and Graf, 2008). The success of ketamine as an antidepressant supports an alternative hypothesis which focuses on glutamate signalling as a key component in the pathology of depression (Sanacora, Treccani and Popoli, 2012).

Earlier research showed that NMDA receptor activation in the mouse CA1 hippocampal region was responsible for behavioural depression in response to inescapable stress (Trullas and Skolnick, 1990). This inspired the investigation into the effects of several NMDA receptor antagonists, which were found to reduce the stress-induced behavioural deficits in a similar way to clinically effective monoaminergic antidepressants, in the same models (Trullas and Skolnick, 1990).

The finding that ketamine could be used as an effective and fast acting alternative to treat depression (Berman *et al.*, 2000) has been described as “arguably the most important discovery in half a century” (Duman and Aghajanian, 2012). As a result, many preclinical and clinical studies have followed in the investigation of ketamine as an antidepressant,

and the evidence base for a role of glutamate as a mechanism in depression has grown (Fond *et al.*, 2014; Newport *et al.*, 2015). Furthermore, the use of advanced imaging techniques has enabled neurochemical and functional evidence to be collected from depressed human patients *in vivo*, which correlates with behavioural, symptomatic, and therapeutic effects observed (Lener *et al.*, 2017).

1.10.10. Suggested mechanisms for the antidepressant effects of NMDA receptor antagonists.

While the exact mechanism by which glutamatergic drugs like ketamine alleviate depressive symptoms remains unknown, two main processes are believed to be important (**Figure 6**).

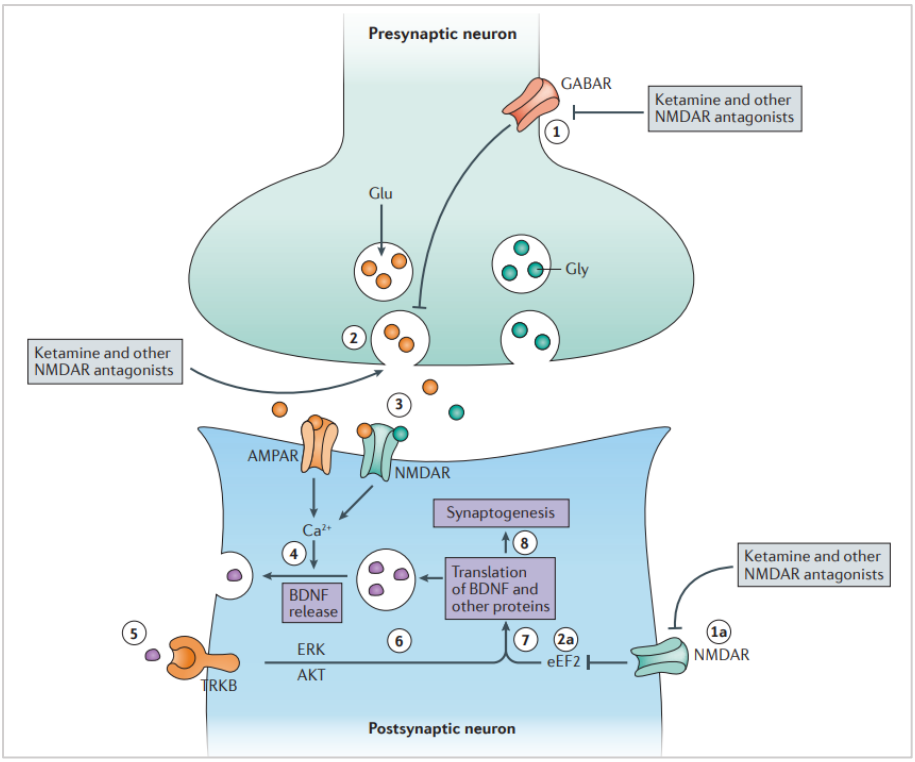

Figure 6. NMDA antagonists are thought to exert their antidepressant effects by restoring glutamate signalling homeostasis and facilitating synaptic plasticity. In animal models, low-dose ketamine promotes beneficial glutamate (Glu) signalling while inhibiting harmful Glu effects, improving synaptic plasticity and reestablishing glutamatergic homeostasis. Ketamine operates by enhancing levels of synaptic proteins and activating key cellular pathways. It also counters the reduction in brain-derived neurotrophic factor (BDNF) caused by chronic stress. Unlike other NMDAR antagonists, ketamine has a wider range of effects, which may explain its better efficacy. However, simply blocking NMDARs does not replicate the therapeutic effects of ketamine; a specific modulation of NMDAR pathways is required. (adapted from: (Murrough, Abdallah and Mathew, 2017).

1.10.11. Restoration of normal glutamate signalling and synaptogenesis

Glutamate is the neurotransmitter responsible for the majority of excitatory signalling in the vertebrate nervous system (Okubo *et al.*, 2010) and as such, plays an important role in several processes including learning, memory, and synaptic plasticity (Riedel, Platt and

Micheau, 2003). Glutamate is also a metabolic precursor to the major inhibitory neurotransmitter, gamma-aminobutyric acid (GABA), and these two neurotransmitter systems work antagonistically in normal brain function, and in the pathophysiology of depression (Petroff, 2002; Lener *et al.*, 2017).

During normal function, glutamate is released from vesicles at the presynaptic terminal and binds to NMDA, α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA), kainite, or metabotropic glutamate receptors (mGluRs) (**Figure 6**). After being removed from the synapse, glutamate is either returned to the presynaptic neuron via astrocytic metabolism or converted into GABA by glutamic acid decarboxylase (GAD) within GABAergic interneurons.

Changes in glutamatergic and GABAergic signalling caused by ketamine occur by the antagonism of NMDA receptors at both the post-synaptic membrane, and on GABAergic interneurons. Glutamatergic neurons in the cerebral cortex are disinhibited as a result of ketamine's effects on the GABAergic interneurons and signalling via the postsynaptic receptors leads to an increase in the synthesis of brain-derived neurotrophic factor (BDNF), thus promoting synaptogenesis. It is also possible that activation of the kinase mammalian target of rapamycin (mTOR) promotes synaptic plasticity as part of ketamine's therapeutic effect (Zunszain *et al.*, 2013).

Investigation into the role of ketamine and glutamatergic signalling in depression continues to be an active area of research.

1.10.12. The cholinergic hypothesis

Around 30 years before glutamate was recognised as a neurotransmitter (Fonnum, 1984), researchers had implicated abnormally high levels of ACh signalling in the

presentation of depressed mood in clinical settings (Rowntree, Nevin and Wilson, 1950; Gershon and Shaw, 1961; Bowers, Goodman, and Sim, 1964). Over the following decades, a role for ACh in the regulation of mood was consistently acknowledged, however theoretical explanations evolved to incorporate NA, and then other catecholamines as the importance of these other neurotransmitters became more evident (Janowsky *et al.*, 1972; Davis and Janowsky, 1974; van Enkhuizen *et al.*, 2015).

More recently, interest in the cholinergic system as a target for the treatment of depression has been renewed by promising results shown with the antimuscarinic drug scopolamine (Janowsky, 2011). Furthermore, because of its direct relevance to organophosphates, the evidence for the cholinergic system's role in mood disorders will now be discussed in more detail.

1.10.13. The cholinergic system

ACh was first recognised for its actions on cardiac muscle in the early 20th century (Dale, 1914). It was identified as a neurotransmitter a few years later, and was the first to be discovered (Loewi, 1921). Since then, it has been well characterised in terms of its synthesis, its function in both the central and peripheral nervous systems, and its role in processes such as memory, neurogenesis, and neurodegenerative diseases (Maurer and Williams, 2017).

1.10.13.1. Synthesis and metabolism of acetylcholine

ACh is synthesised from choline and acetyl coenzyme A (acetyl CoA) via choline acetyltransferase (ChAT) inside nerve terminal cytoplasm (**Figure 7**). Presynaptic ACh is packed into vesicles for release into the synaptic cleft in order to transmit a signal. Unlike with monoamine neurotransmitters, post-signal clearance of ACh is not carried out by

direct reuptake of the neurotransmitter by molecular transporter proteins. Instead, ACh is separated by hydrolysis into choline and acetate, by AChE, and to a lesser extent: butyrylcholinesterase (BChE) (Augustinsson, 1948; Brimijoin *et al.*, 2018). AChE is highly efficient in the clearance of ACh, with one single molecule of AChE being able to hydrolyse 5,000 molecules of ACh per second (Lawler, 1961). As ACh is broken down, the resulting choline is taken back up into the nerve terminal by the high-affinity choline transporter (ChT) (Choudhary *et al.*, 2017) to be resynthesized into ACh (**Figure 7**). The uptake and availability of choline in the presynaptic terminal is the rate limiting factor for ACh synthesis.

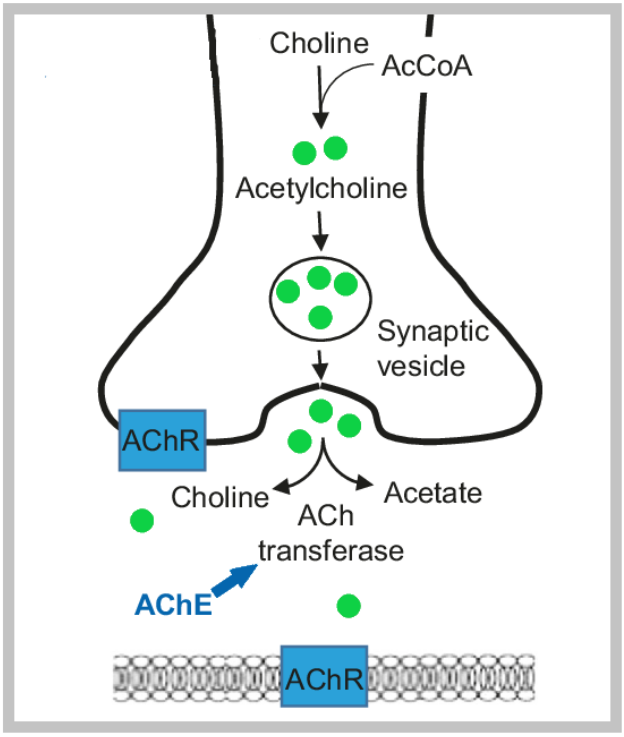

Figure 7. Synthesis and metabolism of acetylcholine. ACh is synthesised from acetyl coenzyme A (AcCoA) and choline, in the presynaptic terminal. It is packaged in and released from synaptic vesicles, then released from the presynaptic membrane. Once released, ACh interacts with acetylcholine receptors (AChR) to produce a signal, which is eventually terminated by AChE. Most of the resulting choline is taken back up into the presynaptic terminal to be resynthesised into ACh (adapted from: Dulawa and Janowsky, 2019)

1.10.14. AChE signalling

Following its release, ACh acts on receptors at sites localised both at and remotely distributed from, synaptic junctions (Sarter, Parikh, and Howe, 2009). There are two main types of cholinergic receptors. Firstly, the nicotinic acetylcholine receptors (nAChRs) are fast acting pentameric ion channels, made up from homomeric or heteromeric combinations of subtypes (**Figure 8**). nAChRs belong to the same ligand-

gated ion channel group as $GABA_A$ receptors and are located at the neuromuscular junction, and also at terminals throughout the nervous system. Therefore, they can be divided into two groups: muscle nAChRs, and neuronal nAChRs (Mulle *et al.*, 1991).

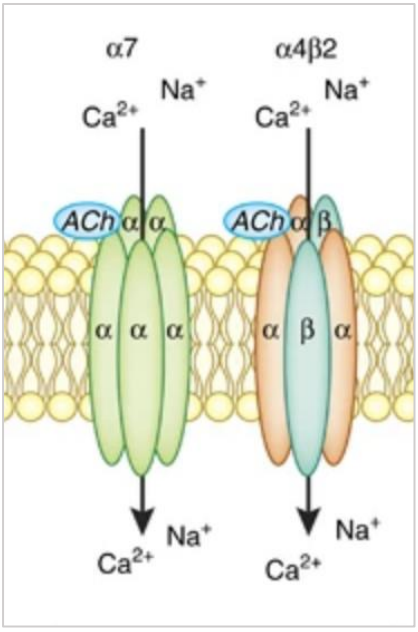

Figure 8. Nicotinic acetylcholine receptors are ligand gated ion channels. The illustration shows examples of two nAChR sub-types; α7-nAChRs (left) are homomeric receptors made up exclusively from α-subunits, and α4β2-nAChRs (right) are heteromeric receptors containing both α and β subunits, are widely expressed in the mammalian brain. nAChRs are activated by the ACh ligand, which allows positively charged ions to pass through and alter the membrane potential (adapted from: Jones, Byun, and Bubser, 2012).

The second type of cholinergic receptors are the muscarinic acetylcholine receptors (mAChRs) (**Figure 9**). These are metabotropic receptors made up from five distinct but highly conserved subtypes, M1-M5. They are Family-A G-protein-coupled receptors (GPCRs) and therefore act more slowly than the nAChR ion channels. However, despite

their slower action, the activation of GPCRs can provide more sustained and even amplified signals, via their effects through second messenger pathways (Warren, Sillivan, and Konradi, 2016). Furthermore, mAChRs show a much higher, nanomolar affinity for the ACh ligand, compared with the micromolar affinity range exhibited by nAChRs (Strang *et al.*, 2015). These characteristics enable mAChRs to respond to concentrations of ACh at an order of magnitude lower than nAChRs, and to respond to signals which may be initiated from distant release sites (Sarter, Parikh, and Howe, 2009; Picciotto, Higley and Mineur, 2012).

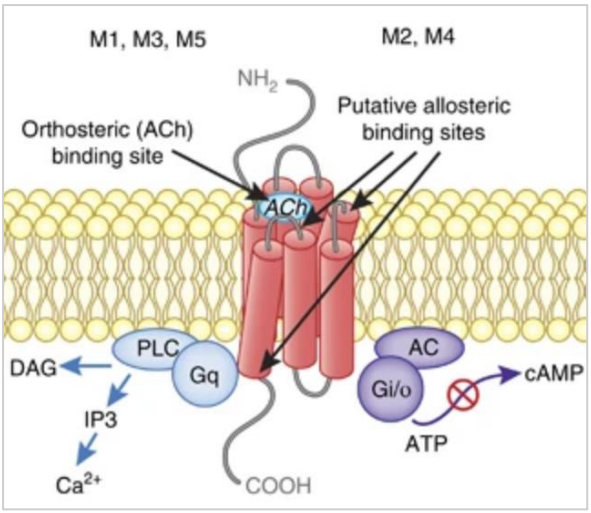

Figure *9.* Muscarinic acetylcholine receptors are G protein-coupled receptors. Muscarinic acetylcholine receptors (mAChRs) are metabotropic receptors comprising several transmembrane subunits. Unlike the nAChR-ion channels (**Figure 8**) mAChRs create signalling cascades by activating G proteins and second messengers within the cell membrane. For example, M1, M3, and M5 subtypes are generally excitatory and signal through the Gq pathway; the Gq protein subunit activates phospholipase C (PLC), which in turn hydrolyses second messengers inositol 1,4,5-trisphosphate (IP3), and diacylglycerol (DAG), increasing intracellular Ca^{2+}, and activating protein Kinase C, respectively. The inhibitory M2 and M4 subtypes signal through the Gi/o pathway, inhibiting adenylyl cyclase (AC), resulting in decreased Cyclic adenosine 3′,5′-monophosphate (cAMP) synthesis from adenosine triphosphate (ATP). (adapted from: Jones, Byun, and Bubser, 2012).

1.10.15. Cholinergic regulation of mood

Early clinical findings that depressed mood appeared to accompany elevations of ACh in the CNS were observed following treatment with, or exposure to, compounds known to inhibit AChE (Rowntree, Nevin and Wilson, 1950; Bowers, Goodman, and Sim, 1964), which included some of the first clinical reports of psychoses and depressive symptoms in agricultural workers exposed to OP pesticides (Gershon and Shaw, 1961). The

evidence for the effects of organophosphates will be discussed in further detail later in this chapter, and throughout this thesis.

In addition to the valuable lessons learned from clinical outcomes and human studies, preclinical models and animal studies offer several advantages, which will now be discussed.

1.11. Evidence from animal models

1.11.1.Strength of animal models in pesticide neurotoxicology

Research regarding the effects of low-level OP exposure is somewhat hindered by lack of control over confounding variables. Furthermore, in pesticide exposure studies reliable exposure data is limited by a lack of exposure measurement tools. These issues can be addressed by design, in the use of experiments with preclinical models. Taking an experimental approach using animal models allows many of the confounds that are associated with epidemiological studies to be removed; exposure/treatment can be deliberately selected and tightly controlled, in terms of compound, dose, timing and exposure route. The study sample and analysis can also control for factors such as age, sex, diet, prior exposure history, and even genotype. This level of experimental control allows researchers to identify and focus on dependent variables of interest with greater confidence. Another important difference between human and animal studies is the shift in priority for medical treatment. Although unnecessary suffering should always be avoided where possible (Festing and Wilkinson, 2007), the treatment and reduction of resulting symptoms is not necessarily the priority in animal experiments, unlike in human case studies for example. From a research perspective, this gives more freedom to investigate negative outcomes, within ethical and regulatory guidelines (Kilkenny *et al.*, 2010), and offers deeper insight into the consequences of exposure.

1.11.2.Acute OP toxicity in animal models

As is the case with existing human data, the vast majority of preclinical studies using OPs have addressed the effects of acute exposure (Moser, 2007, 2011; Pereira *et al.*, 2014). As a result, there are plentiful data to support the reproducibility of toxic effects in animals and humans exposed to OPs (Pereira *et al.*, 2014). This is particularly true for high-dose

exposures which cause cholinergic crisis, following which very similar acute toxicity symptoms are seen across several mammalian species, including rats, mice, guinea pigs, humans, and non-human primates (Deshpande *et al.*, 1986; Albuquerque *et al.*, 2006; Maxwell *et al.*, 2006; Despain *et al.*, 2007). Another common factor between animal models is relative potency, as the LC50 values, which represents the concentration that will kill 50 percent of exposed test subjects, are well correlated between species for different OP compounds tested (Cole, Anderson, and Williams, 2004; Maxwell *et al.*, 2006). Furthermore, clinical data obtained from OP poisoned patients shows that severity of symptoms correlates with AChE activity levels measured in the blood (Rehiman, Lohani and Bhattarai, 2008). Likewise, data from several animal models has confirmed similar correlations for AChE in brain tissue from several regions (Sivam, Hoskins, and Ho, 1984; Fawcett *et al.*, 2009; Chen, 2012; Kazi and Oommen, 2012).

The similar toxic responses make a clear case for the value of animal models to regulatory safety testing and our understanding of acute OP toxicity. Developing our understanding of low-level toxicity poses a much greater challenge, however. Some examples of how animal models have been used to meet that challenge will now be discussed.

1.11.3.Low-level OP toxicity in animal models

One of the factors which complicates our understanding of the effects of low-level toxic exposure, is the lack of a clear definition for the term low-level. Contrary to the relatively simple concept of acute poisoning, several different terms are used to describe exposures and symptoms which fall below that threshold. Whereas acute describes a sudden or rapid onset of symptoms appearing after a single, or multiple exposures, the term subacute describes symptoms that may follow a short delay after a similar pattern of exposure (De Bleecker *et al.*, 1994). Sub chronic toxicity occurs following repeated

exposures which occur over several weeks or months and is perhaps most appropriate to the patterns of occupational exposure experienced by agricultural workers. Chronic toxicity results from months or years of cumulative damage to particular organs, often without being detected or recognised as a toxic injury (Steenland, 1996). Low-level is a term sometimes used to describe any of these types of exposure which do not cause acute symptoms. However, this definition might not be the most useful for experimental work exploring the unknown effects of low-level exposure. Therefore, because acute OP toxicity is known to occur as a direct result of AChE inhibition, identification of the threshold at which inhibition occurs can provide a measure with which to explore low-level effects for each compound (Ray and Richards, 2001). In addition to providing a quantitative measure for low-level exposure experiments, this approach might also uncover novel, non-cholinesterase dependent effects of OP toxicity.

1.11.4.Neurodevelopmental toxicity

One of the ways in which toxins are known to exert harmful effects at sub-acute exposure levels is through developmental toxicity. Nervous systems are particularly vulnerable to toxic exposure during developmental stages, including in utero and postnatal stages (de Graaf-Peters and Hadders-Algra, 2006). Further to the risk added during the complexities of the developmental processes, lipophilic OP compounds such as CPF can be taken up via the placenta into the developing fetus (Bradman Asa *et al.*, 2003), or into neonates via breast milk (Brahmand *et al.*, 2019). Such exposures in humans have been implicated in several adverse health outcomes, including behavioural, learning, motor, and cognitive defects (Bouchard *et al.*, 2011; Rauh *et al.*, 2011, 2015). These suggested effects on children have caused some controversy relating to the continued use of OPs like CPF and have driven efforts to reduce their use (Reynolds, 2016; Hertz-Picciotto *et al.*, 2018).

Despite the epidemiological data, the controversy remains and so data from animal models is important for understanding neurodevelopmental toxicity. In addition to the experimental benefits discussed already in this chapter, similarities between human and rodents, particularly with respect to gestational and neurodevelopmental processes have proven useful (Silva, 2020). Conversely, disparate models such as zebrafish and nematodes have proven useful due to their relatively short generation times, transparent body profiles, and well-defined developmental trajectories (White *et al.*, 1986; Silva, 2020).

The developmental neurotoxicology of OPs is an important and active area of research with the potential to elucidate many of the effects of low-level exposure. However, although it is not impossible for UK agricultural workers to have suffered developmental effects from OP exposure, it is unlikely, and even less likely to have occurred through an adult worker's own occupational exposure. Therefore, research concerning developmental effects of OP exposure will not be a focus in this thesis.

1.11.5.Neurobehavioural toxicity

Another measure which can be sensitive to low-level toxic exposures is behavioural change. Behaviour is often complex, and the changes induced by low-level exposure are usually subtle, thus complicating their identification from epidemiological evidence. This is compounded by difficulties in measuring or isolating exposures to specific toxins. Different animal models have been used to address these issues, resulting in a continually growing range of behavioural paradigms across the range of model species (Anderson, Cole, and Williams, 2004a; Savy *et al.*, 2015; Silva, 2020). Some evidence from animal studies relating to low-level OP exposure, specifically with relevance to anxiety and depression will now be discussed.

1.11.6. Organophosphates and mood in animal models.

Several different animal models have been used to study anxiety and depression, including a range of vertebrate and invertebrate species, each adding different advantages and limitations (Kaletta and Hengartner, 2006a; Narayanan and Rothenfluh, 2016; Gururajan *et al.*, 2019). Rodents have been used extensively to model depression and anxiety, and a range of established behavioural paradigms exist, which have also been used in the context of OP exposure (López-Crespo *et al.*, 2007; Savy *et al.*, 2015; Silva, 2020). Rodent models of anxiety and depression are often based on behavioural responses that are observed in response to salient stimuli within their environment. These may be rewarding stimuli like sucrose, in the sucrose preference test of anhedonia (Savy *et al.*, 2015; Lee *et al.*, 2016; Phillips and Deshpande, 2016), or they may be aversive, such as a raised open platform in the elevated plus maze model of anxiety (**Figure 10**) (Pellow *et al.*, 1985; Silva *et al.*, 2017). Such models can be tested against drugs with known therapeutic, or anxiogenic profiles, which can provide a reference point for testing the effects of organophosphates (Pellow *et al.*, 1985).

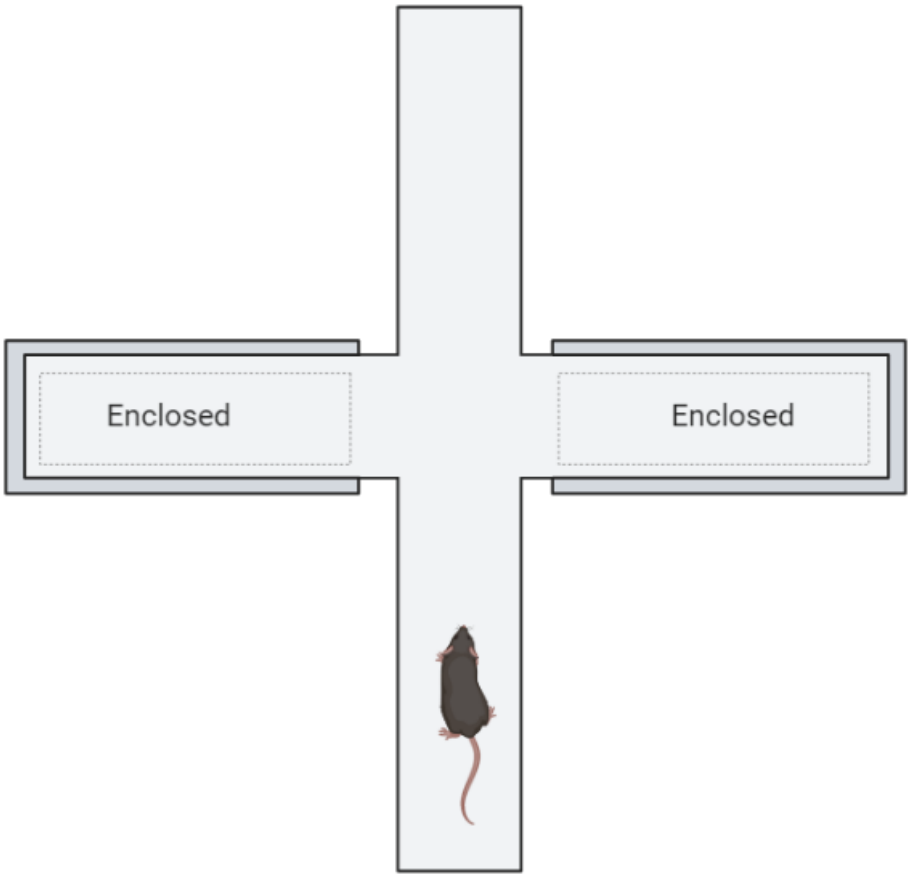

Figure 10. The elevated plus maze is used in rodent models of anxiety. Rats or mice are placed at the intersection of the maze's four arms and their entries/duration in open or enclosed arms are monitored. Anxiety behaviour is characterised by a reduction in open arm activity (Walf and Frye, 2007).

Some studies have reported changes in depression-like, and anxiety-like behavioural responses using these paradigms following low-level exposure to organophosphates, including chlorpyrifos (Savy *et al.*, 2015; Lee *et al.*, 2016; Phillips and Deshpande, 2016). However, low-level effects are often characterized by the absence of acute toxicity symptoms rather than using any biological measure of toxicity. Studies using these criteria have regularly reported increases of depression- and anxiety-like behaviour following treatment with doses of around 250 mg/kg for IV chlorpyrifos, although such

dosages can inhibit brain AChE activity by more than half (De Bleecker *et al.*, 1994; Lee *et al.*, 2016; Phillips and Deshpande, 2016).

In contrast with this, Savy et al. (2015) exposed adult rats to a much lower dose of 1 mg/kg (IP) chlorpyrifos, and the same dose of Diazinon over 5 days. In this case the authors did measure AChE but did not detect any significant inhibition in the blood or any of several brain regions tested. Rats did not show any anxiety in an open field test, or anhedonia in a sucrose preference, but noted an effect of reduced anxiety-like behaviour for both CPF and Diazinon in a marble burying test (Savy *et al.*, 2015). In addition to those anxiolytic effects, CPF caused a decrease in DA levels in the prefrontal cortex and caudate putamen, whereas Diazinon reduced the expression of 5-HT transporters. The results described by Savy *et al.*, (2015) showed detectable behavioural change at much lower exposure levels than those described elsewhere, and showed furthermore that different OP compounds can exert distinct neurochemical effects in the absence of AChE inhibition.

Distinctions between non-cholinergic effects and AChE mediated effects are not always easy to make. This is a common issue in neurodevelopmental processes but also in the adult brain (Aldridge *et al.*, 2005; Slotkin *et al.*, 2006). Even in studies that have measured AChE activity, levels of inhibition as high as 10% have been described as low-level and resulting physiological or behavioural changes have been described as 'non-cholinesterase' mediated effects (Slotkin, Levin and Seidler, 2009; Slotkin and Seidler, 2010). Although, 10% AChE inhibition might not be sufficient to cause overt cholinergic toxicity, there appears to be no consensus on exactly how much inhibition is required to cause subtle signalling or neuromodulatory effects (Picciotto, Higley and Mineur, 2012; Stern, Kirst and Bargmann, 2017).

Judge *et al.*, (2016) neatly demonstrated how small amounts of AChE inhibition caused by relatively low-level OP treatment can indirectly alter glutamatergic and serotonergic signalling in the dorsal raphe nucleus. It is possible that the importance of marginal AChE inhibition in the effects of low-level OP exposure have been underestimated elsewhere.

1.12. *Caenorhabditis elegans* as a model organism

1.12.1. General advantages of using C. elegans.

The soil-dwelling nematode *Caenorhabditis elegans* was originally introduced as a model organism by Sydney Brenner (Brenner, 1974), as a tool for the investigation of development and neurobiology in multicellular organisms. *C. elegans* was the first of such animals to have its whole genome sequenced (Coulson, 1996) and has since been used for a vast range of study purposes, such as the investigation of fundamental biological processes, including ageing, metabolism, apoptosis, gene regulation and cell signalling (Hedgecock, Sulston, and Thomson, 1983; Sternberg and Han, 1998; Kaletta and Hengartner, 2006). The conservation of such processes across different phyla, including humans, has earned *C. elegans* an important place in modern research, thus it remains a valuable tool for the study of diverse topics. Some recent examples include circadian rhythm (Olmedo, Merrow and Geibel, 2017), drug addiction (Engleman, Katner and Neal-Beliveau, 2016), autistic spectrum disorders (Schmeisser and Parker, 2017) and toxicology (Harlow, Perry, Widdison, Daniels, Bondo, Benjamini, *et al.*, 2016). During these and other studies, many of the genes and pathways that have been implicated in human diseases have also been identified in *C. elegans* (Kaletta and Hengartner, 2006).

Several studies have assessed the value of the *C. elegans* model for predicting mammalian toxicity and have shown that it can be used effectively for screening toxicants for both adult and developmental neurotoxicity (Anderson, Cole, and Williams, 2004; Boyd *et al.*, 2010; Harlow *et al.*, 2016). This is partly because many aspects of the sub-cellular and molecular machinery that are implicated in mammalian neurotoxicity are conserved in *C. elegans*, such as the pathways responsible for the synthesis, transport, and release pathways for most neurotransmitters (Bargmann, 1998; Boyd *et al.*, 2010a). Boyd *et al.*,

(2016) tested substances from the ToxCast™ libraries used by the US Environmental Protection Agency (EPA) to prioritise chemical safety testing (Dix *et al.*, 2007), for developmental toxicity in *C. elegans*. In *C. elegans*, 62% of the compounds were categorised as active at low concentrations. Comparing *C. elegans'* chemical activities and potencies to developmental toxicity for rats and rabbit, ranged from 45% to 53%, which was only marginally lower than the between rats and rabbits (58%). This highlights the fact that even mammalian models are not perfect representations of human biology, or indeed each other, however it is important to understand the limitations inherent to the model (described in 1.12.7).

There are a range of practical and economic benefits to the *C. elegans* model, which make it both an attractive and versatile option for the investigation of biological processes. Firstly, it can be reared with relatively little maintenance and cost, because the individuals are small (~ 1.5 mm when fully grown) and survive on a diet of *Escherichia coli*. Their size and low rearing cost also allows the cultivation and inclusion of relatively large numbers of worms, therefore increasing the group sizes and replicates possible, and increasing statistical power. Moreover, worms grow from egg to egg-laying adult in just three days (Kaletta and Hengartner, 2006), after which each individual can produce around 300 offspring, meaning that large scale experiments are possible with relatively short preparation time. Despite its small size however, each hermaphrodite worm has a set of complex functional body parts, including a nervous system comprised of 302 neurons, reproductive and digestive systems, muscle, and hypodermis, all totalling a mere 959 individually characterised cells (Kaletta and Hengartner, 2006). Characterisation is further enabled by the fact that the worm is transparent, allowing microscopic inspection of many internal processes. This can be greatly enhanced by the

use green fluorescent protein (GFP; (Chalfie *et al.*, 1994)) and other fluorescent markers *in vivo* (Fig 3). Importantly, although *C. elegans* reproduce sexually, the proportion of males in a normally maintained laboratory population is only around 0.1-0.2% (Brenner, 1974). The remaining ~ 99.9% is made up of hermaphrodites which, aside from rare encounters with an elusive male, are self-fertile. There is also very little variation between individuals.

Together, the similarity between individuals and the diligence with which the model has already been studied, have resulted in a valuable set of resources for researchers using *C. elegans.* These include a complete cell lineage map and detailed maps of circuit 'wiring' and synaptic connectivity, including a central database of fluorescent and electron micrograph images (Hall and Altun, 2008). There is also a central repository which describes, in detail, all of the major aspects of *C. elegans* biology, including protocols for general maintenance of the organism, microscopy, genetic manipulation, behavioural experimentation and electrophysiology (www.wormbook.org; www.genetics.org).

1.12.2. Genetic tools in C. elegans

A major strength of the model is the ease with which its well-characterised genome can be used to explore biological problems. Extensive libraries exist, from which a vast range of mutant strains can be obtained easily and at negligible cost (cgc.umn.edu). These include not only knockout mutations, as a result of which the gene function becomes completely inactive, but also partial or conditional mutations, providing a more versatile toolset for dissecting gene function (Hodgkin, 2005). The available range of mutations is constantly growing, largely due to the ease with which new strains can be created (Praitis and Maduro, 2011), and also that once created they can be stored indefinitely at -80°C for

later use. Using a 'reverse' genetics approach, these acquired, or newly created mutants can then be used to explore the respective mutation in the context of the organism's behaviour or development, thus providing information regarding the affected gene's function, or location within a pathway (Ahringer, 2006). The alternative method uses a 'forward' genetics approach, where mutants with a specific phenotype are sought by mutagenizing worms (Hobert, 2013) in order to create non-specific lesions in the DNA. At this stage, the location of the mutation is unknown but any worms that display the desired phenotype can be investigated further, using whole genome sequencing. The latter, non-biased approach may not directly uncover known, or partially mapped pathways, however it is essential for finding previously unknown targets involved in a process of interest. Furthermore, if two genes are found to function within the same pathway then it provides valuable reference points for epistasis analysis (Wang and Sherwood, 2011). The reverse approach on the other hand, is well suited to dissecting the function of known genes within partially mapped pathways, and it can often be complimented by environmental or chemical interventions.

1.12.3.Pharmacology in C. elegans

C. elegans has been a useful model for helping to understand the mechanism of action of some drugs, and drugs are also useful for exploring the biology of *C. elegans* (Giunti *et al.*, 2021). However, their use does come with some limitations. As a preclinical model, the complete pharmacodynamic, or pharmacokinetic response to a drug in humans, would not sensibly be predicted by late-stage drug screening with *C. elegans*. This is especially true given that even mammalian models often fail to predict the same (Kaletta and Hengartner, 2006). From this perspective, *C. elegans* is far more useful at the early stage of drug research, where the low-cost and high-throughput capabilities of the model could

help to identify novel drug targets. In addition, if a specific molecular pathway is implicated in a disease, then that pathway can be rigorously investigated using a *C. elegans* model. The advantage of this approach over the use of cell culture is that a fully formed behavioural response can be obtained, rather than a simple drug-receptor interaction. This also provides useful toxicological information for use in future work (Kaletta and Hengartner, 2006). An example of this is the genetic analysis of the antidepressant drug fluoxetine, or Prozac, and its mechanisms (Schafer, 1999).

1.12.4. The C. elegans nervous system

The relatively simple nervous system in *C. elegans* is particularly attractive and useful in the study of neurotoxicology (Mcvey *et al.*, 2012). The system reflects that of higher organisms in that neurons make numerous synapses consisting of a mixture of electrical gap junctions, chemical junctions, and neuromuscular junctions (Chuang *et al.*, 2007), which have been extensively mapped (Hall and Altun, 2008). Each of the worm's 6393 chemical synapses utilises a range of neurotransmitters which are largely conserved with humans. Some notable exceptions are the extensive use of the invertebrate neurotransmitter octopamine, and the absence of its vertebrate analogue adrenalin, in *C. elegans* (Noble, Stieglitz and Srinivasan, 2013). Other neurotransmitters are well conserved including ACh, glutamate, γ-aminobutyric acid (GABA), DA and 5-HT (Bargmann, 2012). The presence of these systems offers many opportunities to investigate their function and to build useful models around them, which are relevant to human disease, and the effects of drugs and toxins (Kaletta and Hengartner, 2006). The behavioural repertoire of *C. elegans* has also been well characterised, in particular with respect to the genetic, signalling, and pharmacological profiles of each behaviour (Rankin,

2002). Together these have helped to establish a coherent background for each neurotransmitter system, which can be used to develop useful models.

1.12.5. C. elegans neurotransmitters relevant to the study of OPs and common mental disorders

Acetylcholine - ACh is a major excitatory neurotransmitter in *C. elegans.* Associated transmission occurs at neuromuscular junctions and modulates contraction of the muscle wall. Additionally, ACh has many modulatory roles and is implicated to some extent in many behaviours, including sexual, feeding, egg laying and defecation. Furthermore, the most evident behavioural role for ACh is in locomotion, including both swimming in liquid and crawling on solid surfaces (Rand, 2007). ACh is a critical factor in the toxicological profile of OP compounds, and its role in the regulation of movement is important for investigating the effects of AChE inhibition (Rand, 2007).

Glutamate - Both excitatory and inhibitory roles can be played by glutamate, depending on the circuit or receptors involved. However, most fast-excitatory transmission involves ionotropic glutamate receptors (iGluRs) (Brockie and Maricq, 2006). Glutamate is directly responsible for spontaneous transitions between forward and backward movement, foraging and long-term memory in *C. elegans* (Brockie and Maricq, 2006). However, it has also been shown to interact with a number of DA-dependent processes and behaviours (Hills, 2004; Hardaway *et al.*, 2015). Both DA and glutamate transmission have been shown to be dysregulated by the OP Chlorpyrifos (Torres-Altoro *et al.*, 2011), and they have been jointly implicated in the pathophysiology of mood disorders (Tomasetti *et al.*, 2017). Furthermore, the glutamatergic system has recently become a

popular focus for the treatment of depression, following numerous demonstrations that glutamatergic drugs, particularly ketamine, offer faster and more effective relief from depression in comparison to current treatments (McGirr *et al.*, 2015).

γ-aminobutyric acid - GABA also plays excitatory and inhibitory roles, and functions mainly at the neuromuscular junction. This is in contrast to its role in humans, where it is mostly found in the CNS. In the worm, GABA is important for defecation behaviour and the coordination of normal movement, via contraction and relaxation of muscles respectively (Jorgensen, 2005). GABA has been heavily implicated in psychiatric disorders but its role in OP, particularly CPF, exposure is less clear (Gonzalez-Burgos, Hashimoto, and Lewis, 2010; López-Granero *et al.*, 2016).

Serotonin - 5-HT is perhaps one of the most well characterised neurotransmitters in *C. elegans*. It is also an important feature in the pathophysiology of mood disorders, evidenced by the fact that serotonin reuptake inhibitors (SSRIs) are currently the most commonly prescribed treatment for depression (MIND, 2016). To the worm, the application of exogenous 5-HT stimulates feeding behaviour and egg laying (Zhang *et al.*, 2008; Jafari *et al.*, 2011). The opposite effect (inhibition) is seen in relation to locomotion and in terms of signalling, this is highly dependent on the environment and experience of the worm. More specifically, when animals have been deprived of food, they will dramatically reduce their rate of locomotion upon their next encounter with bacteria. This "enhanced slowing" response was elegantly demonstrated by Sawin and colleagues (Sawin, Ranganathan and Horvitz, 2000), who showed a defect in this response in

mutants that were defective in 5-HT synthesis, but not those defective in DA synthesis alone. The critical role played by 5-HT was further confirmed by the rescue of the defect by application of exogenous 5-HT (Sawin, Ranganathan and Horvitz, 2000). A great many behaviours, genes, cells, and receptors have been characterised in relation to 5-HT in *C. elegans* (Chase and Koelle, 2007), and these pathways have been extensively investigated in the context of treatments for depression (Kaletta and Hengartner, 2006).

Dopamine - DA is another biogenic amine that is important and well characterised in *C. elegans* (Chase and Koelle, 2007). It is made in 14 different neurons, all of which are thought to have mechanosensory functions (Goodman, 2006). An overriding theme for the function of DA in the worm appears to be modulating its ability to detect and respond to changes in its environment. This is evidenced by the deficiencies in environmental interaction witnessed in mutants deficient in DA release or synthesis, and worms that have had dopaminergic neurons surgically ablated (Chase and Koelle, 2007). As an example, the above-mentioned study that demonstrated the enhanced slowing response in relation to 5-HT (Sawin, Ranganathan and Horvitz, 2000), also found that DA is responsible for a similar but distinct "basal slowing" response. In this case, well-fed worms reduced their rate of locomotion in response to finding bacteria, although the response was much less pronounced than during enhanced slowing. This time, mutants that were deficient in DA synthesis, and worms with ablated dopaminergic neurons, were deficient in the basal slowing response (Sawin, Ranganathan and Horvitz, 2000). It was further shown that worms exhibited a DA-dependent slowing in response to contact with Sephadex gel, which unlike food has no known chemical or nutritional value to *C. elegans*

(Sawin, Ranganathan and Horvitz, 2000). This latter point supports the suggestion for a mechanosensory function in dopaminergic neurons.

1.12.6.Toxicant metabolism differs between mammals and C.elegans

Similar, but not identical, metabolic processes exist between *C. elegans* and mammals for several substances. For example, the stepwise processes of metabolism in general are conserved, however some of the specific enzymes and reactions may differ slightly and not all substances are metabolised in exactly the same way (Harlow *et al.*, 2018). In mammals, OPs are primarily metabolized by enzymes in the liver, such as cytochrome P450 (CYP450) and esterases. These enzymes break down OPs into various metabolites, including detoxified forms, which are then eliminated from the body. *C. elegans* lacks some of the specific CYP450s found in mammals. Instead, it relies on a more limited set of metabolic pathways to metabolize organophosphates. These pathways involve enzymes like glutathione-S-transferases and other enzymes, which help in the detoxification and elimination of organophosphate compounds (Hartman *et al.*, 2021). Nevertheless, despite its relatively simple metabolic machinery around 86 genes encoding CYP450s exist within the *C. elegans* genome, compares with only 60 in humans (Hartman *et al.*, 2021).CYP450s are enzymes with a variety of roles, and are responsible for the metabolism of endogenous substances, drugs, and other exogenous substances, including OPs.

Harlow et al., (2018) compared the function of some of these enzymes in *C. elegans* by testing several non-OP compounds for which the metabolism in mammals is already well understood, such as tolbutamide, amitriptyline and dextromethorphan. They found that

similar metabolites were generated in *C. elegans* to those in mammals and showed that several cytochrome P450 homologs exist between *C. elegans* and mammals (**Table 4**). However, despite broadly similar sequence homology between some CYP-genes for each species, the most closely matched homologs were not always responsible for metabolism of the same compounds, and so sequence homology did not always predict which specific enzyme would be responsible for metabolism of each compound for each species. Furthermore, *C. elegans* lacks some functional, or sequentially similar, homologs for some mammalian enzymes, including for the family of CYP1-like enzymes. These comparative deficiencies in *C. elegans* were evidenced by low levels of the metabolite paracetamol from phenacetin, which requires CYP-1 in mammals (Harlow et al. 2018). Moreover, benzo[a]pyrene is metabolised exclusively by CYP1 in mammals and the process produces genotoxic metabolites, such a metabolic process is absent in *C. elegans* (Leung *et al.*, 2010).

Table 4. *C. elegans* produces metabolites from several compounds that are also produced by CYP450 in mammalian systems. (Adapted from: Harlow et al. 2018).

Compound	Metabolite identified in mammals	Mammalian CYP450 required for metabolite production	Evidence that this reaction can occur in *C. elegans*
Phenacetin	Paracetamol	CYP1A2	yes
Tolbutamide	Hydroxytolbutamide	CYP2C8/9/19	yes
	Carboxytolbutamide	CYP2C8/9/19	yes
Diclofenac	Hydroxydiclofenac	CYP2C9	yes
Amitriptyline	Nortriptyline	CYP2C19	yes
	E-10-hydroxyamitriptyline	CYP2D6	yes
Clomipramine	Norclomipramine	CYP2C19	yes
Dextromethorphan	Dextrorphan	CYP2D6	yes
	3-methoxymorphinan	CYP3A4	yes
Nifedipine	Oxidised nifedipine	CYP3A4	yes

Importantly, *C. elegans*, lacks the enzyme paraoxonase-1 (PON1) that is involved in detoxifying organophosphates in many organisms, including humans (**Figure 11**). Overall, the processes involved in OP metabolism are more sophisticated in mammals than in *C. elegans.* This difference may contribute to variation in susceptibility and toxicity profiles and therefore caution should be applied if making any toxicokinetic comparisons between species.

1.12.7. Some other limitations of the C. elegans model

Despite the many benefits presented by *C. elegans*, there are limitations that must be considered when using the model for applications relating to human health research. It should therefore be noted that the value of the model is restricted to the investigation of molecular interactions and pathways that share some level of conservation with vertebrates, such as receptors, transporter proteins, enzymes, or other such machinery. It is not possible to directly model human conditions, such as common mental disorders, or neurodegenerative diseases. While many molecular components are conserved between mammals and nematodes, the *C. elegans* nervous system lacks the complex physiology of the mammalian brain. As with other models, *C. elegans* is not an anatomically simplified version of the human nervous system and is even more different in that respect than mammalian models. For example, *C. elegans* does not share any equivalent to the amygdala, prefrontal cortex, or the dorsal raphe nucleus, and the neuronal circuitry is very different from the mammalian brain. The utility of *C. elegans* therefore, comes from its comparative simplicity, which makes for simpler

characterisation of the molecular components and their interactions (White *et al*., 1986; Perieira *et al*., 2015).

Some other notable comparisons that may be relevant to this investigation relate to the cholinergic system. Firstly, termination of cholinergic signal transmission relies on AChE in both mammals and *C. elegans.* However, mammalian AChE is encoded by a single gene, with another less-specific ACh substrate: butyrylcholinesterase (BuChE) being encoded by another gene (Darvesh *et al*. 2003). In comparison, *C. elegans* has four different AChE genes known to encode distinct classes (class A, B, and C) of AChE (Combes *et al*. 2001). Class A and class B are encoded by *ace-1* and *ace-2*, respectively, which together account for 95 percent of AChE activity in *C. elegans.* A third gene, *ace-3* encodes class C, and accounts for the remaining five percent. Class D AChE does not appear to be responsible for any enzymatic activity (Combes *et al*. 2001). Importantly, although each of the *C. elegans* ace-genes are orthologues of mammalian BuChE, there is no direct equivalent of BuChE described in the nematode (Combes *et al*. 2001).

Table 5. Some strengths and limitations of the *C. elegans* model. (compiled from: Hunt, 2017).

Strengths	Limitations
A well-known model - the first fully sequenced multicellular organism	Organs, including the eyes, lungs, heart, kidney, and liver, are absent in *C. elegans*.
Extensive genetics, neurology, and cell signalling research.	*C. elegans* have a functioning innate immune system but no adaptive immunity.
Well-conserved neuronal function, genetic homology, metabolic and signalling pathways.	Thick cuticle – can be a poor absorption model for some substances (Xiong, Pears and Woollard, 2017).
Conserved alimentary system, provides a good oral toxicity model	Improper management of stock cultures can lead to changed gene expression patterns and unwanted effects (Hunt, 2017)
In contrast to cell and tissue cultures, *C. elegans* has sensory ability, behaviour, a motor, digestive and reproductive systems.	pH range is wide but limited - liquid culture testing requires soluble test compounds, similar to cell-based models
Can be kept with relatively little cost and maintenance.	Some systems lack genetic homology, but behave or react similarly to equivalent human systems
Multiple concentrations and exposure periods may be evaluated simultaneously using a cheap and small platform.	Adaptive reactions in populations can triggered by small environmental changes
Short life cycle enables whole life, or multigenerational testing in weeks instead of years.	Incorrect handling of stock cultures can result in altered gene expression patterns and the accumulation of dauers or males
Transparent tissues allow morphology and transgene expression at the tissue, cellular, and subcellular levels to be monitored against fully characterised neuronal map (White *et al.*, 1986).	The number of chemicals that may be screened by a single lab is currently limited by issues included above.
High association between *C. elegans* endpoints and rat LD50s (Harlow, Perry, Widdison, Daniels, Bondo, Benjamini, *et al.*, 2016)	

C. elegans tests alone cannot replace in-depth descriptive toxicological assessments in mammals, largely since nematodes lack the majority of human organs. However, while the endpoints of toxicity frequently vary, nematodes and humans share several routes of administration and mechanisms of action. The value of the model therefore lies in early-stage screening, combined with more extensive testing strategies, providing a complementary link between *in vitro* assays and mammalian toxicity testing (Harlow, Perry, Widdison, Daniels, Bondo, Lamberth, *et al.*, 2016; Hunt, 2017).

1.13. Choosing a reference compound

In addition to differences in study design and the measuring of different endpoints, variation in the toxicology literature regarding OPs is caused by differences in precise modes of action between each compound (Slotkin and Seidler, 2007a; Richendrfer and Creton, 2015). This is particularly true where AChE inhibition is not responsible for the measured outcome (Aldridge *et al.*, 2005). To exclude this effect, the development of an experimental model would benefit from using the same compound; a valid model could then be built upon and tested using additional compounds later. To address this, Chlorpyrifos (CPF) was chosen for the following reasons: firstly, CPF appears to be the only specific OP compound shown to have positive, statistically significant associations with human depression and suicide mortality (Freire and Koifman, 2013). Secondly, CPF is the most well-known and well-studied of all agricultural and domestic pesticides (Torres-Altoro *et al.*, 2011; Amani *et al.*, 2016), and would hopefully prove to be the most useful as a reference material for model development. Thirdly, as an example of the second point, previously published microarray data showing transcriptional responses of *C. elegans* to low-level CPF exposure, was identified (Viñuela *et al.*, 2010). This data could be used for direct reference or compared against studies using CPF with different models (Ray *et al.*, 2010; Slotkin and Seidler, 2010; Viñuela *et al.*, 2010; Tilton *et al.*, 2011). Lastly, several reference genes have been identified and evaluated for use in toxicology studies using *C. elegans*, these genes were identified for their relative stability following CPF exposure (Wu *et al.*, 2014).

1.13.1.Background on chlorpyrifos

Chlorpyrifos (CPF) is an OP insecticide that has been used historically in domestic pest control, and more widely for plant protection in agriculture and commercially since its introduction in 1965. Despite its wide use, the use of CPF has been increasingly restricted and authorisations for its use have been withdrawn in the UK in 2016, the EU in 2020, and the USA in 2021 (Hites, 2021). The adoption of CPF as a test item for this experimental investigation is not intended to address the compound's safety profile directly, but because it a relatively well-characterised example of an OP compound that has been studied previously in *C. elegans* (J. Y. Roh and Choi, 2008; Ju *et al.*, 2010; Viñuela *et al.*, 2010a; Roh, Lee and Kwon, 2016a; Silva, 2020).

1.13.2.Inhibition of AChE, following bioactivation and detoxification of CPF

CPF requires bioactivation to exhibit its toxicity. Once bioactivated into chlorpyrifos oxon (CPO), CPF is capable of inhibiting AChE. While the bioactivation of CPF to CPO is a process that confers toxicity, it's noteworthy to mention that CPF can also undergo detoxification, thereby reducing its potential harmful effects. This detoxification typically involves the conversion of CPF to less harmful metabolites, which can then be readily excreted (**Figure 11**).

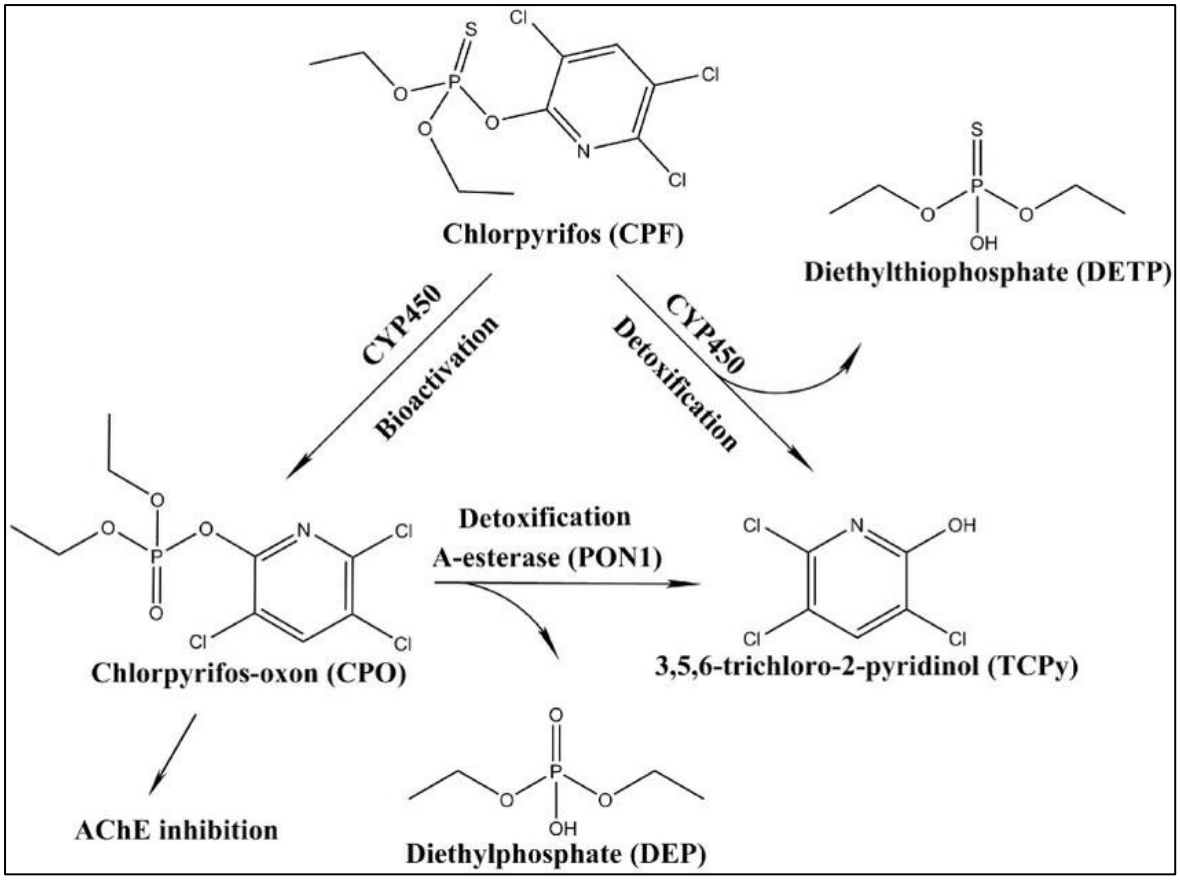

Figure 11 Metabolic pathways are required for bioactivation and detoxification of CPF in humans. The CPF parent compound is a weak inhibitor of AChE. CPF can be detoxified to 3,5,6-trichloro-2-pyridinol (TCPy) and diethyl thiophosphate (DETP), but also converted to its active oxon form (CPO) by cytochrome p450 enzymes (CYP450). CPO is a potent inhibitor of AChE that is detoxified by A-esterases. Paraoxonase (PON 1) is an important A-esterase in CPO detoxification (Adapted from: Timchalk et al., 2002).

1.13.3.Evidence for the effects of chlorpyrifos exposure in C. elegans

Toxicology research has focused on lethality as an endpoint, and thus the commonly quoted measure of toxicity for a given agent is the median lethal dose (LD-50), that is the dose required to kill half of a sample. Correspondingly, early OP research using nematodes was aimed at finding ecological measures of toxicity and focused mainly on lethality (Mcvey *et al.*, 2012). A true dose cannot be determined for *C. elegans* because it

is difficult to determine how much of the substance, if any, has been ingested by the organism, and so a median lethal concentration (LC-50) is used instead. A more recent focus on sub-lethal endpoints, has enabled sophisticated investigations to be performed in the well characterised *C. elegans* model. Although the figure is not always reported, the EC-50 value depicts a concentration at which a 50% 'effect' is seen in the sample and can relate to virtually any chosen endpoint. A few studies have reported such values for CPF in *C. elegans*, but they are not always consistent (Meyer and Williams, 2014). One study (Anderson, Cole, and Williams, 2004) compared a range of non-neuronal, and neuro-toxicants including CPF, for levels of toxic effects. Interestingly, they reported that the order of toxicity for all tested toxicants was exactly the same for *C. elegans* as was found for rats and mice (P = 1), which is consistent with studies that have made similar comparisons using different toxicants (Anderson, Cole, and Williams, 2004b; Rajini, Melstrom and Williams, 2008). For locomotory inhibition in *C. elegans* these authors reported an EC-50 of 5 μM for CPF (Anderson, Cole, and Williams, 2004). This was five times higher than the 1 μM EC-50 reported for locomotory inhibition by Boyd et al (Boyd *et al.*, 2010), who reported an even lower EC-50 (0.09 μM) for reproduction. These authors also reported a decrease in body length, but at a much higher concentration of 22.9 μM (Boyd *et al.*, 2010). Another study reported a significant difference in locomotion versus control at 1 mg/L CPF (Ruan *et al.*, 2009), which was more pronounced after 24 hours exposure than it was at 72 hours, however no effect on reproduction was found at 1 mg/L (Ruan *et al.*, 2009). Indeed, Martin et al (Martin, Svendsen, Lister, Gomez-Eyles, *et al.*, 2009) found that a higher concentration of 3.5 mg/L was necessary to inhibit reproduction via egg laying. Interestingly, a study which measured AChE enzyme inhibition in *C. elegans* (J. Y. Roh and Choi, 2008) reported that around 50% was inhibited

by 10 and 100 mg/L CPF. This would seem to be important to consider in the investigation of sub-AChE threshold effects. However, the same authors report an LC-50 of 0.966 mg/L CPF (J. Y. Roh and Choi, 2008), which seems low considering the behavioural endpoints reported elsewhere at equivalent, or higher concentrations (Anderson, Cole, and Williams, 2004b; Ruan *et al.*, 2009a; Boyd *et al.*, 2010). Conversely, Jadiya and Nazir (Jadiya and Nazir, 2012) did not find such a lethal effect at 0.966 mg/L, when using a transgenic *C. elegans* model for Parkinson's disease, including wild-type controls (Jadiya and Nazir, 2012). In light of these concentration-related discrepancies, an important part of building a new model would be to establish appropriate and consistent working concentrations, relative to the endpoints observed.

1.14. Aims and objectives.

The overall aim of this project is to investigate the neurotoxic effects of low-level OP exposure in *C. elegans*, and help to clarify how, and if, they contribute to human psychiatric disorders in UK agricultural workers. In particular, the effects of low-level chronic exposure remain relatively understudied, with frequent gaps and inconsistencies in the literature. Thus, the project will focus on low-level level exposure and attempt specifically to determine any effects which occur below the threshold for AChE inhibition. The experimental component of this project will use the invertebrate model: *C. elegans* to screen for any effects of CPF on behaviour which may give clues to and enable further investigation of any aspects of neurotransmission that may be involved.

The project has three main aims:

1. To identify any target or targets of Chlorpyrifos using the *C. elegans* model, and to discover and report any novel, or non-cholinergic molecular pathways that are directly affected by low-level treatment with this compound.

2. To develop and present a robust experimental model using *C. elegans*, which can be used to test organophosphate compounds or other neurotoxicants, for their potential to alter molecular signalling that may be relevant to psychiatric disorders.

3. To explore the relationship between organophosphate exposure and mood disorder using a questionnaire-based survey, in a human cohort. Consideration is given to other potential confounds and chronic life-stressors which may also contribute to such conditions, and those relationships explored using statistical tools, including moderation and mediation analyses.

2. Materials and Methods

2.1. Introduction

This section of the thesis outlines the materials and methods used across this body of research. As this project was multi-disciplinary in its approach, working with both *C. elegans* and humans, the chapter is split broadly into two sections covering each approach separately.

2.2. *C. elegans* studies

2.2.1. C. elegans strains

The different *C. elegans* strains used in this project are listed in **Table 6**.

Table 6. *C. elegans* strains used.

Strain	Genotype	Description/notes	Source
N2	Wild type	Bristol WT (Brenner, 1974)	*Caenorhabditis* Genetics Centre (CGC)
VC505	*ace-1(ok663)* X	Lacking class A AChE	CGC
RB1942	*ace-2(ok2545)* I	Lacking class B AChE	CGC
GG202	*ace-2(g72)* I	Lacking class B AChE (Combes *et al.*, 2000)	CGC
PR1300	*ace-3(dc2)* II	Lacking class C AChE (Combes *et al.*, 2000)	CGC
RM2702	*dat-1(ok157)* III	DA transporter deficient (Nass *et al.*, 2002)	CGC
CB1112	*cat-2(e1112)* II	Catecholamine absent (Lints and Emmons, 1999)	CGC
LC33	*bas-1(tm351)* III	5-HT-deficient, likely DA-deficient	CGC
JD269	*gar-2(by124)* III; *gar-3(*lg1201) V; *gar-1(ad1676)* X	Triple mutant, lacks all muscarinic receptor signalling (Steger and Avery, 2004)	CGC
VC657	*gar-3(gk305)* V	Muscarinic receptor (*gar-3*) deletion	CGC
DA521	*egl-4(ad450)* IV	Spontaneously enters a sleep-like state and ceases feeding. (Raizen *et al.*, 2006)	CGC

KP2018	*egl-21(*n476) IV	Deficient in a carboxypeptidase E (CPE)-like protein. Reduced ACh release at NMJ via inability to process endogenous neuropeptides (Jacob and Kaplan, 2003)	CGC
RB918	*acr-16(ok789)* V	Lacks α7-like subunit of homomeric nicotinic ACh receptor (Consortium, 2012)	CGC
MT15434	*tph-1(mg280)* II	Tryptophan hydroxylase deficient. Cannot synthesize 5-HT. Behaviour mimics starvation (Sze *et al.*, 2000)	CGC
CB1072	*unc-29(e1072)* I	Lacks nicotinic receptor subunit (human β2 & β4 ortholog) necessary for positive regulation of ACh secretion (Fleming *et al.*, 1997)	CGC
RJM195	vjIs50 [*ttx-3p:*:mRFP, *unc-129p*::GAR-3-GFP]	GFP tagged GAR-3 cDNA overexpressed in cholinergic neurons	Derek Sieburth (University of southern California)
RJM194	vjIs50 [*ttx-3p:*:mRFP, *unc-129p*::GAR-3-GFP];*gar-3(ok305)*	GFP tagged GAR-3 cDNA expressed in cholinergic neurons of *gar-3(ok305)* deletion strain	Alex Dawson

2.2.2. Maintenance of C. elegans stocks

2.2.2.1. Rearing conditions

All strains were maintained at 20°C and grown on standard nematode growth media (NGM) plates seeded with *E. coli* OP50 bacteria as previously described (Brenner, 1974). Details on reagents and solutions can be found in Reagents used.(8.2).

Standard 55mm rearing plates were poured using a peristaltic pump to dispense 13ml NGM per plate and left to dry overnight. *Escherichia coli* strain OP50 was provided as the main food source. OP50 is unable to synthesise uracil which limits its rate of growth on NGM plates, thus preventing overgrowth. A bacterial lawn of approximately 200 µl OP50 was seeded onto each plate and left to dry overnight at room temperature before use. OP50 was the only food source provided unless otherwise stated. Plates of nematodes

were stored at 20°C in a dark and ventilated incubator, inverted with the lid down to prevent the escape of moisture, and plates from drying out. Nematodes were transferred between plates, or otherwise moved, using a worm pick made from sterilised platinum wire, regularly sterilised in a flame to prevent contamination, or mixing of strains (Brenner, 1974). During maintenance, larger groups of nematodes or eggs were transferred by 'chunking', where a section of NGM was cut from an existing plate and transferred face down onto a fresh plate to prevent plates from deterioration and overcrowding.

2.2.2.2. Low-bactopeptone tracking plates.

Behaviour tracking of *C. elegans* relies on detectable contrast between nematode silhouettes and the plate surface. Image acquisition can be hindered by thick and uneven bacterial lawns, risking sub-optimal tracker performance. Plates used for tracking assays were modified by increasing the agar content from 17 g/L to 20 g/L and reducing the peptone from 2.5 g/L to 0.13 g/L. The low-peptone content slows bacterial growth, leading to a thinner lawn and improving image acquisition (Yemini, Kerr, and Schafer, 2011).

2.2.2.3. Freezing C. elegans strains

For each strain, >10 plates were grown to starvation. Once starved, worms were washed into a 15ml falcon tube with 10 ml M9 buffer and left on ice for 15 minutes until gravity settled. Once worms were settled at the bottom, the buffer was reduced to 2 ml and gently agitated to resuspend the worms. 2ml of molten freezing agar was mixed into the suspension, which was then halved into 2 x 2 ml aliquots and stored in 2 ml cryovials

(Corning). Strains added to the laboratory collection were maintained at -80°C and catalogued using FileMaker (Claris International Inc.).

2.2.2.4. Thawing C. elegans strains

After identifying the catalogued location of each required strain, one cryobox at a time was removed from the -80 °C freezer and kept on dry ice. A small volume of agar was scraped from one aliquot per strain using a sterilised spatula and added to a seeded NGM plate. Plates were left at 20°C, directly on the incubator shelf or in an open box to prevent excessive humidity, for 48 hours. Worms were grown for at least two generations before being used in experiments.

2.2.2.5. Age Synchronisation by Bleaching

To reduce experimental variation from population variability, age synchronous *C. elegans* cultures were obtained by 'bleaching' (Stiernagle, 2006). For each strain > 6 plates of gravid adults were washed into 3.5 ml dH_2O in a 15ml falcon tube, using a glass Pasteur pipette. 0.5 ml 5 M NaOH and 1 ml NaOCl (bleach) were added, before vortexing and leaving at room temperature for 10 minutes, vortexing every 2 minutes. Once worms were dissolved, eggs were pelleted by centrifugation at 1300 x g for 1 minute. Supernatant was removed to a final volume of 1 ml, and eggs were resuspended in dH_2O to a volume of 10 ml. Eggs were re-pelleted by spinning at 900 x g for a further 1 minute, supernatant removed and resuspended in dH_2O, for a total of three washes. After the final wash, approximately 100 µl was left in the tube and transferred to 25 ml M9 buffer in a 50 ml falcon tube and shaken gently at 20°C, overnight. The following morning worms had arrested as L1 larvae due to the absence of food. L1 larvae were centrifuged for 2

minutes at 900 x g and supernatant was removed. Final volume was dependent on experimental requirements and was distributed by pipetting onto seeded plates.

2.2.3. Single Worm Polymerase Chain Reaction (PCR) for checking deletion alleles and screening progeny when crossing strains.

10µg proteinase K was added to 100 µl single worm PCR lysis buffer immediately prior to use and 2.5 µl of the resulting mixture added to each flat capped PCR tube (ABgene EayStrip; Thermo Scientific). Single, adult worms were picked into each tube with a sterile platinum wire pick. A negative control tube, containing no worm DNA, was included in all experiments. N2-wild-type and strains carrying the deletion of interest were included as positive controls.

Sealed samples were frozen in the PCR tubes at - 80°C for at least 30 min prior to lysis.

To lyse worms sample tubes were thawed at RT, before running the following programme in a thermal cycler (Bio-Rad C1000):

1. 60°C for 60 min
2. 95°C for 15 min

Following lysis sample tubes were kept on ice, and the PCR reaction mix was added. 10pmol of each primer (**Table 7**), 0.2mM dNTP mix, 2.5 units Taq polymerase and 1mM $MgCl_2$ were used unless otherwise stated. PCR reactions were run in a total volume of 25 µl.

The same PCR programmes was used for all reactions. The annealing temperature was determined for each pair of primers by performing a temperature gradient with N2-wild

type and the appropriate deletion strain. In all cases, a temperature of 60°C gave the optimal results, with the predicted band size and no non-specific bands.

1. 95°C for 3 min
2. 95°C for 45 sec
3. 60°C for 40 sec
4. 60°C for 1 min per Kb
5. Return to step 2 and repeat 2, 3, and 4 for 35 repetitions
6. 72°C for 10 min
7. Hold at 4°C

2.2.3.1. Agarose gel electrophoresis

4 µl of 6x DNA loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll] was added to each PCR reaction and 10µl of PCR products were run on 1% (w/v) agarose gels made with TBE buffer containing 10µl 10,000x Sybr-safe DNA gel stain to visualise the DNA. Samples were run alongside a DNA ladder (Invitrogen: 10787-026) at 100V for 1 hr. Gels were visualised on a standard UV transilluminator.

2.2.3.2. PCR primers

Table 7. PCR primers used to confirm genotype of AChE and mAChR mutants.

Name	Sequence	WT product size (bp)	Deletion allele size (bp)
ace-1.1	GATCGCAAAATATACACAATCTG	1084	266
ace-1.2	CCTGAGATTGCCAACTGGTGG		
ace-2.1	GTTATCCGATATCAAATCAGC	2060	874
ace-2.2	CGTGGTCAATGTGAATTACAG		
gar-3.1	GATTGAGGCGTAACTATTGTTG	1040	578
gar-3.2	GTGCTTGAAGTCGTCATTTTC		

2.2.3.3. Creating a gar-3 rescue strain.

To confirm the requirement for GAR-3 in the foraging phenotype a rescue strain was created by crossing the *gar-3(gk305)* deletion strain with *vjIs50 [ttx-3p:mRFP, Punc-129::GAR-3-GFP]* animals, which express GFP tagged GAR-3 cDNA in cholinergic motor neurons.

2.2.3.4. Crossing worms

55mm standard NGM plates were seeded with a very small lawn of *E. coli* OP50 bacteria in the centre of the plate. Five larval stage 4 (L4) *gar-3(gk305)* hermaphrodites were transferred onto the lawn and 10 young *vjIs50* adult males were transferred to the

perimeter of the lawn. Males were generated by heat shocking hermaphrodites at 30°C overnight and selecting males from the F1 progeny. Cross plates were left for 2 days at 22°C. The hermaphrodites were then each separated to individual plates and the progeny examined after 3 days. The presence of the *gar-3(gk305)* was confirmed using single worm PCR.

2.2.4. Measuring AChE activity

Cholinesterase enzymatic activity was measured using a modified version of Ellman's method (Ellman, 1961), adapted for use in *C. elegans* and most closely resembling the protocol described by Moulton et al. (1996). Reagents were purchased as kits (Abcam: ab138871), stored in single use aliquots at -20°C and thawed overnight before use. The stages of the enzyme assay are described in the sections below.

2.2.4.1. Washing and freezing worms

Worms were washed from 10 plates per condition into a 15 ml falcon tube with 10 ml dH_2O. Worms were left to settle on ice for 5 mins before removing supernatant and resuspending in 10 ml dH_2O. The suspension was gently mixed using bubbles from a Pasteur pipette and allowed to settle on ice for a further 5 minutes. Wash steps were repeated for a total of 3 washes, before centrifuging at 4°C for 10 minutes at 2000 x g in an Eppendorf 5424 centrifuge. The supernatant was removed, and the pellet flash frozen in a cryovial using liquid nitrogen. Samples were stored at -80°C overnight or until analysed.

2.2.4.2. Sample homogenisation

Samples were removed from the -80°C freezer and kept on ice. 400 µl cold phosphate buffered saline (PBS) was added to each sample before transferring to tubes filled with

Zirconium beads (Sigma Z763810). Samples were homogenised at 400 strokes per minute (SPM), for 30 seconds in a Beadbug™ microtube homogeniser, and supernatant transferred to a 1.5 ml microcentrifuge tube on ice. The bead tube was washed with a further 200 µl cold PBS at 400 SPM for 20 seconds, and the remaining liquid transferred to the sample tube. The sample was centrifuged at 2000 x g for 5 minutes at 4°C.

2.2.4.3. Colorimetric assay

Assays were performed in 96 well plates. For each 96 well plate 5 ml of acetylthiocholine reaction mixture was prepared in a 15 ml falcon tube, wrapped in foil to protect from light. The reaction mixture comprised of 4.5 ml assay buffer, 250 µl 20X dithiobis (2-nitrobenzoic acid) (DTNB) stock solution and 250 µl 20X acetylthiocholine stock solution.

AChE standards were prepared by diluting 20 µl AChE stock solution (Abcam) in 980 µl 0.1M sodium phosphate buffer solution. Serial dilutions were performed to give final concentrations of 300, 100, 30, 10, 1, and 0 mU/ml. 50 µl of each AChE standard was added to individual wells of a 96 well, clear flat-bottomed plate (Corning®) in duplicate. 50 µl assay buffer was included as a blank for each set of standards, and 50 µl of each test sample was added to wells in triplicate. 50 µl of acetylthiocholine (Abcam) reaction mixture was then added to each blank, standard and test sample to make a total volume of 100 µl per well, before covering the plate in foil to protect from light and incubating at room temperature for 10 minutes. Increase in absorbance was measured over 90 seconds at 405 nm, in a FLUOstar Optima fluorescence microplate reader. Blank well values were subtracted from each sample measurement and the resulting value measured against the standard calibration curve.

2.2.4.4. Measuring total protein

Total protein concentrations were determined for each sample using a Pierce BCA Protein Assay Kit (Thermo Scientific: cat 23225) using bovine serum albumin as the protein standard. Samples were measured at 562 nm in the microplate reader. Final values for AChE activity were divided by protein content value from each sample and given as milliunits AChE activity/milligrams protein (mU/mg).

2.2.5. Chlorpyrifos treatment

Chlorpyrifos (PESTANAL®45395-100MG) was purchased from Sigma Aldrich. Solutions and plates containing CPF were protected from light whenever possible to prevent photodegradation.

2.2.5.1. CPF stock solution

A stock solution of CPF was created by dissolving 100 mg CPF into 20 ml acetone (final stock concentration: 5 mg/ml or 14.26 mM). The stock solution was stored in 1 ml aliquots and protected from light at -20°C. Preliminary experiments indicated that the effect of CPF on *C. elegans* behaviour was reduced by repeated freeze thaw cycles of CPF (**See Appendix 8.4**) therefore each aliquot was thawed, protected from light, and maintained at room temperature for the duration of each experiment or until depleted. Even after several weeks, this produced more consistent results than repeatedly freezing the stock after each use (A. Dawson, unpublished observation). The final concentrations used for treatments were mixed into molten NGM at 0.05 mg/L, 0.1 mg/L, 0.5 mg/L, 1 mg/L and 2.5 mg/L CPF. Alongside each CPF concentration, an equivalent volume of acetone was mixed into NGM to produce solvent control plates as described below.

2.2.5.2. CPF exposure plates

All plates containing CPF were poured by hand. NGM was mixed to either standard or low-bactopeptone recipes, as described above. Before pouring, 30 ml molten NGM was transferred to a 50 ml Falcon tube and CPF stock solution added to the specified final concentration inside a fume cupboard. The tube was mixed by inverting 20 times and 13ml was added to each 55 mm petri dish. Plates were removed from the fume cupboard once dry to prevent evaporation and protected from light at room temperature overnight. Plates were seeded with 200 µl OP50, left to dry overnight and used within 48 hours.

2.2.5.3. Vehicle control plates

Control plates were poured using the method described above for CPF plates by replacing CPF stock solution with the same volume of acetone as a solvent control.

2.2.5.4. Exposure of C. elegans to CPF

Nematodes were added to CPF, or vehicle control plates, as age synchronous L4 larvae unless otherwise stated. This life stage was chosen because all neurons have been generated by the L4 larval stage (Sulston and Horvitz, 1977; Taylor *et al.*, 2020), and because potentially confounding factors of aging and production of progeny are yet to occur. Depending on the number of individuals required for each experiment, worms were either picked individually, 5-10 to each plate, or washed from standard rearing plates in M9 buffer and ~ 50 nematodes transferred with a micropipette, onto each CPF or control plate. During exposure, plates of nematodes were incubated at 20°C in the dark. Unless otherwise stated, L4 larvae were exposed for 24 hours, and behaviour was assessed in one day old adult hermaphrodites as described for each experiment. To remove any confounding effects of starvation, any plates with no visible food remaining

after 24 hr exposure were discarded and excluded from each experiment. Where indicated animals were removed from CPF plates after exposure and allowed to recover for 24 hours at 20°C on standard NGM plates seeded with OP50 before behaviour was assayed as described in sections 2.2.6.7 and 2.2.6.8.

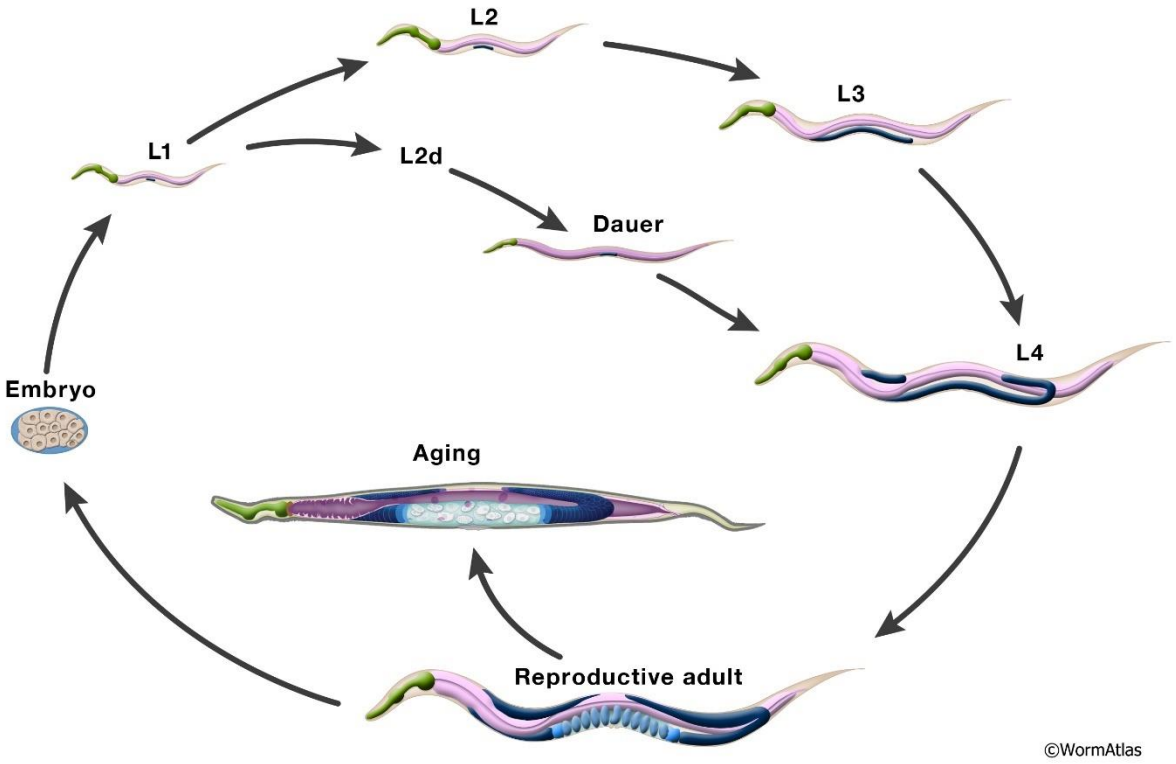

Figure 12. The *C. elegans* life cycle includes four larval stages between the embryo and adult stage. Between each larval stage a moult occurs, during which the animal enters a period of inactivity while a new cuticle is synthesised. In response to unfavourable conditions, such as starvation, overcrowding, or extreme temperature, larvae can arrest at the L1 stage, or following the L2-moult, can enter dauer diapause, in which they can survive for several months before developing into adults on return to favourable conditions. Without dauer formation, the average lifespan of *C. elegans* is around three weeks (Fielenbach and Antebi, 2008).

2.2.5.5. Recovery following removal from CPF.

To test whether animals could recover from the effects of low level CPF treatment, 15-20 wild-type L4 larvae were exposed to CPF or vehicle control for 24 hr as described in 1.8.4. Animals from each treatment were picked into micro-arenas for 30 min in the absence of food, before behaviour was recorded using, as described in 2.2.6.8. These animals were then split into three groups: first, animals from the vehicle control group were picked onto a fresh vehicle control plate seeded with OP50 (control group); secondly, half of the animals from the CPF treated group were picked onto a fresh vehicle control plate seeded with OP50 (ex-CPF group); and the other half of the CPF treated animals were transferred to a fresh CPF treated plate seeded with OP50 (CPF group). Animals were incubated under these conditions for a further 48 hr at 20°C, before being picked to fresh plates and assayed as described in section 2.2.6.8

2.2.6. Behavioural assays

2.2.6.1. Assessing egg-laying behaviour

The percentage of early-stage eggs laid, and number of unlaid eggs retained by adult nematodes were quantified using methods described previously (Koelle and Horvitz, 1996). Assays are briefly described in sections 2.2.6.2 and 2.2.6.3. Egg laying assays were performed exclusively with wild-type nematodes. Each experiment was performed in triplicate.

2.2.6.2. Constitutive egg-laying assay

Age synchronous populations were created as described in section 2.2.2.5. 25-30 L4 larvae were picked onto 3 standard plates seeded with OP50 and incubated for 24 hr at 20°C. One day old adult animals were picked from these plates onto OP50 seeded

treatment plates containing CPF. Standard NGM plates and plates containing acetone were included as controls. From each treatment, 20 animals were transferred taking care not to transfer any previously laid eggs. After 30 min at 20°C, eggs were counted under a Nikon SMZ-1500 dissecting microscope and recorded using a multi-channel mechanical counting device. To help keep track of plate position during counting, a lined grid was drawn onto a plate lid and placed under each plate on the microscope. Each egg was examined and categorised as shown in **Table 8**. More than 99% of N2 embryos are laid after reaching the 9-cell stage, therefore any eggs laid with 8 or fewer cells were considered to have been laid early.

Table 8. Classification categories for egg stage assays. Embryos counted in the egg-stage assay were recorded based on the number of cells observed in each. Embryos containing fewer than 9 cells are considered to have been laid earlier than normal (Schafer, 2005).

1 Cell embryos
2 Cell embryos
3-4 Cell embryos
5-8Cell embryos
>9 Cell embryos

2.2.6.3. Quantifying eggs inside nematodes

Following the egg-laying assay described above, 10 animals from each treatment were picked individually into wells of a 96 well clear flat-bottomed plate (Corning®), containing 50 µl 20% (w/v) NaOCl in dH_2O. After 8-10 min, or after the adult in each well

had dissolved, the total number of eggs remaining in each well was counted and recorded under a Nikon SMZ1500 dissecting microscope.

2.2.6.4. Aldicarb and levamisole paralysis assay

Sensitivity to aldicarb or levamisole following CPF exposure was determined by analysing the onset of paralysis as described previously (Nurrish, Ségalat and Kaplan, 1999). Staged, L4 wild-type larvae were transferred onto plates treated with CPF or acetone as control for 24 hr as described in 2.2.5.4. For aldicarb and levamisole treatment, plates were prepared to final concentrations of either 1 mM Aldicarb in NGM (Greyhound Chromatography) or 0.2 mM levamisole in NGM (Sigma) and left to cool overnight. A spot of 5 µl OP50 was added to the middle of each plate to prevent the worms from leaving and allowed to dry for 30 min. 25 young adults from each treatment were picked onto paralysis assay plates containing aldicarb or levamisole. Paralysis assay plates were kept in separate boxes and code marked as a blinding measure. During the assay, nematodes were checked and scored for paralysis at 10 min intervals. Individuals were scored as paralysed when they failed to move over a period of 10 seconds and did not respond to being gently prodded with the end of a worm pick. Paralysed animals were removed from the plate once scored to aid counting. Aldicarb and levamisole assays were repeated in triplicate.

2.2.6.5. Swimming induced paralysis (SWIP)

Analysis of locomotory behaviour in liquid media was performed as described previously, to test for the effect of CPF on swimming induced paralysis (Matthies *et al.*, 2006; McDonald *et al.*, 2007). Wild-type L4 larvae were exposed to CPF or acetone as a vehicle control for 24 hr as described in 2.2.5.4. 50 µl M9 buffer was added to each well of a clear,

flat bottomed 96-well plate (Corning®). 10x one day-old adult nematodes from each treatment were added to plate wells and monitored under a Nikon SMZ1500 dissecting microscope. The number of nematodes paralysed after 10 minutes was recorded. Paralysis was counted when no thrashing movements were observed over a five second period. SWIP assays were repeated in triplicate. SWIP is dependent on normal clearance and reuptake of synaptic DA and is known to occur in untreated *dat-1(ok157)* mutants following normal swimming. Therefore, *dat-1(ok157)* mutants were also subjected to the SWIP assay under the same conditions as wild-type animals. This provided a positive control for the assay.

2.2.6.6. Swimming

In addition to SWIP, locomotion in liquid was measured by counting the number of thrashes from side to side made by nematodes in M9 buffer. Nematodes were exposed to CPF or control and observed for 10 minutes in liquid as described above for SWIP. The number of thrashes performed by each nematode was recorded for the first minute, and again for the tenth minute.

2.2.6.7. Measuring locomotion by counting body bends

Locomotion was first quantified by manually counting individual body bends (Hart, 2006). Following treatment, 12 adult worms were picked onto a recently seeded, standard NGM plate. Plates of worms were gently placed onto a dissecting microscope and allowed to habituate for 10 min. One at a time, worms were observed, and the number of body bends recorded for 3 minutes each. A single body bend was counted when the area behind the pharynx reaches its fullest extension from one side to the other during the nematode's characteristic sinusoidal movement (**Figure 13**).

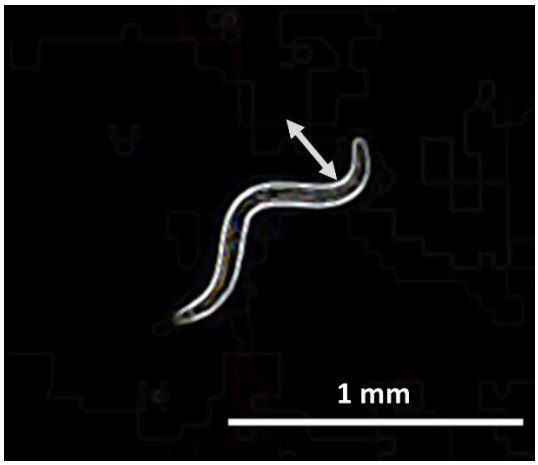

Figure 13. Body bend movement in *C. elegans*. Body bends are counted during normal 's-shaped' movement. As the worm's body shifts from side to side, one body bend is counted for each time the area behind the pharynx switches from one side to the other (Hart, 2006).

Movement in reverse was counted, but only after one full body bend in the opposite direction (i.e., half bends back and forth were not counted). Once counted, the individual was removed from the plate to prevent accidentally counting the same animal twice. The total number of body bends counted was divided by 3 to give a score of body bends per minute for each animal. 10 animals were counted from each treatment and experiments were performed in triplicate.

Locomotion was also scored using body bends in the absence of food. In this case nematodes were picked from treatment plates onto unseeded plates but were otherwise scored as described above.

2.2.6.8. Measuring locomotion with automated tracking

After the adoption of the WormLab behaviour tracker various aspects of locomotion, including locomotory speed, were measured automatically from video recordings (described in more detail in section 2.2.7). In these cases, the manual counting of body bends was replaced by measuring locomotory speed. Justification for the use of this measure is presented in the results section (4.4.3). Where automated tracking was used, the blinding procedure became obsolete and was not used.

2.2.6.9. Decreased locomotion on food.

To test whether CPF affects behaviour under different feeding conditions, treated nematodes were assessed for basal and enhanced slowing response as previously described (Sawin, Ranganathan and Horvitz, 2000). Nematodes were reared under standard conditions as described above (Brenner, 1974), but plates were seeded with *E. coli* strain HB101, instead of OP50, and incubated overnight at 37°C instead of drying at room temperature. Plates were left to cool for at least 1 hr at room temperature before use. HB101 grown overnight produces a more uniform bacterial lawn and more reliable results in basal and enhanced slowing assays (Sawin, Ranganathan and Horvitz, 2000). Some plates were not seeded with bacteria but were incubated and cooled together with the seeded plates for uniformity. Wild-type L4 larvae were exposed to CPF or vehicle control for 24 hr as described in 2.2.5.4 and assayed as one day old adults. Basal and enhanced slowing assays were performed in triplicate.

2.2.6.10. Basal slowing assay

To measure the locomotory response of well-fed nematodes to a new food source (basal slowing response), 5 animals from each treatment were removed from treatment plates

and washed twice in S basal buffer (Brenner, 1974). Animals were transferred to a bacteria free area on each assay plate, in a drop of buffer using a glass Pasteur pipette. Excess buffer was absorbed with a Kimwipe. For each treatment one assay plate was seeded with HB101, and another assay plate contained no food. After 5 min, the plates were recorded for 2 min and scored for locomotory speed using the worm tracker. Basal slowing was measured as the difference in locomotory speed between well-fed animals on plates with food, compared with animals on plates without food. Basal slowing is dependent on normal dopamine signalling and *cat-2(e1112)* mutants which are deficient in the synthesis of DA, do not show a normal basal slowing response (Sawin, Ranganathan and Horvitz, 2000). Therefore, these BSR assays were validated using *cat-2(e1112)* mutants as negative controls.

2.2.6.11. Enhanced slowing assay

To measure the locomotory response of food-deprived nematodes to a new food source (enhanced slowing response), 10-15 animals per treatment were washed twice in S basal to remove bacteria and transferred to plates without food. Animals were transferred in a drop of S basal using a glass Pasteur pipette and excess buffer absorbed with a Kimwipe. To prevent animals from trying to escape by climbing the plastic petri dish, the outer rim of each plate was lined with a ring of 0.5M copper sulphate pentahydrate ($CuSO_4$-$5H_2O$). Animals were kept deprived of food on these plates for 30 min at 20°C, before being picked onto assay plates. Half of the assay plates were seeded with HB101, and the other half contained no food. After 5 min, animals were recorded for 2 min and scored for locomotory speed using the worm tracker, as described above for the basal slowing assay. Enhanced slowing response was measured as the difference in locomotory speed between food-deprived animals on plates with food, compared with animals on plates

without food. The enhanced slowing response is dependent on normal 5-HT signalling and mutants carrying the *tph-1(mg280)* allele, which are deficient in 5-HT production (Sze et al., 2000), do not show a normal enhanced slowing response (Sawin, Ranganathan and Horvitz, 2000). Therefore, these ESR assays were validated using *tph-1(mg280)* mutants as negative controls.

2.2.6.12. Maximum Euclidean distance (Rmax)

To capture the phenotype observed as reduced foraging range, position plots showing the location of each worm throughout the course of the recording were extracted for each video using WormLab. Plot scaling was equalised to increments of 2500 μm on the Y axis, and 6000 μm along the X axis. Rmax was defined as the furthest point each animal reached from the beginning of the track (**Figure 14**) and was measured using ImageJ.

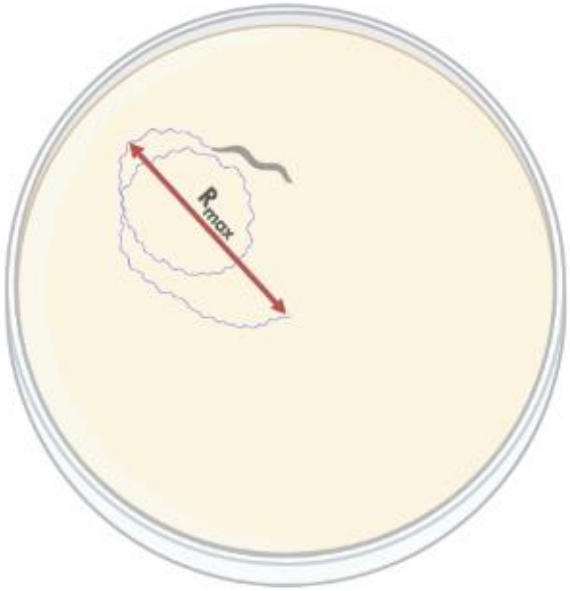

Figure 14. An illustration of maximum Euclidean distance from the start of *C. elegans* tracks. Rmax represents the furthest point each animal reaches from the beginning of each track. This is a measurement of the maximum Euclidean distance travelled by the worm and represents its individual foraging range during tracking (Dittman and Kaplan, 2008).

2.2.7. Worm behaviour tracking

Having detected no effect of low-level CPF treatment that could be used in any of the established behavioural assays that we had tested so far, a multipurpose, computerised behaviour tracker was utilised to assist in measuring the effects of low-level CPF exposure. Animals were exposed to treatments as described for each experiment, and video recordings were made for future analysis using the worm tracking software (**Figure 15**).

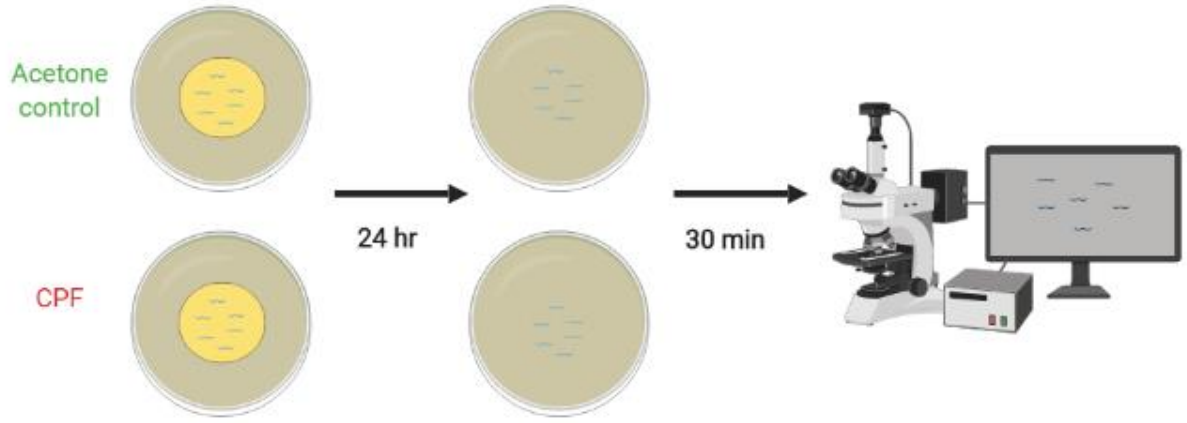

Figure 15. Schematic representation of the experimental setup used to investigate the effects of CPF treatment on *C. elegans* foraging behaviour. Worms were treated with 0.05 mg/L CPF or vehicle control for 24 hours on plates with food. Worms were then moved to food-free plates for a 30-minute period, which was necessary recreate the subtle effects of low-level CPF on foraging. Worms were recorded for 2 minutes under a light microscope. The captured video files were analysed using WormLab tracking software to quantitatively assess foraging behaviour changes.

2.2.7.1. Micro-arena preparation

To prevent the worms from leaving the field of view, a single copper washer of 12 mm internal diameter was pressed onto the surface of a 55 mm low-peptone worm plate to serve as a micro-arena. L4 larvae were exposed to CPF or vehicle control for 24 hr as described in 2.2.5.4. 5-10 worms were transferred into the centre of each copper washer and left to habituate for 30 minutes at 20°C, in the absence of food (**Figure 15**).

2.2.7.2. Video acquisition

Each micro-arena was recorded using a Nikon SMZ1500 stereomicroscope with camera attachment. A Nikon Plan Apo 0.5x/WF objective was used to capture the widest field of

view possible, at 3X magnification. The image was recorded using a Nikon Digital Sight DS-Qi1Mc digital camera with DS-U2 camera control unit (Nikon). Imaging software NIS-Elements Basic Research version 5.01.00 (32bit) was used to collect 2-minute, monochromatic (AVI/MP4) files recorded at 5 frames per second. Image size was set to 1440 (width) x 1024 (height). Ambient light was kept to a minimum and contrast settings were adjusted to provide maximum contrast between the worms and the background, while increasing the background lighting to minimise background appearance.

2.2.7.3. Setting scaling

Before each experiment, a clear plastic ruler was placed on a lid taken from a 55 mm plate, placed in the microscopic field of view. A 10 second video image was taken of the ruler scale and used to calibrate the scaling during WormLab setup described below.

2.2.8. Video processing using WormLab

Video files were processed and analysed using WormLab tracking and analysis software (MBF Biosciences, version: 2019.1.1.). Each video was imported into the WormLab desktop. Tracker settings were set up as determined during consultations with MBF Biosciences (2017) and were as follows:

2.2.8.1. Sequence settings

The captured frame rate was set to 5 fps. Scaling was set using the scale measure function, by measuring 10 mm across the ruler scale in the calibration image described above.

2.2.8.2. Image adjustment

The brightfield option was selected, and the threshold slider adjusted so that each worm's body area showed as a green silhouette, with the background remaining as white

as possible. Small object filtering was used to remove any eggs or artefacts from the adjusted image, then gap-filling and Gaussian-smoothing tools were used to balance the image, so that only individual worms where highlighted. Once optimised, these settings were applied and then saved.

2.2.8.3. Detection and tracking

Width fitting was deactivated, and width uniformity enforced. Detection frequency was set to every 10 frames, and fitting iterations set to 80. The tracker uses a machine learning approach based on the settings applied and by pressing 'Detect worms' the tracker highlights any worms it has detected in the current image. If all visible worms were highlighted, then the video was skipped forward a few frames and the tracker was asked to detect worms again. If any worms in view were not highlighted, then they were selected manually with the cursor, thus teaching the tracker to recognise those parameters. If necessary, this process was repeated until all worms were detected, noting that colliding worms are not highlighted during any frames where two or more worms are in direct contact.

To help the tracker to resolve collisions between worms correctly, the following settings were applied:

'Frames worms can overlap': 30

'Max tracked hypotheses': 3

'Use back tracking': on

Once optimised, the above details were saved as a settings profile which was used for the entire experiment. The profile and settings were checked for detection accuracy for each

new set of videos and adjusted as necessary. Once accurate settings were applied, each video and its matching profile were saved as a WormLab project file. The 'Batch tracking' function was then used to track multiple videos automatically.

2.2.8.4. Checking tracker output quality

For each video analysed, a new project file was created and opened to resolve any conflicts or errors within the file. Using the 'repair' function in the user interface, continuity of each track was ensured by joining any interrupted tracks and splitting any erroneous tracks which followed incorrect collision resolution. Head and tail orientation were checked and corrected if necessary and any artefacts from incorrectly identified objects removed.

2.2.8.5. Track analysis.

To extract data from WormLab project files, after batch tracking had completed each file was reopened in the WormLab desktop and 'Analyse Data' selected. Endpoints of interest were selected from the analysis menu, exported into Microsoft Excel for further processing. Where whole track summaries were used, the entire data table was copied and transposed into a new tab to simplify data extraction.

2.2.9. Statistical analysis

2.2.9.1. Indications of significance

Statistical significance is presented in the text and on figures as follows; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, n.s. $P > 0.05$.

Wherever possible, one-way, or two-way independent Analysis of Variance (ANOVA) tests were used to reduce the risk of Type I errors. Where ANOVA tests were not

appropriate, for instance where only one exposed treatment group was compared against a single vehicle group, differences in mean scores were analysed using unpaired t-tests, or Mann-Whitney U-tests as a non-parametric alternative.

Where more than one t-test was conducted simultaneously, for instance in the production of heatmaps, multiple unpaired t-tests were performed using Welch's correction to account for any inconsistencies in standard deviation between groups. In order to decide which effects warranted further investigation multiple comparisons were made using the False Discovery Rate (FDR) approach. The Two-stage Step-up method of Benjamini, Krieger and Yekutieli (Benjamini, Krieger and Yekutieli, 2006) was used with a desired FDR (Q-value) of 1%. Individual Q values representing the difference in each endpoint, between CPF-treated groups and their corresponding vehicle control, were used to produce the colour gradient represented in each cell of the heatmaps.

2.3. Human study

Just as the previous sections in this chapter give an overview of the methods and materials used in the *C. elegans* studies, the following sections outline those used for the research conducted with humans.

2.3.1. Ethical approval and consent

Ethical approval for this study was granted by the Human Research Ethics Committee of the Open University (HREC/2711/Dawson). Written informed consent was collected from each participant by the survey software before they could continue with the survey questions. All responses were anonymised at the point of collection and no personally identifying information was collected. A unique personal identifier known only to the participant was created to enable exclusion or destruction of an individual's data at that participant's request. In addition to managing data rights of potentially sensitive information, some of the content of the survey relating to their health or life situation could be difficult or upsetting for the participants to think about. In case of any general concern, contact details were provided for the author and the supervisory team from the outset. Furthermore, any participants that reported moderate or severe distress during any of the survey questions were automatically presented with the details of several mental health helplines.

2.3.2. Study design

This was a cross-sectional study in which UK agricultural workers with previous history of pesticide exposure, alongside a control group consisting of UK construction workers with no known history of exposure to organophosphates, were assessed for evidence of

mood disorder using established clinical assessment questionnaires. First, between group comparisons were made between the exposed agricultural workers and non-exposed construction workers as controls. Next, within-group analyses looking for correlations between pesticide exposure, symptoms of mood, and other influencing factors such as chronic daily stressors and physical health status, were performed. Finally, any observed relationships would be tested for mediation effects, to uncover any indirect effects that may be related to covariates.

Using the effect sizes found by Harrison and Mackenzie-Ross (2016) (ηp2 range = 0.11 - 0.16) a moderate-large effect size was assumed. A power analysis was conducted to determine the necessary sample size to detect differences of similar magnitudes between groups. Using this information, power analyses indicated that a sample size of between 21 and 45 individuals per group would be required to have 80% power to detect a relationship of this magnitude between exposure group and mood using 2-tailed tests. Data was collected between July 2017 and December 2018 using the Qualtrics Core XM survey platform (www.qualtrics.com).

2.3.3. Participants

2.3.3.1. Exposed cohort

Participants were asked to report their experiences with OP pesticides. They were asked whether they had ever worked with OPs and in what context, or if they had been exposed in some other way and asked to describe circumstances of exposure. Respondents were asked if they had ever worked with OPs, or if they had been exposed to them in any way then they were asked to specify how. Those who reported historical exposure were

included in the exposed cohort. They were also asked about distinct types of agricultural activities they had been involved in. Any respondents that answered "don't know" when questioned specifically about OP exposure were included in the exposed cohort if they reported having dipped sheep, sprayed crops, mixed or prepared insecticides, or any other specified activity which would be highly likely to involve OP compounds described in the Committee on Toxicity of Chemicals in Food, Consumer Products, and the Environment Report on organophosphates (CoT, 1999). Respondents were asked to specify compound or product names wherever possible. Agricultural workers who answered "no" when asked whether they had worked with or otherwise been exposed to OPs were completely excluded from the study.

Information regarding previous acute exposure was collected, including whether participants had ever suffered from 'dipper's flu', or been diagnosed with any other organophosphate related illness. Participants who reported to have experienced acute OP poisoning were not immediately excluded, but the information was collected so that it could be considered during analysis. The basic inclusion and exclusion criteria are presented in **Table 9**.

Table 9. Inclusion and exclusion criteria

	Exposed group	Unexposed group
Inclusion	Over 18 years of age. Currently working or having previously worked in agriculture in the United Kingdom. Exposure to organophosphate pesticides, either occupationally or via another specified route.	Over 18 years of age. Currently working or having previously worked in the construction industry in the United Kingdom. No known exposure to organophosphates.
Exclusion	Individuals having never been exposed to organophosphates.	Individuals with any known historical exposure to organophosphates.

2.3.3.2. Control cohort

Since many UK farmers have experienced some exposure to organophosphates at some point during their career, finding sufficient unexposed individuals for an occupationally-matched control group is difficult (Harrison and Mackenzie Ross, 2016). Therefore, an alternative occupational group was sought which was closely matched on variables such as gender, educational attainment, lifestyle and working environment. Importantly, the control group were also unlikely to encounter OP pesticides through their work. It is possible that some OPs may be present on construction sites, since they are used in industrial processes and may therefore by found in building materials, as flame retardants, for example. However, key differences in uses between pesticides and most building materials, mean that pesticides are designed to be dispersed, absorbed and/or consumed by biological organisms. Flame retardants by comparison, are most effective

when they remain part of the article or mixture in which they are required to function. This means construction workers are less likely to be sprayed, splashed, or to inadvertently inhale or ingest the material, when compared with people who dip, spray, or mix volatile pesticide mixtures. Some OPs have been detected in indoor dust; however, this is as true for domestic homes and childcare centres, and exposure of OPs through dust is considered to be comparatively low (Langer *et al.*, 2016; Bi *et al.*, 2018).

For this survey, construction workers who had no previous agricultural experience or known exposure to organophosphates were recruited as a control group. Workers in the construction industry undertake a variety of roles ranging from skilled or unskilled labouring to project management, in addition to engineering or more technical roles. These diverse roles require varying educational requirements, leading to similar educational variation to that seen within the agricultural workforce. Agriculture and construction work is also frequently physically demanding, often carried out outdoors, and work progress can be sensitive to adverse weather conditions. Traditionally, both industries have shown a male gender bias, which may be related to high risks of accident and injury in both industries (Grazier and Sloane, 2008; HSE, 2020).

Basic demographic information was collected to test for successful matching between the exposed and control groups.

2.3.4. Recruitment

2.3.4.1. Purposive sampling

Health and safety and workplace practices can vary between different countries, and therefore only UK agricultural and construction workers were asked to participate in this

study. This was also intended to reduce any confounding factors resulting from cultural, environmental, or regulatory differences.

A combination of sampling methods was used to reach the target population:

1. Purposive sampling - written (email) or direct telephone contact
2. Purposive sampling - online participant recruitment service
3. Advertising and social media

2.3.4.2. Purposive sampling - written (email) or direct telephone contact

Business and contractors working within UK agriculture were identified by searching the internet. Some business contact details were acquired from the National Association of Agricultural Contractors (NAAC) website, directly from contractors' own websites, or self-published contact details available through Google search results. Each business was sent an email explaining the purpose of the study or given an explanation over the telephone using a prewritten script. Participation and/or dissemination of the survey was requested and a shareable web link to access the survey was provided on request.

2.3.4.3. Purposive sampling - online participant recruitment service

A second phase of recruitment was conducted using an online participant sourcing service provided commercially by a company called Prolific (www.prolific.co). Prolific hold a database of registered participants who provide information relating to their basic demographics, health status, occupation, and other variables. Participants meeting the required inclusion criteria for age, occupation, and geographic location (**Table 9**) were identified using the information supplied. Information relating to organophosphate exposure was not previously collected during Prolific's existing screening questions. Therefore, a set of custom questions exploring historical use of pesticides and known

organophosphate exposure was developed and presented to all participants that otherwise met the criteria for inclusion in the exposed or control study groups. For screening from Prolific, participants were required to select organophosphate exposure from a list of other possible occupational exposures, such as industrial solvents or fertilisers, which reduced the likelihood of them falsely claiming to have been exposed to access the survey for payment. These screening steps were carried out by Prolific during their own participant recruitment steps, before they were given access to the survey. A more detailed history of OP exposure was confirmed during the questionnaire.

2.3.4.4. Advertising and social media

Links and a description of the study were posted on several Open University social media accounts and were shared by other accounts and individuals within those networks, to increase awareness of the survey and encourage participation. Hashtags including #farming, #pesticides, #organophosphates and #sheep were included in posts in order to reach potentially interested parties. In addition, external accounts belonging to relevant individuals and organisations were identified by searching keywords relating to organophosphates, farming and general agriculture using Google search and on Twitter. Relevant parties were asked to disseminate information on how to take part in the survey to their readership, members, and followers.

During the initial phase of data collection, we encountered an issue when an industry organisation agreed to share the survey access link through their social media channels. Unfortunately, this led to a significant influx of suspicious survey responses originating from IP addresses located outside of the United Kingdom. These responses contained

consistently nonsensical answers and were completed within unrealistically short timeframes, indicating the involvement of automated bots. It was evident that these fraudulent responses aimed to exploit the promised compensation for survey participation. To address this situation, we immediately suspended the collection of responses. We took measures to identify vulnerabilities and removed all fraudulent responses from our dataset. The incident was reported to the Open University Human Research Ethics Committee. Once the identified vulnerabilities were addressed and the fraudulent responses were eliminated, we resumed data collection. To mitigate the risk of future attacks, we implemented several protective measures. Firstly, we adjusted the survey flow settings to automatically prevent multiple responses from the same IP address. This prevents individuals from submitting multiple entries and prevents skewing of the collection process. Secondly, we implemented a restriction on responses originating from IP locations outside of the UK, which helps maintain the focus on the intended target population. We also provided contact information for further inquiries, enabling participants to report any concerns they may have, particularly relating to the exclusion of valid responses, from UK agricultural workers currently outside the UK, or who's IP may not be visible through use of a virtual private network (VPN) for example. Furthermore, we implemented monitoring mechanisms to detect and flag responses with unrealistic completion times and nonsensical answers. By doing so, we aimed to filter out non-genuine submissions. Additionally, for questions that required text-based answers, we prevented participants from skipping those sections to discourage hasty and superficial responses.

2.3.5. Measures and procedures

All study participants could complete the questionnaires in their own time, at home or any convenient location, with any capable computer or mobile device with access to the internet. Questionnaires were designed to capture demographic data, along with information relating to pesticide exposure, health, and mood status.

2.3.5.1. Pesticide exposure history

Being able to accurately determine pesticide exposure history among individuals is critically important when trying to determine any associated adverse health effects. This presents a challenge when investigating the effects of longer-term, low-level exposure, where it is assumed that the effects may accumulate over prolonged use. Unlike with acute exposure, biomarkers found in samples such as blood or urine, including AChE activity levels or pesticide metabolites respectively, do not provide meaningful measures of ongoing exposure due to their transient nature (Wessels, Barr and Mendola, 2003). Therefore, estimates of exposure have historically been made using self-reported occupational experience. At the most basic level, this can simply involve directly comparing farm workers as an exposed group, against people who have never worked on farms as controls. However, in attempts to quantify OP exposure, more complicated self-report metrics have been proposed. Some of these consider factors like use of personal protective equipment (PPE), the type of work activities performed, or the number of animals kept, for example (Mackenzie Ross *et al.*, 2013). While such factors could be important and affect the likelihood and extent of exposure, the values and weightings assigned to each variable and calculations included in such formulae may be arbitrary and potentially misleading if not accurate.

In this study, in addition to separating exposed and control groups, a relatively simple metric was used to estimate the total number of hours for which each participant had been exposed over their lifetime (Mackenzie Ross *et al.*, 2013). This would allow comparison between exposed individuals and was determined by asking how long participants had worked with pesticides on a typical day, over how many days per week, weeks per year and how many years in total. Information was collected about the specific job roles and activities performed, which could indicate likely compounds and concentrations used in cases where participants did not know or remember such information. Rather than trying to calculate a value for any effect of PPE use on total exposure, participants were asked how often they used such equipment as a distinct variable. Not all the exposed group were currently working with pesticides, and so the time elapsed since their last exposure was recorded, as were details of any reported history of acute poisoning or diagnosis of pesticide specific illness. Participants were also asked to report how they perceived their own general health while working with pesticides, and how they felt after they had stopped working with them. Due to the diverse range of occupations and experiences with pesticides it was not possible to reliably assess the levels, concentrations, or often even the types of compounds had been used. For most of the participants it could be assumed that dermal exposure would be the most likely route, however, unless specifically stated this could not be directly assessed beyond self-reported activities and PPE use.

Construction workers were asked whether they had ever worked on farms or knowingly worked with pesticides, or if they had reason to believe that they had been exposed to OP compounds in any other way. Any indication or suspicion of OP exposure in individual construction workers resulted in them being excluded from the study.

2.3.5.2. Physical health

Since physical wellbeing and mental health state are closely interlinked (Ohrnberger, Fichera and Sutton, 2017) participants were asked about various aspects of their physical health. Firstly, they were asked to rate their own overall health on a five-point scale ranging from 'excellent' to 'terrible.' Participants were also asked to report any occurrence of several specific physical symptoms and their severity, some of which relate to cholinergic toxicity, such as muscular spasms or respiratory problems, and some likely-unrelated symptoms such as toothache. They were also asked to report any bodily pain they were experiencing on a scale of 0 (none) to 5 ('very severe').

2.3.5.3. Mood state

Assessment of participants' mood state was carried out via the questionnaire, using the 21-question version of the Depression Anxiety and Stress Scale (DASS-21: Lovibond and Lovibond, 1996). The DASS scale is a standardised psychometric scale which has been widely evaluated for research and clinical screening. Originally, the DASS-42 was developed as a 42-item scale split into three subscales of 14 items designed to measure symptoms relating to depression, anxiety, and stress, respectively. More recently the scale has been refined to an equally reliable 21-item version (DASS-21; (Lovibond and Lovibond, 1996; Henry and Crawford, 2005).

The DASS-21 is divided into subscales, with the 7 questions relating to depression concerning items such as dysphoria, anhedonia, and hopelessness. The anxiety scale assesses symptoms relating to physical arousal and a sense of fear or panic, while the stress scale explores participants' ability to cope with stressful situations, general irritability, and tendency to overact. Each item is scored on a scale of '0: did not apply to

me at all', 1: 'applied to me to some degree, or some of the time', 2: 'applied to me to a considerable degree, or a good part of the time' or 3: 'Applied to me very much, or most of the time'. The resulting scores for each scale can be doubled and compared against the same recommended cut-offs for the likelihood of disorder severity as are used in the DASS-42 scale (**Table 10**). Alternatively, the raw scores could be used as a continuous variable to compare relationships with other factors. These aspects of the scale make it a particularly useful tool for estimating the likely prevalence of mood related symptoms in agricultural and construction workers, and for investigating the relationship between OP exposure and mood related symptoms, respectively.

Table 10. Cut-off scores for the Depression Anxiety and Stress Scales DASS-21 and DASS-42. (Lovibond and Lovibond, 1996).

	Depression	Anxiety	Stress
Normal	0-9	0-7	0-14
Mild	10-13	8-9	15-18
Moderate	14-20	10-14	19-25
Severe	21-27	15-19	26-33
Extremely Severe	≥28	≥20	≥34

The DASS-21 has a Cronbach's alpha reliability score of 0.88 for the depression scale, 0.82 for anxiety, and 0.90 for the stress scale, and 0.93 overall (Henry and Crawford, 2005). These scores suggest that the DASS-21 and its subscales are reliable measures of mood related symptoms.

Furthermore, the relatively short form of the DASS-21 makes it a preferable option for inclusion in longer and more comprehensive questionnaires than other, longer measures. Questionnaire response rates are negatively affected by lengthy surveys, particularly

where questions are of a sensitive nature (Edwards *et al.*, 2009; Sahlqvist *et al.*, 2011). Therefore, due to the relatively wide scope and content of the present study the shorter DASS-21 set was chosen.

In addition to the symptomatic information provided by the DASS-21, participants were asked to rate their own depression, anxiety, and stress levels. They were asked to what extent their mood state caused them distress, and to what extent it impaired their work, home, leisure, or private lives, to better understand the relative impact of any effects on each individual.

2.3.5.4. Acutely stressful 'major events'

When measuring mood, it is important to consider potential life events that may result in mood changes. It is therefore important to consider such external factors with respect to how they influence mood, to avoid confounding study findings. Some life events which are known to cause stress will be experienced by most people at some point during their lives, such as starting a new job or moving to a new home. Other such events most people would try to avoid, such as having contact from debt collectors, or being fired from work. To measure how participants in this study had each been affected by such events the Social Readjustment Rating Scale (SRRS: Holmes and Rahe, 1967) was included in the online survey. The SRRS is a checklist of 43 known stressful events rated and scored in order of their perceived impact. For example, death of a spouse or long-term partner attracts the highest possible score of 100 points. Conversely, a change in eating habits earns a lower score of 15 points. The sum of events over the previous 12 months was tallied to produce a final score representing 'major stressful events' for each participant. The SRRS has been used previously to measure recent stressful experiences in OP

research and provides critical information relating to depression and anxiety (Harrison and Mackenzie Ross, 2016). Importantly however, the scale only accounts for individually occurring events, most of which are unlikely to regularly reoccur. It does not account for the potential effects of ongoing issues leading up to or following each event, or factors which may not be attributable to any specific event. For example, losing one's job could result in financial hardship, but financial hardship can also exist in the absence of any recent or specific causal event.

2.3.5.5. Day to day stressors

Life as a farmer can involve several unique lifestyle factors which may impact psychiatric health. While previous OP studies have considered the importance of stressful life events, none appear to have considered the psychological impact of chronic, ongoing, or everyday stressors which may be particularly evident in farming cohorts. The inclusion of such factors is critical. For example: 'a change in social activities' according to the SRRS would count as a stressful event, scoring 18 points. Whereas an individual who had experienced complete social isolation over several years would score no points for this, because the SRRS only concerns events which have occurred in the previous 12 months, and only when circumstances have changed. This does not consider ongoing situations which may also cause stress. Studies have shown strong links between social isolation and psychiatric disorders, including anxiety and depression (Matthews *et al.*, 2016; Santini *et al.*, 2020), which highlights the importance of considering ongoing stressors alongside emerging events.

To investigate the impact of 'day-to-day' stressors the Daily Hassles and Uplifts Scale (Kanner *et al.*, 1981) was adapted for use in this study. The original scale consisted of 117

daily hassles, which are "irritating, frustrating, distressing demands that to some degree characterise everyday transactions with the environment" (Kanner *et al.*, 1981). Included hassles are items such as: 'the weather,' 'doing paperwork,' 'feeling lonely,' 'financial issues,' and 'children.' The uplifts scale asks about factors which could be seen to alleviate stress levels and includes some of the same items as the hassles scale, including 'children,' and 'the weather.' From a sample of 100 people, Kanner et al. (1981) found that the hassles scale more accurately predicted stress than the SRRS. Furthermore, although uplifts did seem to alleviate stress in females, the same effect was not detectable in male participants. Consequently, the hassles section of the scale was selected for use in this study in addition to the SRRS. To minimise questionnaire length, and to account for the gender related disparity, the uplifts scale was not used.

2.3.6. Statistical analysis

To test whether the exposed and non-exposed groups were matched according to key demographic characteristics, between-groups t-tests or chi squared tests were used as appropriate.

To explore the effect of exposure to organophosphates on mood and other health outcomes, between-group comparisons were made between the exposed and control cohorts using independent multivariate analysis of variance (MANOVA) with both depression and anxiety scores as outcome measures representing mood as the dependent variable. Follow-up univariate ANOVAs were then performed separately on each mood component. Independent group comparisons between the exposed and control cohort were also made for measures of health, more generally, using between-groups t-tests, one-way ANOVAs or chi squared tests as appropriate.

To identify any common themes among the physical symptoms or stressors participants reported experiencing, factor analysis (principal components analysis) was used. Follow up t-tests were used to identify any differences on factor scores between the exposure group and the controls.

For the exposure group, the relationship between factor scores, mental health and exposure was explored further by correlating factor scores with mood, stress, health, and lifestyle variables, along with measures relating to OP exposure using Spearman's rank correlations. To explore these relationships in more depth, and to better understand the possible direct and indirect relationships between these variables, mediation analyses were carried out.

3. Characterising the effect of low-level CPF exposure on *C. elegans* behaviours associated with mood related neurotransmitters.

3.1. Introduction

3.1.1. Choosing a 'low-level' concentration from the literature

In order to study the effects of low-level OP exposure on neurotransmitter systems related to mood it is necessary to define a low-level OP concentration that does not exert acute effects on AChE system. High concentrations of OP that acutely inhibit AChE have previously been shown to cause developmental defects and paralysis which prevent the study of *C. elegans* behaviours associated with altered mood-related neurotransmission. A universally accepted concentration for low-level CPF exposure in *C. elegans* has been difficult to establish and can depend on several factors such as the exposure method, or the endpoint being measured. Several methods for exposing *C. elegans* to CPF have been reported (Roh and Choi, 2008; Ruan *et al.*, 2009; Boyd *et al.*, 2010; Ju *et al.*, 2010; Fischer *et al.*, 2016; Roh, Lee and Kwon, 2016). Exposing worms in liquid media helps to measure the actual concentration that worms come into contact with however, despite some practical benefits of liquid exposure, the method limits the possible treatment duration and can lead to starvation or lethality from prolonged immersion in liquid. Roh, Lee and Kwon (2014) reported body deformation and death by body rupture in worms that were immersed in liquid for longer than 10 hours. Body rupture was also observed during preliminary experimentation during this project and so exposure in liquid was deemed unsuitable.

Mixing CPF into NGM before pouring plates and allowing them to set allows otherwise standard laboratory conditions to be maintained during exposure. This includes the provision of food, and a solid surface on which worms can behave freely, thus allowing a greater range of behaviours to be tested under low-level CPF treatment. Exposure of *C. elegans* to CPF in NGM plates has been demonstrated previously (Viñuela *et al.*, 2010). Taken together this suggests that mixing CPF into NGM plates would be more suitable to studying low-level exposure, and so a concentration was chosen that had previously been described as "low-dose" (Viñuela *et al.*, 2010). Viñuela *et al.* (2010) mixed 0.5 mg/L CPF into NGM plates and used this concentration to explore processes affected other than AChE inhibition in *C. elegans*. They reported transcriptional responses relating to several processes, including stress, immunity, and lipid metabolism, despite 0.5mg/L CPF being well below established EC50s for measures such as growth or egg-laying behaviour (Anderson, Cole and Williams, 2004a; Martin, Svendsen, Lister, Gomez-Eyles, *et al.*, 2009; Boyd *et al.*, 2010; Viñuela *et al.*, 2010).

3.1.2. Choosing an exposure duration

The chosen exposure duration needed to be long enough for CPF to affect behaviour, especially because the route of administration is difficult to determine, and the effects of low-level exposure may be more difficult to detect than those following higher-level exposure. Due to the difference in life cycle, and life span, between nematodes and mammals, there was no intention to directly model the exposure time against the equivalent lifespan for humans. However, depending on genotype and rearing conditions, the average life span of *C. elegans* is around 18-20 days (Zhang *et al.*, 2020). Therefore, 24 hours exposure would equal around five percent of the worm's expected life. In 2018-2020, the UK life expectancy for human females was 82.9 years, and 79.0 for males (Office

for National Statistics, 2021). A direct comparison would suggest that a 24-hour exposure in *C. elegans* would represent around 4 years in a human life. Although the toxicodynamic effects may share similarities at a molecular level, it is likely that the longer-term effects would continue to diverge over several years, due to a multitude of confounding internal and environmental factors. Therefore, such direct comparisons may not be useful. Therefore, the intention was not to create a realistic model of human exposure duration, but rather to use *C. elegans* to explore the effects of low-level exposure only. The selection of the 24-hour exposure period was therefore based on practical considerations; this represents the maximum possible exposure time between the nervous system being fully-generated at the L4 larval stage (**Figure 12**), and the optimal stage for assaying behaviour as a 1-day old adult. After this stage, several potentially confounding factors emerge, such as the production and presence of new eggs and progeny laid by gravid-adults, and the effects of aging.

3.1.3. The relevance of exposure method to route of administration.

Investigations into the order of toxicity of substances have shown good agreement between mortality in *C. elegans* exposed to toxicants mixed into NGM plates, and both mouse and rat oral LD50 rankings, (Williams and Dusenbery, 1988). Similarly, close correlations were found between the order of effects on locomotory speed in *C. elegans* treated with 15 OP compounds in liquid, and the rank order of mouse and rat LD50 (Cole, Anderson, and Williams, 2004). Despite these correlations, it is difficult to determine whether the route of administration for *C. elegans* exposed to toxicants via NGM plates, or in liquid media, could most accurately be compared to oral or dermal exposure in mammals. Regarding exposure in liquid, it is known that *C. elegans* ceases to feed while swimming (Vidal-Gadea *et al.*, 2012). Such cessation of feeding could suggest that dermal

exposure during drug treatment in liquid is most likely. Vidal-Gadea *et al*., (2012) showed that worms will readily ingest a fluorescent dye solution while crawling on a solid NGM surface for 10 minutes, regardless of whether a food source is present. However, worms swimming in water for the same amount of time ingested only trace amounts of the dye. Nevertheless, if even a small amount of a substance is ingested, then a strictly dermal administration route cannot be determined, particularly over longer treatment periods.

In comparison to liquid, the *C. elegans* behavioural repertoire is less restricted on the solid surface of an NGM plate, including a higher rate of feeding behaviour. *C. elegans* is a filter feeder, meaning that it eats by drawing in bacteria suspended in liquid, then filters the bacteria, and expels the liquid (You and Avery, 2012). When CPF is mixed into molten NGM and then dried to form plates, it would seem unlikely that the exposure follows a directly oral route, since the CPF is not added to the bacterial solution used to seed the plates as a food source. CPF is not soluble in water (PubChem, 2022), but it should be noted that some bacteria interact with xenobiotics. Notably, this is true for *E. coli*, which is able to degrade CPF, producing: chlorpyrifos-oxon, which is the more toxic metabolic end product of CPF, and diethyl phosphate (Harishankar, Sasikala and Ramya, 2013).

Little is known about exactly how CPF is taken up by *C. elegans* (Hartman *et al.*, 2021; **Figure 16**). It is therefore difficult to specify a particular route of administration, but both oral and dermal are likely to some extent, regardless of exposure method (Kudelska *et al.*, 2017). It is therefore difficult to extrapolate toxicokinetic properties of exposure to humans or other mammals. However, the value of the *C. elegans* model is drawn partly from the physiological differences from humans; the relative simplicity of the animal means the toxicokinetic mechanisms are not directly comparable, but at the same time, these attributes offer a level of practicality that enables relatively high-throughput

investigations of the toxicodynamic interactions of toxicants with receptors, proteins, and other molecules that are conserved.

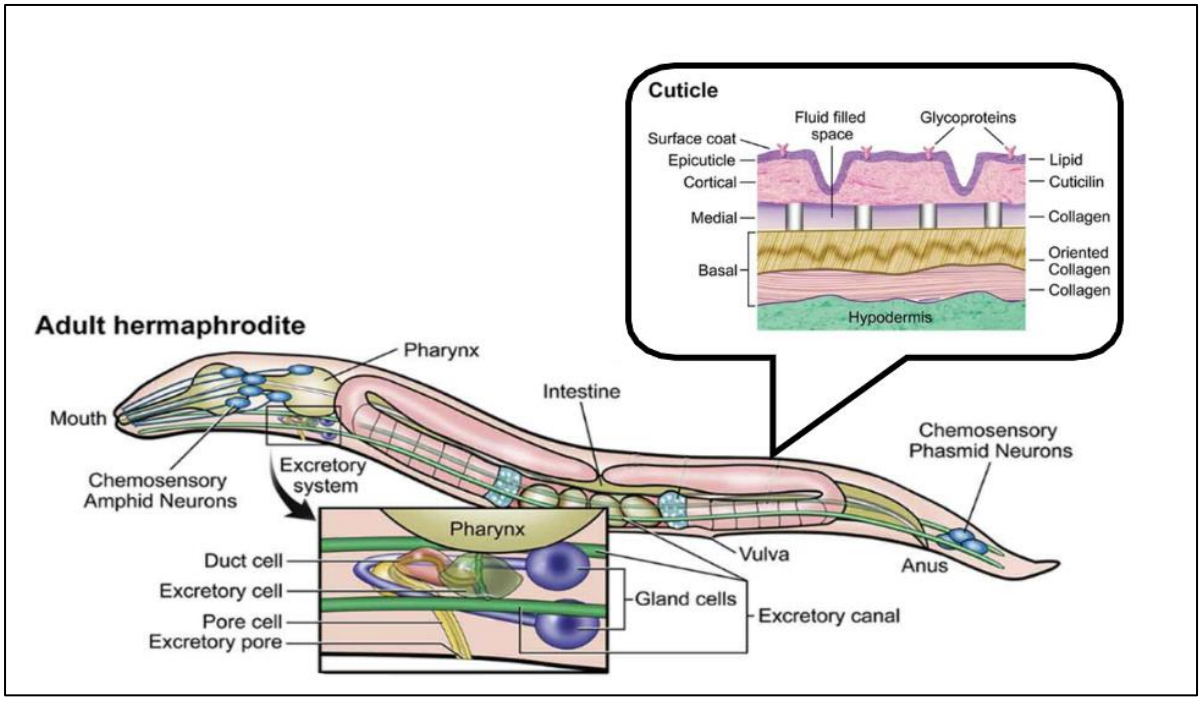

Figure 16. Several routes of entry for xenobiotics in *C. elegans* have been proposed. The cuticle provides the primary method of protection from the environment. Some substances and toxicants are more readily taken up through the cuticle than others (Harlow, Perry, Widdison, Daniels, Bondo, Benjamini, *et al.*, 2016), but the feeding apparatus, excretory system, and sensory cilia, are additional possible routes for entry. The anatomy of these components is illustrated (Adapted from: Hartman et al., 2021).

3.1.4. Using C. elegans to investigate mood-related neurotransmission.

C. elegans is a useful model for investigating neurotoxic effects of OPs, having shown parallels with mammalian neurotoxicity (Cole, Anderson, and Williams, 2004). The model is useful in part because it shares common neurotransmitter systems such as DA, 5-HT, and acetylcholine, among others (Daniel L. Chase and Koelle, 2007). In addition, the

surprisingly rich behavioural repertoire of *C. elegans* has been well characterised, particularly with respect to how behaviours depend on neurotransmitter function. As a result, *C. elegans* has often been used to study drugs with therapeutic use in the treatment of mood disorder (Choy and Thomas, 1999; Petrascheck, Ye and Buck, 2007; Bermingham *et al.*, 2016; Weeks *et al.*, 2018). Whilst no attempt is made to ascribe 'depression-like' or 'anxiety-like' behaviours to *C. elegans*, there are well established behavioural assays for investigating DA and 5-HT pathways, which will now be discussed.

3.1.5. Swimming induced paralysis as a paradigm to investigate the DA signalling pathway in C. elegans.

In addition to being able to crawl along solid surfaces, wild-type *C. elegans* can swim for long periods in water. Nematodes swim by thrashing from side-to-side at relatively high frequency compared with normal crawling (Vidal-Gadea *et al.*, 2011). This swimming ability is impaired in mutants deficient in DA transporter (DAT-1) expression, which fail to swim continuously for more than a few minutes without experiencing temporary and reversible paralysis (McDonald *et al.*, 2007). This behaviour has been termed swimming induced paralysis (SWIP). Originally discovered in *dat-1(ok157)* loss-of-function mutants by McDonald et al. (2007), who showed the paralysis is mediated by an excess of DA in the synaptic cleft. This is caused in part by the rapid thrashing movement and the lack of reuptake and clearance of DA by missing DAT-1 transporters. The excessive build-up of DA leads to hyperactivation of DOP-3, D2-like, G-protein coupled receptors at the postsynaptic terminal leading to paralysis. Consequently, drugs such as the dopamine receptor antagonist azaperone, or genetic tools which impair DOP-3 receptor function, or DA release or synthesis can prevent SWIP (Refai and Blakely, 2019). In addition to the well-characterised mechanisms described for *dat-1* mutants, SWIP can also be observed

in wild-type animals following DA-related pharmacological interventions. For example, several drugs that block DAT-1 in *C. elegans*, such as: imipramine, nisoxetine or methylphenidate can induce SWIP, although these also notably affect the noradrenaline transporter in mammals (Carvelli, Blakely and DeFelice, 2008; Bermingham *et al.*, 2016). Whereas amphetamine can lead to concentration dependent onset of SWIP through increased presynaptic DA release, in addition to any effect on DAT-1 mediated reuptake (Carvelli, Matthies and Galli, 2010).

The SWIP behaviour is well characterised and offers a useful tool for probing for effects of neuroactive compounds on DA pathway function. Altered DA transmission has been linked to depression (Dunlop and Nemeroff, 2007), and most of the drugs described above as modulating the SWIP response, are also known to moderate mood in people (Lemberger *et al.*, 1976; El-Mallakh, 2000; Bruijn *et al.*, 2001). It would seem therefore, that the SWIP assay could be a valuable tool with which to investigate the effects of OPs on DA function.

3.1.6. Egg-laying: a well-characterised behaviour involving the 5-HT signalling pathway.

Egg-laying in *C. elegans* has proven to be a useful system with which to investigate neuronal signalling and the molecular mechanisms required to perform specific behavioural functions (Koelle and Horvitz, 1996). The relative simplicity of the egg-laying behaviour, and its suitability for genetic analysis, has encouraged its widespread use, and as such the mechanisms involved in egg-laying are particularly well-characterised (Lints and Hall, 2004; Schafer, 2005; Ruan *et al.*, 2012).

Conveniently, egg-laying has helped to characterise several mood-therapeutic drugs and their interactions with relevant conserved neurotransmitter pathways (Dempsey *et al.*, 2005). One of the key components of egg-laying behaviour in *C. elegans* is 5-HT signalling (Schafer, 2005), and drugs which target 5-HT are the most common of those prescribed for psychiatric disorders, including mood disorder (Dempsey *et al.*, 2005). Consequently, several antidepressant drugs, exogenous neurotransmitters, and genetic manipulations have been used to elucidate the mechanisms of egg-laying in *C. elegans*, and vice versa (Dempsey *et al.*, 2005; Schafer, 2006; Brewer *et al.*, 2019; Fernandez *et al.*, 2020).

Treatments or mutations can affect egg laying in two opposing ways: laying rates can be increased, resulting in eggs being laid prematurely, before they have developed to the 8-cell stage (**Figure 17**). Assuming that the rate of egg production is unchanged this will also lead to fewer eggs being retained within the uterus than normal. In comparison, wild-type animals typically lay fewer than 10% early-stage eggs and retain ~10-15 eggs in the uterus (Bany, Dong and Koelle, 2003). Increased egg laying can be induced by the application of exogenous 5-HT, and also with treatment of serotonin reuptake inhibitors such as fluoxetine (Prozac), or tricyclic antidepressants such as imipramine (Dempsey *et al.*, 2005).

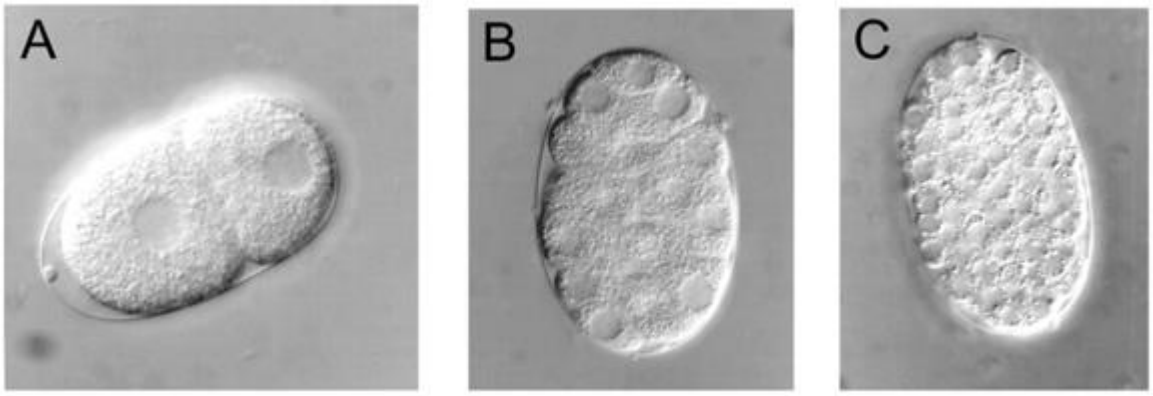

Figure 17. *C. elegans*. eggs can be staged at different stages of development. A) shows an early stage, 2-cell embryo. B) Eggs containing more than 8 cells are considered as beyond 'early stage'. C) Most wild-type eggs contain between 30-100 cells when laid (adapted from: Bénard et al., 2001).

The alternate egg-laying phenotype is seen in animals which are defective in egg laying. This leads to a reduction in egg-laying frequency and, assuming that the rate of egg production remains normal, more than the normal number of eggs being retained within the uterus. These animals will appear bloated by the abnormal number of late-stage eggs being carried inside, and in extreme cases progeny can hatch inside the adult (Schafer, 2006). Aside from the numerous mutant strains which cause some level of egg laying defect (termed: Egl, short for egg-laying-deficient; Schafer, 2006), egg-laying frequency is reduced in the absence of available food, and also following exogenous DA treatment (Dempsey *et al.*, 2005).

Egg-laying behaviour therefore offers another well-characterised paradigm with which to explore potential interactions of CPF with neurotransmitter systems, which might help to elucidate any potential interactions with conserved targets related to human psychiatric disorder.

3.1.7. Using locomotory behaviour as a measure for low-level CPF exposure

Locomotion in *C. elegans* is regulated by multiple conserved neurotransmitter pathways. For example, ACh is the major excitatory neurotransmitter responsible for muscle contraction, and can therefore initiate movement through intermittent signals, or cause paralysis if the signal is prolonged, such as in cholinergic toxicity (Rand, 2007a). Glutamate is another largely excitatory neurotransmitter, which works antagonistically with the mostly inhibitory neurotransmitter GABA; this pair are responsible for the characteristic sinusoidal movement of *C. elegans*, during which glutamate and GABA alternately cause the body-wall muscles to rhythmically contract and relax. Other conserved neurotransmitters such as 5-HT and DA moderate behaviour in various ways depending on the internal state of the animal or the environment, and the difference in whether most neurotransmitters are excitatory or inhibitory is often decided by the different types of receptors present (Brockie *et al.*, 2001; Dittman and Kaplan, 2008; Gürel *et al.*, 2012; Omura *et al.*, 2012). This could be of use for detecting possible interactions of CPF with neurotransmitter systems that have been implicated in mood disorder (Möhler, 2012; Sanacora, Treccani and Popoli, 2012; Saricicek *et al.*, 2012; Cowen and Browning, 2015; Belujon and Grace, 2017). Furthermore, CPF has previously been shown to affect locomotory behaviour in *C. elegans* (Anderson, Cole, and Williams, 2004a; Ruan *et al.*, 2009b; Boyd *et al.*, 2010).

Different forms of movement and their underlying mechanisms have been well-characterised. *C. elegans* moves primarily by crawling with a sinusoidal movement along a solid surface (**Figure 18**).

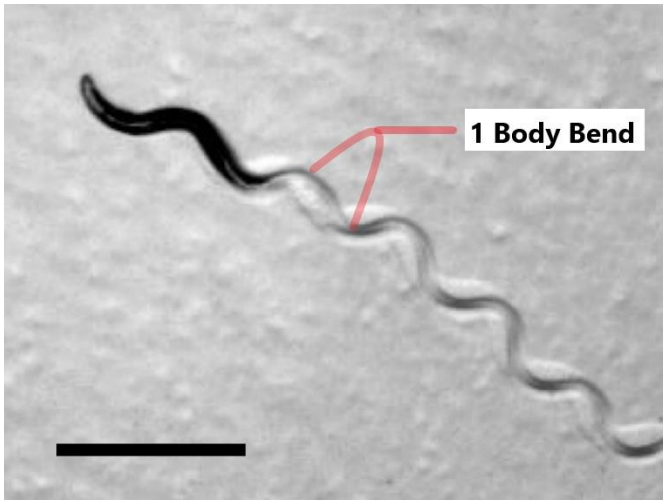

Figure 18. *C. elegans* exhibits a distinct sinusoidal locomotory pattern. The sinusoidal movement is captured during movement on a solid NGM plate seeded with *E. coli*. The track marks left behind by the nematode on the bacterial surface clearly depict the characteristic 'S' shaped movement pattern achieved through a series of body bends. The inclusion of a scale bar (1 mm) provides a reference for the observed behaviour (adapted from: Park et al., 2008).

The traditional method for quantifying locomotory behaviour on solid surfaces is to measure body bends (Hart, 2006). Body bends are measured by observing linear movement and counting each time the area behind the head moves from side to side, as evidenced by the track shown in **Figure 18**. Locomotion can also be measured using computerised tracking and this will be discussed further in the next chapter.

C. elegans can also swim in liquid by performing a side-to-side 'thrashing' movement and crawling and swimming require distinct patterns of neuromuscular activity. Whereas DA regulates the transition from swimming to crawling, 5-HT is necessary and sufficient to switch from crawling to swimming (Pierce-Shimomura *et al.*, 2008; Vidal-Gadea *et al.*, 2011).

The role of DA in swimming induced paralysis as discussed earlier, in combination with crawling and wild-type swimming behaviour present opportunities to investigate the effects of CPF in relation to DA and 5-HT signalling. Additionally, the known roles of acetylcholine and other neurotransmitters in locomotion could provide indications of any interaction of those pathways with low-level CPF treatment.

3.1.8. Body length as a measure of CPF exposure and AChE activity

C. elegans body length can be used to detect the effects of exposure to some pesticides, including CPF. Reduced body size can occur as a result of impaired developmental processes impacting normal growth rate (Boyd *et al.*, 2009). However, although a high degree of conservation exists between *C. elegans*' and mammalian developmental processes, these are perhaps less relevant to occupational exposure in adult farm workers. Outside of developmental effects, body length in *C. elegans* can be reduced by hypercontraction of the body wall muscle, which is induced by pesticides that inhibit AChE , and not by those which do not inhibit the enzyme (Kearn *et al.*, 2014).

Studies using CPF have produced mixed results, notably with no effect on body length being reported at lower concentrations (J.-Y. Roh and Choi, 2008), and reductions in body length in animals exposed to higher concentrations (Ruan *et al.*, 2009b; Boyd *et al.*, 2010).

Measuring body length could therefore be useful for two reasons: Firstly, since CPF appears to affect body length at higher, but not lower concentrations, the measure could provide some indication as to whether the concentration being used is appropriate for studying the effects of low-level exposure. Secondly, changes in body length could provide useful information when trying to dissect the mechanisms responsible for any change in locomotory behaviour. One of the ways in which locomotion can be regulated

is through altered muscle tone caused by cholinergic signalling, and such changes in muscle tone are accompanied by altered body length (Petzold *et al.*, 2011; Hwang *et al.*, 2016). The biomechanics of the motor system comprise nerve cords made up of motor neurons which span the length of the worm's body (**Figure 19**), terminating at neuromuscular junctions (White *et al.*, 1986). Contraction of the body wall muscles is caused by acetylcholine (Lewis *et al.*, 1980; Fleming *et al.*, 1997) and the same muscles are relaxed by GABA signalling (**Figure 19**) at these junctions (Mclntire *et al.*, 1993; Hernando and Bouzat, 2014). These excitatory and inhibitory effects can be mimicked by drugs which target the respective pathways: the GABA receptor agonist muscimol can cause flaccid paralysis and slight body-lengthening in *C. elegans* (Mclntire *et al.*, 1993; Hernando and Bouzat, 2014), whereas the acetylcholine receptor agonist levamisole causes spastic paralysis and body-shortening (Petzold *et al.*, 2011). A similar body-shortening effect is seen in response to increased acetylcholine signalling as a by-product of treatment with the AChE inhibitor aldicarb (Nguyen *et al.*, 1995; Kearn *et al.*, 2014). Therefore, since CPF also inhibits AChE it is likely that it would also cause body-shortening with significant inhibition, perhaps explaining some of the changes in body length reported at higher CPF exposures (Ruan *et al.*, 2009b; Boyd *et al.*, 2010).

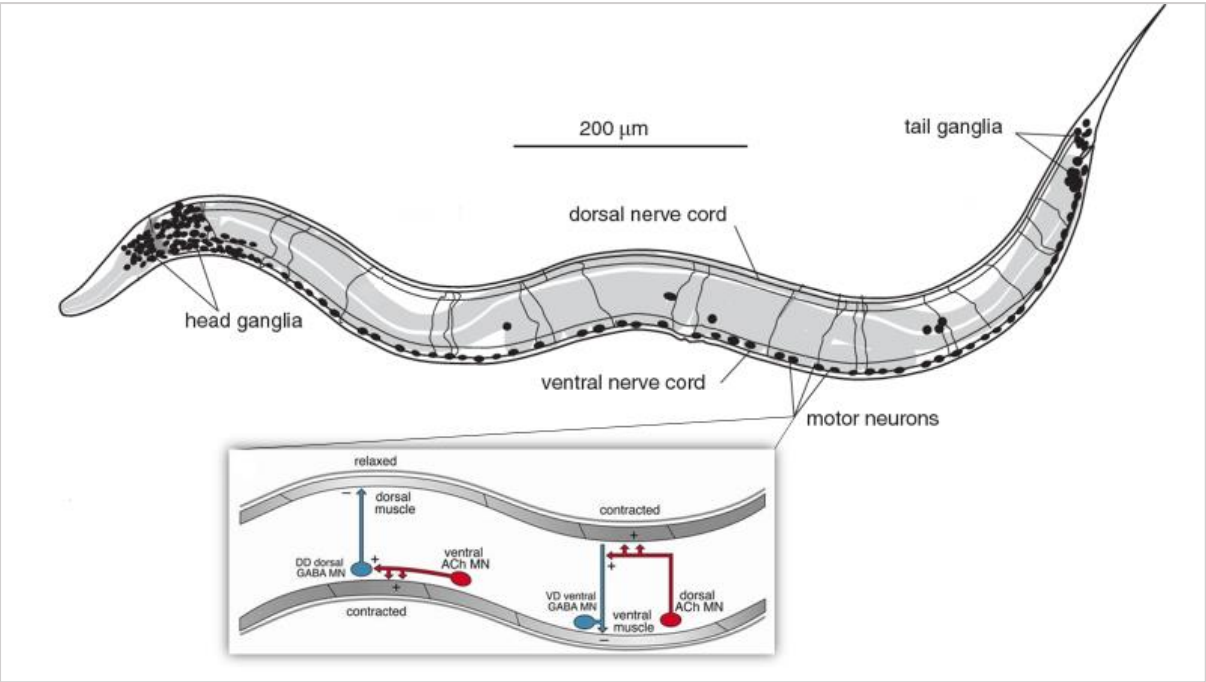

Figure 19. Shows *C. elegans* nerve cords in relation to body wall muscle contraction. The ventral nerve cord houses motor neuron (MN) cell bodies, as shown. The inset reveals cholinergic motor neurons (red) that innervate GABAergic motor neurons (blue) along with the ventral and dorsal body wall muscles. During normal locomotion, the coordinated alternation of acetylcholine (ACh) release contracts muscles on one side while stimulating GABA release to relax muscles on the other side (Adapted from Schuske, Beg and Jorgensen (2004) and Hobson, Yook, and Jorgensen (2017).

Taken together these points suggest that it could be useful to test whether and how body length is affected at 0.5 mg/L CPF. This could provide some insight into the cause of any observed effect on locomotion, and whether cholinergic or GABAergic signalling is involved.

3.2. Chapter objectives

1. To test the behavioural response of *C. elegans* to 0.5 mg/L CPF, which has been described previously as a "low dose" concentration in nematode growth media (Viñuela *et al.*, 2010).
2. To investigate the effects of CPF treatment in response to neurotransmitter pathways relevant to human mood disorder using established and well-characterised behavioural assays.
3. To evaluate the use of *C. elegans* body length as a physiological measure of CPF treatment and/or AChE inhibition.

3.3. Results

3.3.1. 0.5mg/L CPF does not cause SWIP in wild-type C. elegans

To explore whether 0.5mg/L CPF exerts any effects through transmission in the DA pathway some behavioural assays with known sensitivities to DA signalling were selected to test for any noticeable effects. Swimming-induced paralysis in *C. elegans* is a behaviour which is sensitive to perturbations of several aspects of DA signalling (3.1.5). Wild-type nematodes do not paralyse within 10 min of transfer to water, and so wild-type animals exposed to 0.5 mg/L CPF or vehicle control for 24 hr were tested for SWIP response to determine whether CPF induced SWIP behaviour.

More than 80% of wild-type animals did not paralyse within 10 min of swimming and this phenotype was not affected by treatment with CPF (**Figure 20**). Moreover, CPF treated, and vehicle control wild-type worms were observed to still be swimming at least 30 min later (personal observation), providing no evidence for CPF induced SWIP.

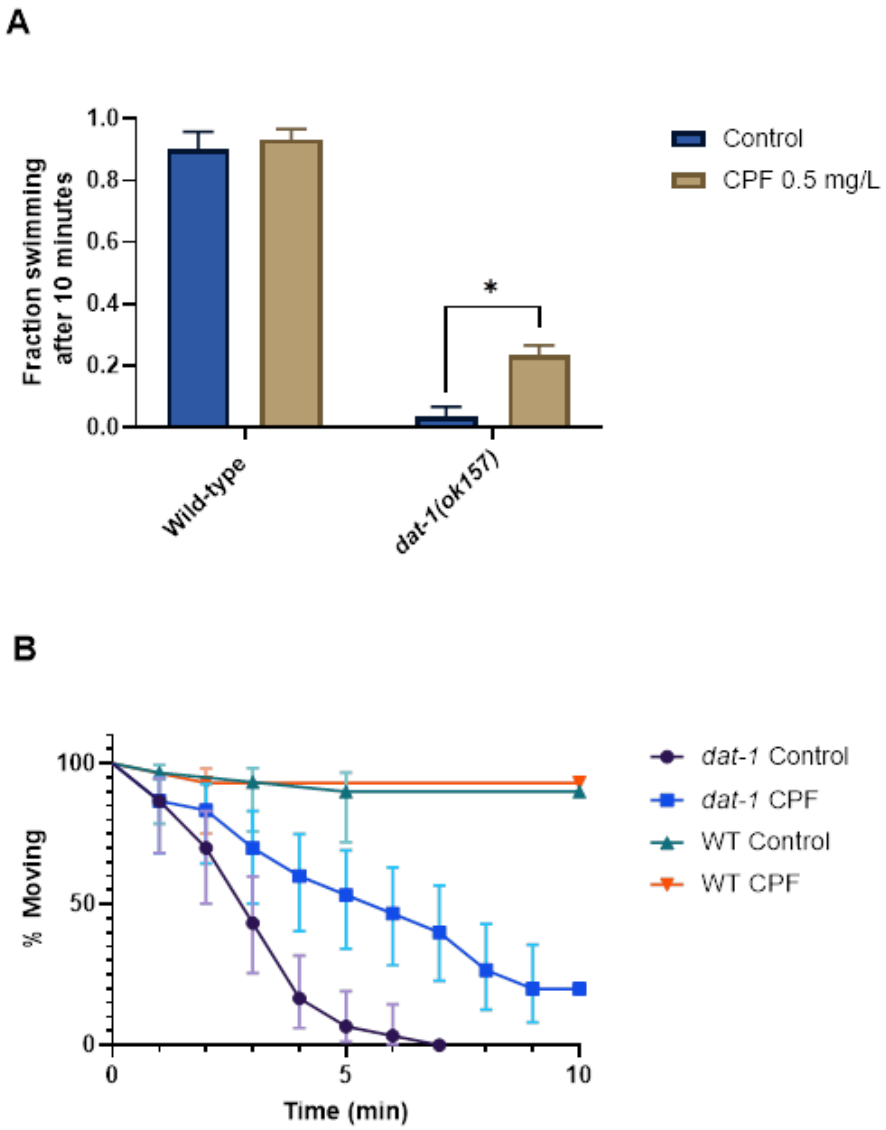

Figure 20. CPF does not induce SWIP in wild-type animals but does reduce SWIP observed in a DAT-1 mutant. Panel (A) shows CPF treatment does not affect sustained swimming behaviour in wild-type animals. Swimming induced paralysis (SWIP: Refai and Blakely, 2019) naturally occurs in untreated *dat-1(ok157)* mutants. SWIP was observed in *dat-1(ok157)* mutants but was reduced by CPF treatment (Panel A: right). In Panel (B), CPF treatment significantly delays the onset of paralysis in *dat-1(ok157)* mutants, highlighting its effect in reducing SWIP. Data gathered in triplicate with n=10 per strain and treatment; mean values are represented, and error bars denote 95% confidence intervals. * = $p < 0.05$.

C. elegans deficient in the DA transporter DAT-1 are known to exhibit SWIP within 10 min (McDonald *et al.*, 2007), and so *dat-1(ok157)* mutants were exposed to the 0.5 mg/L CPF and observed to test whether CPF suppressed or enhanced the SWIP response (**Figure 20**).

Nearly all *dat-1(ok157)* mutants exposed to acetone paralysed within 10 min of swimming (**Figure 20**). Onset of SWIP in *dat-1(ok157)* was significantly reduced by CPF as although all *dat-1(ok157)* animals exposed to acetone as a vehicle control had paralysed by the 10 min point, 10-20% of CPF-exposed *dat-1(ok157)* nematodes continued to thrash beyond 10 min (**Figure 20**).

These results indicate that CPF does not induce SWIP in wild-type animals, suggesting that it is unlikely that its effects are due to inhibition of DA reuptake. Interestingly exposure to CPF did appear to partially supress the SWIP phenotype of *dat-1(ok157)* animals suggesting that CPF may have some effect in animals deficient in DA reuptake although the observed effect was relatively small.

3.3.2. Locomotory rate in liquid was not significantly affected by 0.5 mg/L CPF

Although swimming wild-type animals did not show full paralysis following exposure to 0.5 mg/L CPF, it was still possible that other measures of *C. elegans* swimming behaviour were affected. It was even considered possible that inhibition of AChE was causing some level of muscular paralysis, and consequently preventing the vigorous motor activity necessary to induce SWIP in *dat-1(ok157)* mutants (McDonald *et al.*, 2007).

To test for other effects of CPF on swimming locomotion, wild-type animals were exposed to either 0.5mg/L CPF or acetone vehicle control and observed for 10 min while swimming as described above. The frequency of head-thrashes was counted, for the first

and last minute to determine whether there was any change in swimming rate over time (**Figure 21**).

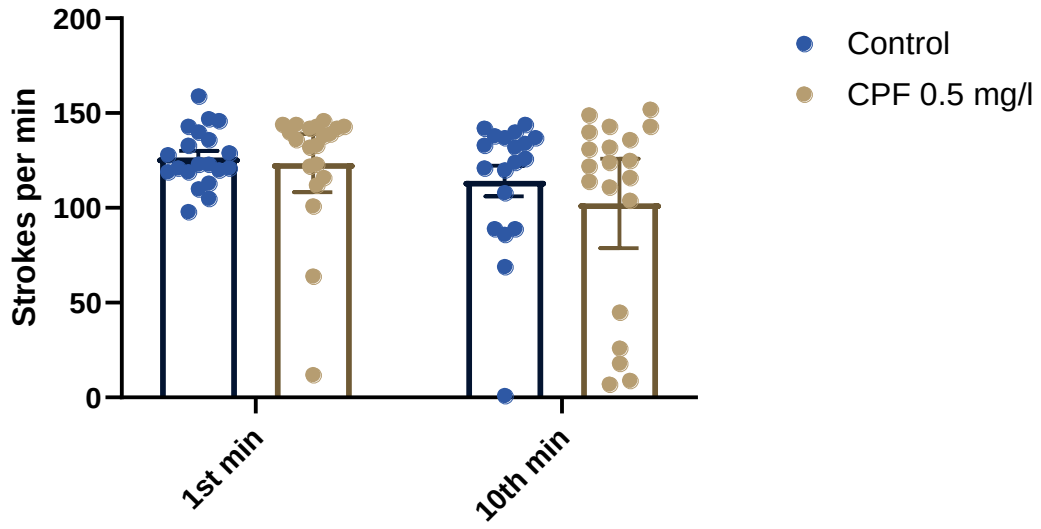

Figure 21. Swimming rate of wild-type *C. elegans* is not affected by treatment with 0.5 mg/L CPF. *C. elegans* exposed to 0.5 mg/L CPF or vehicle control swam for 10 minutes. The number of thrashes was recorded for the first, and the last minute to test for any change in swimming activity over time (n = 20 control, n = 20 CPF). Bars show mean and 95% confidence intervals.

Overall, the mean number of strokes was lower in the tenth minute than in the first minute of swimming across both treatments ($F(1, 75) = 4.413$, $p < 0.05$. 95% CI [0.87, 32.82], however this seemed to be driven by a few individuals rather an effect on the whole group (**Figure 21**). CPF did not cause any significant change in swimming rate measured by strokes per minute, and although a few individuals in the CPF treated group

swam more slowly (**Figure 21**), this did not cause a significant difference between treatments overall .

These results suggest that swimming behaviour, measured by thrashing frequency at least, may not be an effective measure of low-level CPF exposure in *C. elegans*. It is possible that the slower swimming speeds observed in a small number of CPF-treated individuals is somehow linked to the small proportion of animals that did not paralyse during the SWIP assays (3.3.1), perhaps by a threshold effect of cholinergic muscle paralysis.

3.3.3. Egg laying as a measure of low-level CPF exposure in C. elegans

To test whether CPF caused any observable egg-laying phenotype, wild-type hermaphrodites were exposed to 0.5 mg/L CPF or vehicle control for 24 hours. Egg-laying behaviour was assessed using two standard methods: firstly, the number, and developmental stage, of eggs laid by worms over a 30-minute period was recorded. The number of eggs being carried inside the uterus was also determined, by bleaching gravid adults to release unlaid eggs (**Figure 22**).

3.3.4. Egg-laying rate and developmental stage were not affected by 0.5 mg/L CPF

After 30 mins, the total number of eggs laid by animals treated with 0.5 mg/L CPF was not significantly different from the vehicle control group (**Figure 22**). Both groups laid more than 90% late-stage eggs with 9 or more cells. Vehicle control animals laid 7.5% at the 5-8 cell stage, compared with 9.95% in CPF-treated animals. There were no eggs laid at any earlier stage than the 5-8 cell stage in either group.

3.3.5. Egg-retention was not affected by 0.5 mg/L CPF

A similar number of eggs were retained in animals treated with 0.5 mg/L CPF and the vehicle control group (**Figure 22**), with vehicle control animals retaining an average of 11.33 eggs per animal, compared with an average of 10.67 in the CPF-treated group.

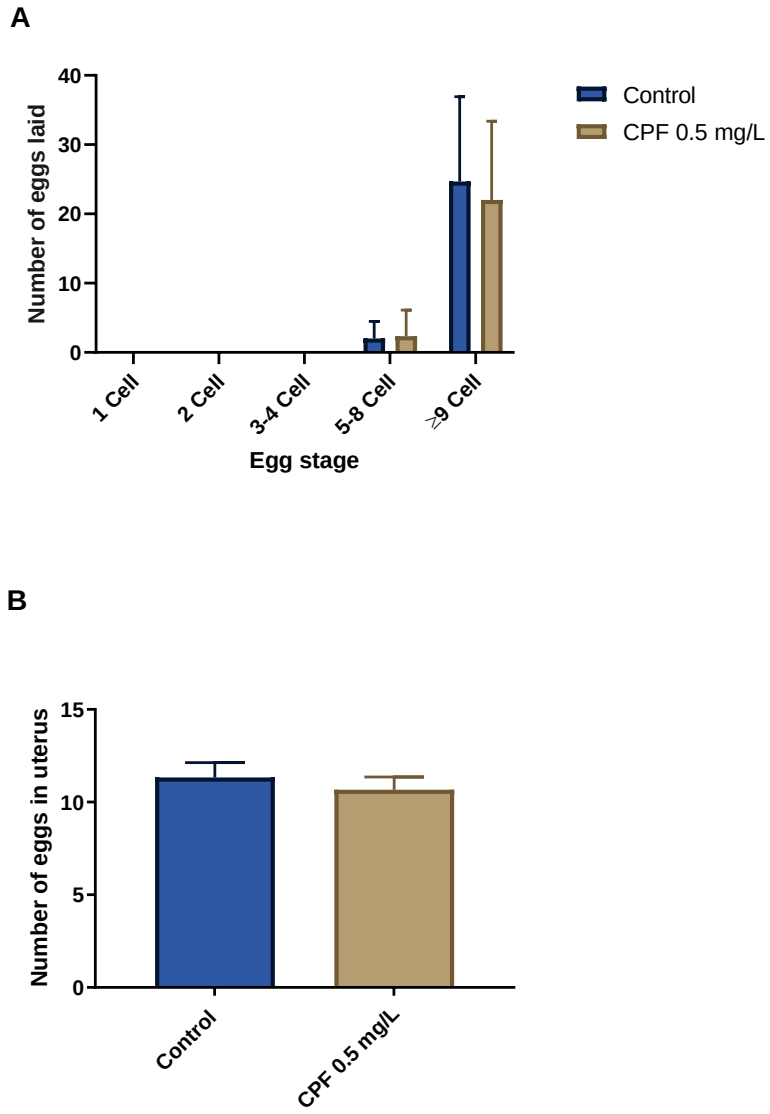

Figure 22. CPF treatment does not affect egg laying behaviour in wild type *C. elegans*. Egg-laying behaviour was measured by counting the number of eggs-laid in 30 minutes (A) and the number of eggs retained in each animal (B). There was no significant difference between animals treated with 0.5 mg/L CPF and vehicle controls in either assay. For the egg-stage assay 10 animals were assayed from each treatment and the assay was repeated on three separate days. The egg-retention assay included 8 individuals from each treatment and was repeated on three separate days. Bars show mean with 95% confidence intervals.

Taken together, these results suggest that exposure to 0.5 mg/L CPF does not alter egg laying behaviour and is not a useful measure for low-level exposure in *C. elegans*.

3.3.6. 0.5 mg/L CPF does not significantly affect body-bend frequency in C. elegans.

Locomotory rate can be used to measure the effects of toxic exposure (Boyd *et al.*, 2010) and so movement behaviour was measured by counting body bends of animals that had been exposed to 0.5 mg/L CPF or vehicle control. No distinguishable phenotype had so far been observed in wild type animals treated with 0.5 mg/L CPF for 24 hours, and so animals were exposed to 0.5 mg/L CPF or vehicle control for 24, 48 or 72 hours, to test whether a longer exposure time might be necessary to observe an effect at this concentration. Animals were scored on plates seeded with an OP50 lawn.

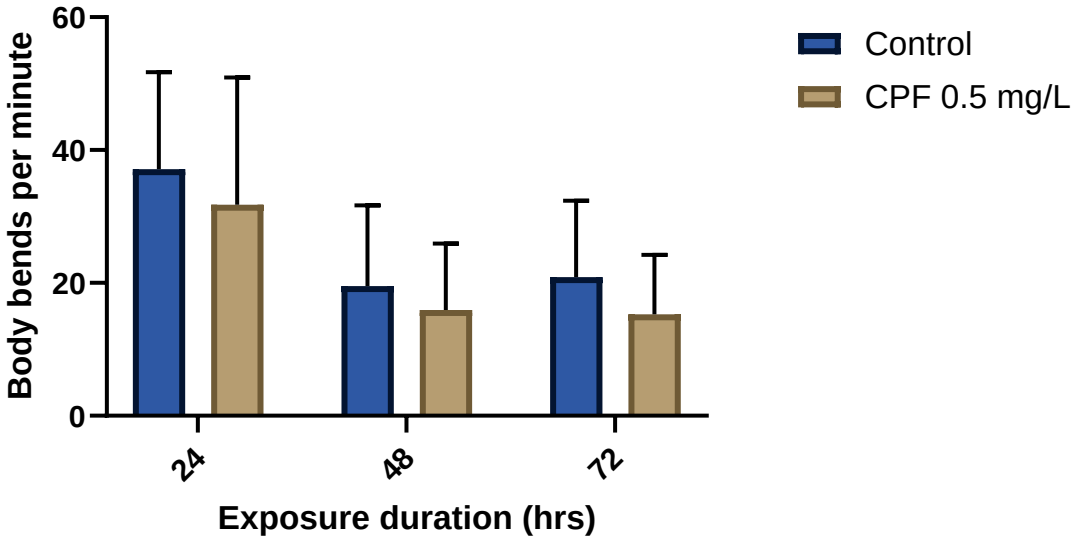

Figure 23. CPF treatment does not affect *C. elegans* locomotory rate, as measured by body bends. The impact of CPF treatment on the locomotion rate of *C. elegans*, was assessed by counting body bends per minute. Regardless of whether animals were exposed to CPF or the vehicle control, and regardless of the duration of exposure (24, 48, or 72 hours), locomotion rates remained consistent when assayed on OP50 bacteria. (n = 20 control, n = 20 CPF, for each timepoint). Bars represent mean and 95% confidence intervals.

There was no overall effect of CPF on body bend frequency, regardless of how long animals were exposed (**Figure 23**).

Conversely, there was significant reduction in body bends after 24 hours, however this also occurred in animals exposed to acetone as a vehicle control ($F(2, 114) = 4.614$. $p < 0.05$).

These body bend results did not indicate any effect of 0.5 mg/L CPF on locomotion in *C. elegans*. Moreover, since the decrease in body-bend frequency observed following 48- and 72-hour exposures occurred in both CPF treated and vehicle control groups, it is

unlikely to have been a consequence of CPF treatment. This locomotory inhibition was most likely due to normal ageing (Glenn *et al.*, 2004).

3.3.7. C. elegans body length is significantly reduced by 0.5 mg/L CPF

So far, no observable behavioural effect of CPF had been observed in wild-type *C. elegans.* However, body length in *C. elegans* can be a useful indicator of AChE activity (Petzold *et al.*, 2011; Roussel *et al.*, 2014). To test whether CPF affects body length in *C. elegans,* animals were treated with 0.5 mg/L CPF or acetone as a vehicle control, as described in methods (2.2.5), and their body length was measured from video files using WormLab software.

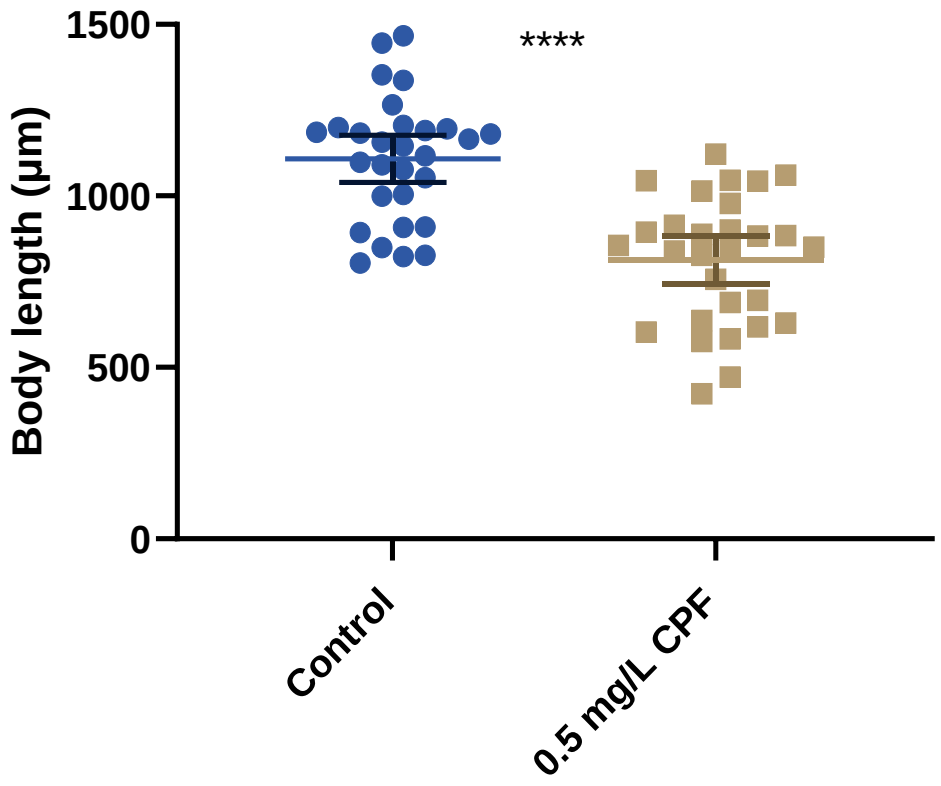

Figure 24. Body length is reduced in *C. elegans* following exposure to 0.5 mg/L CPF. Animals exposed to 0.5 mg/L CPF (brown squares) were significantly shorter than those exposed to acetone as vehicle controls (blue circles) (n = 29 control, n = 29 CPF, mean body length 812.7 µm versus 1108 µm, respectively). Bars show mean with 95 % CI. **** = $p < 0.0001$).

The mean body length was significantly shorter in animals treated with 0.5 mg/L CPF than in those treated with acetone as a vehicle control ($t = 6.114$, df = 56, $p < 0.0001$) (**Figure 24**).

This change in body length was important since it was the first effect observed following treatment with CPF at 0.5 mg/L. It also suggests that 0.5 mg/L CPF might cause muscle contraction by inhibiting AChE , the implications of which will now be discussed.

3.4. Discussion

Having set out to investigate the effects of low-level CPF exposure using *C. elegans* as a model, a concentration of 0.5 mg/L was selected from previous work that used the same exposure regime, by adding CPF to nematode growth media (Viñuela *et al.*, 2010). Exposure to this concentration was then used to test for effects in behavioural assays with known relevance to neurotransmitter pathways related to human mood disorders, including 5-HT, DA, GABA and ACh. However, apart from the physiological measure of body length, no behavioural phenotype was observed in wild-type animals as a result of CPF exposure.

3.4.1. Locomotory rate in liquid was not significantly affected by 0.5 mg/L CPF

Wild-type animals did not paralyse after 10 minutes of swimming in the SWIP assays, which is normal (McDonald *et al.*, 2007), and did not seem to be affected in any way by prior treatment with CPF (**Figure 20**). However, *dat-1(ok157)* mutants, which are deficient in DA reuptake, did paralyse as previously reported (McDonald *et al.*, 2007). There was a noticeable effect of CPF treatment in *dat-1(ok157)* mutants, in that around 10-20% of individuals were not completely paralysed by the end of 10 minutes. This was the most interesting behavioural result so far, but the reasons for it were not clear. SWIP behaviour occurs as a result of excessive DA in animals that cannot clear it effectively due to DA-transporter deficiencies, therefore it is possible that paralysis could be prevented by altering the DA signal. This could occur presynaptically, in response to impaired DA

synthesis or release, or postsynaptically by antagonising DA receptors, and the D2-like DA receptor DOP-3 more specifically (Refai and Blakely, 2019). Azaperone is a drug which binds to, but does not activate DOP-3, and has also been shown to prevent SWIP in *dat-1(ok157)* mutants (Refai and Blakely, 2019). Similarly, reserpine reduces presynaptic DA release and also prevents SWIP behaviour (Refai and Blakely, 2019). However, these authors reported a much lower rate of paralysis (less than 20%) in response to those drugs than ~ 80% seen following 0.5 mg/L CPF treatment in this chapter (**Figure 20**). They also observed a slight paralysing effect of azaperone on wild-type animals, which was not observed following treatment with 0.5 mg/L CPF. The concentration of azaperone was ~1000-fold higher than the CPF concentration used in this chapter, which could explain the difference in effect. This brings into question whether the relatively mild and inconsistent effect seen in CPF treated animals could be useful for investigation of low-level CPF exposure. There was no significant difference in swimming rate between wild-type animals treated with 0.5 mg/L CPF or acetone vehicle control (**Figure 21**), but there were a few CPF-treated individuals that swam more slowly than the rest of the group without fully paralysing. SWIP occurs in *dat-1(ok157)* in response to vigorous motor activity such as swimming, but not crawling on solid surfaces (McDonald *et al.*, 2007). Crawling occurs at a lower frequency than swimming (**Figure 21** and **Figure 23**) and *dat-1(ok157)* mutants can swim for several minutes before paralysing. It is therefore possible that slow-swimming individuals do not reach the threshold for paralysis, although the reasons why some animals swam more slowly remains unclear. The potential DA mediated effect on locomotory slowing is explored further in the next chapter.

3.4.2. Egg-laying behaviour was not affected by 0.5 mg/L CPF

Treatment with 0.5 mg/L CPF in these experiments did not have any effect on brood size or egg-retention (**Figure 22**). Previous studies which have measured egg-laying behaviour in response to CPF treatment have used varying exposure methodology, in terms of concentration, exposure time, and delivery method. Thus, there is no identical study with which to compare this result. However, Ruan et al. (2009, 2012) tested a range of CPF concentrations from 0.003 - 3 mg/L and did not find any effect on brood size following a 24-hour exposure time. They did observe a reduction in brood size following a 48-hour exposure to 3 mg/L CPF, which is a much higher concentration, and double the exposure time used in this study. Those results are in line with the findings described in this chapter, and together suggest that egg laying behaviour may not be an appropriate measure for modelling low-level CPF exposure in *C. elegans*. Conversely, Boyd et al. (2010) did report a reduction in brood size following treatment with 0.031 mg/L CPF, which is much lower than used in this study, however they exposed the animals for 48 hours in a liquid medium, highlighting the influence of different exposure times and methodologies. In agreement with the data from this study, Roh and Choi (2008) did not find any effect of CPF treatment on egg-laying behaviour before 96 hours of exposure, and only found effects on reproductive behaviour when AChE inhibition was also detected.

Taken together, the results of this study and context provided by previous studies suggest that egg-laying behaviours are not suitable for the purpose of this project. There is no doubt that CPF can affect reproductive behaviour under certain conditions, however it seems unlikely that any useful change in egg-laying could be harnessed while avoiding AChE inhibition and altering developmental processes.

3.4.3. 0.5 mg/L CPF did not significantly affect body-bend frequency in C. elegans.

There appeared to be no effect of 0.5 mg/L CPF on locomotion when measured by body bends (**Figure 23**), despite the inclusion of longer exposure times in this experiment. As has already been discussed, longer exposure times can increase the detection rate of behavioural changes (such as egg-laying described above). In this case the opposite appeared to be true, since body bend frequency was reduced in groups that had been exposed for longer durations, which leaves a smaller margin for any potential changes to be detected. This could explain why Ruan et al. (2009) found assays using 24-hour exposures to be more sensitive than those using 72-hour exposures. The reduced frequency of body bends after 48 and 72 hours of exposure was not a consequence of CPF treatment itself, evidenced by the fact that it also occurred in the vehicle control groups. This reduction in locomotory behaviour is most likely a consequence of normal ageing (Glenn *et al.*, 2004) and illustrates one of the reasons for favouring a 24-hour exposure regime for modelling low-level CPF exposure in *C. elegans*. In addition to the practical convenience and potential for higher throughput, the model would also avoid the potential of confounding factors associated with ageing.

These results did not support the need for longer exposure durations, nor did they present any useful phenotype for use in the model. The most obvious possible explanation for the absence of any locomotory effect is that 0.5 mg/L could be too low a concentration. This is possible, although some studies have reported locomotory effects in *C. elegans* treated with lower concentrations than 0.5 mg/L CPF, these studies used liquid media for the exposure method (Anderson, Cole, and Williams, 2004a; Ruan *et al.*, 2009). Others report no effect on locomotion following exposure to concentrations below

3 mg/L CPF (Boyd *et al.*, 2010; Ju *et al.*, 2010), but none of these studies measuring locomotion used the NGM exposure method for CPF as was used in this study.

There are other factors that can affect locomotory behaviour, such as the presence or absence of food (Sawin, Ranganathan and Horvitz, 2000), temperature (Parida, Neogi and Padmanabhan, 2014) or even ambient lighting (Abdel-Rahman *et al.*, 2017), which might explain some of the discrepancies observed in the literature. Furthermore, the measure used to quantify locomotion can also vary. While body bends are a well-accepted method, there remains a risk of human measurement error while manually counting. Some studies have used automated methods for quantifying locomotory behaviour, which not only removes the risk for human error, but can also detect multiple locomotory endpoints with high sensitivity (Ramot *et al.*, 2008; Roussel *et al.*, 2014; Husson *et al.*, 2018).

3.4.4. C. elegans body length is significantly reduced by 0.5 mg/L CPF

Despite no behavioural phenotype having been observed in animals treated with 0.5 mg/L CPF, body length was significantly reduced at that concentration (**Figure 24**). The difference in body length is a possible indication of AChE inhibition at this concentration since AChE inhibition can result in hypercontraction of body wall muscles leading to reduced body length (Hwang *et al.*, 2016). Roh and Choi (2008) found no effect on body length in response to CPF treatment, at different exposure concentrations in liquid media. Although the maximum concentration they tested was only 0.1 mg/L CPF, they reported 50% AChE inhibition from that treatment (J.-Y. Roh and Choi, 2008). Conversely, reports of reduced body length following CPF exposure from other studies have been associated with higher concentrations, such as 1 mg/L CPF (Ruan *et al.*, 2009) and even reporting an EC50 of 8 mg/L CPF for body length reduction (Boyd *et al.*, 2010).

While the different exposure methodologies make direct comparisons of the effects of CPF at specific concentrations difficult, it does seem that reduced body length is unlikely to occur in the absence of AChE inhibition. Biomechanically, body shortening due to body wall muscle contraction, resulting from excess ACh at the neuromuscular junction, is a known effect of AChE inhibitors (Kearn *et al.*, 2014). Moreover, previous studies have detected AChE inhibition in *C. elegans* treated with concentrations of CPF that did not cause any reduction on body length (Roh and Choi, 2008; Roh, Lee and Kwon, 2016). It is therefore unlikely that 0.5 mg/L CPF is an appropriate concentration with which to test the effects of low-level exposure in *C. elegans*, due to the high chance of AChE inhibition at that concentration.

3.5. Conclusions

Taken together with previously reported work, the results of this chapter suggest that 0.5 mg/L CPF is likely too high a concentration for studying low-level exposure in *C. elegans*. However, since previous studies have used different exposure methodologies biochemical measurement of AChE activity would be useful to clarify how CPF treatment in solid NGM media compares to the other methods reported.

It is also clear that the behavioural assays used in this chapter are unsuitable for detection of effects of low-level CPF exposure. The measurement of alternative behavioural endpoints would be necessary, in particular for detection of effects at lower concentrations.

4. Sensitive behavioural measures of the effect of low-level CPF exposure.

4.1. Introduction

4.1.1. Establishing an appropriate concentration for testing low-level CPF exposure in C. elegans.

To investigate the effects of low-level OP exposure on neuronal function using *C. elegans* it is necessary to measure effects which occur in the absence of acute cholinergic toxicity. Acute OP toxicity is caused by exposure to OP compounds at sufficient concentrations to cause overt physiological or behavioural changes through inhibition of AChE. Identifying a suitable concentration for testing low-level effects is, by definition, complicated somewhat by the absence of such overt changes in animals exposed to smaller concentrations (Silva, 2020). Consensus on which concentrations could be classed as low-level is lacking in the literature, largely because the few studies investigating low-level CPF exposure in *C. elegans* have employed a range of exposure methodologies and reported different results (Roh and Choi, 2008; Ju *et al.*, 2010; Viñuela *et al.*, 2010; Roh, Lee and Kwon, 2016). The results presented in the previous chapter suggest that 0.5 mg/L CPF delivered in solid NGM plates does not cause any detectable effect on brood size, swimming-induced paralysis, or locomotion measured by swimming rate or counting body bends. However, 0.5 mg/L CPF did cause a reduction in body length, which suggests that significant inhibition of AChE may still be present at this concentration. Exposure of *C. elegans* to 0.5 mg/L CPF in NGM plates has been demonstrated previously (Viñuela *et al.*, 2010), however AChE enzyme activity was not measured. It would

therefore be useful to measure AChE activity in *C. elegans* to determine the inhibitory effects of CPF at a similar range of concentrations, and to establish a concentration at which AChE was not inhibited.

This chapter describes the identification of a more suitable CPF concentration for investigating low-level exposure in *C. elegans*. This was achieved by measuring AChE enzyme activity, and the use of more sensitive behavioural measures.

4.1.2. Measuring AChE enzyme activity in C. elegans.

Interpretation of reported effects of different CPF concentrations on *C. elegans* is hindered by inconsistencies with the range of concentrations used, methodology, and lack of AChE activity data in many cases (Anderson, Cole, and Williams, 2004a; Ruan *et al.*, 2009b; Boyd *et al.*, 2010; Viñuela *et al.*, 2010; Ju *et al.*, 2014). Furthermore, since individual studies are often concerned with different toxic endpoints, there is little consensus regarding the sub-lethal effects of specific CPF concentrations. Roh and Choi (2008) reported reduced AChE activity in *C. elegans* exposed to 0.1 mg/L CPF in liquid media, but saw no effect on body length or behaviour at 0.1 mg/L. In a later study, a more advanced method for exposing *C. elegans* to CPF in liquid known as 'passive dosing' was used, with the authors reporting some AChE inhibition following treatment with 0.03 mg/L CPF (Roh, Lee and Kwon, 2016). Passive dosing has been shown to increase the uptake of chemicals of interest by *C. elegans* considerably, even when compared to standard liquid exposure methods (Fischer *et al.*, 2016), and is likely more sensitive than exposure treatment via solvent spiking in NGM (Escher, Hermens and Schwarzenbach, 2005). Although Roh, Lee and Kwon, (2016) did not report any behavioural changes during their study, they did report a lowest observable effect level (LOEL) of 0.06 mg/L CPF for changes in gene expression related to toxic metabolism.

Exposure in liquid, including passive dosing, is unsuitable for prolonged exposure beyond 10 hours (J.-Y. Roh and Choi, 2008; Roh, Lee and Kwon, 2014). Therefore, a better understanding of AChE activity in *C. elegans* exposed to CPF concentrations mixed into NGM plates would be of great benefit to experiments using low concentrations and longer exposures. Standard methods for measuring AChE enzyme activity in biological samples are generally based on the method of Ellman (1961). The Ellman assay provides an indirect colorimetric indication of enzyme activity. Acetylthiocholine is added to the sample and is hydrolysed by any AChE present in the biological material being tested, producing thiocholine and acetate. Ellman's reagent (5,5-dithio-bis-(2-nitrobenzoic acid): DTNB) is added, which reacts with thiocholine producing a visible yellow colouration (**Figure 25**). This colour change can be measured at ~ 410 nm and is proportionate to the quantity of thiocholine hydrolysed by AChE (Ellman, 1961).

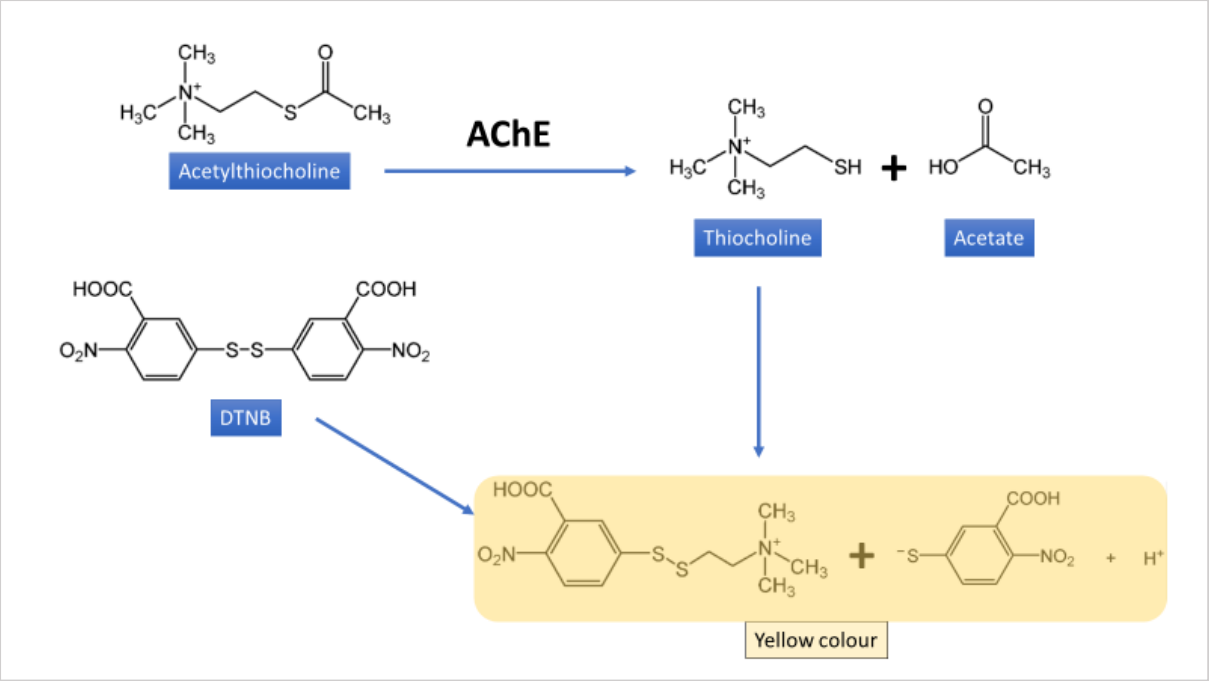

Figure 25. Ellman's reaction is used to measure AChE enzyme activity. This biochemical reaction is a method widely used for the quantification of AChE activity in biological samples. The process involves the reaction of the substrate acetylthiocholine (ATCh) with AChE, resulting in the production of thiocholine and acetate. Thiocholine then reacts with the Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB), leading to the formation of 5-thio-2-nitrobenzoate anion (TNB-) which can be detected spectrophotometrically at 412 nm. The intensity of the yellow colour produced corresponds to the level of AChE activity in the sample (Ellman, 1961).

Assays based on the Ellman reaction can be used to measure AChE activity in a range of samples, such as erythrocytes and plasma, or homogenates of tissues such as brain or liver (Dingova *et al.*, 2014). Researchers have also adapted the assay for use with invertebrate tissues, including *C. elegans* (Moulton, Fleming, and Purnell, 1996; Anderson, Cole and Williams, 2004; Melstrom and Williams, 2007b). A notable difference between the methods used for measuring AChE activity in mammals compared to nematodes, is the requirement to differentiate between AChE and BuChE activity. The

presence of BuChE in mammalian samples can lead to false positives, therefore a positive control may be used, such as donepezil, which is highly selective for AChE rather than BuChE (Sugimoto *et al.*, 2002). However, only AChE is present in *C. elegans* and therefore all of the cholinesterase activity is measured.

AChE enzyme activity data provides a useful frame of reference alongside other endpoints of interest, such as behavioural or gene expression changes resulting from OP treatment to define a concentration of CPF that can be classified as low-level.

4.1.3. Establishing a relationship between AChE enzyme activity and body length in CPF-exposed C. elegans.

The most notable effect of 0.5 mg/L CPF reported in the previous chapter was a change in nematode body length. Based on the evidence described above and the known role of cholinergic hyperexcitation in body contraction (Kearn *et al.*, 2014), it is likely that the observed difference in body length was due to significant inhibition of AChE following exposure to 0.5 mg/L CPF. Although this excludes *C. elegans* body length as a phenotype with which to directly measure the effects of low-level CPF treatment, the observed incidence of body shortening could indicate that treatment of *C. elegans* with 0.5mg/L CPF results in acute inhibition of AChE, making it too high to investigate the chronic effects of CPF. In addition to defining a low-level concentration at which AChE activity is minimally inhibited, measuring AChE enzyme activity and body length in *C. elegans* following exposure to a range of different concentrations will confirm whether changes in *C. elegans* body length observed following exposure to CPF are associated with altered AChE activity.

4.1.4. Detection of an observable phenotype caused by low-level CPF treatment.

In addition to finding a concentration of CPF that has minimal effect on AChE activity, a model of low-level CPF exposure requires a reliable and detectable phenotype with which to measure and explore the effect of exposure. However, the results presented in the previous chapter revealed no effect of CPF on reproductive behaviour, locomotion on solid surfaces or in liquid, or SWIP in wild type animals exposed to 0.5 mg/L CPF. This is despite having used a relatively high concentration compared with some other studies. In agreement with these findings, behavioural effects have rarely been reported following exposure to 0.5 mg/L CPF, regardless of exposure methodology (Boyd, McBride, and Freedman, 2007; Roh, Lee and Kwon, 2014). Nonetheless, it is clear that low-level exposure to CPF exerts some biological effect on *C. elegans*. For example, both altered gene expression and impaired development have been detected following exposures equal to or lower than 0.5 mg/L CPF (J.-Y. Roh and Choi, 2008; Viñuela *et al.*, 2010; Roh, Lee and Kwon, 2014). In order to find a more sensitive behavioural measurement with which to measure low-level effects, computerised behaviour tracking was used next.

4.1.5. Automated behaviour tracking in C. elegans.

In recent years experimental behaviour has increasingly been measured using computerised tracking software and video analysis (Husson *et al.*, 2018). There are numerous benefits to taking this approach. Firstly, video files of behaving nematodes can be saved for later analysis. This adds convenience, but more importantly allows treated groups to be reanalysed, and additional hypotheses to be tested retrospectively without further data collection. Modern trackers are increasingly able to handle various common file formats and so good quality images could even be used by other researchers using

different tracking software (Javer *et al.*, 2018). Any combination of mutants, drug-exposure or other conditions used can be available for future analysis.

Another benefit is that many computer trackers record multiple behavioural endpoints simultaneously, some of which would be difficult to measure with the human eye. The values extracted are also free from observer bias or human measurement error. These features might be particularly useful for capturing any subtle behavioural effects resulting from low-level CPF treatment.

4.1.6. Choosing a worm tracker

There are clear benefits in adopting an automated behaviour tracking approach for the purpose of testing the effects of low-level OP treatment, however consideration needed to be given to which type of tracker would suit the needs of this project. No established behavioural paradigms have been specifically associated with low-level OP treatment in *C. elegans*, and so having the versatility to test worm behaviour across multiple conditions was a priority. Also important was having high enough sensitivity to detect subtle behavioural differences, preferably with the potential to scale up tracking for a medium to high throughput system.

To date, several different systems have been created to capture record and analyse *C. elegans* behavioural data (reviewed in Husson *et al.*, 2018). Existing trackers range from large custom-built hardware arrangements to standalone software packages which can be used with existing microscopes and digital imaging setups, or a combination of hardware and software. For the most part, trackers are usually developed to measure a specific aspect of behaviour, which is likely aligned with behavioural endpoints relevant to the research group who have developed each tool. For example, some trackers are

designed to analyse movement of *C. elegans* in liquid and would be unsuitable for tracking behaviour on solid surfaces (Feng *et al.*, 2004; Matthies *et al.*, 2006; Buckingham and Sattelle, 2009; Krajacic *et al.*, 2012; Zheng *et al.*, 2012). Others specialise in measuring foraging behaviour (Moy *et al.*, 2015), or tracking worms throughout three-dimensional environments (Kwon *et al.*, 2013).

Perhaps the most common distinction between types of trackers is whether they track a single animal or multiple animals simultaneously. A benefit of the former approach is that a relatively large amount of data, and therefore a high-resolution account of a single individuals' behaviour, can be created with relatively low demands on image processing and processing power (Feng *et al.*, 2004; Husson *et al.*, 2018). Usually, the tracking of a single worm is achieved with the use of a motorised stage to follow the worm along its track, rather than trying to include the whole movement area within frame, and an outline/skeleton image of the worm is used to measure movement parameters (**Figure 26**).

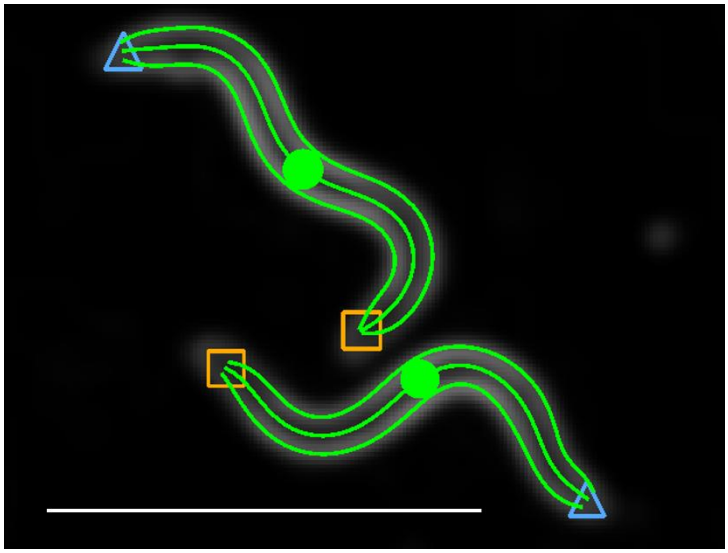

Figure 26. The skeletal outline and specific features of *C. elegans* are used in some tracking software to quantify movement parameters. The green outline represents the worm's body, with a green circle marking the midpoint. The head is marked with a square orange symbol, while the tail is represented by a blue triangle. These visual cues allow accurate tracking of worm movements, providing data for behavioural analysis (WormLab).

Some trackers only follow the change in position of the centroid (shown in **Figure 26** as the green circle in the middle of each worm), which measures the position of the worm in space. Other trackers, as shown here, measure additional features such as the body outline and centre line (green lines along the length of each worm), or the head (yellow square) and tail (blue triangle). The additional points of measurement allow more complex postural and physiological information to be acquired.

The main disadvantage associated with single worm trackers is that they are less amenable to high-throughput upscaling. Despite being anatomically identical to individual cell level (Sulston, Dew, and Brenner, 1975) behaviour in *C. elegans* can vary greatly between individuals and across time (Stern, Kirst and Bargmann, 2017).

Experiments which require large sample sizes may find a different approach more suitable, especially where time is limited such as in screening large drug libraries (Ikenaka *et al.*, 2019). To address this issue several multi-worm trackers have been developed (Ramot *et al.*, 2008; Swierczek *et al.*, 2011; Perni *et al.*, 2018). Earlier versions of these multi-worm trackers would track several worms simultaneously by measuring the position of the centroid (**Figure 26**). Tracking only the centroid does not provide any postural or physiological data, and so results are limited to endpoints such as velocity, track-length, or direction changes. This approach requires comparatively less computing power per-worm tracked compared with more detailed approaches and require fewer overlapping objects and collisions to be resolved. Recent advances in computing power and software development, however, have enabled multiple worms to be tracked simultaneously at levels of detail and resolution previously offered exclusively by single worm trackers (Roussel *et al.*, 2014; Kiel *et al.*, 2018; Perni *et al.*, 2018).

The range of available trackers presents options for use with diverse behaviours in *C. elegans* (Husson *et al.*, 2018). However, since a phenotype for low-level CPF exposure is yet to be established for *C. elegans,* a versatile tracker with a range of options would be more suited to this investigation than one specialised for measuring any specific behaviour or endpoint. To this end, a 'wish list' was created based on the features deemed useful for finding and measuring phenotypes for modelling low-level OP exposure in *C. elegans*. The main requirements were as follows:

- It should measure skeleton and outline (not just centroid position) to ensure sensitivity to individual postural differences, to test for effects on locomotory phenotypes. Measuring the outline also includes body-length and size measurements.

- The ability to detect more complex behavioural phenotypes, such as foraging, which requires information about the animal's movement in relation to its environment, as well as changes in body posture.
- The versatility to measure behaviour both on solid surfaces and in liquid, to extend the range of testable phenotypes.
- Multi-worm tracking capabilities, to measure subtle, low-level effects at the population level. This would help to account for individual variation, and to upscale to higher throughput.

Some features of some existing trackers are shown in **Table 11**.

Table 11. A comparison of features from a selection of worm trackers. (Adapted from: Husson et al., 2018). * = Estimated setup costs do not include camera, microscope, or third-party software.

Tracker name	**Worm Tracker 2.0 (Schafer lab)**	**Nemo (Tavernarakis Lab)**	**The Parallel Worm Tracker (Goodman lab)**	**The Multi-worm tracker (Kerr lab)**	**WormLab MBF Biosciences**
Single or multi-worm tracking	Single	Single	Multi-worm	Multi-worm	Multi-worm
Parameters measured	Skeleton outline	Skeleton outline	Centroid	Skeleton outline	Skeleton outline
Track info	Yes	Yes	Yes	Yes	Yes
Solid or liquid tracking	Both	Solid	Solid	Solid	Both
Hardware required	X-Y stage, camera	Camera	Camera	Camera, frame grabber, backlight	Camera only OR (optional) proprietary X-Y stage, camera, and microscope setup
Software required	Java, MATLAB or MCR	MATLAB + Image Processing Toolbox	MATLAB + Image acquisition and Image Processing Toolbox	LabVIEW (+ Vision), C++ (custom), Java	None/standalone
Setup costs * (GBP)	~2,700	~300	~300	~5,400	~5000 (software only)
Reference	(Yemini, Kerr, and Schafer, 2011)	(Tsibidis and Tavernarakis, 2007)	(Ramot *et al.*, 2008)	(Swierczek *et al.*, 2011)	(Roussel *et al.*, 2014)

4.1.7. WormLab: Micro Brightfield (MBF Biosciences)

WormLab is a commercially available worm tracking platform developed by MicroBrightField Inc. (MBF Biosciences).

WormLab has been used previously to perform assays relating to dopaminergic neurotransmission, including the basal slowing response which is described in some detail in the next section of this chapter (Sawin, Ranganathan and Horvitz, 2000; Nagarajan *et al.*, 2014). Nagarajan et al. (2014) specifically used the centroid tracking feature of WormLab to detect changes in locomotory speed caused by DA dependent neurodegeneration in *C. elegans*. Another study used WormLab to detect several locomotory effects of sublethal acrylamide exposure, which the authors showed were also caused by neurotoxic effects on dopaminergic function (Li *et al.*, 2016). Li et al. (2016) likewise, used locomotory movement data from WormLab, but also the software to measure postural information and swimming behaviour in liquid.

In addition, WormLab has been compared to two other trackers during an investigation into the effects of chronic, low-level nicotine exposure on *C. elegans* (Polli *et al.*, 2015). The dual aims of that study were, firstly to test the effects of 24-hr exposure to 6.17 μM nicotine, by measuring locomotory behaviour on nicotine treated NGM plates in relation to cholinergic receptor expression. The exposure and behavioural methodology described by Polli et al. (2015) are somewhat similar to the requirements of the current project for low-level CPF exposure. It is therefore helpful that the second aim of their study was to compare the behavioural analysis capabilities of WormLab against those of the Worm Tracker 2.0 (Schafer lab), and the Parallel Worm Tracker (Goodman lab) (Polli *et al.*, 2015), each of which were considered or trialled for use in the current project, respectively.

Worm Tracker 2.0 is an open-source software package which requires a self-assembled moving stage to follow a single worm at high magnification. This enables more sensitive behavioural analysis per individual worm than WormLab, but WormLab eliminates the need for the moving stage and enables multi-worm tracking, which the authors considered advantageous for detecting locomotory behavioural changes associated with low-level nicotine exposure (Polli *et al.*, 2015). As an alternative the same authors assessed the Parallel Worm Tracker but found the higher behavioural resolution of WormLab to be more useful, stating that WormLab "combines the advantageous features of both the Worm Tracker (2.0) and the Parallel Worm Tracker." (Polli *et al.*, 2015). As a result, they managed to demonstrate nicotine induced stimulation, adaptation and nicotine withdrawal response using locomotion parameters extracted from videos using WormLab (Polli *et al.*, 2015).

Based on the matching of the features required for testing low-level OP exposure with the feature sets offered by WormLab, it was considered to be a suitable option for the purposes of this project. WormLab was therefore used to test for phenotypes using behavioural assays with established associations with mood-related neurotransmission. Having chosen a tracking setup, the next objective was to identify experimental conditions that could uncover any effects of low-level CPF exposure. To address this, *C. elegans* behavioural responses to food were tested.

4.2. The basal and enhanced slowing response

Information can be gained by observing *C. elegans*' behavioural responses to changes in its environment or internal state. Food is a salient stimulus which can alter behaviour by its presence or absence within the environment. Behaviour can also change significantly depending on an organism's current nutritional state, or recent experience with food (Angstman, Frank, and Schmitz, 2016). Manipulation of these factors can be used to induce behavioural changes which are known to relate to DA and 5-HT signalling in *C. elegans.* The basal and enhanced slowing responses (BSR and ESR, respectively) were selected as potentially useful examples, and these will now be discussed.

4.2.1. C. elegans locomotory rate is moderated by the presence of food via dopaminergic signalling.

Preliminary experiments presented in chapter one described how wild-type *C. elegans* treated with a relatively high concentration (0.5 mg/L) of CPF did not alter normal egg-laying behaviour, SWIP, swimming, or normal locomotory behaviour in the presence of food. However, some of the nuances of behaviour can be revealed by manipulations such as the removal of access to food, both in the short or longer term. Such experiments have previously been used to demonstrate distinct mechanisms for defined behavioural responses at the molecular, cellular and circuit level. Sawin et al. (2000) demonstrated that the presence or absence of an existing food source, and whether *C. elegans* had experienced food recently, differentially modulate locomotory rate. Moreover, they showed that behavioural responses of different magnitudes, which are appropriate under different circumstances, depend on separate neurotransmitter pathways relevant to mood (Sawin, Ranganathan and Horvitz, 2000).

The first behaviour identified by Sawin et al. (2000) showed that wild-type *C. elegans* move relatively quickly when a food source is not present, but slow down measurably when encountering a food source. This reduction in speed is termed as the 'BSR' and is reliant on a DA operated circuit (**Figure 27**). Mutant strains that are deficient in DA signalling fail to show the BSR, including *cat-1(e1111)* mutants, which are deficient in presynaptic vesicular loading for monoamines (Duerr *et al.*, 1999), and *cat-2(e1112)* mutants which are deficient in the synthesis of DA (Sawin, Ranganathan and Horvitz, 2000).

The BSR is an established paradigm for testing the effects of various genetic and pharmacological perturbations on dopaminergic neuronal function. It was therefore considered as a potentially useful tool with which to explore any effects of CPF on dopaminergic signalling.

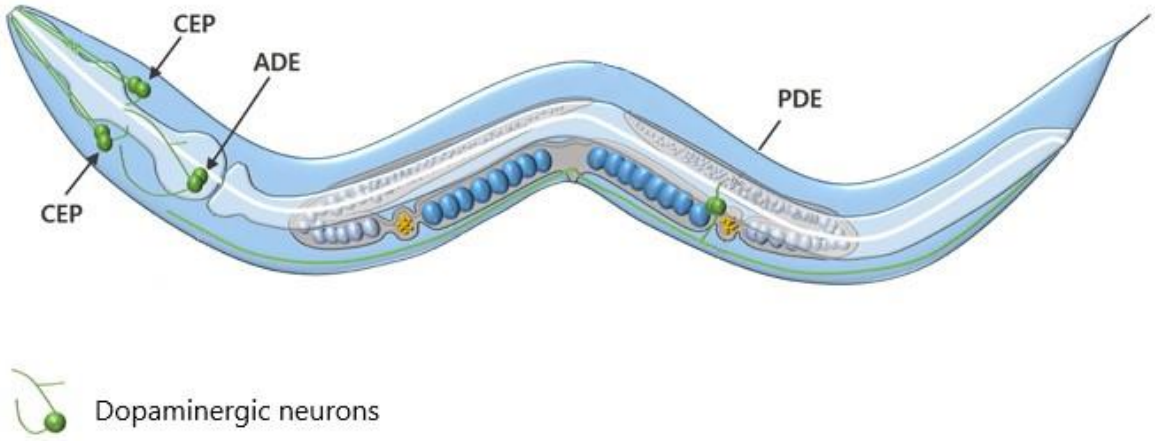

Figure 27. Illustration of the dopaminergic circuit in *C. elegans* involved in the Basal Slowing Response (BSR). Hermaphrodite *C. elegans* possess a total of eight dopaminergic neurons: four cephalic neurons (CEP), two posterior deirid neurons (PDE), and two anterior deirid neurons (ADE). These neurons extend ciliated sensory endings within the cuticle and play a pivotal role in mechanosensation (Perkins et al., 1986; Sawin, Ranganathan and Horvitz, 2000; Hills, Brockie and Maricq, 2004) (Adapted from: Chege and McColl, 2014)..

4.2.2. Change in locomotory rate in response to food is dependent on prior experience via serotonergic signalling.

Further to the BSR, Sawin et al. (2000) also demonstrated that the locomotory slowing in response to the presence of a food source was more pronounced when worms had been deprived of food for 30 minutes prior to reintroduction of the food source. This behaviour is termed the 'ESR' (**Figure 28**) and is dependent on serotonergic signalling rather than DA, and unlike basal slowing is not initiated by mechanosensory stimulation (Sawin, Ranganathan and Horvitz, 2000).

While investigating the mechanism responsible for enhanced slowing, the authors noted that food-deprived and well-fed animals moved at a similar rate in the absence of food.

This eliminated the possibility that the enhanced slowing was due to energetic depletion or exhaustion, indicating that some other factor relating to the experience of food deprivation must be responsible for the altered response (Sawin, Ranganathan and Horvitz, 2000).

Mutants that are deficient in DA synthesis alone - *cat-2(e1112)*, do show enhanced slowing, but a reduced BSR, however mutants that are deficient in DA and 5-HT synthesis - *bas-1(ad446)* and *cat-4(e1141),* are deficient in both basal and enhanced slowing (Sawin, Ranganathan and Horvitz, 2000). This indicates that DA and 5-HT are differentially responsible for basal and enhanced slowing, respectively (**Figure 28**). This is further supported by the fact that basal slowing in mutants deficient in the enzymes required for DA synthesis is rescued by the application of exogenous DA (Sawin, Ranganathan and Horvitz, 2000). Moreover, enhanced slowing in 5-HT deficient mutants is rescued by exogenous 5-HT (Sawin, Ranganathan and Horvitz, 2000).

Although the exact neurons responsible for the ESR are yet to be identified (Mori *et al.*, 2019), the behaviour is modulated by 5-HT signalling through the 5-HT-gated chloride channel, MOD-1 (Ranganathan, Cannon, and Horvitz, 2000; Sawin, Ranganathan and Horvitz, 2000). Enhanced slowing is somewhat inhibited by ablation of the neurosecretory-motor neurons (NSMs), which are the main serotonergic neurons in the *C. elegans* nervous system (Sawin, Ranganathan and Horvitz, 2000). However, although the NSMs produce 5-HT and take up 5-HT from the extracellular space via the 5-HT reuptake transporter - MOD-5/SERT (Ranganathan *et al.*, 2001), they do not synapse directly onto any other serotonergic neurons. Rather, the NSMs release 5-HT from extra-synaptic release sites (extra-synaptic neuro-secretory terminals: ENTs), which signal via volume transmission to distal targets (Fuxe *et al.*, 2010).

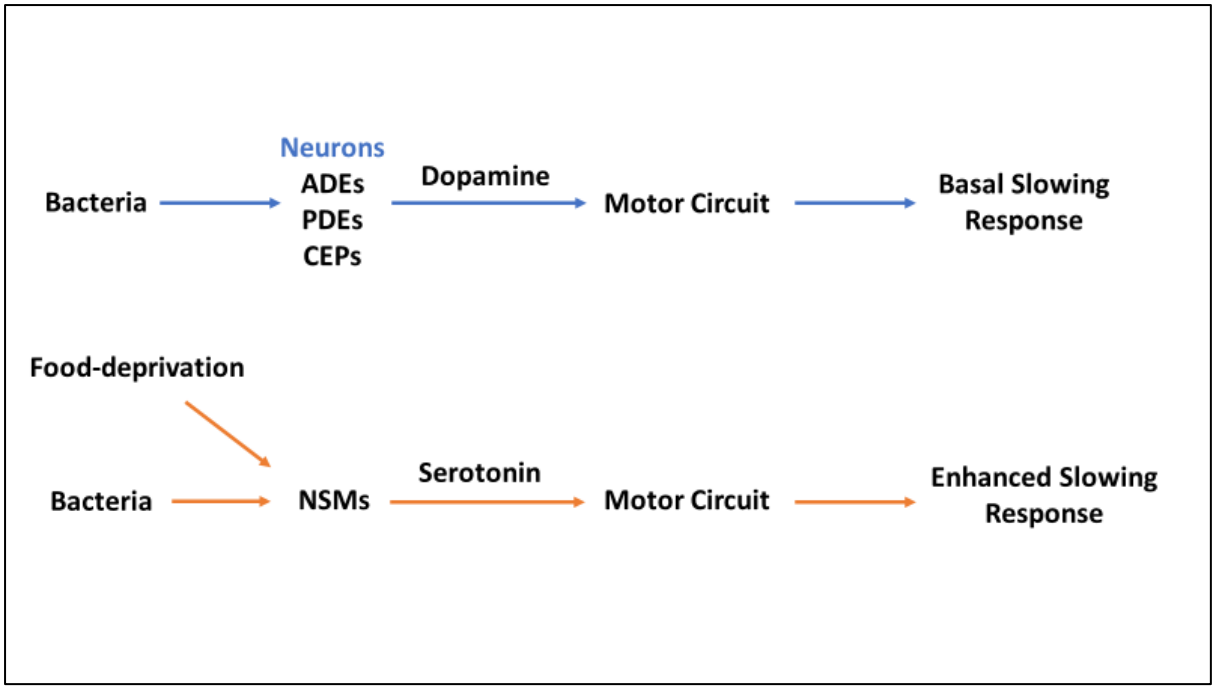

Figure 28. Schematic illustrating the role of dopamine and serotonin signalling in basal and enhanced slowing responses (BSR and ESR) in *C. elegans*. The basal slowing response (BSR) and the enhanced slowing response (ESR) are key mechanisms through which *C. elegans* modulates its locomotion in response to food availability. In the presence of food, *C. elegans* exhibits the BSR: sensory neurons (ADE, PDE, and CEP) detect the bacteria, triggering the release of dopamine (DA), which acts on the motor circuit to slow down the worm's movement, enabling efficient feeding. When the worm is food-deprived, it exhibits the ESR: the NSM sensory neurons sense the reintroduction of food, leading to the release of serotonin (5-HT), which induces a pronounced slowing of locomotion, allowing the worm to remain in the food-rich environment. This interplay between DA and 5-HT signalling pathways enables *C. elegans* to adapt its foraging behaviour based on food availability, ensuring survival in fluctuating environments (Sawin et al. 2000).

This type of volume transmission during which neurotransmitters are released by ENTs occur across different species, including mammals (Fuxe *et al.*, 2010). Extra-synaptic neurotransmission is responsible for the modulation of arousal state, and response to

rewards and other salient stimuli across a diversity of phyla (Fuxe *et al.*, 2010; Jafari *et al.*, 2011). This raises interesting parallels to traits such as anhedonia and impaired reward sensitivity in depression (Alloy *et al.*, 2016), or hypervigilance and maladaptive processing of salient cues in anxiety disorders (D'Hondt *et al.*, 2014; Richards *et al.*, 2014). These aspects of complex human behaviour are unlikely to have evolved using exactly the same neurobiological adaptations as the phenotypes observed in *C. elegans*, and so care must be taken to avoid making higher level behavioural comparisons. However, there is considerable value in exploring the interactions with drugs which are known to be relevant to mood, and their targets, within a defined biological system with conserved features (Lanzo *et al.*, 2018).

Pharmacological manipulations with antidepressant drugs known to act on serotonergic neurotransmission also interact with the ESR, but not basal slowing. Mianserin and methiothepin are 5-HT receptor antagonists that have both been used clinically to treat depression and anxiety in humans (PubChem), and both drugs block enhanced slowing, but not basal slowing, in *C. elegans* (Ranganathan, Cannon and Horvitz, 2000; Sawin, Ranganathan and Horvitz, 2000; Rivard *et al.*, 2010). Another common antidepressant - fluoxetine (Prozac), which works by inhibiting 5-HT reuptake and therefore prolonging serotonergic signals, also potentiates the ESR (Ranganathan, Cannon, and Horvitz, 2000; Sawin, Ranganathan and Horvitz, 2000; Ranganathan *et al.*, 2001).

The basal and enhanced slowing assays could prove to be valuable tools for investigating low-level CPF treatment with respect to its interaction with neurotransmitter systems relevant to mood for several reasons. The SWIP assay was already used to test for any effect on a part of the *C. elegans* dopaminergic system (3.1.5). The SWIP assay and the BSR address different aspects of DA signalling and can therefore be used together to

comprehensively explore the DA pathway (Lanzo *et al.*, 2018). In contrast, the ESR offers an opportunity to investigate any possible interaction of low-level CPF treatment with serotonergic volume transmission, in a system that has already been shown to interact with several mood therapeutic drugs. This makes ESR and BSR a highly suitable choice in the search for a relevant phenotype.

4.3. Chapter objectives

The three main objectives of this chapter are as follows:

1. To establish an exposure regime and working concentration for low-level CPF exposure in *C. elegans*, having a minimal effect on AChE inhibition that affects *C. elegans* physiology and locomotion behaviour.
2. To compare the use of WormLab tracking software against manual methods for measuring behaviour and physiology in *C. elegans* treated with low-level CPF concentrations.
3. To identify a phenotype resulting from CPF treatment at concentrations which have a minimal effect on AChE enzyme activity, using measurements extracted using WormLab and the *C. elegans* BSR and ESRs.

4.4. Results

4.4.1. Treatment of C. elegans with 0.05mg/L CPF does not result in significant AChE inhibition.

To test different CPF concentrations for their effect on AChE activity it was necessary to establish an optimal exposure concentration. Previously, 0.5 mg/L has been reported as an acceptable "low-dose" concentration for CPF exposure in *C. elegans* (Viñuela *et al.*, 2010). However, the results from behavioural experiments presented in the previous chapter do not show a behavioural phenotype at 0.5 mg/L CPF. A reduction in body length was detected which suggested that AChE enzyme activity may be inhibited by exposure to 0.5 mg/L CPF. To test this, and to establish a working concentration for a low-level exposure model, AChE was measured using a method based on the Ellman assay (Ellman, 1961).

Five CPF concentrations were chosen and compared against a vehicle control group representing baseline AChE activity. The range tested was 0.05 mg/L, 0.1 mg/L, 0.5 mg/L, 1 mg/L and 2.5 mg/L CPF which includes concentrations above and below those previously described as "low-dose" (Viñuela *et al.*, 2010). L4 nematodes were exposed to the each of these concentrations for 24-hr before being processed and measured for AChE activity.

The Ellman enzyme activity assay showed evidence for inhibition of AChE by CPF overall (**Figure 29**) (Welch's ANOVA: $F(5, 27.96) = 85.02$, $p < .0001$). *Post-hoc* analysis using Dunnett's T3 multiple comparisons test showed significant AChE inhibition at 0.1 mg/L, 0.5 mg/L, 1 mg/L and 2.5 mg/L CPF, but not at the lowest CPF concentration of 0.05 mg/L (**Table 12**). An IC02 value, which indicates the CPF concentration inhibiting 2% of

baseline AChE activity, was calculated as 0.044 mg/L CPF [95% CI: 0.025, 0.082] (n = 70), which suggests that our closest concentration of 0.05 mg/L CPF is predicted to inhibit AChE by approximately 2% (**Figure 30**).

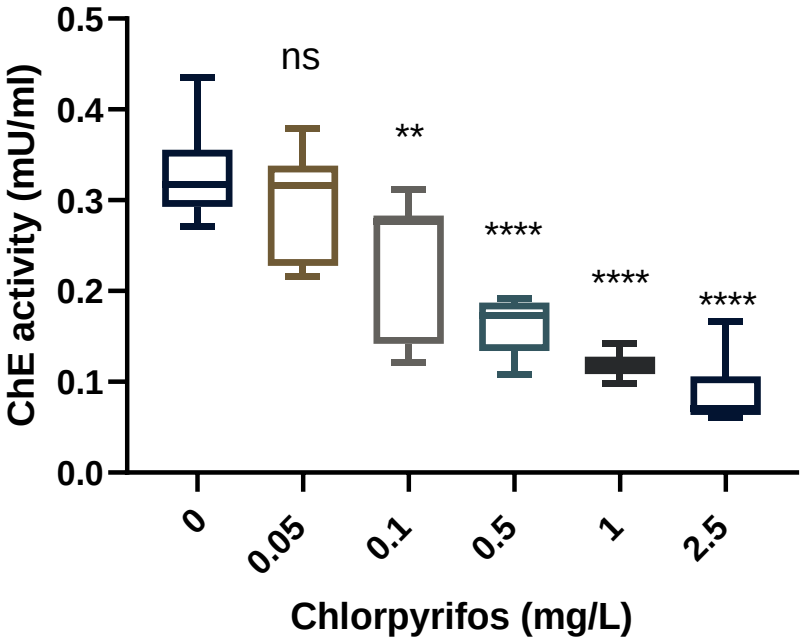

Figure 29. 0.05 mg/L CPF does not significantly inhibit *C. elegans* AChE activity. AChE activity, expressed as milliunits per millilitre (mU/ml) normalised against total protein concentration (AChE activity/TP ratio), was measured using the Ellman assay (Ellman, 1961) after treating *C. elegans* with varying concentrations of CPF. Six plates containing >100 worms were used for each treatment/replicate, and assays were performed in triplicate (n = 72). All concentrations of CPF significantly inhibited AChE activity, except 0.05 mg/L. Brown-Forsythe and Welch's ANOVA tests revealed significant differences among means ($p < 0.0001$). Dunnett's T3 multiple comparisons test showed significant differences between the vehicle control and treatment groups of 0.1, 0.5, 1, and 2.5 mg/L CPF (**$p < 0.005$; ****$p < 0.0001$), but not 0.05 mg/L CPF (ns).

Table 12. Pairwise comparisons between different CPF concentrations and vehicle control and their effect on AChE inhibition. Following up from a significant one-way ANOVA, each concentration tested was compared pairwise with vehicle control using Dunnet's T3 multiple comparisons test. ****s and corresponding P-values match those shown in **Figure 29**.

Comparison	Mean Diff.	95.00% CI	Significant?	Summary	Adjusted P Value
0 vs. 0.05 mg/L CPF	0.031	-0.032 to 0.096	No	ns	0.4875
0 vs. 0.1 mg/L CPF	0.101	0.019 to 0.182	Yes	**	0.0052
0 vs. 0.5 mg/L CPF	0.166	0.122 to 0.211	Yes	****	<0.0001
0 vs. 1 mg/L CPF	0.210	0.174 to 0.245	Yes	****	<0.0001
0 vs. 2.5 mg/L CPF	0.237	0.190 to 0.285	Yes	****	<0.0001

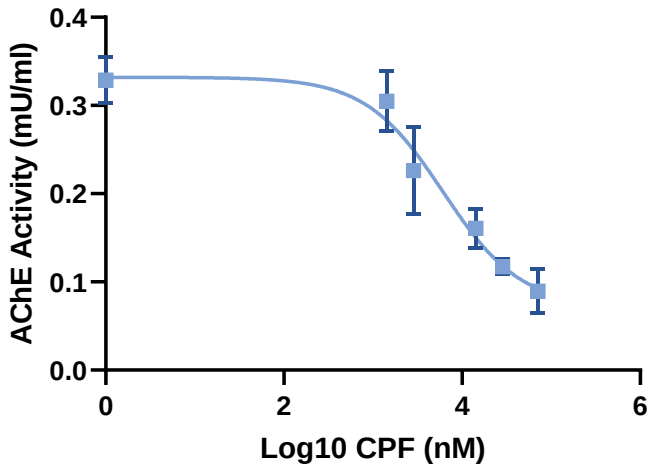

Figure 30. Dose-response curve for AChE inhibition by chlorpyrifos (CPF) in *C. elegans*. The concentration curve was plotted using log-transformed CPF concentrations in nanomolar values. An IC02 value, which indicates the CPF concentration inhibiting 2% of baseline AChE activity, was calculated as 0.044 mg/L CPF [95% CI: 0.025, 0.082] (n = 70) using the 'Find ECanything' function in GraphPad Prism software.

4.4.2. Physiological and behavioural changes associated with AChE inhibition are observed at 2.5, 1, 0.5 and 0.1mg/L but not at 0.05mg/L.

Reduced body length and locomotory behaviour are associated with acute AChE inhibition in *C. elegans* (Nguyen *et al.*, 1995; Melstrom and Williams, 2007b; Petzold *et al.*, 2011). Preliminary experiments in section 3.1.8 identified a reduction in body length following treatment with 0.5 mg/L CPF, which suggests that CPF at this concentration is sufficient to alter *C. elegans* physiology. The lower concentration of 0.05 mg/L CPF did not significantly alter AChE in the enzyme activity assay (**Figure 29**). Therefore, it was hypothesised that 0.05 mg/L CPF would not cause effects on *C. elegans* physiology or behaviour associated with AChE inhibition. Conversely, it was predicted that body length and behaviour would be altered at higher concentrations where AChE inhibition was observed. To test this hypothesis, body length and locomotory behaviour were measured following 24 hr exposure to 0.05, 0.1, 0.5, 1 and 2.5 mg/L CPF.

4.4.2.1. Body-length results

Computer generated body length measurements confirmed the occurrence of shortening due to CPF-exposure (**Figure 31**; one-way ANOVA ($F(3, 101) = 20.14$, $P < 0.0001$). Nematodes exposed to 0.5 mg/L CPF were significantly shorter in length than controls (Tukey's test: 0 vs 0.5mg/L CPF, $P < 0.001$). The 0.1 mg/L CPF treated group were also shorter than controls (Tukey's test: 0 vs 0.1 mg/L CPF, $P < 0.01$), but not significantly longer than the higher concentration (Tukey's test: 0.1 vs 0.5 mg/L CPF, ns). Nematodes exposed to 0.5 mg/L were not significantly shorter than those exposed to 0.1 mg/L, potentially showing a ceiling effect of body-wall muscle contraction (Caldwell, 2009).

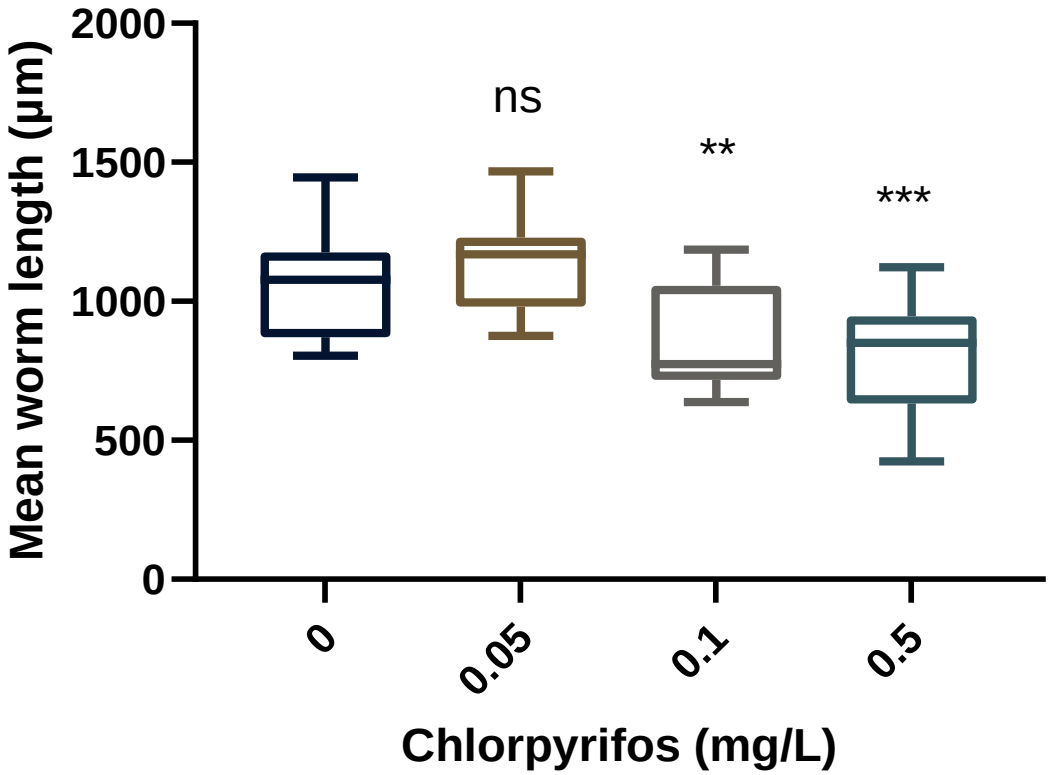

Figure 31. 0.05 mg/L CPF did not have a significant effect on mean body length of *C. elegans*. Treatment groups included 0.05 (n = 29), 0.1 (n = 30), and 0.5 mg/L CPF (n = 29), and an acetone control (n = 17). The body length of worms treated with 0.05 mg/L CPF was not significantly different from the control (ns), whereas body length was notably reduced at 0.1 mg/L CPF (n = and even more significantly decreased at 0.5 mg/L CPF. The box represents the interquartile range (IQR), the line inside the box is the median, and the whiskers depict the minimum and maximum values of the data. Significance is indicated as: ** = $p < 0.01$, *** = $p < 0.001$, and ns = not significant.

The lowest concentration (0.05 mg/L) CPF did not cause significant body-length shortening compared with the control group (Tukey's test: 0 vs 0.05 mg/L CPF, ns). There was no evidence of significant AChE inhibition or body-length shortening at 0.05 mg/L

CPF, therefore this concentration was considered as potentially suitable for modelling low-level CPF exposure.

4.4.3. Manual versus automated body length

As an additional validation step, 15 body length measurements were taken from WormLab' s output and compared against measurements taken from still frames of the same video using ImageJ (see methods).

Manual measurement of *C. elegans* body length using ImageJ resulted in significantly shorter length measurements than those produced by WormLab (Welch's t test: $t(18.17) = 3.787$, $p < 0.01$) and resulted in greater variability between measurements (WormLab SD: 32.76, manual measurement SD: 83.91;**Figure 32**). The difference in variation between each method demonstrates the higher level of precision achieved by WormLab over manual measurement.

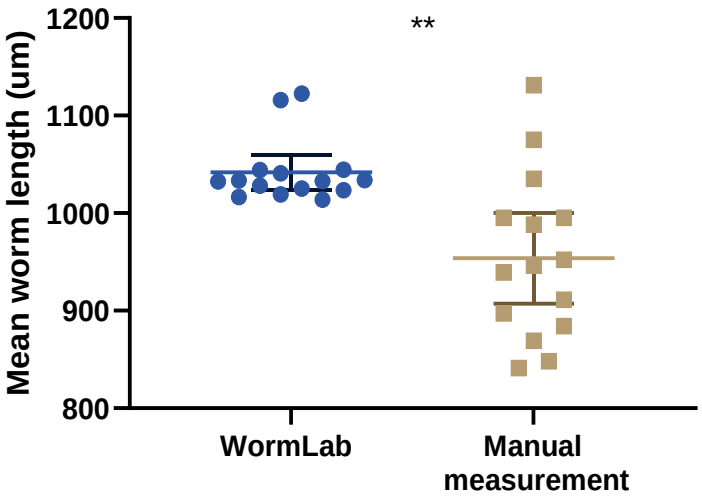

Figure 32. Wormlab body length measurements are less variable than manual measurement. WormLab measurements, taken continuously across the full range of movement in video frames (n = 15), showed less variability compared to manual measurements from still images (n = 15), which could not account for changes in posture. The difference in measurements between the two methods was statistically significant (Welch's t-test: t(18.17) = 3.787, p = 0.0013). The variance was also significantly different between the two groups ($F(14, 14) = 6.561$, p = 0.0012). Bars represent mean values, and error bars denote the 95% confidence interval. ** indicates $p < 0.01$.

4.4.4. Locomotion

Acetylcholine is a key component of neuromuscular function and disruptions of cholinergic signalling, such as treatment with AChE inhibitors, can inhibit locomotion in *C. elegans* (Hunt, 2017). Due to the fact that significant reductions in both AChE activity and body length were observed in animals treated with 0.1 mg/L CPF or higher, but not at 0.05 mg/L , 0.05 mg/L CPF seemed like a suitable concentration with which to model low-level CPF exposure in *C. elegans*. However, a behavioural phenotype with which to

explore the effects of CPF at this concentration was still required, and it was unknown at this stage whether locomotion is affected by treatment with 0.05 mg/L CPF.

To test whether locomotory speed could be used to measure the effects of low-level CPF treatment and how this related to AChE activity and body length, animals were exposed to 0 (vehicle control), 0.05, 0.1 and 0.5 mg/L CPF for 24 hours. They were then recorded and their locomotory speed measured using WormLab.

Overall, locomotory speed was affected by CPF treatment (independent one-way ANOVA: $F(3, 60) = 32.28$, $P < 0.0001$). At the highest concentration of 0.5 mg/L a significant decrease in speed was observed when compared to untreated controls (Dunnett's multiple comparison test: $p < 0.0001$). A smaller but still significant decrease was observed in animals treated with 0.1 mg/L ($p < 0.05$); however, no significant difference was observed in animals exposed to 0.05 mg/L (**Figure 33**). This suggests that 0.05 mg/L CPF does not cause the levels of muscular paralysis which are seen at higher concentrations and are associated with AChE inhibition.

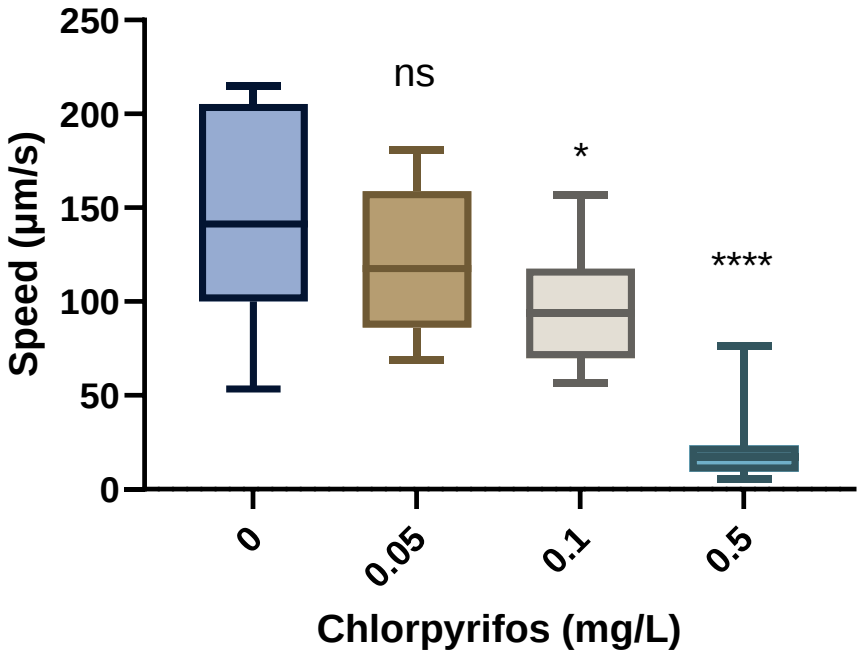

Figure 33. 0.05mg/L CPF does not have a significant effect on *C. elegans* locomotory speed. Treatment groups include 0.05 (n = 20), 0.1 (n = 10), and 0.5 mg/L CPF (n = 19), and an acetone control (n = 15). The speed of worms treated with 0.05 mg/L CPF is not significantly different from the control (ns), whereas locomotory speed is notably reduced at 0.1 mg/L CPF and even more significantly decreased at 0.5 mg/L CPF. The box represents the interquartile range (IQR), the line inside the box is the median, and the whiskers depict the minimum and maximum values of the data. Significance is indicated as: * = $p < 0.05$, **** = $p < 0.0001$, and ns = not significant.

4.5. WormLab software measures more reliably than manual measurement techniques.

4.5.1. Validation of locomotory speed measurement

Movement behaviour is commonly used as a behavioural measure of the effects of acute OP toxicity in *C. elegans* (Meyer and Williams, 2014b). However, although the results so far confirmed an effect on locomotion at concentrations of CPF ≥ 0.1 mg/L, the same was not observed at 0.05 mg/L CPF using speed measurements obtained with WormLab. Traditionally, some *C. elegans* researchers have advocated the counting of individual body bends as a more useful measure of locomotory effort in several circumstances (Sawin, Ranganathan and Horvitz, 2000; Hart, 2006; Roussel *et al.*, 2014). We therefore wanted to test the possibility that body bend counts might be more sensitive to changes in locomotory behaviour caused by CPF treatment. To compare the sensitivity of WormLab versus manual body bend counts, worms were exposed to 0.1 mg/L CPF, which was the lowest concentration at which locomotory differences had been observed, or vehicle control. They were then scored for both body bends and locomotory speed for comparison (**Figure 34**).

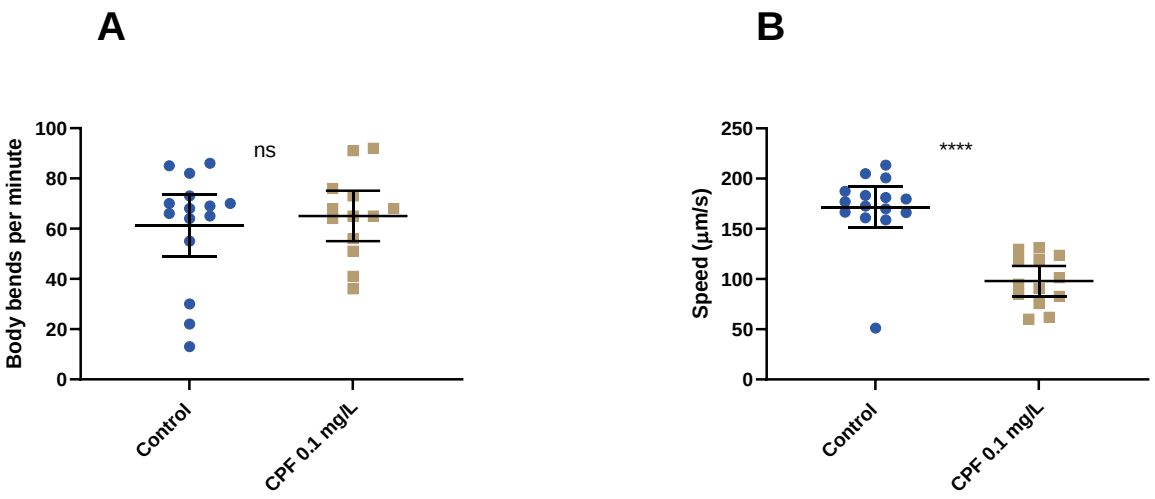

Figure 34. Wormlab speed measurements reveal differences in locomotion between CPF treated and control *C. elegans* not observed using manual body bend measures. (A) Body bend frequency count reveals no significant difference in locomotion in CPF-treated animals (t(26) = 0.515, ns). (B) Conversely, WormLab software records a marked decrease in locomotory speed in the same CPF-treated group (t(26) = 6.075, p < 0.0001). n = 15 control, n = 13 CPF. Bars represent mean values and 95% confidence intervals. 'ns' indicates not significant, while asterisks (****) denote a p-value < 0.0001.

No difference in locomotion was detected by counting body bends in animals treated with 0.1 mg/L CPF (t(26) = 0.515, ns). WormLab however, recorded a noticeable difference in locomotory speed from the same group of animals (t(26) = 6.075, p < 0.0001). That WormLab was able to detect this difference and manual counting of body bends could not, suggests that worms were covering smaller distances on average per number of body bends. The discrepancy between the results of the two techniques also suggests that counting body bend frequency may not be sensitive to low-level CPF exposure in *C. elegans* under these experimental conditions. This discrepancy suggests that worms

cover smaller distances per body bend under CPF exposure and that body bend count may not accurately reflect locomotory changes due to low-level CPF exposure. Previously, measuring body bends has been described as a measure of locomotory effort (Hart, 2006), whereas locomotory speed measured here may be considered as locomotory success. This distinction is important as locomotory speed appears to be the more sensitive to low-level effects in this case.

4.6. Nematodes treated with 0.05 mg/L CPF do not show any behavioural, physiological, or biochemical evidence of acute AChE inhibition.

A critical objective in this chapter was to identify an exposure concentration that does not cause significant inhibition of AChE (objective one). Taken together, the results of the AChE enzyme assay, body-length measurements, and observed normal locomotory rate in the presence of bacteria all indicate that treatment with 0.05 mg/L CPF has a relatively small effect on AChE activity in *C. elegans* when compared to higher concentrations. 0.05 mg/L CPF was therefore considered to be an appropriate concentration with which to explore behaviours relating to neurotransmitters associated with mood. Having previously looked at SWIP and egg laying behaviours and observed no effect at the higher concentration of 0.5 mg/L CPF, a set of associated behaviours which are sensitive to internal state in *C. elegans* was selected - the BSR and ESR.

4.7. Basal and enhanced slowing

A well-characterised behavioural paradigm which both neatly demonstrates and separates the roles of dopaminergic and serotonergic signalling, is the BSR and ESR described by Sawin et al. (2000). These behaviours are sensitive to *C. elegans*' perception of its environment, as well as the organism's historical experience and internal state relating to food. The BSR and ESR are also modulated by DA and 5-HT, respectively, and have been shown to interact with several mood-related and behaviourally therapeutic drugs (Izquierdo, Calahorro and Ruiz-Rubio, 2013). They were therefore considered suitable to test for any interaction with low-level CPF exposure and mood-related neurotransmitter function.

4.7.1. Wild-type BSR and ESR were observed in untreated nematodes.

First, to confirm that basal and enhanced slowing could be replicated in wild type animals, untreated nematodes were tested for each response (**Figure 35**). A two-way independent ANOVA was performed to test for differences between well-fed and food deprived animals, with or without the presence of OP50 bacteria. As expected, the presence of bacteria caused a reduction in locomotory speed overall ($F(1, 396) = 97.33$, $p < 0.0001$), and this was also significantly affected by whether the animals had been well fed (F (1, 396) = 24.94, p <0.0001). However, there was no significant interaction between the two factors (F (1, 396) = 2.496, ns). Well-fed animals moved 29% more slowly on plates containing bacteria than well-fed animals on plates without food, with Tukey's multiple comparison test indicating a significant BSR ($p < 0.0001$). An enhanced slowing rate of 45.62% ($p < 0.0001$) was observed in animals that had been deprived of food for 30 mins, upon reintroduction to food (Tukey's test of food-deprived/bacteria present vs food-deprived/no bacteria: $p < 0.0001$). Moreover, food-deprived animals were significantly

slower than well-fed animals when both groups were presented with bacteria (p < 0.0001), confirming an ESR in wild type untreated animals. Locomotory speed was not significantly different between well-fed and food deprived animals when bacteria were not present, which also mirrored the paradigm described by Sawin et al. (2000).

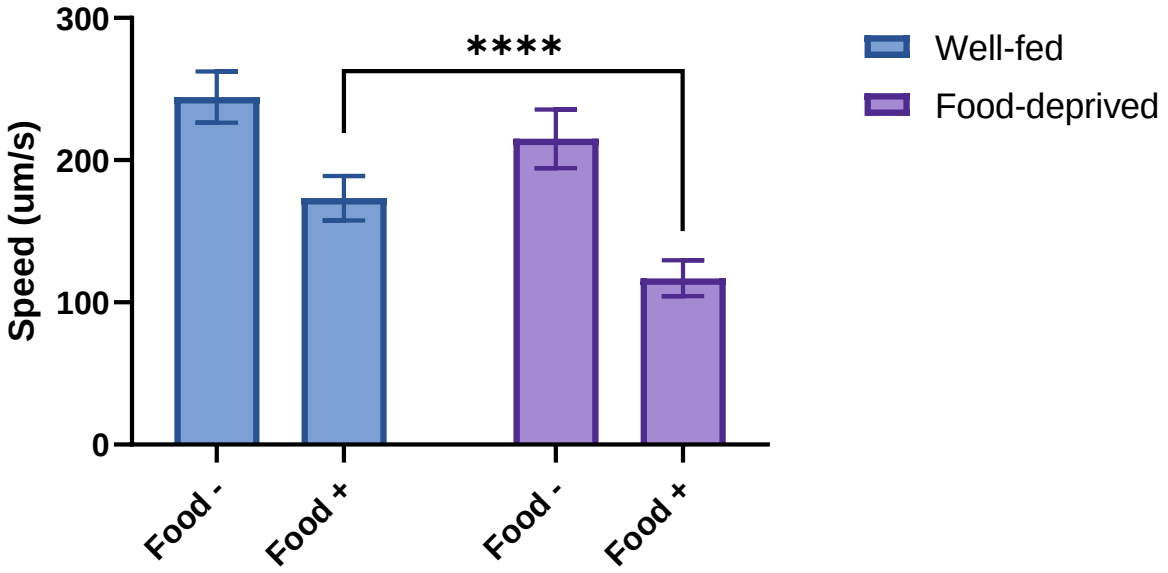

Figure 35. Wild type *C. elegans* undergo basal and enhanced slowing responses to food. Well-fed and food-deprived nematodes were tested for BSR and ESR, as described by Sawin et al. (2000) but using locomotory speed measured by WormLab rather than counting body bends. Previously well-fed animals moved more slowly in the presence of OP50 bacteria (n = 100) than in the absence of bacteria (n = 100) (mean difference: 71.02, 95% CI [39.75, 102.3]). The slowing response was enhanced when nematodes were deprived of food for 30 minutes before being moved to plates with (n = 100) or without bacteria (n = 100) (mean difference: 98.10, 95% CI [66.83, 129.4]). Bars represent mean and 95% CI. **** = p < 0.0001.

4.7.2. 0.05 mg/L CPF reduces locomotory speed in well-fed nematodes.

Having established that basal and enhanced slowing could be replicated in untreated wild-type *C. elegans*, the next step was to test whether CPF treatment had any effect on those behaviours. This would help to determine any involvement of dopaminergic or serotonergic signalling in the effects of low-level CPF exposure (**Figure 36**).

First, to test for any effect of low-level CPF on the BSR which is mediated by DA, wild type animals exposed to 0.05mg/L CPF or vehicle control were measured for locomotory speed both with and without OP50 bacteria present. A two-way independent ANOVA showed an overall difference in locomotion caused by the presence of bacteria ($F(1, 476) = 45.84$, $p < 0.0001$, 95% CI [35.46, 64.46]). **Figure 36** compares animals exposed to acetone as a control with and without bacteria (blue bars, + and - respectively), with CPF treated animals with and without bacteria present (tan coloured bars, + and -). There was a significant overall effect of CPF treatment ($F(1, 476) = 45.84$, $p < 0.0001$, 95% CI [55.46, 84.46]). There was a significant interaction between bacterial presence and CPF treatment (F (1, 476) = 13.65. p=0.0002. Post-hoc analysis using Tukey's test revealed that the significant main effect of bacterial presence was driven mainly by the (31.74%) BSR observed in the control group ($p < 0.0001$). Conversely, although CPF-treated nematodes moved more slowly (15.52%) on food than the CPF-treated group without food this was not significant at the 95% confidence level. Interestingly, locomotory speed did not differ significantly between either of the CPF treated groups, or the control group with bacteria present. Therefore, the most striking difference was that CPF treated animals moved more slowly than controls in the absence of food ($p < 0.0001$). While still interesting, this was a reduction in speed in the absence of food, rather than an effect on basal slowing which occurs in the presence of food. The absence of an effect on basal

slowing suggested that that the effects of 0.05 mg/L CPF on locomotion may not act directly through the same DA-mediated motor circuit.

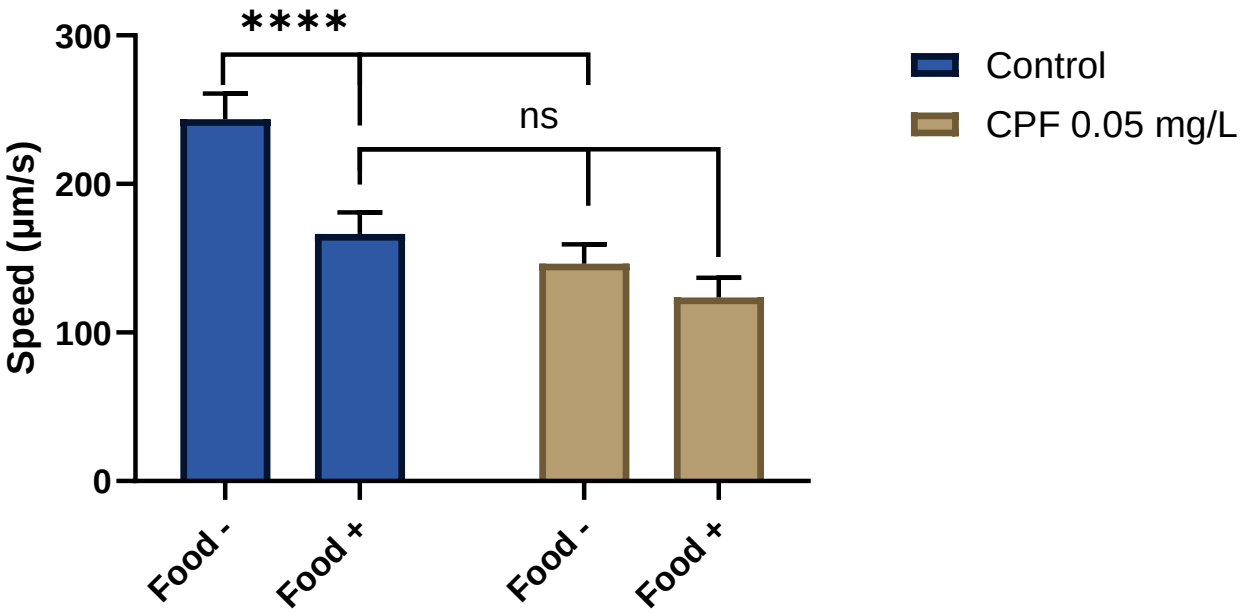

Figure 36. Basal Slowing Response (BSR) was not demonstrated in wild-type *C. elegans* exposed to CPF. Wild-type BSR was tested in nematodes exposed to 0.05 mg/L CPF (n = 120) or vehicle control (n = 120). Basal slowing was observed in control animals (mean difference: 77.22, 95% CI [50.31, 104.1]). A small but non-significant speed reduction was observed in CPF treated animals on food (mean difference: 22.7, 95% CI [-4.20, 49.61]). The experiment was run in triplicate with 30 animals in each condition and data pooled for analysis. Bars represent mean and 95% CI. **** = $p < 0.0001$, ns = not significant.

4.7.3. 0.05 mg/L CPF reduces locomotory speed in food-deprived nematodes.

Next, to test for effects of CPF on the 5-HT-mediated ESR, nematodes treated for 24 hours with 0.05 mg/L CPF or vehicle control, were deprived of food for 30 min before being assayed for locomotory speed (**Figure 37**). A two-way independent ANOVA showed an overall difference in locomotion based on both the presence of food ($F(1, 476) = 77.91$, p

< 0.0001, 95% CI [48.75, 76.67]) and CPF treatment ($F(1, 476) = 20.72$, $p < 0.0001$, 95% CI [18.38, 46.30]), with a significant interaction (F (1, 476) = 45.82, p<0.0001). An enhanced rate of slowing (50.03%) was observed in the control group with bacteria present ($p < 0.0001$). However, slowing rate (10.35%) was not significant in the CPF exposed group. The locomotory speed of food-deprived animals did not differ significantly between the CPF treated and control groups when food was present. However, in contrast to the basal slowing results, control animals with bacteria present were significantly slower than CPF treated animals without bacteria ($p < 0.05$).

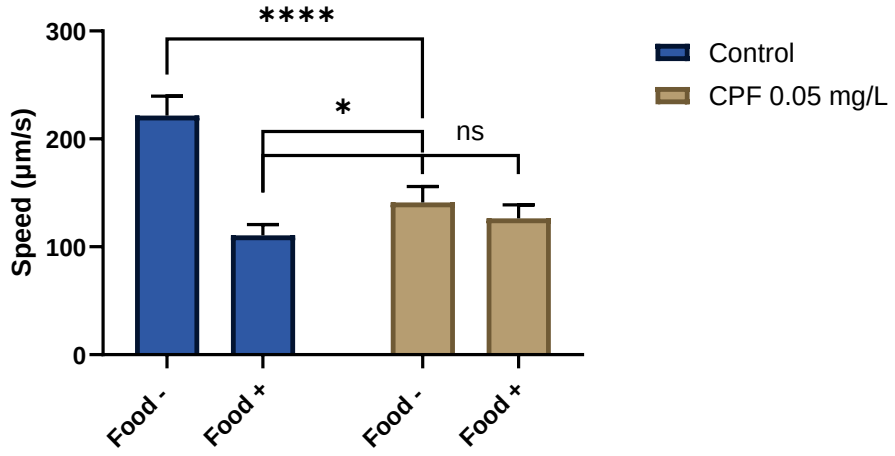

Figure 37. Enhanced Slowing Response (ESR) was not demonstrated in wild-type *C. elegans* exposed to 0.05 mg/L CPF. Wild-type ESR was tested in nematodes exposed to 0.05 mg/L CPF (n = 120) or vehicle control (n = 120) and deprived of food for 30 min. Enhanced slowing was observed in control animals (mean difference: 110.8, 95% CI [84.90, 136.7]. A smaller difference observed in animals treated with 0.05 mg/L CPF which was not significant (mean difference: 14.62, 95% CI [-11.29, 40.52]). The experiment was run in triplicate with 30 animals in each condition and data pooled for analysis. Bars represent mean and 95% CI. **** = p < 0.0001, * = p < 0.05.

Importantly, although this phenotype emerged under the conditions set out for testing basal and enhanced slowing rates, the observed difference is not a genuine change in either BSR or ESR, because animals treated with 0.05 mg/L CPF showed similar locomotory behaviour to animals treated with acetone as a control when food was present (**Figure 37**). Instead, the difference seen in animals treated with 0.05 mg/L CPF is more accurately described as a failure to increase speed in response to the absence of food (**Figure 36**; **Figure 37**). This distinction suggested that the mechanism of action is likely different from the BSR and ESR and therefore required further investigation.

4.7.4. BSR and ESR in a 5-HT synthesis deficient mutant (tph-1(mg280))

So far, any effects of CPF treatment on BSR and ESR were difficult to determine. This was because locomotory rate in the absence of food serves as the baseline against which slowing response is measured. It was therefore the baseline that was altered by 0.05 mg/L CPF, rather than the slowing response. These results did not suggest or exclude any specific role for DA or 5-HT in the effect of low-level CPF treatment, and so to investigate this further, mutants with deficiencies in 5-HT and DA synthesis were assayed for BSR and ESR.

4.7.5. 5-HT is not required for the effect of CPF on locomotion in the absence of food.

Mutants carrying the *tph-1(mg280)* allele, which are deficient in 5-HT production (Sze *et al.*, 2000), were exposed to 0.05 mg/L CPF, or vehicle control for 24 hours, before being assayed for BSR and ESR as described above (**Figure 38**).

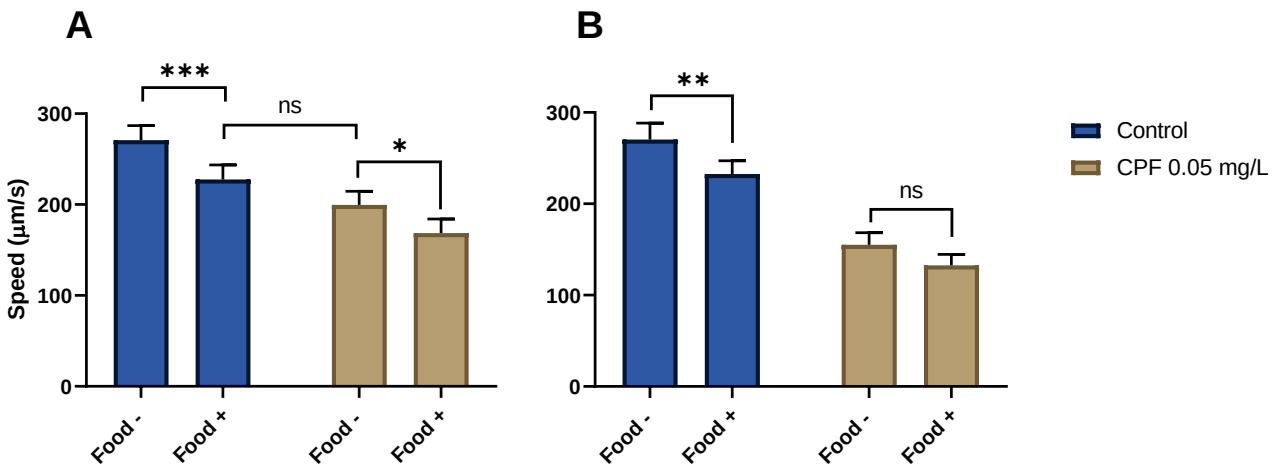

Figure 38. Enhanced slowing response (ESR) is suppressed in 5-HT-synthesis deficient *tph-1(mg280)* mutants and the effect is not changed by CPF treatment. Basal (**A**) and enhanced (**B**) slowing response were tested in 5-HT synthesis deficient *tph-1(mg280)* mutants exposed to 0.05 mg/L CPF (n = 120), or vehicle control (n = 120). Well-fed control animals showed a BSR (mean difference: 43.30, 95% CI [14.51, 72.10]), as did the well-fed CPF-treated group (mean difference: 31.04, 95% CI [2.246, 59.84]). Enhanced slowing was not observed (**B**) in these 5-HT deficient animals, as described previously (Ben Arous, Laffont and Chatenay, 2009) . Bars represent mean values and 95% confidence intervals. *** = p-value < 0.001, ** = p-value < 0.01, * = p-value < 0.05, 'ns' = not significant.

4.7.5.1. Basal slowing is not affected by CPF treatment or 5-HT synthesis.

Well-fed *tph-1(mg280)* mutants displayed normal BSR in response to bacteria (**Figure 38 A**) ($F(1, 476) = 22.15$, $p < 0.0001$, 95% CI [21.65, 52.69]). A similar BSR was observed in control animals ($p < 0.001$) to that seen in animals treated with 0.05 mg/L CPF ($p < 0.05$) (15.99% and 15.53% slowing, respectively). CPF treatment therefore did not affect BSR in the absence of 5-HT signalling.

The reduced baseline speed in the absence of food following treatment with CPF 0.05 mg/L was similar in *tph-1(mg280)*mutants to that seen earlier in the wild type (**Figure 36**; **Figure 38 A**). Therefore, since the same effect was observed in the absence (*tph-1(mg280)*mutant) and presence (wild type) of 5-HT signalling, 5-HT was not responsible for this effect of CPF.

4.7.5.2. Suppression of ESR in 5-HT deficient mutants is unaffected by CPF treatment.

Locomotory speed of food-deprived *tph-1(mg280)* mutants was reduced by the presence of bacteria ($F(1, 476) = 16.92$, $p < 0.0001$, 95% CI [15.70, 44.41]), and by treatment with 0.05 mg/L CPF ($F(1, 476) = 217.2$, $p < 0.0001$, 95% CI [93.33, 122.0]) (**Figure 38 B**). However, the slowing rate in the control group (13.95% slowing) was slightly lower than the baseline BSR response in *tph-1(mg280)* mutants (15.99% slowing, **Figure 38 A**). Therefore, in line with previous reports, ESR was not observed in *tph-1(mg280)* mutants, (Sawin, Ranganathan and Horvitz, 2000; Ben Arous, Laffont and Chatenay, 2009). Furthermore, the reduced speed in the presence of food caused by treatment with 0.05 mg/L CPF was unaffected by 30 mins of food deprivation, or by impaired 5-HT synthesis in tph-1(mg280) mutants.

Having found no effect of impaired 5-HT synthesis on the locomotory rate change caused by low-level CPF treatment, the experiment was repeated using a DA deficient mutant.

4.7.6. BSR and ESR in a DA synthesis deficient mutant (cat-2(e1112))

Mutants carrying the *cat-2(e1112)* allele, which are deficient in DA synthesis but normal for 5-HT (Sulston, Dew and Brenner, 1975; Loer and Kenyon, 1993) were exposed to 0.05

mg/L CPF or vehicle control for 24 hours, before being assayed for BSR and ESR as described above (**Figure 39**).

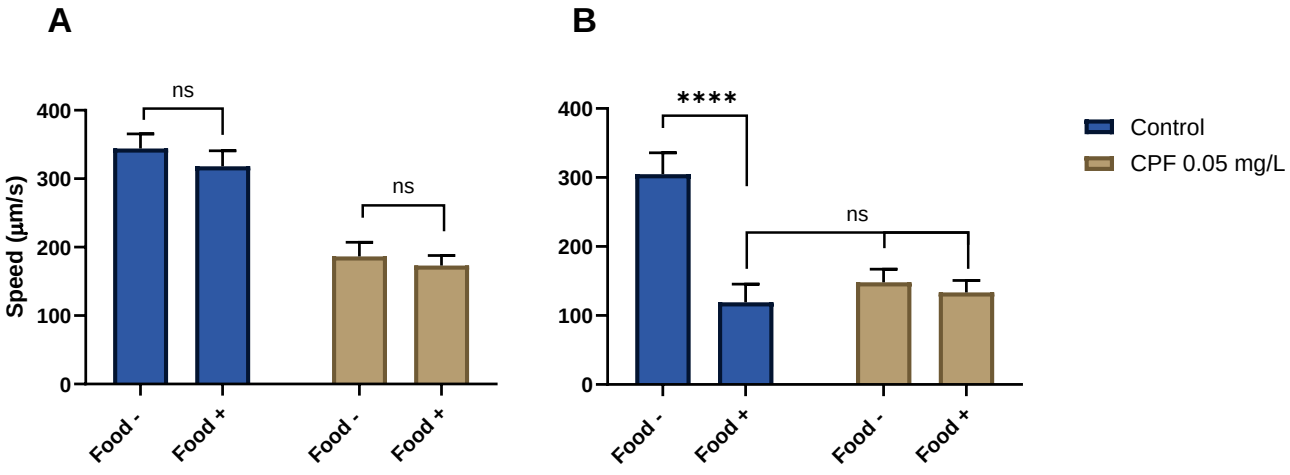

Figure 39. Enhanced slowing response (ESR) is not demonstrated in DA-synthesis deficient cat-2*(e1112)* exposed to CPF, but baseline speed is affected. (A) Well-fed *cat-2(e1112)* mutants do not exhibit a BSR in line with results reported by Sawin, Ranganathan and Horvitz (2000). Slowing response was not affected by CPF treatment. (B) Food-deprived vehicle control animals (n = 87) demonstrate an ESR (mean difference: 185.9, 95% CI [136.2, 235.5]), an effect not observed in food-deprived CPF-treated animals (n = 115). Bars represent mean values and 95% confidence intervals. **** = p-value < 0.0001, 'ns' = not significant.

4.7.6.1. Suppression of BSR in cat-2(e1112) mutants is not affected by CPF treatment.

In agreement with previous work (Sawin, Ranganathan and Horvitz, 2000) there was no reduction in locomotory speed in response to the presence of bacteria (BSR) in well-fed *cat-2(e1112)* mutants. The same was true for both the control, and for CPF-treated groups (**Figure 39 A**).

An overall effect of CPF treatment was observed in well-fed animals ($F(1, 476) = 223.7$, $p < 0.0001$, 95% CI [131.5, 171.3], with CPF-treated animals moving significantly more slowly than controls, with or without bacteria present ($p < 0.0001$, respectively).

Treatment with 0.05mg/L CPF did not affect the suppression of BSR in DA deficient *cat-2(e1112)* animals. However, CPF treatment did reduce locomotory speed in *cat-2(e1112)* mutants when food was not present, in a similar way to that seen in wild type. DA signalling was therefore not responsible for this effect of CPF.

4.7.6.2. Baseline speed but not ESR is reduced in cat-2(e1112) mutants exposed to CPF.

Food-deprived *cat-2(e1112)* mutants exposed to solvent control showed ESR ($F(1, 198) = 64.51$, $p < 0.0001$, 95% CI [75.66, 124.9]). Overall, locomotory speed was reduced by treatment with 0.05 mg/L CPF ($F(1, 198) = 32.46$, $p < 0.0001$, 96% CI [46.51, 95.76]). Food-deprived animals were slower than the well-fed group when food was present (**Figure 39 A & B**) confirming that 5-HT mediated slowing was present in the control group (Sawin, Ranganathan and Horvitz, 2000).

CPF treatment did not significantly affect speed in food-deprived *cat-2(e1112)* mutants when food was present. However, CPF-treatment prevented *cat-2(e1112)* mutants from increasing speed when food was not present (**Figure 39 B**).

The inhibition of basal slowing in DA deficient *cat-2(e1112)* is in line with previous reports (Sawin, Ranganathan and Horvitz, 2000) and the general reduction in speed following treatment with 0.05 mg/L CPF was not dependent on DA signalling.

Absence of ESR in *cat-2(e1112)* mutants treated with 0.05 mg/L CPF was similar to that observed in CPF-treated wild-type animals (**Figure 37**). This was not a direct effect on enhanced slowing but was instead a failure to increase speed in the absence of food.

4.8. Discussion

4.8.1. 0.05 mg/L CPF is a suitable concentration for modelling low-level exposure in C. elegans.

To meet the first objective set out in this chapter, AChE was measured in treated animals and the results showed that 0.05 mg/L CPF did not significantly inhibit AChE. This supports the use of 0.05 mg/L as a suitable concentration for modelling low-level CPF exposure in *C. elegans*. This result was surprising because 0.05 mg/L is tenfold lower than the concentrations previously described as low level for *C. elegans* treated with CPF in NGM plates (Viñuela *et al.*, 2010). However, the results presented in this chapter appear to be the first time that AChE activity has been reported for *C. elegans* using this exposure method. This is useful because exposure in NGM makes longer treatment durations possible by allowing otherwise standard feeding and rearing conditions to be maintained during treatment. Furthermore, the range of worm-behaviours performed on solid surfaces is richer and more diverse than in liquid, which extends the range of assays and pathways that can be tested. Therefore, these results are beneficial to this investigation, and extend the lower end of the concentration range at which changes in behaviour may be detected following CPF treatment in *C. elegans*.

Interestingly, Roh, Lee and Kwon (2016) reported 0.06 mg/L CPF as a lowest observed effect level LOEL (the dose or concentration at which an effect can be detected) for changes in gene expression relating to toxic metabolism in *C. elegans,* but without detectable changes in behaviour. Those results seem in line with a low-level effect occurring in the absence of AChE inhibition and at an exposure concentration very similar to 0.05 mg/L CPF, which did not inhibit AChE in this study. Together these results support the use of 0.05 mg/L CPF as a low-level exposure concentration, even though they do not

provide any evidence for an effect of CPF on mood-related neurotransmission at this concentration.

It may be possible that the absence of AChE inhibition could be affected by the feeding state of the nematodes in these experiments. During periods of food deprivation, several factors could increase the likelihood of AChE inhibition as a result of OP exposure. This could result from an increase in absorption and increased bioavailability, impaired detoxification and elimination through reduced energy resources. The processing of nematodes for the AChE activity assay includes several washes to remove food, and therefore the worms would have been food deprived for around 30 minutes prior to homogenization, and this was true for all treatment groups. We did not test for the effect of extended periods of food deprivation substantially longer than 30 minutes, or for effects of worms exposed to CPF entirely in the absence of food. However, 30 minutes of food deprivation did not lead to any measurable inhibition of AChE in worms treated with 0.05 mg/L CPF.

Further support for the use of 0.05 mg/L CPF as a low-level concentration comes from the physiological changes evident in the body length measurements. In a similar pattern to that observed for AChE inhibition, body length was not significantly affected by treatment with 0.05 mg/L CPF, but worms were shorter after being exposed to higher concentrations. Acute AChE inhibition is known to cause contraction of the body-wall muscles (Kearn *et al.*, 2014) and so reduced body length was predicted. Most importantly, the hypothesis that CPF concentrations that do not cause significant AChE inhibition would not have any effect on body length was also supported by these results. This was useful because it suggests that 0.05 mg/L CPF does not cause acute cholinergic toxicity and therefore meets the objective criteria for a low-level working concentration.

Furthermore, body-length is simple to measure and so could provide a convenient visual check measure for AChE inhibition in future experiments. Body-length measurements would not remove the need for measuring enzyme activity completely. However, once a working concentration has been established, data showing body shortening in CPF exposed animals could indicate unacceptable levels of AChE inhibition and provide a criterion for exclusion.

4.8.2. Automated body-length measurement is more efficient and more accurate than manual measurement.

The potential usefulness of body-length measurements as a criterion for exclusion is increased if length can be measured automatically and presented alongside behavioural data for each sample. WormLab was able to measure body-length from every video frame, following each worm through each change of body posture and producing an average length across the whole video. This provided much more consistent and accurate measurements than measurements taken manually using ImageJ. This was not surprising because manual measurements were very basic head-to-tail measurements and were only taken over 3 frames for each worm. The accuracy of the manual measurements could therefore be improved; however even the basic measurements are considerably more time-consuming than the automated measurements, which are available instantly, and there is nothing to be gained in terms of accuracy. Moreover, automated body-length measurements can be presented alongside behavioural data for each sample, which will be discussed further in the next chapter.

5. Low-level chlorpyrifos treatment reduces foraging range in *C. elegans.*

5.1. Introduction

The results described in the previous chapters show that even 0.1 mg/L and 0.5 mg/L CPF, which have previously been described as 'low-level', can inhibit AChE activity in *C. elegans*. The behavioural effects described in previous chapters were difficult to separate from AChE inhibition at 0.1, 0.25 and 0.5 mg/L CPF. Treatment with 0.05 mg/L CPF did not significantly inhibit AChE activity or cause any overt physiological or behavioural changes, thus satisfying our criteria for modelling low-level exposure. The absence of overt effects presents a major challenge for detecting effects of low-level OP exposure in clinical settings (Blain, 2001) and this was mirrored here in *C. elegans.* Assays designed to investigate specific neurotransmitter systems, including DA and 5-HT, failed to show any recognisable phenotype in well-fed animals. However, while performing enhanced slowing assays, food-deprived animals showed a CPF-induced reduction in baseline locomotory speed that did not conform to the enhanced slowing behaviour described previously (Sawin, Ranganathan and Horvitz, 2000). In this chapter, the observed locomotory effect is described further and results from the investigation of potential causal mechanisms are discussed.

5.1.1. Food deprivation mediates the effects of CPF exposure in C. elegans.

The newly observed phenotype was discovered serendipitously during BSR and ESR assays, notably because those assays impose restrictions on the animals' access to a bacterial food source (Sawin, Ranganathan and Horvitz, 2000). While the observed

phenotype was not caused by changes in BSR or ESR directly, it was observed consistently in each of the strains tested so far under CPF treatment when the bacterial food source was removed. The absence of food was therefore a conditional requirement for the observed effect of CPF treatment at 0.05 mg/L. This presented clues to the possible causal mechanisms and the experimental conditions under which the behaviour could be reproduced for investigation. Potential mechanisms that might influence behaviour in the absence of food were therefore considered, to help guide the experimental approach.

5.1.2. C. elegans behavioural states relating to food availability.

C. elegans is known to increase locomotory speed when food is scarce (Fujiwara, Sengupta, and McIntire, 2002; Ben Arous, Laffont and Chatenay, 2009; Flavell *et al.*, 2013; McCloskey *et al.*, 2017). This speed increase forms part of a wider behavioural state known as 'roaming', which is also characterised by a relatively wide search area and low turn frequency (Fujiwara, Sengupta, and McIntire, 2002). Worms are less likely to roam when a suitable food source is present in the environment and are instead more likely to cover a smaller local area, making frequent turns and moving more slowly, which are collectively known as 'dwelling' (Ben Arous, Laffont and Chatenay, 2009). Distinct from dwelling, a third state known as 'quiescence' can occur when animals reach satiety after feeding, and is characterised by cessation of feeding, and low levels of movement (You *et al.*, 2008).

Dwelling, roaming, and quiescence have been studied extensively in *C. elegans*, and are known to be regulated by a combination of neuropeptide and biogenic amine signalling (Ben Arous, Laffont and Chatenay, 2009; Flavell *et al.*, 2013; McCloskey *et al.*, 2017; McClanahan *et al.*, 2020). Therefore, if the change in locomotory speed induced by 0.05 mg/L CPF was indicative of a reduction in roaming and an increase in dwelling or

quiescence, it could provide clues to the underlying mechanisms responsible for the effect of low-level CPF treatment. This could be tested by observing food-deprived worms exposed to 0.05 mg/L CPF, to see if their behaviours fit the profiles described for dwelling or quiescence.

5.1.3. A possible effect on cholinergic signalling?

The change in speed observed following treatment with 0.05 mg/L CPF was dependent on the availability of food. Therefore, a link to food-related drive and foraging behaviours seemed plausible. However, it remained possible that CPF treatment simply reduced the worms' ability to move quickly, irrespectively of food, drive, or motivational state. Hypothetically, reduced locomotory capacity due to CPF treatment might only become apparent after certain speed thresholds are met, incidentally in this case, associated with food seeking behaviour. Such an effect might possibly relate to altered cholinergic signalling as a result, or independently, of residual AChE inhibition (Lewis *et al.*, 1980; Nguyen *et al.*, 1995; Melstrom and Williams, 2007a; Liu *et al.*, 2018).

Locomotory behaviour in *C. elegans* relies more heavily on cholinergic signalling than any other behaviour (Rand, 2007). Moreover, inhibition of AChE is the most widely reported mechanism of action of CPF and most other OP compounds (Ballantyne and Marrs, 2017), and although a growing number of studies report non-cholinergic effects at low-level OP exposures, the evidence remains equivocal. AChE enzyme activity has not always been measured in studies that have reported low-level effects (Anderson, Cole, and Williams, 2004a; Ruan *et al.*, 2009b; Boyd *et al.*, 2010; Ju *et al.*, 2014). Some effects have been reported as occurring in the absence of AChE inhibition, based on comparisons between different studies, some measuring only AChE activity, and others measuring behaviour in isolation, sometimes ignoring critical methodological differences, or misinterpretation of

study results (e.g., Roh and Choi, 2008; Ju et al., 2014; & Roh, Lee and Kwon, 2016 in: Silva, 2020).

The disregarding of changes in AChE inhibition from some reported behavioural or physiological effects of OP treatment could also be problematic because effects are often described as 'non-cholinergic', even in the presence of "little", "minimal", or "barely-detectable" AChE inhibition (McDaniel, Phillips and Moser, 2003; Slotkin and Seidler, 2007; Timofeeva *et al.*, 2008; Slotkin, Levin and Seidler, 2009; Carr *et al.*, 2014; Savy *et al.*, 2018; Dawson, this study). In this context, levels of AChE inhibition reported in studies can vary between 0 - 10% (Slotkin *et al.*, 2006; Timofeeva *et al.*, 2008), the contribution of which could be argued to be significant.

Although no statistically significant inhibition of AChE was detected following exposure to 0.05 mg/L CPF in this study, we chose to consider the possibility that some biologically significant change in cholinergic signalling might nevertheless be involved. This would therefore form a part of the investigations described in this chapter.

5.1.4. Dopamine and serotonin

The previous results chapters described several behavioural assays which have been linked to mood-related neurotransmitter pathways, including BSR and SWIP for DA signalling, and ESR and egg laying behaviours for 5-HT signalling pathways. Although CPF treatment did not affect those specific behaviours, the possibility that those pathways were involved in its effects could not be excluded. Moreover, changes in DA and 5-HT systems have been heavily implicated in alterations in human mood (Cosci and Chouinard, 2019), in animal models of mood related behaviours, and in neurochemical responses to OP exposure in several studies (Slotkin *et al.*, 2006; Kobayashi *et al.*, 2008;

Venerosi *et al.*, 2010; Lima *et al.*, 2011; Bermingham *et al.*, 2016; Judge *et al.*, 2016; Carr, Alugubelly and Mohammed, 2018; Silva, 2020). Furthermore, in rodent models, different behavioural paradigms that are modelled on human depression or anxiety specifically can yield different results, and detect distinct mechanisms (Kobayashi *et al.*, 2008; Savy *et al.*, 2015). Taken together, this suggests that mood-related neurotransmitter systems such as DA and 5-HT could still be important to the effects of low-level CPF treatment and were therefore included in the ongoing investigation.

5.2. A three-pronged approach

5.2.1. Following the observed phenotype

Having discovered a behavioural difference in nematodes exposed to 0.05 mg/L CPF that was below the threshold for significant AChE inhibition, the aim was to clarify the mechanisms surrounding this behaviour. To do this, a three-pronged approach was employed to investigate the effects of low-level CPF treatment on *C. elegans* behaviour, to enable subsequent investigation of the genetic mechanisms responsible. Firstly, since the behaviour was not immediately identifiable within the paradigms tested so far in this study; it was studied further to understand its possible functional context in line with known *C. elegans* behaviours. The behaviour was affected by the presence of food within the environment, and so known foraging behaviours in *C. elegans* were considered in the first part of this three-pronged approach.

5.2.2. Following the known mechanism of action of OPs: the cholinergic system

For the second part of the approach, consideration was given to the fact that although AChE inhibition was not statistically different from vehicle control levels in this study, behavioural effects reported in the literature have rarely occurred alongside absolute AChE inhibition. Furthermore, since acetylcholine acts as both a neurotransmitter and a neuromodulator (Picciotto, Higley and Mineur, 2012), interactions between cholinergic signalling and other neurotransmitter systems are difficult to separate. Therefore, because CPF is most known for inhibiting AChE, and altering cholinergic signalling, the observed effect on foraging behaviour was tested further for interactions with cholinergic signalling in *C. elegans*.

5.2.3. Following suggested links to mood-related neurotransmitter pathways.

Since DA and 5-HT signalling have been heavily implicated in mood, behaviour and the neurochemical effects of OP exposure, the behaviour was also considered in the context of those systems.

5.3. Chapter objectives

The three main objectives of this chapter are:

1) To use WormLab behavioural tracking software to measure and quantify the behavioural phenotype induced by 0.05 mg/L CPF.

2) To investigate biological mechanisms associated with the observed phenotype using *C. elegans* nematodes with mutations in genes known to affect AChE and cholinergic signalling, DA and 5-HT signalling, and foraging behaviour.

3) To create a method for visualising subtle changes in behaviour caused by low-level toxic exposures in *C. elegans,* which could be used for screening different toxicants or genetic mutants of interest in future investigations.

5.1. Results

To explore the locomotion phenotype induced by 0.05 mg/L CPF in the absence of food further, wild-type worms were exposed to 0.05 mg/L CPF or acetone as a vehicle control for 24 hours, as described in the materials and methods chapter (2.2.5). Behaviour was recorded for 2 min for each condition and visualised using WormLab (**Figure 40**).

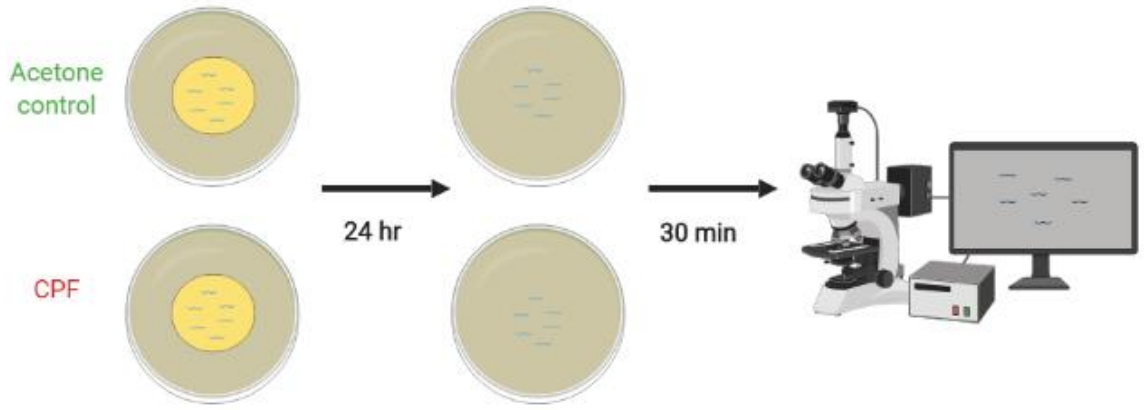

Figure 40. Schematic representation of the experimental setup used to investigate the effects of CPF treatment on *C. elegans* foraging behaviour. Worms were treated with 0.05 mg/L CPF or vehicle control for 24 hours on plates with food. Worms were then moved to food-free plates for a 30-minute period, which was necessary recreate the subtle effects of low-level CPF on foraging. Worms were recorded for 2 minutes under a light microscope. The captured video files were analysed using WormLab tracking software to quantitatively assess foraging behaviour changes.

5.1.1. Exposure to 0.05 mg/L CPF reduces speed and maximal Euclidean distance in food-deprived wild-type C. elegans.

Track plots were generated in WormLab showing the direction and distance over which worms moved relative to their starting point during the recordings. Track plots for each

condition showed that wild-type worms covered relatively large distances, whereas wild-type worms treated with 0.05 mg/L CPF covered a more restricted area (**Figure 42** & **Figure 43**). Such differences in movement have been quantified previously using the maximal Euclidean distance, or Rmax, which measures the farthest point in the track reached from the starting point, independently from the track length (**Figure 41**) (Dittman and Kaplan, 2008).

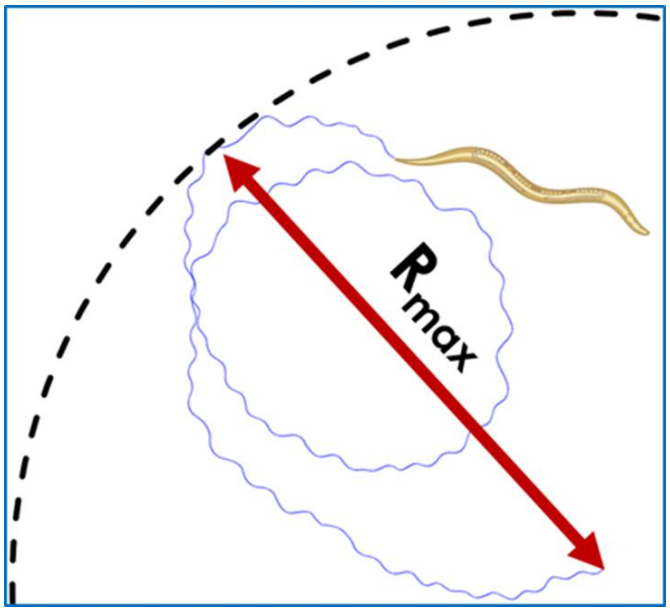

Figure 41. Illustrative depiction of the Maximum Euclidean Distance (Rmax) measurement in *C. elegans* foraging analysis. Rmax represents the greatest straight-line distance from the starting point of a track to the furthest point reached by the worm during each observation period. This parameter provides a quantifiable metric of foraging range, enabling the assessment of behavioural changes under different treatment conditions (Dittman and Kaplan, 2008).

Using the same measure here, an unpaired t test showed that Rmax was significantly reduced in CPF-treated wild-type animals compared to untreated vehicle controls (**Figure 44**) ($t(32) = 6.856$, $p < 0.001$). Track measurements taken from WormLab also

confirmed that speed was significantly reduced in CPF-treated worms ($t(35) = 4.842$, $p < 0.001$). This supported the suggestion that the phenotype observed following treatment with 0.05 mg/L CPF was related to a decrease in foraging range. Wild-type *C. elegans* are known to increase both foraging range and speed when food is scarce, and treatment with 0.05 mg/L CPF appeared to be preventing that transition.

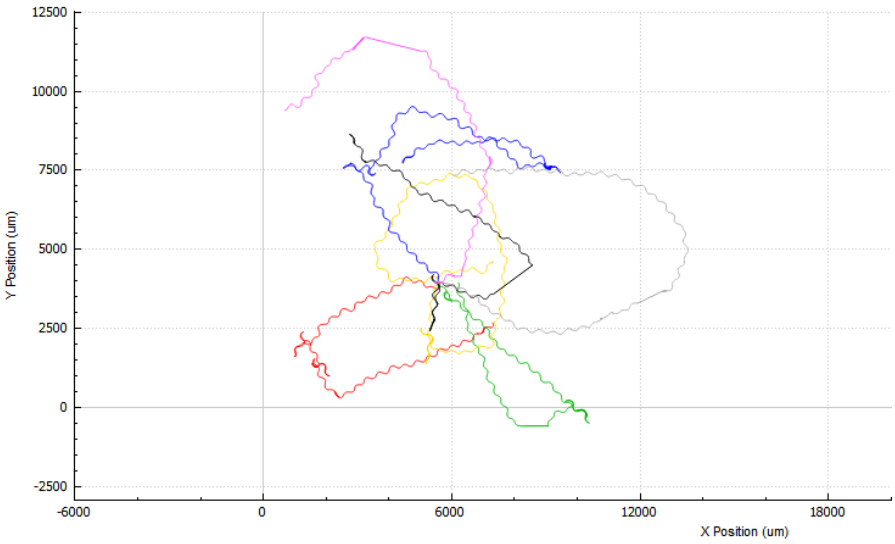

Figure 42. Wild type *C. elegans* displays foraging behaviour in the absence of food. Representative track plots showing the movements of wild-type *C. elegans* following exposure to vehicle control. Each colour corresponds to an individual worm's track recorded over a period of 2 minutes. The trajectories were captured and plotted using WormLab software, highlighting variations in the foraging patterns of the mutants. Tracks are centred meaning the tracks all begin from the same starting point.

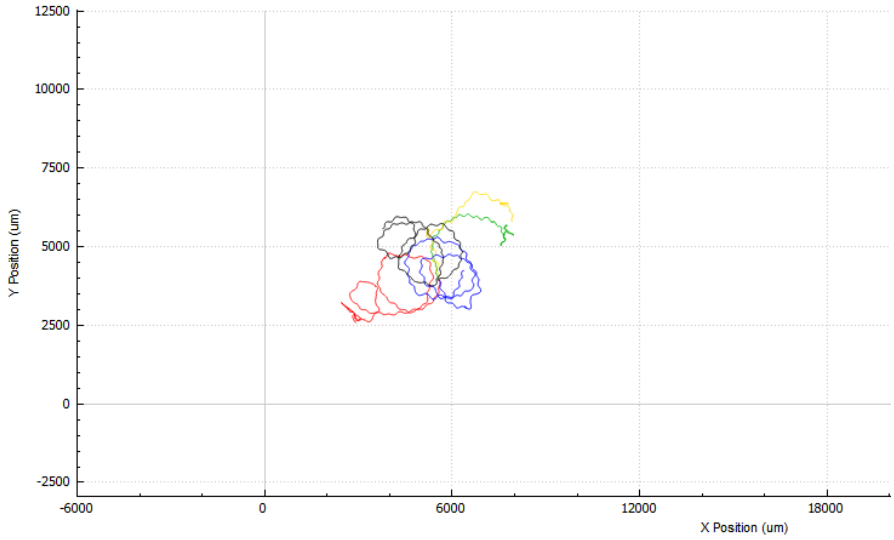

Figure 43. CPF treatment reduces foraging behaviour in the absence of food. Representative track plots showing the movements of wild-type *C. elegans* exposed to 0.05 mg/L CPF. Each colour corresponds to an individual worm's track recorded over a period of 2 minutes. The trajectories were captured and plotted using WormLab software, highlighting variations in the foraging patterns of the mutants. Tracks are centred meaning the tracks all begin from the same starting point.

Measuring Rmax proved to be an effective method for quantifying the effect of CPF treatment on foraging behaviour in wild-type animals, thus satisfying Objective 1 for this chapter. To investigate the underlying mechanisms using the three-pronged approach described earlier in this chapter, Rmax was measured in *C. elegans* carrying genetic mutations relevant to AChE, because AChE is a known target for OPs, mutations affecting DA or 5-HT signalling as known mood-related neurotransmitter systems, or in genes known to be involved in *C. elegans* foraging behaviour (**Table 13**). To test for the effects of CPF, worms with the respective mutations were treated with either 0.05 mg/L CPF, or acetone as a vehicle control. Wild-type *C. elegans* were exposed to the same conditions for comparison.

5.1.2. Neuronal acetylcholinesterase is necessary for increased foraging range in wild-type C. elegans.

To test whether AChE was a factor in the reduced foraging phenotype caused by CPF treatment, mutants with deficiencies in class-A AChE (*ace-1(ok663)*), class-B AChE (*ace-2(ok2545)*), class-C AChE (*ace-3 (dc2)*) and wild-type *C. elegans*, were treated with CPF or acetone as a vehicle control and tested for the foraging phenotype observed previously in wild type.

Both *ace-1(ok663)* and *ace-3 (dc2)*, which are deficient for class-A and class-B AChE, respectively (Combes *et al.*, 2003), showed similar reductions in foraging range to that seen in wild-type worms in response to CPF treatment (**Figure 44**).

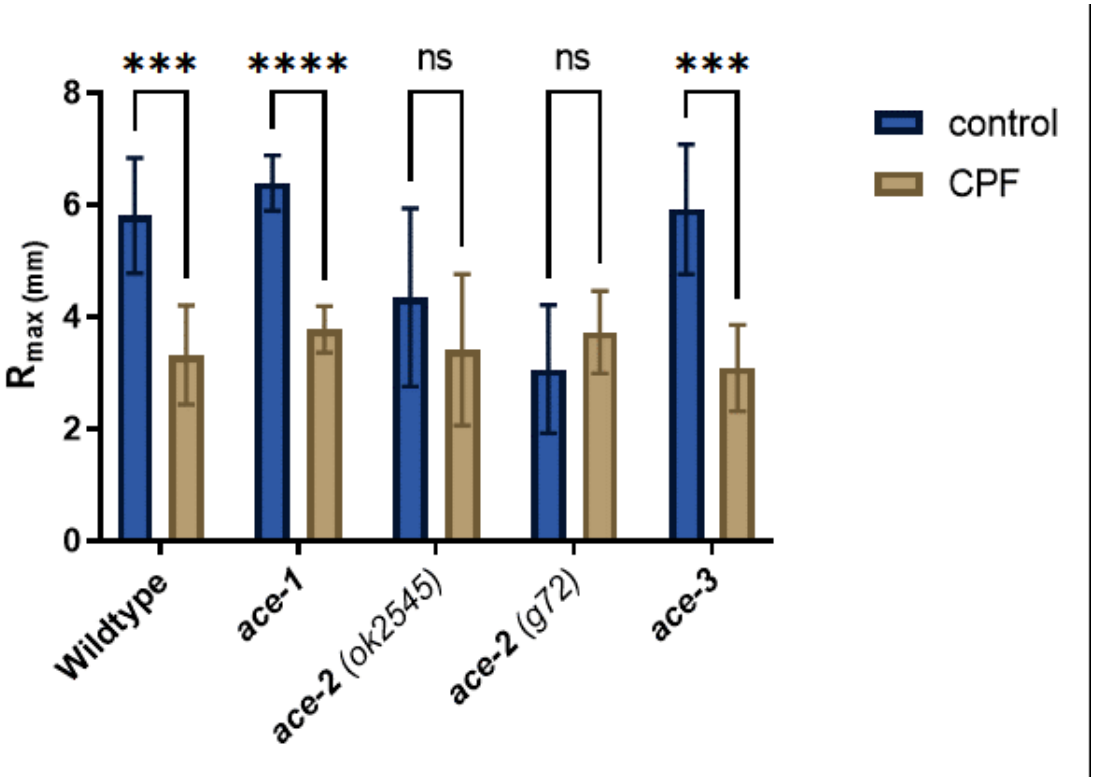

Figure 44. Knockout of *ace-2* mimics the effect of CPF on foraging. The figure shows a comparison of foraging ranges among wild-type *C. elegans* and AChE mutants treated with 0.05 mg/L CPF or acetone as a vehicle control. Strains tested include wild-type, *ace-1(ok663)* (class-A AChE deficient), *ace-2(ok2545)* (class-B AChE deficient), and *ace-3(dc2)* (class-C AChE deficient). Both *ace-1(ok663)* (n = 26 control, n = 28 CPF) and *ace-3(dc2)* (n = 16 control, n = 16 CPF) mutants exhibited CPF-induced reductions in foraging range similar to wild-type (n = 15 control, n = 15 CPF). However, the foraging range of CPF-treated *ace-2(ok2545)* mutants (n = 10) did not differ significantly from vehicle controls (n = 10). This was confirmed using another allele, *ace-2(g72)*, which also showed no significant changes in foraging behaviour (as measured by Rmax) following CPF treatment (n = 21 control, n = 20 CPF), suggesting a specific role of neuronal AChE in modulating CPF's impact on foraging range. Bars represent mean values and 95% confidence intervals. Statistical significance is denoted by *** ($p < 0.001$). **** ($p < 0.0001$), ns (not significant).

However, CPF-treated *ace-2(ok2545)* mutants, which are deficient specifically in neuronal AChE, did not decrease their foraging range significantly compared to acetone-treated vehicle controls (**Figure 44**). To confirm that this was an effect of ACE-2 and not some background mutation in the strain carrying *ace-2(ok2545)*, the experiment was repeated, this time including worms carrying mutations at a different allele of ACE-2: *ace-2(g72).* The results showed that worms with mutations at either allele: *ace-2(g72)* or *ace-2(ok2545)*, both failed to show the change in foraging behaviour measured by *Rmax* values, regardless of treatment with CPF (**Figure 45**).

These results showed that *ace-2* neuronal AChE is necessary for the decreased foraging range shown following treatment of wild type with 0.05 mg/L CPF. This was interesting and suggested that AChE activity could still be a principal factor in the effect of CPF treatment at such low concentrations.

Importantly however, the observed difference in *ace-2* mutants was not a direct reversal of the CPF-induced foraging phenotype seen in wild-type animals. Rather, the expected increase in foraging performed by vehicle control animals appeared to be absent in animals with *ace-2* mutations. ACE-2 deficiency therefore seemed to mimic, or suppress, the foraging phenotype induced by CPF treatment, rather than reverse it. If deficiencies in ACE-2 and CPF treatment caused similar effects, it could indicate ACE-2 as a potential target of CPF. This was interesting, but insufficient to reliably implicate *ace-2* as the target of CPF at this stage.

To gain a clearer picture of the effects and possible targets of CPF, more evidence would need to be considered.

5.1.3. Using a heatmap to visualise CPF induced behavioural effects in several different mutants simultaneously.

So far, the effects of CPF treatment had been tested using individual behavioural endpoints in AChE-deficient mutants and wild-type animals. This provided useful insights and suggested a role for AChE in the effects of low-level CPF treatment but did not exclude or indicate any other mechanistic explanations.

To gain a clearer picture of the mechanisms underpinning the CPF-dependent foraging phenotype, it would be useful to compare the effects of CPF on a greater number of behavioural endpoints, and in context with a range of potentially relevant mutations simultaneously. This would help to search for any emerging patterns in behavioural response. Given the possible implication of ACE-2, it would also be useful to view any emergent changes in body length, because significant inhibition of AChE that exceeds our criteria for low-level exposure was shown in the previous chapter to cause shortening of body length. Being able to visualise body length data from each treatment group would allow scrutiny and exclusion of treatment groups that showed signs of significant AChE inhibition. The use of a heatmap would help to visualise multiple endpoints as required.

To achieve this, several strains were selected based on either: deficiencies in cholinergic signalling, deficiencies in human mood-related neurotransmitter signalling, deficiencies in *C. elegans* foraging behaviour, or a combination of any of these characteristics (**Table 13**). The mutants and wild-type animals were exposed to 0.05 mg/L CPF or acetone as a vehicle control for 24 hours as describe previously, and behaviour was recorded for 2 min for each condition. Selected readouts were recorded for each strain, including body size, locomotory speed, and Rmax values. Body wavelength, amplitude, and the number of turns and reversals were all included in this example (**Figure 45**).

To visualise the effects of CPF exposure, multiple t-tests were used to compare the CPF and vehicle control group score for each endpoint. Where more than one strain was tested together, multiple t-tests were carried out across the strains for one endpoint at a time, using the two-stage step-up procedure of Benjamini, Krieger and Yekutieli, (2006). The false discovery rate (FDR) adjusted p values (Q-values), representing the difference between CPF-treated and untreated vehicle control groups for each endpoint, were colour coded and added to each cell of a heatmap (**Figure 45**).

Table 13. *C. elegans* mutant strains (column 1) presented in Figure 45. The Reason for inclusion column contains information on the trait or attribute for which each strain was included in the experiment, in line with one or more of the 'three-pronged' approaches in the experimental inquiry (column 3); a) AChE: associated with the known target of OPs/CPF, b) Associated with mood-related NT systems, and/or c) related to the foraging phenotype observed following treatment with 0.05 mg/L CPF.

Strain	Reason for inclusion	Approach	References
***N2* Bristol**	Wild type	N/A	(Brenner, 1974)
ace-1(ok663) X	Lacking class A AChE	AChE	(Combes et al., 2000, 2003)
ace-2(ok2545) I	Lacking class B AChE	AChE	(Combes et al., 2000, 2003)
ace-2(g72) I	Lacking class B AChE (alternative to ok2545)	AChE	(Combes *et al.*, 2000, 2003)
ace-3(dc2) II	Lacking class C AChE	AChE	(Combes et al., 2000, 2003)
tph-1(mg280) II	Tryptophan hydroxylase deficient. Cannot synthesize 5-HT	Mood-associated	(Sze *et al.*, 2000)
dat-1(ok157) III	DA transporter deficient	Mood-associated	(Nass *et al.*, 2002)
gar-3(gk305) V	Muscarinic receptor (*GAR-3*) deletion. Mediates starvation response.	AChE Observed phenotype	(You *et al.*, 2006)
acr-16(ok789) V	Lacks α7-like subunit of homomeric nicotinic ACh receptor	AChE	(Touroutine *et al.*, 2005)
egl-21(n476) IV	Reduced ACh release at NMJ Inability to process endogenous Neuropeptides. Reduced ACh release at NMJ, important in locomotion and feeding behaviour.	AChE Observed Phenotype	(Jacob and Kaplan, 2003) (Bhat *et al.*, 2021)
egl-4(ad450) IV	Spontaneously enters a sleep-like state and ceases feeding	Observed phenotype	(Raizen *et al.*, 2006)

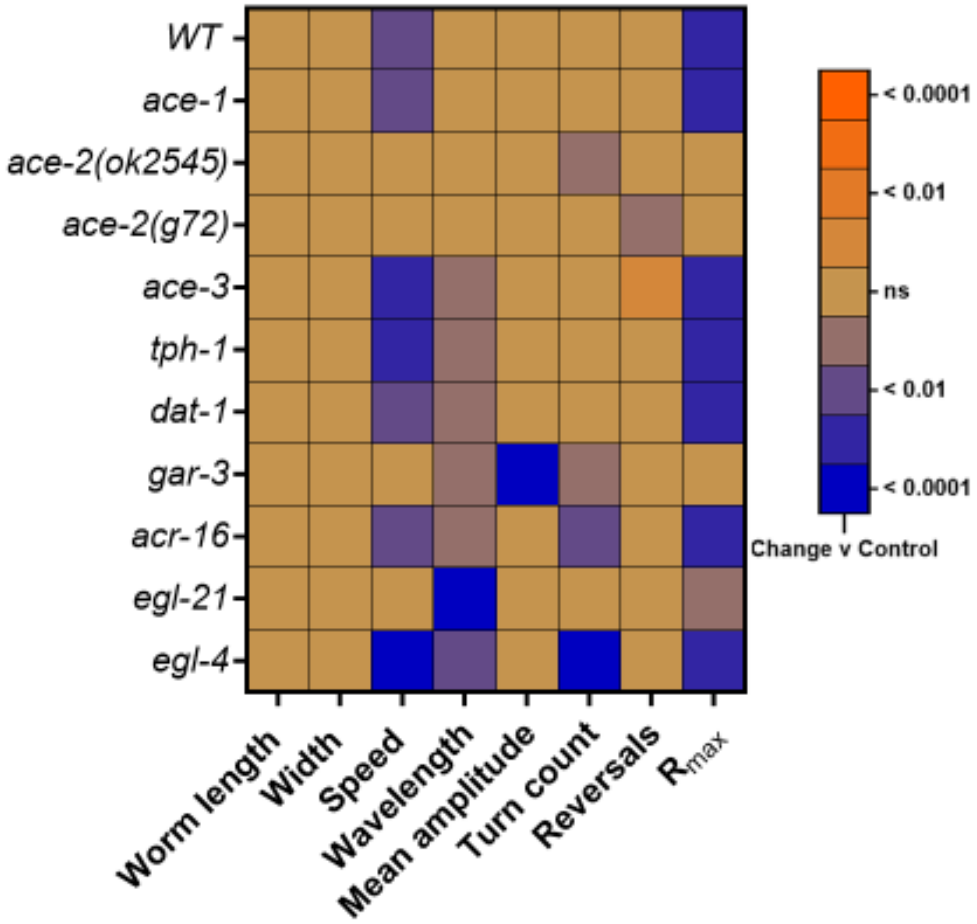

Figure 45. A heatmap of behavioural endpoints can be used to identify behaviour signatures and the mechanisms underpinning them. This heatmap illustrates the significant differences between 0.05 mg/L CPF-treated and vehicle control groups across 11 different *C. elegans* strains for various endpoints, as measured using WormLab. Bristol N2 (wild type), *ace-1(ok663), ace-2(ok2545), ace-2(g72), ace-3(dc2), tph-1(mg280), dat-1(ok157), gar-3(gk305), acr-16(ok789), egl-21(n476),* and *egl-4(ad450).* Each cell represents the False Discovery Rate (FDR) adjusted p-value for a specific endpoint in a given strain, with colour indicating the direction of the effect: blue signifies lower values in CPF-treated animals compared to controls, while orange denotes higher values. The intensity of the colour correlates with the degree of difference.

None of the strains tested showed any notable change in body length following CPF exposure, indicating that the level of exposure was appropriate in each case. Use of the heatmap allowed the foraging phenotype associated with CPF exposure in wild type, or its absence, to be quickly identified, as demonstrated with the *ace*-mutant data. However, in addition to confirming the effect of CPF identified previously, visualising the data in this way revealed additional effects, such as a change in turning in *ace-2(ok2545)* and reversal behaviour in *ace-2(g72)* (**Figure 45**). CPF-induced changes in turn counts or reversals were not observed in wild-type animals, but were seen in some other mutants, including *ace-3 (dc2), acr-16(ok789), egl-4(ad450)* and *gar-3(gk305)* (**Figure 45**), making the cause of these changes difficult to isolate.

5.1.4. Visualising the CPF induced foraging phenotype.

The wild-type foraging response to CPF exposure is represented clearly in the top row of **Figure 45**, represented by blue cells, which correspond to treatment-dependent differences in speed ($q < 0.01$), and Rmax ($q < 0.0001$). The phenotype is easily detected in wild-type animals, which were not significantly affected by 0.05 mg/L CPF treatment by any other measure, including body length (ns). The similar body-length measurements indicated that AChE was not overtly inhibited in these groups. The second row of the heatmap shows that *ace-1(ok663)* mutants show a similar behavioural response overall to that seen in wild type, with only speed ($q < 0.01$) and Rmax ($q < 0.0001$) affected by CPF exposure. In contrast, neither of the two *ace-2* mutant strains: *(g72)* or *(ok2545),* showed any difference in speed (ns) or Rmax (ns) in response to CPF treatment. The distinct behavioural pattern of *ace-2* mutants stands out clearly against other strains, which showed profiles more like wild type animals (**Figure 45**). However, there was a

slight difference in turning behaviour between the two *ace-2* alleles; *ace-2(ok2545)* performed fewer turns when exposed to CPF ($q < 0.05$), whereas CPF exposed *ace-2(g72)* performed fewer reversals ($q < 0.05$). These were the only observable differences between CPF-treated and untreated *ace-2* mutants and will be explored in further detail in the discussion. In line with results presented earlier (**Figure 44**), the CPF-induced decreases in speed ($q < 0.0001$) and Rmax ($q < 0.0001$) were observed in *ace-3 (dc2)* mutants, in addition to reduced wavelength ($q < 0.05$) and an increase in reversals ($q < 0.05$).

The remaining genotypes and their respective interactions with the CPF mediated foraging phenotype are detailed in the following sections.

5.1.5. The alpha-type nicotinic receptor subunit acr-16 is not required for the effect of low-level CPF on foraging behaviour.

Since AChE and cholinergic signalling were suspected to interact with the foraging phenotype, we wanted to test whether low-level CPF might act via cholinergic signalling at the neuromuscular junction (NMJ). ACR-16 is an ortholog of the mammalian alpha-7 nicotinic receptor, which has previously been associated with depression-like behaviour (Mineur *et al.*, 2018). In *C. elegans*, ACR-16 is responsible for fast cholinergic signalling at the NMJ and is therefore important to normal locomotory behaviour (Touroutine *et al.*, 2005).

To test for any interaction with CPF, the foraging phenotype of mutants carrying the *acr-16(ok789)* deletion was tested following exposure to vehicle control or 0.05 mg/L CPF and the results were represented in the heatmap (**Figure 45**). Despite showing a slight

reduction in wavelength ($q < 0.05$), and a decrease in turns ($q < 0.01$), *acr-16(ok789)* mutants did show a reduction in speed ($q < 0.01$) and Rmax ($q < 0.001$) like those seen in wild type animals when exposed to CPF. This suggests that ACR-16 receptor signalling is not required for the effect of CPF on foraging behaviour.

5.1.6. Neither dat-1 or tph-1 are necessary for the effect of low-level CPF on foraging behaviour

In the previous chapters, potential roles for DA and 5-HT signalling were explored using behavioural assays known to reveal specific aspects of DA and 5-HT signalling (Sawin, Ranganathan and Horvitz, 2000; McDonald *et al.*, 2007). Those experiments did not indicate any interactions with CPF, but we wanted to test whether this tracker protocol might be able to detect some effect. To test this, *dat-1(ok157)* and *tph-1(mg280)* were exposed and tested as described for inclusion in the heatmap (**Figure 45**).

Both *dat-1(ok157)* and *tph-1(mg280)* showed similar reductions in speed and Rmax to that seen in wild type. In addition, however, they each displayed a slight decrease in wavelength ($q < 0.05$, respectively). Reduced wavelength in CPF-treated animals was common to all the strains tested in this experiment except for both ace-2 mutants, and wild type. Since the effects of CPF on *dat-1(ok157)* and *tph-1(mg280)* appeared to be shared in wild type animals, or other mutants, these results did not suggest any specific role for DA or 5-HT signalling in the effect of CPF on *C. elegans* foraging behaviour.

5.1.7. Following the phenotype: a potential role for appetite and hunger signals

Upon first observing the phenotype caused by exposure to 0.05 mg/L CPF, it did not appear to resemble a typical basal slowing, or enhanced slowing response, which are each characterised by a decrease in speed in animals that encounter a new food source (Sawin, Ranganathan and Horvitz, 2000). Instead, these CPF-treated animals failed to show a normal increase in speed when food was removed, particularly under starved conditions. Furthermore, the effect was undetectable whenever a bacterial food source was present. This excluded the possibility of using established BSR and ESR assays to investigate the effects of CPF treatment. However, the apparent interaction between CPF treatment and the animal's access to, or recent experience with, food raised questions as to whether the behaviour may be linked to food-related behaviours, such as dwelling, roaming, and quiescence (Ben Arous, Laffont and Chatenay, 2009; McCloskey *et al.*, 2017). If the effect of low-level CPF treatment is distinct from cholinergic paralysis, then perhaps the signals involved in switching between behavioural states could be responsible for the effect on foraging behaviour.

5.1.8. Hunger and satiety signalling in C. elegans.

To see whether any such effect could be detected, we investigated some pathways known to modulate food-related behavioural states. Food seeking behaviour in *C. elegans* has been extensively studied and is driven largely by the animal's metabolic state, ranging between two main opposing states: hunger and satiety (You *et al.*, 2006, 2008; Davis *et al.*, 2017) (**Figure 46**). Following periods without eating, hungry animals show an increase in active food-seeking. The animal's behaviour will change on discovering food, depending on the quality of the food source and the internal nutritional state of the

animal; as seen in the previous chapter, hungry animals slow very quickly on discovering a high-quality food source, such as bacteria that are easy to consume and contribute well to the growth of the worm. As an example, *E. coli* strain HB101 is more nutritious than the commonly used OP50 *E. coli* strain, and consequently produces a more distinct slowing response when encountered by *C. elegans* (Shtonda and Avery, 2006; Sawin, Ranganathan and Horvitz, 2000) In addition, foraging activity is reduced even further as animals enter a 'quiescent' state after becoming satiated (Sawin, Ranganathan and Horvitz, 2000; You *et al.*, 2006, 2008; Ben Arous, Laffont and Chatenay, 2009; McClanahan *et al.*, 2020).

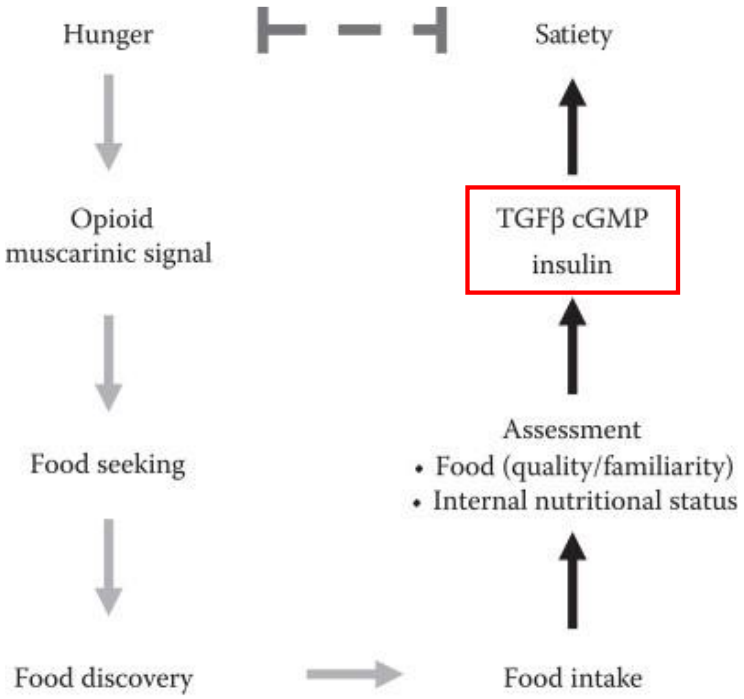

Figure 46. *C. elegans* behavioural responses to food are driven by two behavioural states: hunger and satiety. This illustrative flow diagram shows the interconnected processes regulating feeding behaviour, from hunger to satiety. Initial hunger triggers opioid/muscarinic signalling that drives food-seeking behaviour, leading to food discovery and intake. Subsequently, an assessment process takes place considering factors like food quality, familiarity, and the organism's internal nutritional status. Ultimately, feedback via TGFβ, cGMP, and insulin signalling contributes to the state of satiety. (Adapted from: Davis et al., 2017).

Firstly, EGL-4 is a *C. elegans* ortholog of cGMP-dependent protein kinase (PKG), also known as 'foraging' in *Drosophila melanogaster* (Hao *et al.*, 2011; Allen *et al.*, 2017). In *C. elegans*, EGL-4 was previously known as EAT-7, because it is involved in the regulation of food intake, satiety, and quiescence (You *et al.*, 2008). Conversely, *egl-21* mutants are also unable to regulate satiety and quiescence because they are unable to regulate peptide

signalling which is required to control those behavioural states (Husson *et al.*, 2007). To test whether the CPF-induced foraging phenotype was dependent on signals relating to satiety or quiescence, *egl-4(ad450)* and *egl-21(n476*) mutants were tested for interactions with CPF (**Figure 45**).

5.1.9. CPF-dependent reduction in foraging range is not dependent on EGL-4 or EGL-21

Treatment of *egl-4(ad450)* mutants with 0.05 mg/L CPF resulted in a similar reduction of speed and Rmax to that observed in wild-type animals treated with CPF ($q < 0.0001$) and Rmax ($q < 0.001$). However, in contrast to wild-type animals, CPF treatment also resulted in a significant reduction in wavelength and turn frequency in *egl-4(ad450)* mutants. ($q < 0.0001$) (**Figure 45**).

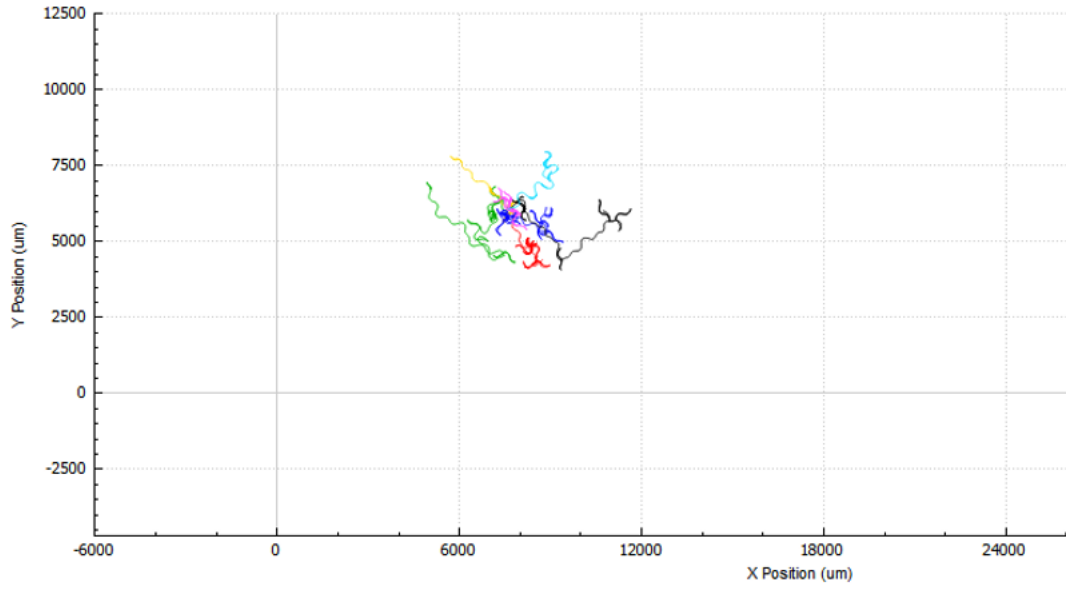

Figure 47. Representative track plots showing the movements of *egl-4(ad450)* mutants exposed to vehicle control. Each colour corresponds to an individual worm's track recorded over a period of 2 minutes. The trajectories were captured and plotted using WormLab software, highlighting variations in the foraging patterns of the mutants.

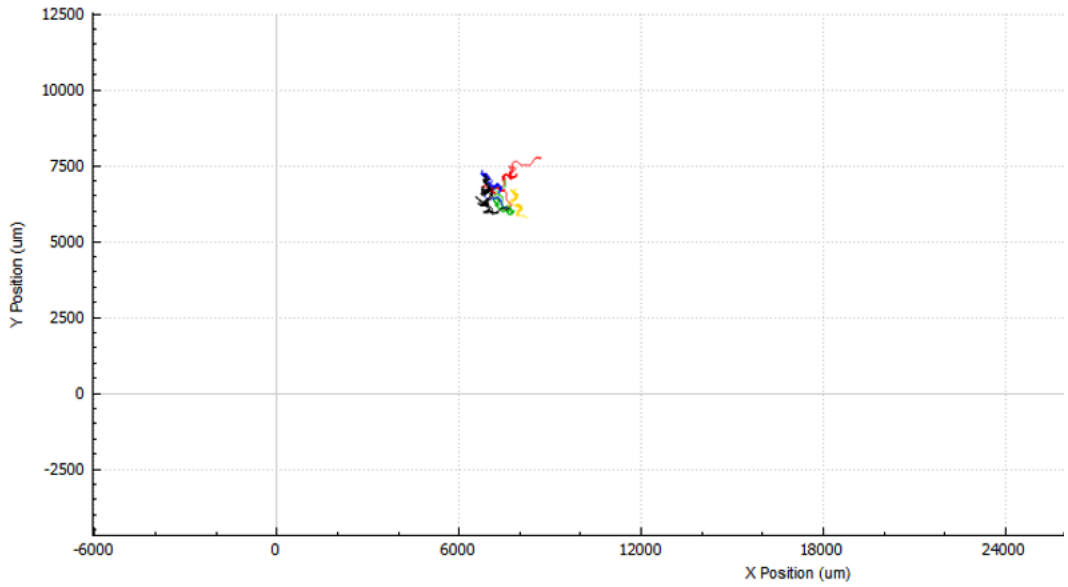

Figure 48. Representative track plots showing the movements of *egl-4(ad450)* mutants exposed with 0.05 mg/L CPF. Each colour corresponds to an individual worm's track recorded over a period of 2 minutes. The trajectories were captured and plotted using WormLab software, highlighting variations in the foraging patterns of the mutants.

Interestingly, *egl-21(n476)* mutants did not show a significant change in speed when exposed to CPF (ns), suggesting that peptide signalling could be involved in the foraging phenotype induced by CPF treatment to some extent. There was, however, a reduction in foraging range in *egl-21(n476)* mutants treated with CPF compared to untreated *egl-21(n476)* mutants ($q < 0.05$) (**Figure 50**), suggesting that the CPF-dependent foraging phenotype observed in wild-type animals was not completely abolished in *egl-21(n476)* mutants. This was accompanied by a reduction in wavelength ($q < 0.0001$).

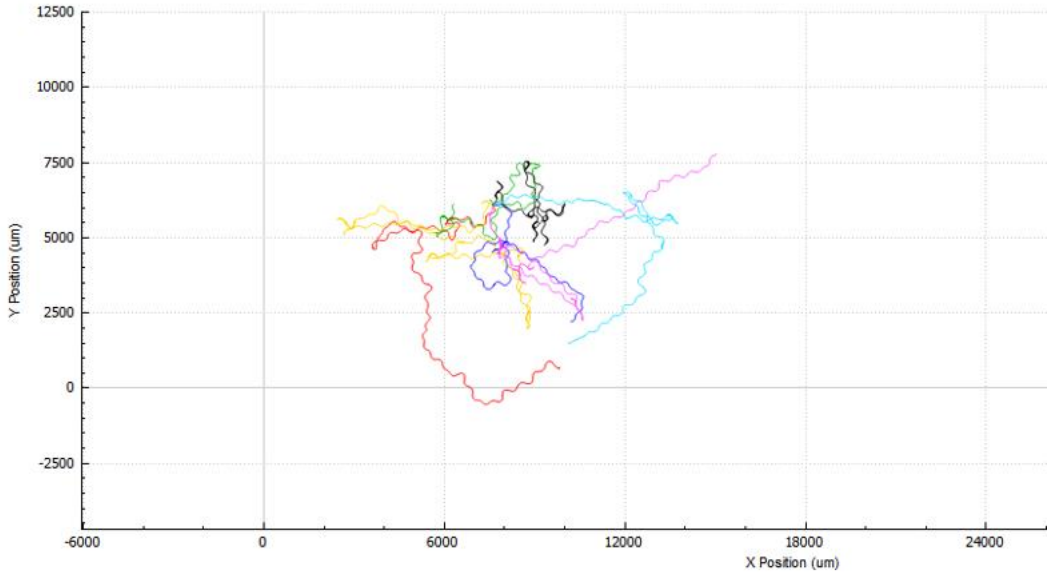

Figure 49. Representative track plots showing the movements of *egl-21(n476)* mutants exposed to vehicle control. Each colour corresponds to an individual worm's track recorded over a period of 2 minutes. The trajectories were captured and plotted using WormLab software, highlighting variations in the foraging patterns of the mutants.

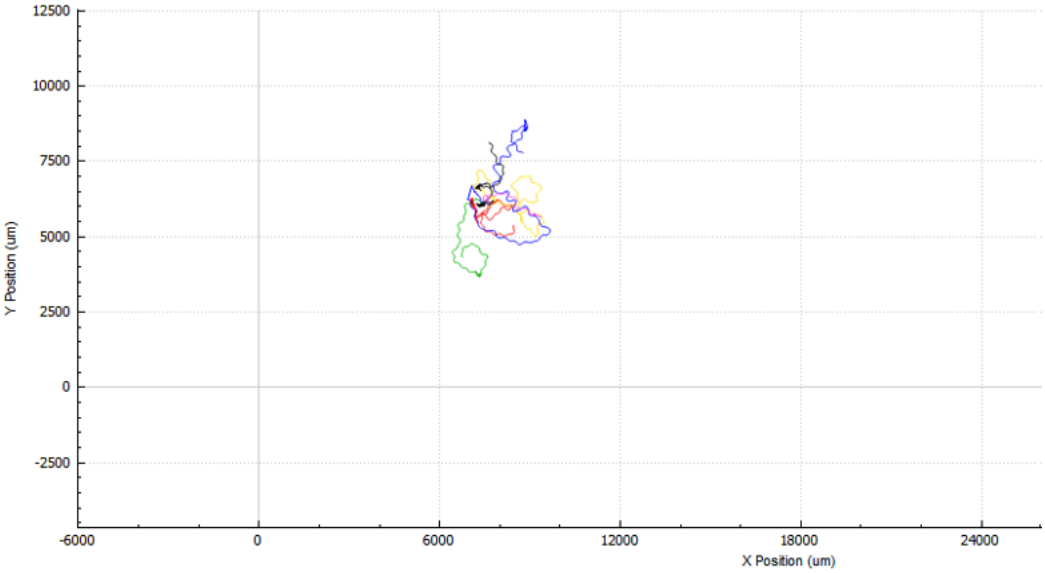

Figure 50. Representative track plots showing the movements of *egl-21(n476)* mutants exposed to 0.05 mg/L CPF. Each colour corresponds to an individual worm's track recorded over a period of 2 minutes. The trajectories were captured and plotted using WormLab software, highlighting variations in the foraging patterns of the mutants.

5.1.10.Muscarinic signalling mediates response to starvation via GAR-3

In addition, the *C. elegans* response to starvation is regulated in part by ACh; more specifically by muscarinic signalling via the muscarinic receptor: GAR-3, via the mitogen-activated protein kinase (MAPK) pathway (Steger and Avery, 2004; You *et al.*, 2006). GAR-3 is one of three genes which encode muscarinic receptors in *C. elegans*, and is expressed in body-wall muscles, pharyngeal muscles, and cholinergic motor neurons (Steger and Avery, 2004). The GAR-3 receptor has a high affinity for the ACh ligand and can respond to small concentrations of ACh that escape the synapse (Dittman and Kaplan, 2008; Chan *et al.*, 2013). In contrast to the ACh gated ion channels (nAChRs), that mediate fast transmission at synapses, GAR-3 is enriched at extra synaptic locations where it can function as an auto receptor and can modulate locomotion in response to distal and indirect ACh signals, known as volume transmission, on cholinergic motor neurons (Chan *et al.*, 2013).

Interestingly, the closest mammalian homolog to GAR-3 is the mammalian muscarinic receptor M3, which when disrupted in mice, causes a decrease in food consumption comparable to that seen in *gar-3* defective nematodes (Yamada *et al.*, 2001; You *et al.*, 2006; You and Avery, 2012; Davis *et al.*, 2017).

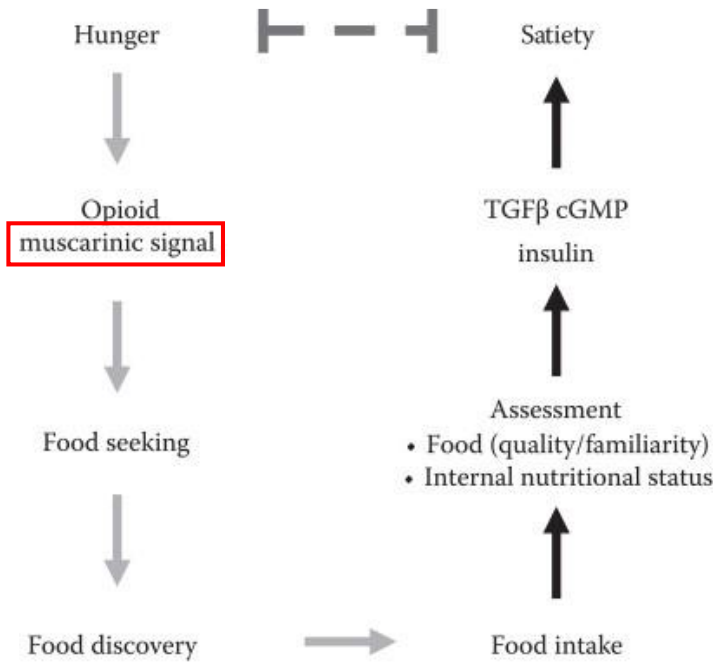

Figure 51. Muscarinic signalling through GAR-3 is known to mediate food seeking behaviour in response to hunger under starved conditions.This illustrative flow diagram shows the interconnected processes regulating feeding behaviour, from hunger to satiety. Initial hunger triggers opioid/muscarinic signalling that drives food-seeking behaviour, leading to food discovery and intake. Subsequently, an assessment process takes place considering factors like food quality, familiarity, and the organism's internal nutritional status. Ultimately, feedback via TGFβ, cGMP, and insulin signalling contributes to the state of satiety (Adapted from: Davis et al., 2017).

The phenotype observed in nematodes following treatment with 0.05 mg/L CPF appeared to relate to foraging behaviour and was dependent on a period of food deprivation. It was possible that this could relate to the GAR-3-mediated starvation response described previously (You and Avery, 2012). Moreover, since GAR-3 is highly sensitive to fluctuations in ACh concentrations, it was suspected that altered function in *gar-3* mutants could be sensitive to subtle changes in AChE, occurring below limits of

detection by the Ellman assay, following treatment with 0.05 mg/L CPF. The implication of GAR-3 would fit with a change in feeding behaviour, and with the cholinergic function hypotheses described in this chapter. Therefore *gar-3(gk305)* muscarinic receptor deletion mutants were an important candidate for investigating this CPF phenotype.

5.1.11. Muscarinic receptor signalling is required for low-level effects of CPF treatment in C. elegans

An interesting result from CPF treatment was observed in a muscarinic receptor signalling mutant. Animals lacking a muscarinic receptor: *gar-3(gk305)*, were the only group, other than the *ace-2* mutants, not to display the CPF-induced foraging phenotype (**Figure 52** & **Figure 53**). However, deletion of ACE-2 prevented the increase in activity induced by food deprivation, whereas in contrast, deletion of GAR-3 prevented the reduction of activity induced by CPF-treatment. Moreover, *gar-3(gk305)* showed similar foraging activity to wild-type, untreated animals, but unlike wild-type animals, that activity was not impaired by treatment with 0.05 mg/L CPF. This indicates that the effect of low-level CPF treatment in *C. elegans* could be somehow mediated by muscarinic receptor signaling via GAR-3.

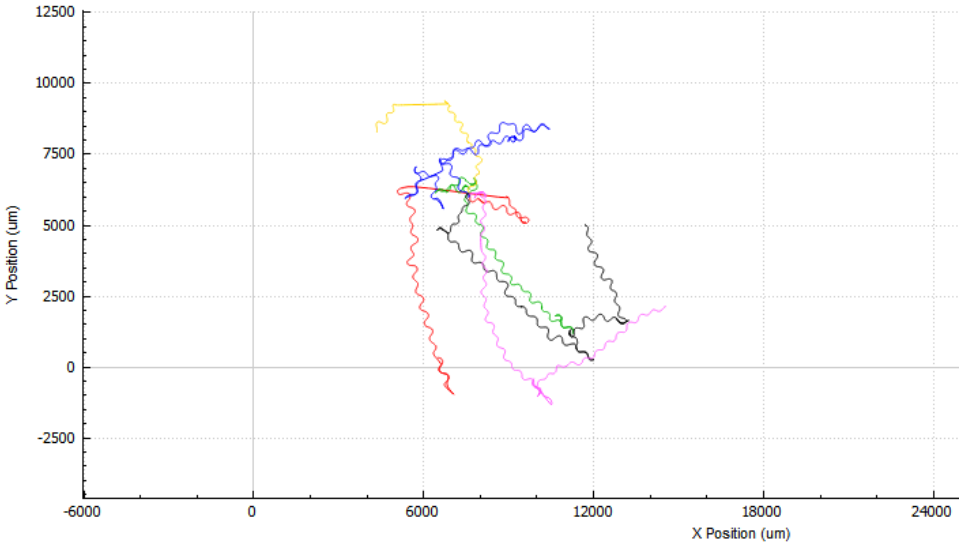

Figure 52. Representative track plots showing the movements of *gar-3(gk305)* muscarinic receptor deletion mutants exposed to vehicle control. Each colour corresponds to an individual worm's track recorded over a period of 2 minutes. The trajectories were captured and plotted using WormLab software, highlighting variations in the foraging patterns of the mutants.

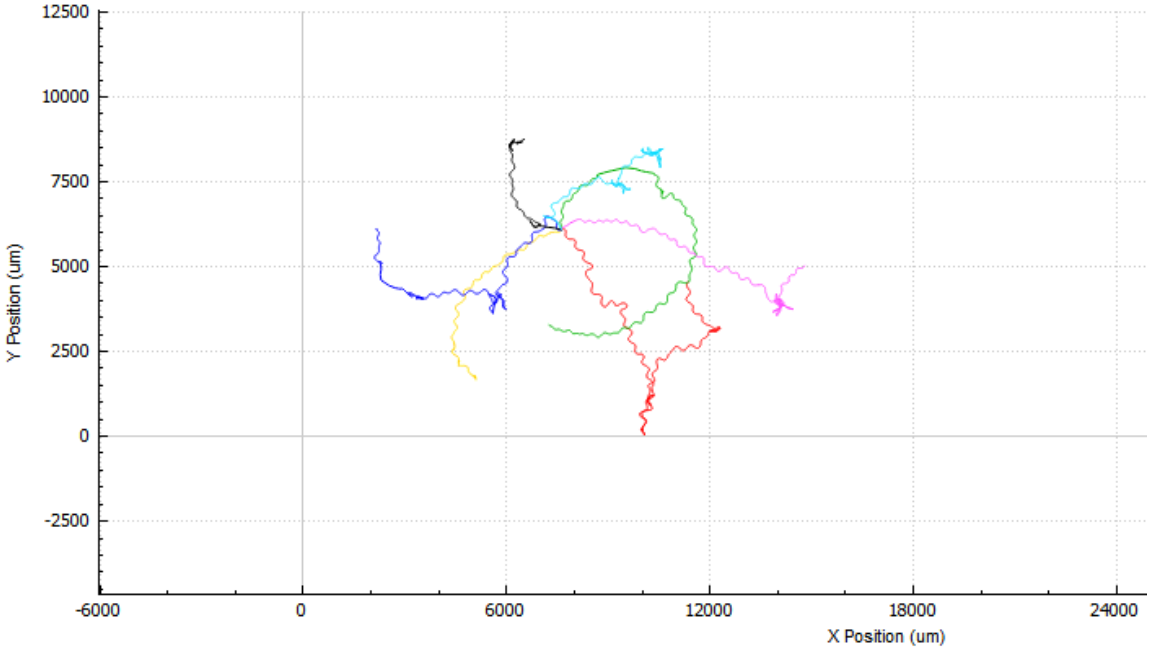

Figure 53. Representative track plots showing the movements of *gar-3(gk305)* muscarinic receptor deletion mutants exposed to 0.05 mg/L CPF. Each colour corresponds to an individual worm's track recorded over a period of 2 minutes. The trajectories were captured and plotted using WormLab software, highlighting variations in the foraging patterns of the mutants.

5.1.12. Gar-3 expressed in cholinergic neurons rescues the foraging phenotype in a muscarinic receptor deletion mutant.

The CPF-induced phenotype seen in wild-type animals was not present in *gar-3(gk305)* muscarinic receptor deletion mutants (**Figure 53**). To confirm that GAR-3 is required for the phenotype, animals expressing GFP tagged GAR-3 cDNA in cholinergic neurons: *vjIs50 [ttx-3p:mRFP, Punc-129::GAR-3-GFP],* were crossed with the *gar-3(gk305)* mAChR deletion strain, as described in the method section. The resulting animals were exposed to 0.05 mg/L CPF or vehicle control, in parallel with wild-type animals, and each of the parent strains: *gar-3(gk305)* and *vjIs50 [ttx-3p:mRFP, Punc-129::GAR-3-GFP]*, and assayed for the foraging phenotype using the worm tracker (**Figure 54**).

Expression of GAR-3 in cholinergic neurons did appear to rescue the reduced foraging phenotype caused by treatment with 0.05 mg/L CPF in the *gar-3(gk305)* deletion mutants ($q < 0.0001$) (**Figure 54**). As before, *gar-3(gk305)* deletion mutants' foraging range was not significantly different from untreated wild-type animals, regardless of whether *gar-3(gk305)* were treated with CPF (ns), or not (ns). In contrast, the CPF-induced decrease in foraging range was restored when GAR-3 was expressed in cholinergic neurons in the *gar-3(gk305)*-deletion background (*gar-3(gk305);vjIs50*: $q < 0.0001$).

Similarly, animals with GAR-3-GFP expressed in cholinergic neurons, but in the wild-type background (*vjIs50*), also displayed the CPF-induced reduction in foraging range, with Rmax values similar to both *gar-3(gk305);vjIs50* (ns), and wild-type animals (ns), when treated with 0.05 mg/L CPF. This suggests that the GAR-3 muscarinic receptor, specifically in cholinergic neurons, is required for the effect of low-level CPF treatment in this phenotype.

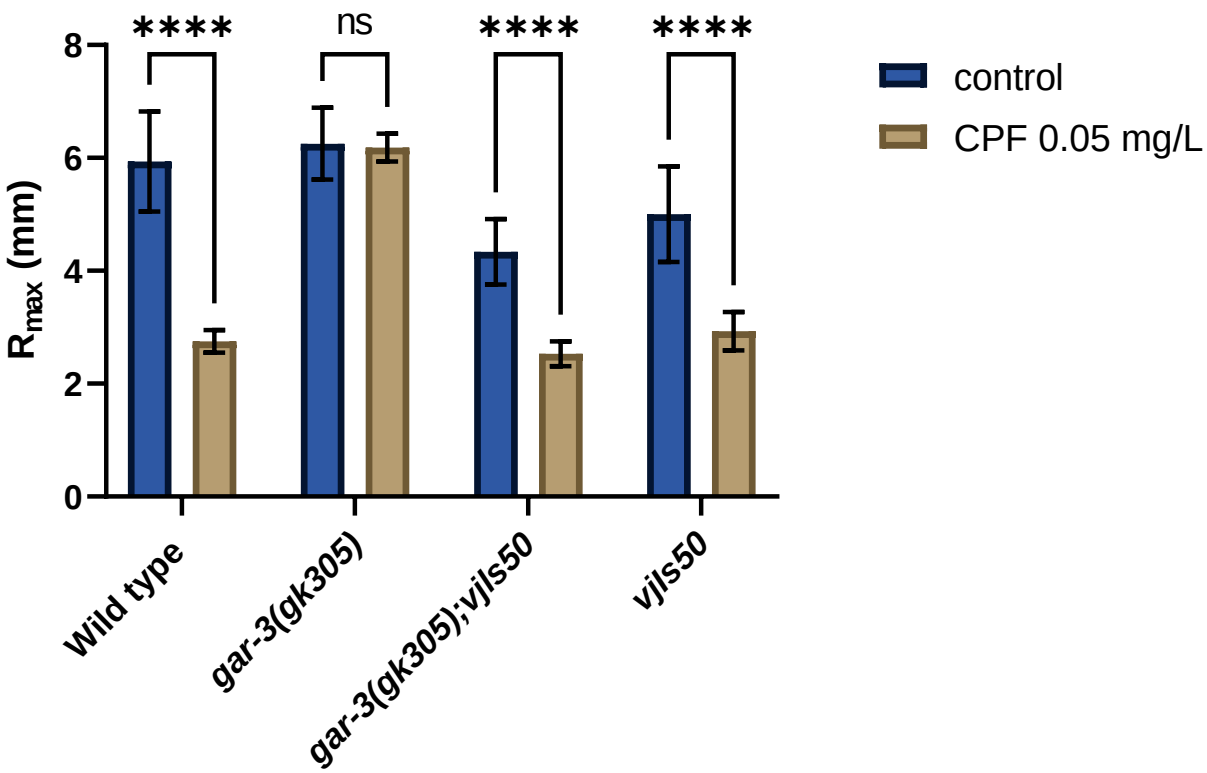

Figure 54. The effect of CPF on foraging behaviour is absent in a *gar-3* deletion mutant. The graph demonstrates the impact of CPF treatment on the foraging behaviour of wild-type (n=34), *gar-3(gk305)*(n=50), *vjIs50* (n=40), and *gar-3(gk305);vjIs50* (n=30) strains, as measured by maximum Euclidean distance (Rmax). GAR-3 expression in cholinergic neurons appears to rescue the reduced foraging phenotype caused by CPF treatment in *gar-3(gk305)* deletion mutants ($q < 0.0001$). However, untreated *gar-3(gk305);vjIs50* mutants show a significantly reduced foraging range compared to untreated wild-type or *gar-3(gk305)* animals ($P < 0.05$). Bars represent mean values, with 95% confidence intervals. **** = $p < 0.0001$. ns = not significant.

Notably, although foraging range in the CPF-treated GAR-3 rescue strain was restored to similar levels to the CPF-treated wild-type group, foraging range in the untreated GAR-3 rescue group (*gar-3(gk305);vjIs50*) was also reduced compared to untreated *gar-3(gk305)* deletion, and untreated wild-type animals (**Figure 54**). The difference in

mutant vehicle control groups from wild type was tested using one-way ANOVA ($F(3, 74) = 6$), $P = 0.001$). Tukey's multiple comparisons test confirmed that this was driven by the rescue strain which had a significantly lower Rmax than wild-type or *gar-3(gk305)* deletion animals ($P< 0.05$). This is possibly because the rescue strain used multicopy integration and consequent overexpression of GAR-3 in the cholinergic neurons, which would cause hypersensitivity to endogenous ACh (Dittman and Kaplan, 2008). Alternatively, the difference could be caused by a background mutation linked to the *vjIs50* transgene. Nevertheless, the reduction in Rmax following CPF treatment was restored by reintroduction of GAR-3 and therefore rescue of the phenotype seems to have been achieved, suggesting that GAR-3 is necessary for the reduction in foraging range induced by treatment with 0.05 mg/L CPF.

5.2. Discussion

The aim of this chapter was to identify, measure and investigate the reduction in foraging range observed in *C. elegans* following treatment with 0.05 mg/L CPF, and to try to determine the underlying mechanisms responsible for its effect. Data presented in previous chapters showed that the effect of 0.05 mg/L CPF was only observable in animals that had experienced a period of food-deprivation. More specifically, CPF-treatment prevented the increase in foraging behaviour that normally occurs in response to hunger in *C. elegans* (Davis *et al.*, 2017). Based on these observations, several genes with known roles in the *C. elegans* hunger response and known effects on foraging behaviour were chosen to investigate the effect of low-level CPF exposure.

In addition to the apparent importance of feeding state, animals that were deficient in neuronal AChE (ACE-2) did not show the CPF-induced foraging phenotype that was observed in wild-type animals. Therefore, and because inhibition of AChE is a widely acknowledged mechanism of action for OP toxicity, a possible role for AChE inhibition in the effect of low-level CPF-treatment remains difficult to exclude. We found that whole-body AChE inhibition following treatment with 0.05 mg/L CPF was not statistically significant (Chapter 2). Nevertheless, even very small changes in ambient ACh can produce biologically significant effects (Picciotto, Higley and Mineur, 2012). Analysis based on whole-body, combined AChE activity might fail to detect small, localised changes in ACE-2-specific activity that may be relevant to neuronal function. We therefore continue to consider the possibility that cholinergic signalling might play a role in the effects of CPF, even at treatment concentrations as low as 0.05 mg/L.

Alongside the potential cholinergic effects of CPF treatment, and the impact on foraging behaviour, consideration of neurotransmitter signalling pathways that have previously been associated with human mood was the third aspect of the 'three pronged' approach used to investigate low-level CPF effects in this chapter. Before discussing the results of each part of this approach, an evaluation of the measures used to measure and quantify the behaviour induced by treatment with 0.05 mg/L CPF is presented below.

5.2.1. Objective one: measuring and quantifying the CPF-induced effect on foraging behaviour using WormLab tracking software.

So that the mechanisms underlying low-level CPF toxicity could be investigated, characterisation of the CPF-induced behavioural phenotype was required. This would allow comparison of the effect of CPF treatment across selected *C. elegans* genotypes, relevant to feeding, mood-associated neurotransmitters, and cholinergic signalling. The phenotype that emerged following treatment with 0.05 mg/L CPF did not directly resemble any previously reported behavioural paradigm, and so a quantitative unit with which to measure the effect was required. Using WormLab tracking software to measure different endpoints simultaneously proved useful for this purpose. The endpoints selected for this investigation were intended to capture changes in locomotory behaviour and worm-body measurements. A critical aim of this project was to differentiate between distinguishable 'low-level' effects of CPF exposure, and those caused by acute inhibition of AChE. It is known that acute AChE inhibition causes hypercontraction of *C. elegans* body-wall muscles, leading to a relative shortening of body length (Izquierdo *et al.*, 2020). Results presented in the previous chapter confirmed that this was the case following treatment with 0.1 mg/L CPF and above, and consequently body-length measurements

were used to show that acute AChE inhibition did not occur in animals treated with 0.05 mg/L CPF overtly in the remaining experiments.

5.2.1.1. Treatment with 0.05 mg/L CPF affects foraging behaviour in C. elegans.

The behavioural changes following treatment with 0.05 mg/L CPF were dependent on a period of food-deprivation. However, they did not fit with a classic enhanced slowing response (Sawin, Ranganathan and Horvitz, 2000) and so additional foraging behaviours were considered. These included 'dwelling', 'roaming' and 'quiescence', which *C. elegans* switches innately between, depending on the availability of food and the individual's internal feeding state (You *et al.*, 2008; Ben Arous, Laffont and Chatenay, 2009). Ben Arous, Laffont and Chatenay (2009) describe 'dwelling' as a state of reduced locomotory speed, accompanied by an increase in directional changes. Conversely, 'roaming' is described as an increase in locomotory speed, with less-frequent directional changes (Ben Arous, Laffont and Chatenay, 2009). While the results presented in this chapter did show a CPF-dependent reduction in speed in food-deprived wild-type animals, suggesting a possible increase in dwelling or reduction in roaming, significant changes in the frequency of turns or reversals were not detected in these animals. This could suggest that CPF treatment was not affecting dwelling or roaming directly.

An alternative explanation is that the 2-minute recording time used here may not have been long enough to detect a significant difference in directional change in wild-type animals, because turns and reversals are relatively infrequent events. By comparison, Ben Arous, Laffont and Chatenay (2009) used a much longer 2-hour recording period to capture differences between dwelling and roaming behaviour. The additional recording

time would be more sensitive to infrequent behavioural changes but would be less practical for the purpose of screening multiple conditions with strict exposure and food deprivation requirements. The shorter recording time was therefore considered more suitable for this investigation.

It is also possible that the metrics by which turns are quantified vary between WormLab, and the custom MATLAB tracking code used by Ben Arous, Laffont and Chatenay (2009). Therefore, due to the different measurement techniques used, a specific effect on dwelling and roaming could not be confirmed or excluded. In any case, the functional importance of the behaviour to the animal, although interesting, was less important than identifying a quantifiable measure of the effect of CPF treatment.

Regardless of its functional importance, a significant change in locomotory speed was detected in food-deprived wild-type animals following treatment with 0.05 mg/L CPF. This was a potentially useful result, although the reliability of locomotory speed as a sole measure of low-level CPF treatment could be impaired by natural variability (Angstman *et al.*, 2015; Stern, Kirst and Bargmann, 2017), particularly over relatively short 2-minute recording times. It was therefore useful to note that wild-type animals treated with 0.05 mg/L CPF also tended to cover a more restricted area than vehicle controls. While not explicitly a measure of dwelling or roaming, this type of measurement has previously been used to quantify movement behaviour *C. elegans* (Dittman and Kaplan, 2008). Together, these measurements offered a more comprehensive behavioural fingerprint than a simple measure of speed in isolation. Moreover, maximum Euclidean distance (Rmax) and locomotory speed were the only differences observed between 0.05 mg/L CPF-treated and untreated wild-type animals, and while Rmax appeared to be the most reliable, both measurements together provided a quantifiable reference point for the

CPF-induced foraging phenotype, with which to compare genetic manipulations. Together, these measures were considered sufficiently sensitive and reliable to represent the effect of CPF treatment at this level, and the first objective of this chapter was considered to have been met.

5.2.2. Objective two: Investigating the biological mechanisms of low-level CPF treatment using a three-pronged approach.

The identification of a quantitative measure for the effect of low-level CPF treatment in this chapter enabled further investigation of the biological mechanisms underpinning its observed effect. This was important, since understanding the biological effects of CPF could help to inform whether low-level exposure could plausibly affect human psychiatric health. However, in contrast to vertebrate models, analogous behaviours and models for depression have not been established in *C. elegans* (Wang *et al.*, 2017). Likewise, links between OP exposure and human mood disorders are also not well established. Consequently, an exploratory approach, using the conserved molecular attributes shared between *C. elegans* and vertebrates, was followed. The characteristics of *C. elegans* as a model organism were well suited to such an approach and the exploration of the effects of treatment with 0.05 mg/L CPF on cholinergic signalling pathways, in addition to some neurotransmitter pathways known to be important to human mood, and some candidate genes known to be involved in the regulation of *C. elegans* foraging behaviour.

5.2.3. The effect of low-level CPF treatment on C. elegans foraging behaviour does not require 5-HT biosynthesis.

Despite the seemingly separate aspects described for the three-pronged approach used to select candidate genes, it is often the case that a single gene interacts with more than one important biological function. This means that several of the candidates used in these

experiments can be associated with more than one part of the approach as described. For example, 5-HT signalling is commonly associated with depression and is a key feature of the pharmacology of commonly prescribed antidepressants (Cowen and Browning, 2015). 5-HT signalling also regulates a response to finding food in *C. elegans* after a period of starvation (Sawin, Ranganathan and Horvitz, 2000), and organophosphate exposure has been linked to 5-HT signalling in rodent models (Slotkin *et al.*, 2006; Lima *et al.*, 2011; Judge *et al.*, 2016). Therefore, while 5-HT signalling was considered here primarily for its association with depression, its inclusion in this study links equally to each part of the three-pronged approach. Despite all of this, the results in this chapter did not indicate any direct relationship between 5-HT signalling and treatment with 0.05 mg/L CPF in this *C. elegans* model. This was evidenced using the *tph-1(mg280)* deletion mutant, which showed a similar CPF-induced change in foraging behaviour to wild-type animals, despite being unable to synthesise 5-HT (Sze *et al.*, 2000). 5-HT therefore appeared not to be required for the effect of low-level CPF treatment, which would explain why the effect of CPF did not directly affect the 5-HT-dependent enhanced slowing response tested in the previous chapter. The apparent absence of a 5-HT mediated effect here does not exclude the possibility that 5-HT signalling may play a role in low-level OP exposure in humans or other mammals. However, the lack of a direct effect would support the suggestion that links between 5-HT signalling and OP exposure described elsewhere could emerge secondarily via some indirect route, such as developmental impairment (Slotkin *et al.*, 2006), or another signalling pathway (Judge *et al.*, 2016). These possibilities are beyond the scope of this investigation however, and so any role for 5-HT signalling in the effect of 0.05 mg/L CPF treatment was not considered further here.

5.2.4. Deletion of the conserved DA transporter DAT-1 does not block the effect of 0.05 mg/L CPF on C. elegans foraging behaviour.

Similarly, DA signalling in humans, which is negatively moderated via clearance by the DAT transporter (Kristensen *et al.*, 2011), is associated with mood, reward-signalling, and movement (Nieoullon and Coquerel, 2003). In mice, DA signalling is disrupted by acute CPF treatment (Torres-Altoro *et al.*, 2011). There are several examples in which switching between different movement-oriented behavioural states is regulated by DA signalling in *C. elegans*, and via the conserved DA transporter DAT-1 specifically (Sawin, Ranganathan and Horvitz, 2000; McDonald *et al.*, 2007; Torres-Altoro *et al.*, 2011; Belujon and Grace, 2017).

Taken together, this justified the use of the *dat-1(ok157)* DA transporter-deletion mutant in the investigation of the CPF-induced effect on foraging behaviour. Particularly because CPF treatment in wild-type animals also appeared to manifest as the interruption of a switch from one movement-oriented behavioural state to another. However, DAT-1 deletion mutants showed a similar change in speed and foraging range to wild-type animals when treated with CPF, which suggests that normal DA reuptake is not a requirement for its effect. While this alone does not exclude a role for DA signalling in the effects of low-level OP exposure, it does support findings presented in the previous chapters, through which no specific role for DA signalling was identified. Together, these results also suggest that the change in foraging behaviour in food-deprived wild-type animals, which is prevented by CPF treatment, depends primarily on different signalling pathways from the switch from swimming to crawling (Vidal-Gadea *et al.*, 2011), or the basal-slowing response (Sawin, Ranganathan and Horvitz, 2000), both of which are DA-dependent.

The agreement between these results and those from the SWIP, and BSR assays indicate that a direct effect on DA signalling by low-level CPF treatment is unlikely. Therefore, alternative pathways relating to foraging behaviour were explored.

5.2.5. CPF-induced effects on wild-type foraging behaviour are not mediated by EGL-4

Foraging behaviour is driven by the internal state of the animal, and so it was possible that the CPF-induced foraging phenotype resulted from alterations to some related behavioural drive in *C. elegans*. A known regulator of feeding behaviour in several species including *C. elegans*, is an orthologous cGMP-dependent kinase, which is coded by EGL-4 in *C. elegans* (Husson *et al.*, 2007; You *et al.*, 2008; Hao *et al.*, 2011; Allen *et al.*, 2017). However, since the CPF-induced reductions in speed and foraging range were also observed in CPF-treated *egl-4(ad450)* mutants, there was no indication that EGL-4 was required for the effect of CPF on wild-type foraging behaviour. There was, however, a significant decrease in wavelength in CPF-treated *egl-4(ad450)* mutants that was not observed in CPF-treated wild-type animals. This was not exclusive to *egl-4(ad450)* however, as reduced wavelength was observed in several other strains, making the causal mechanism difficult to identify. Similarly, a CPF-induced reduction in turn count was observed in *egl-4(ad450)*, and also in other mutants: *ace-2(ok2545)*, *acr-16(ok789)* and *gar-3(gk305)*, which are each deficient in neuronal AChE, an acetylcholine-gated ion channel subunit, and a muscarinic receptor subtype, respectively. All of which, apart from *egl-4(ad450)*, are principal components of the cholinergic signalling pathway (Nguyen *et al.*, 1995). It has previously been suggested that EGL-4 functions downstream of cholinergic signalling to modulate locomotory activity and quiescence (Ghosh and Emmons, 2010), which would support the suggestion that cholinergic signalling may be

involved in the effects of CPF observed here. Nevertheless, EGL-4 did not seem to be required for the wild-type response to CPF treatment, and since no other unique effects of CPF were observed exclusively in the *egl-4(ad450)* mutant, EGL-4 was not investigated here further. Possible upstream effects, including cholinergic effects, will now be discussed.

5.2.6. CPF-induced reduction in speed, but not foraging range, is affected by impaired neuropeptide signalling.

Satiety and quiescence in *C. elegans* are regulated by neuropeptide signalling (Husson *et al.*, 2007). The *egl-21(n476)* mutant is deficient in carboxypeptidase activity and cannot synthesise most neuropeptides, therefore *egl-21(n476)* animals exhibit abnormal satiety-quiescence behaviour (Jacob and Kaplan, 2003; Husson *et al.*, 2007). EGL-21's role in satiety quiescence was the primary reason for its inclusion in this investigation. However, neuropeptides are highly diverse in form and function, and are broadly disrupted in the *egl-21(n476)* mutant (Burbach, 2011; Bhat *et al.*, 2021). This presented an opportunity to investigate whether normal neuropeptidergic function more widely, could be implicated in the effect of low-level CPF treatment. If normal neuropeptide signalling was required for the effect or CPF, and/or if some component of one or more neuropeptide signalling pathways was the direct target, then we could expect the effect of CPF to be absent in the *egl-21(n476)* mutant. This suggestion was only partly supported, because only the wild-type CPF-induced change in locomotory speed was abolished by altered neuropeptide synthesis. However, foraging range was reduced in response to CPF-treatment in *egl-21(n476)*, similarly to the reduction observed in wild-type animals. The shared effect on foraging range suggests that at least some of the effect of 0.05 mg/L CPF treatment is independent of the neuropeptides disrupted by this mutation. Conversely, the abolished

effect on locomotory speed could originate from some downstream target, mediated by the *egl-21(n476)* mutation. Many neuropeptides function as neuromodulators as well as directly as neurotransmitters, (Burbach, 2011; Hu *et al.*, 2011). From that perspective, the broad-ranging nature of EGL-21's role in neuropeptide signalling becomes less helpful for purpose of narrowing down the effects of CPF, particularly when the effects are not fully abolished in *egl-21(n476)* mutants.

One modulatory role that may be relevant to the investigation of low-level CPF treatment, is the modulation of cholinergic neurotransmission by EGL-21. Jacob and Kaplan (2003) demonstrated that the same *egl-21(n476)* mutant strain is resistant to paralysis observed in wild-type animals treated with the AChE inhibitor: Aldicarb. This resistance occurs because EGL-21 is required for the synthesis of endogenous neuropeptides needed to stimulate acetylcholine release at neuromuscular junctions (Jacob and Kaplan, 2003). It is therefore possible, that the absence of the wild-type reduction of locomotory speed caused by CPF treatment in the *egl-21(n476)* mutant, may be analogous to the aldicarb resistance demonstrated by Jacob and Kaplan (2003). This would make sense if AChE were also inhibited by treatment with 0.05 mg/L CPF, albeit at statistically non-significant levels. Interpretation of the heatmap showed that the wild-type effects of 0.05 mg/L CPF treatment seen were abolished more completely by mutations in the cholinergic signalling pathways, therefore those pathways were investigated more closely.

5.2.7. Low-level CPF-treatment appears most closely related to cholinergic signalling pathways.

The results presented in previous chapters demonstrated that most detectable effects of CPF treatment are difficult to separate from inhibition of AChE. This is mirrored in the

literature, where behavioural or physiological effects attributed to CPF treatment are rarely, if ever, reported to occur explicitly or reliably in the absolute absence of AChE inhibition (Silva, 2020). We showed that treatment with 0.05 mg/L CPF did not inhibit AChE to a statistically significant extent in *C. elegans*. However, it was considered possible that the threshold for statistical significance could differ from that of biological significance in this case, particularly if any effect were due localised AChE inhibition. Notably, we could only measure AChE activity from whole animals. Moreover, none of the results presented here have so far indicated any explicitly non-cholinergic effects caused by 0.05 mg/L CPF treatment. For these reasons, mutations relating to different components of cholinergic signalling in *C. elegans* and their interactions with 0.05 mg/L CPF-treatment were investigated further.

5.2.8. The CPF-dependent effect on C. elegans foraging behaviour does not require fast nicotinic excitatory current at neuromuscular junctions.

From investigating genes known to be associated with appetite and feeding behaviour we learned that neuropeptide deficient *egl-21(n476)* mutants appeared to be resistant to some, but not all of the effects of low-level CPF-treatment. EGL-21 is known to modulate acetylcholine signalling at the neuromuscular junction, and the deletion causes resistance to another AChE inhibitor: aldicarb (Jacob and Kaplan, 2003; Hu *et al.*, 2011). While the similarities in response to aldicarb and CPF treatment could be coincidental, both substances are known AChE inhibitors, and so some shared mechanism seemed likely. We tested to see whether fast nicotinic-receptor signalling at neuromuscular junctions was necessary for the effects of CPF on *C. elegans* foraging behaviour, using an *acr-16* deletion mutant. ACR-16 is homologous to the vertebrate α7 nicotinic acetylcholine receptor subunit and is absolutely required for fast nicotinic signalling at *C. elegans*

neuromuscular junctions (Francis *et al.*, 2005). However, CPF-treated *acr-16* deletion mutants showed similar reductions in locomotory speed and foraging range to those seen in wild-type animals. This indicates that fast nicotinic current at neuromuscular junctions is not required for those effects. This finding is important, firstly because nicotinic signalling is major component of the cholinergic signalling pathway (Rand, 2007), and consistently with this, excessive nicotinic signalling is an important mediator of acute organophosphate toxicity in humans, and *C. elegans* (Slavica, Dubravko and Milan, 2018; Izquierdo, Charvet, *et al.*, 2021). The finding that ACR-16 is not required for the observed effect of 0.05 mg/L CPF on *C. elegans* foraging behaviour does not exclude the possibility of an effect within the cholinergic signalling pathway. However, it helps to separate the effect of CPF at this level from those known to occur through acute exposure to greater OP concentrations.

5.2.9. Neuronal AChE is necessary for increased foraging range in wild-type C. elegans.

Although neuromuscular, fast nicotinic signalling did not seem to be necessary for the observed effect of CPF at this concentration, other aspects of the cholinergic signalling pathway did seem to be important. *C. elegans* has three distinct classes of catalytically-active AChE, each coded by a different gene and with individually distinct expression patterns (Combes *et al.*, 2003). Our results showed that mutants deficient for one of these: *ace-1(ok663)*, and another: *ace-3(dc2)* expressed similar CPF-induced reductions in locomotory speed and foraging range to wild-type animals. This indicates that neither ACE-1 nor ACE-3 are required for these particular effects of CPF treatment at this level of exposure. Conversely, the remaining AChE: ACE-2, did appear to be important. Firstly, because mutants with a deletion at *ace-2(ok2545)* did not show any significant reduction

in either locomotory speed, or foraging range. This result, and the importance of ACE-2, were supported by similar results in an *ace-2(g72)* substitution mutant. Notably, these were the first results showing complete abolishment of the CPF-induced wild-type phenotype. This seemed to support the suggestion that AChE inhibition could be important for the effect of CPF-treatment, even at exposure concentrations as low as 0.05 mg/L. This possibility raises questions, such as why *ace-1(ok663)*, or *ace-3(dc2)* mutants still seemed to be affected. One possible explanation is that CPF could interact differently due to structural differences between the classes of AChE; such pharmacological differences have been acknowledged previously (Combes *et al.*, 2000). It is also possible that independent localisation of each AChE class could explain the apparent difference in effect; *ace-1* is expressed mostly in muscle cells, *ace-2* mainly in motoneurons, and *ace*-3, which only accounts for around 5% of total AChE activity, is found in very few cells (Combes *et al.*, 2000). The data presented here cannot confirm or exclude either of these explanations, however the demonstration of wild-type-like, CPF-induced foraging phenotypes in both ACE-1, and ACR-16-deficient mutants, based on their known expression in *C. elegans* muscle, points toward a neuronal rather than a primarily muscular effect. That suggestion is further supported by the absence of the phenotype in mutants that are deficient in the neuronally-expressed ACE-2. If ACE-2 is inhibited by treatment with 0.05 mg/L CPF, then the CPF-dependent foraging phenotype would presumably be blocked, which is precisely what was seen in the results presented here. This likely indicates an anti-cholinesterase effect.

5.2.10. Muscarinic signalling via GAR-3 is required for the effect of low-level CPF treatment on C. elegans foraging behaviour.

Anti-cholinesterase toxicity is primarily caused by inhibition of AChE, and consequent hyperactivation of acetylcholine gated receptors by elevated synaptic acetylcholine (Slavica, Dubravko and Milan, 2018). The apparent resistance of *ace-2* AChE-deficient mutants to CPF-treatment seemed to suggest the presence of an anti-cholinergic effect. However, the presence of the phenotype in *acr-16* nAChR-deficient mutants indicated that fast nicotinic signalling was not required for the effect of 0.05 mg/L CPF treatment on foraging behaviour. Moreover, aside from the two ACE-2-deficient strains, the only other mutant that showed resistance to the CPF-induced foraging phenotype was the *gar-3(gk305)* muscarinic receptor deletion mutant. This was interesting, because although the symptoms with fastest onset in human OP toxicity are mediated by excessive nicotinic receptor activation, muscarinic receptor activation also mediates some OP poisoning symptoms, with muscarinic symptoms emerging more slowly in comparison (Slavica, Dubravko and Milan, 2018).

Muscarinic signalling in *C. elegans*, particularly with regard to OP toxicity, has been relatively less-well studied. For this project, the GAR-3- mAChR was chosen primarily due to its role in *C. elegans* feeding behaviour (Steger and Avery, 2004; Davis *et al.*, 2017), and concurrently as a component of cholinergic signalling pathways. The resistance of *gar-3(gk305)*-mAChR-, and *ace-2*-AChE-deletion mutants to the effect of low-level CPF treatment indicates, firstly, that the observed effect is mediated to some extent by the cholinergic signalling pathway. If *ace-2* is indeed inhibited by treatment with 0.05 mg/L CPF, then consequently, small increases in ACh concentration could conceivably activate GAR-3 mAChRs, even in the absence of increased nicotinic signalling. This is possible

because mAChRs have approximately 1000-fold higher affinity for the ACh ligand, than do nAChRs (Hille, 1992; Park, Cho, and Cho, 2006; Dittman and Kaplan, 2008). The higher binding affinity enables mAChRs that are located extrasynaptically, to respond to relatively low concentrations of ACh released from distal sites (**Figure 55**), as part of the conserved function of volume transmission (Sarter, Parikh, and Howe, 2009; Fuxe *et al.*, 2010). AChE is highly efficient in the hydrolysis of ACh and is highly enriched at synaptic junctions in close proximity to nAChRs (Pereira *et al.*, 2015; Blotnick-Rubin and Anglister, 2018). Therefore, following low-level inhibition of neuronal AChE: *ace-2* by CPF, most of the functional AChE would remain active at the synapse. The efficiency and abundance of the remaining AChE thus hydrolysing most, or all of the ACh within the synapse, and preventing hyperactivation of relatively low-affinity synaptic nAChRs.

It is also likely that inhibition of small amounts of AChE would have a greater relative effect at extra-synaptic sites where AChE is more sparsely distributed (**Figure 57** & **Figure 58**). The high-affinity of mAChRs for the ACh ligand, together with the potential for GPCRs to amplify a relatively small signal in comparison to ionotropic nAChRs, offers a plausible explanation for a CPF-induced effect on foraging behaviour mediated by low-level AChE inhibition. It has been shown previously in *C. elegans* that extrasynaptic GAR-3 can respond to ACh from distal release sites (Chan *et al.*, 2013), and furthermore, spillover of ACh from cholinergic synapses has been demonstrated following treatment with other AChE inhibitors (Stanchev and Sargent, 2011; Petrov *et al.*, 2014).

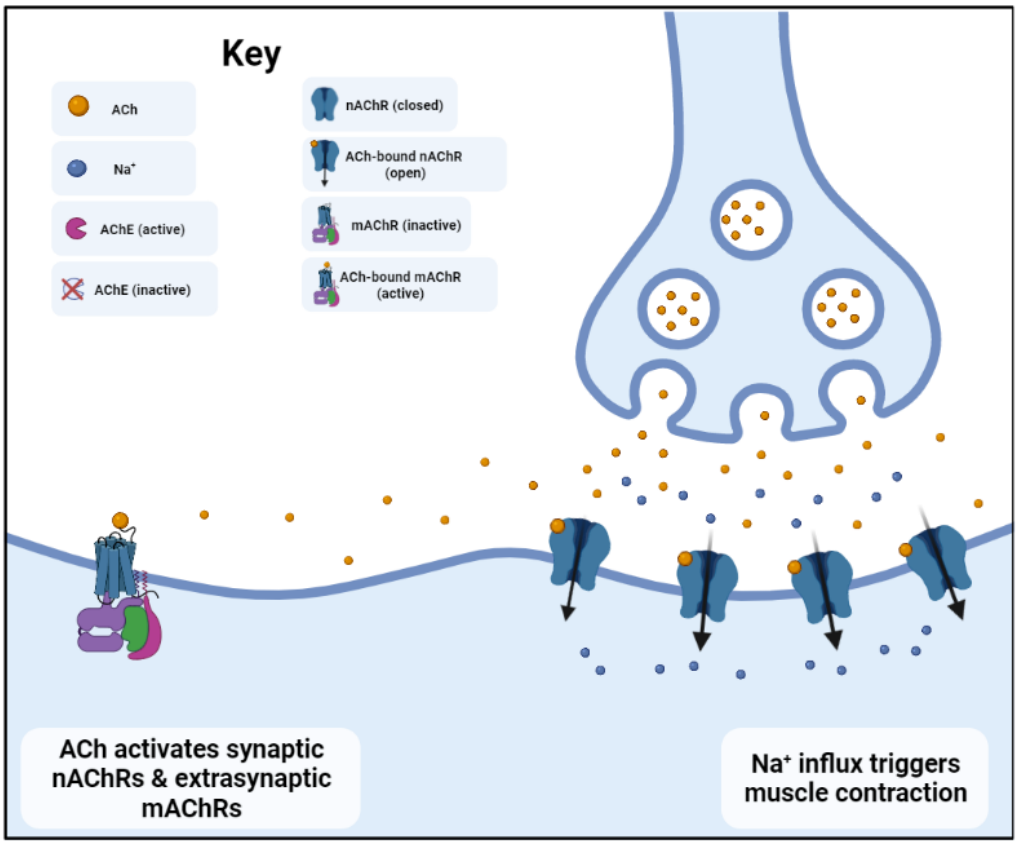

Figure 55. Normal cholinergic signalling following synaptic AChE release. Synaptically-released ACh concentrated at the synaptic terminal activates post-synaptic ionotropic nAChRs, leading to sodium influx and muscle contraction. Spillover of relatively small amounts of ACh activates the relatively high-affinity mAChRs sited at distal locations.

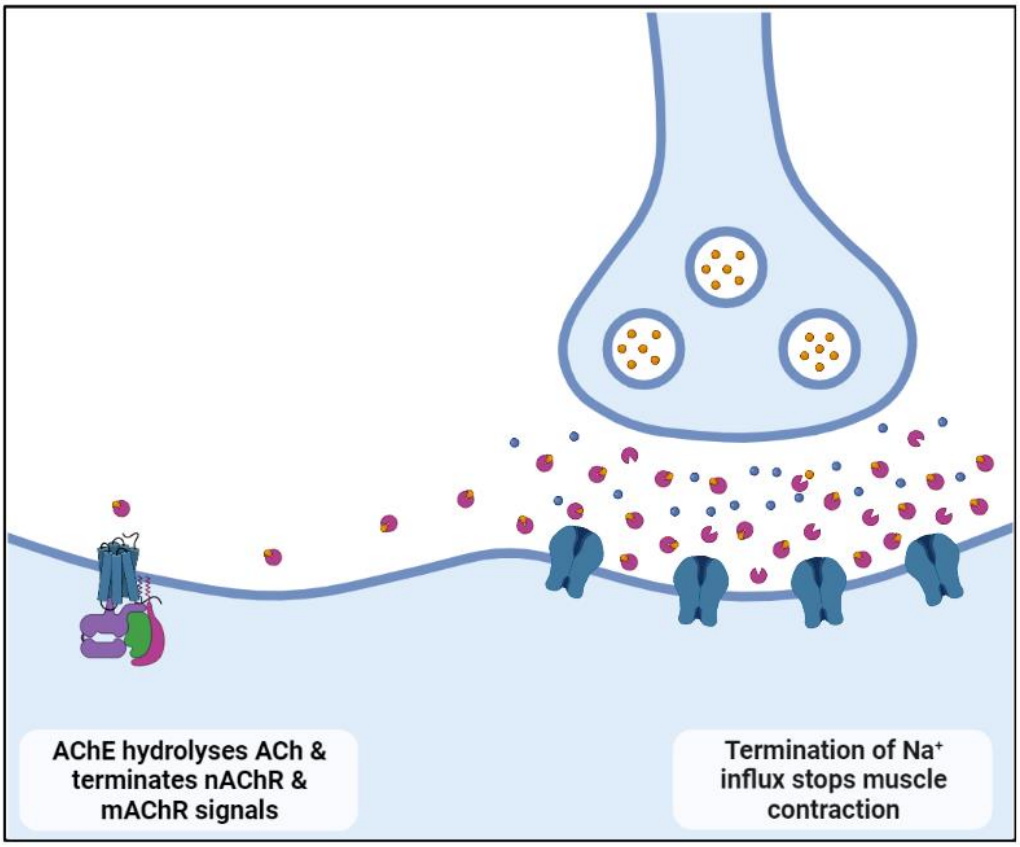

Figure 56. Normal inactivation of the cholinergic signal by AChE. AChE is highly efficient in the hydrolysis of ACh and is concentrated mostly at the synapse. During normal function this terminates the signal, ceasing muscle contraction and preventing overspill to nearby receptors.

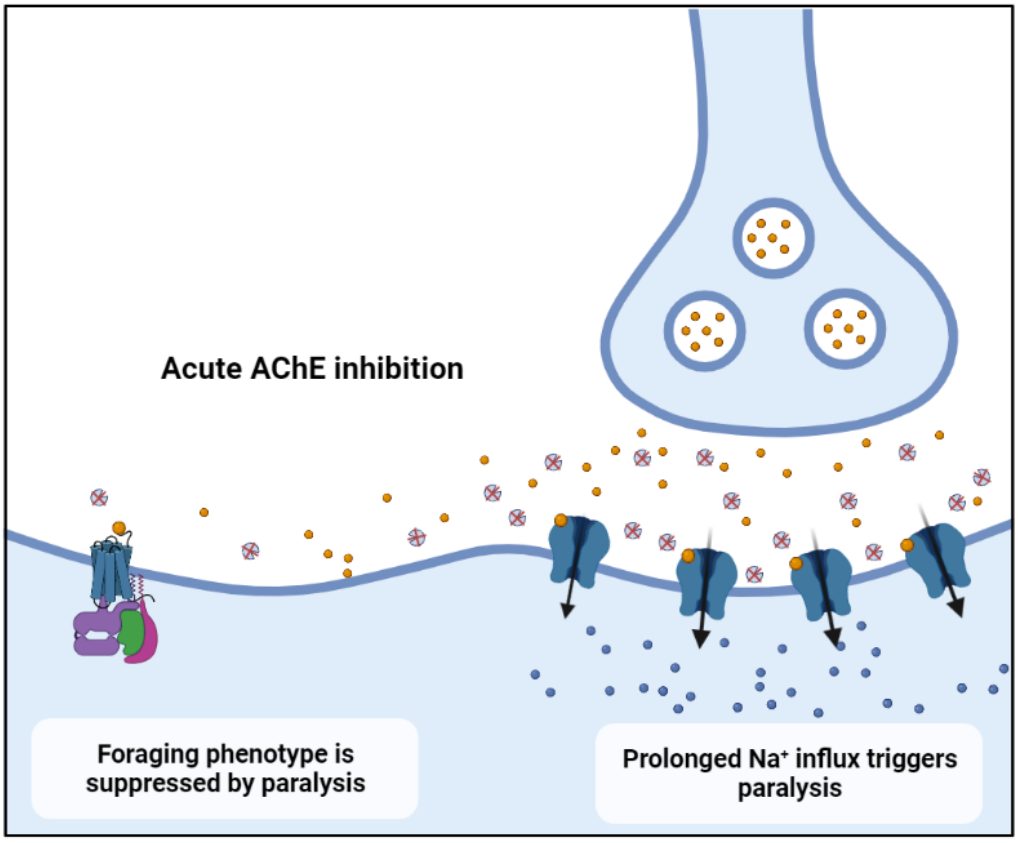

Figure 57. Cholinergic effect of acute AChE inhibition. Massive inhibition of AChE such as is caused by acute OP poisoning, leads to the accumulation of ACh at the synapse and consequent hyperexcitation and muscular paralysis. Muscarinic effects are likely present in *C. elegans* but would be difficult to detect in paralysed worms.

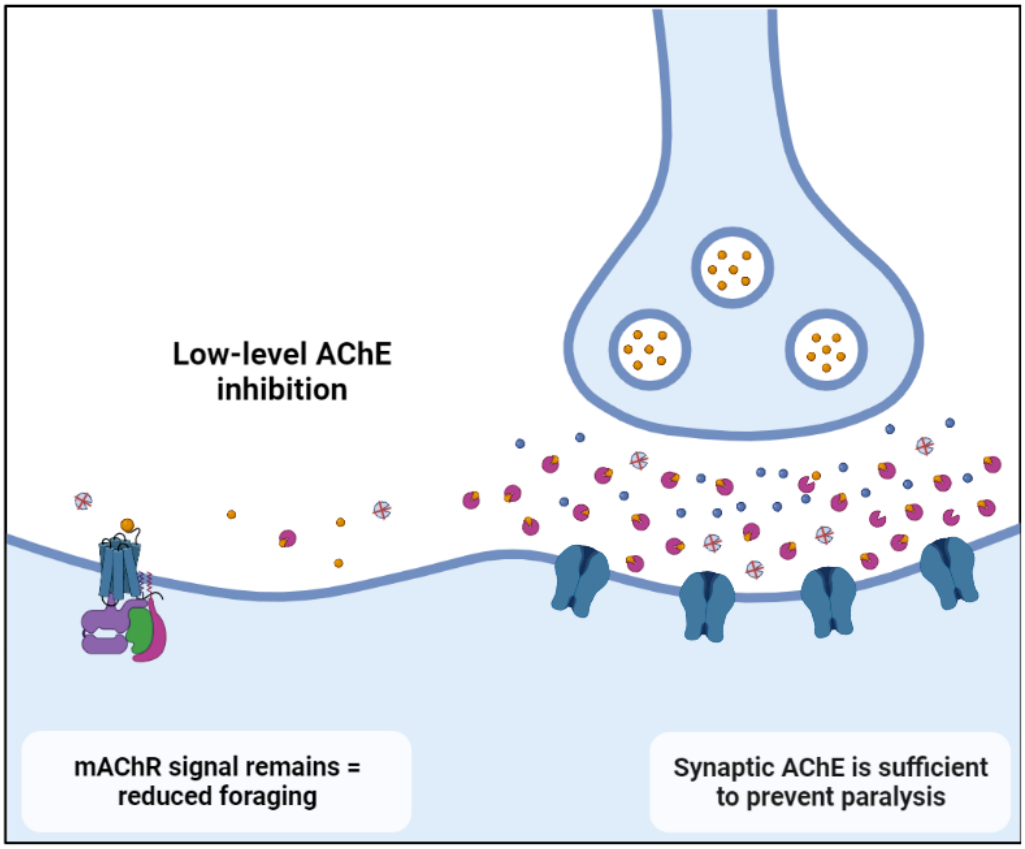

Figure 58. Cholinergic effect of low-level AChE inhibition. The suggested explanation is that small but biologically relevant levels of AChE inhibition occur as a result of treatment with 0.05 mg/L CPF . The resulting change in ACh concentration would be insufficient to depolarise the postsynaptic membrane via activation of relatively low-affinity nAChRs but could presumably activate high-affinity mAChRs leading to more nuanced effects on foraging behaviour.

5.2.11. Expression of GAR-3 in cholinergic neurons rescues the CPF-induced foraging phenotype in the gar-3(gk305) deletion mutant.

The requirement of GAR-3 for the effect of low-level CPF treatment on foraging behaviour was further confirmed by the rescue experiment. The foraging range displayed by CPF-treated *gar-3(gk305)* mutants was similar to that displayed by untreated wild-type animals, suggesting that deletion of the GAR-3 mAChR caused resistance to low-level CPF

treatment. However, foraging range was reduced to levels similar to CPF-treated wild-type animals when GAR-3 was inserted in cholinergic neurons, in the CPF-treated *gar-3(gk305);vjIs50* rescue mutant. The reduction in foraging range to wild-type levels indicates that GAR-3 is necessary for the effect of low-level CPF treatment and an effect of CPF was clearly reinstated, suggesting a full rescue. However, expression of GAR-3 in cholinergic neurons also appeared to cause a slight reduction in foraging range in untreated animals, as both the *gar-3(gk305);vjIs50* rescue and *vjIs50* mutants showed reduced foraging range compared to wild-type and *gar-3(gk305)* deletion mutants, meaning that the difference caused by CPF was marginally greater in wild-type animals. It could be argued that the phenotype was not fully rescued, however the difference in the size of the effect was driven by differences in the behaviour of the respective vehicle control groups. The *vjIs50* mutant, and hence also the *gar-3(gk305);vjIs50* rescue mutant, express multiple copies of GAR-3 and consequently it is overexpressed in the cholinergic neurons of those mutants (Chan *et al.*, 2013). Overexpression of GAR-3 mAChRs would likely increase baseline sensitivity to endogenous ACh, which could explain the reduced foraging range in untreated *vjIs50,* and *gar-3(gk305);vjIs50* vehicle controls relative to untreated wild-type animals. The possibility that the behavioural difference between untreated mutants could be caused by a background mutation specific to the *gar-3(gk305);vjIs50* rescue strain is unlikely, since the *vjIs50* mutants showed similar differences.

5.3. Conclusions

Low-level CPF treatment results in a subtle foraging phenotype in *C. elegans*, which is detectable only following food deprivation and with the use of computerised behavioural tracking tools. To the best of our knowledge, 0.05 mg/L is the lowest concentration of CPF at which an effect has been observed in *C. elegans* following exposure in NGM. Even so, despite such low exposure levels, we found no effect of CPF treatment that was detectably mediated by anything other than cholinergic signalling pathways. These findings, therefore, do not support a primarily non-cholinergic effect of CPF exposure, even at considerably low-levels. This was important, because the possibility that non-cholinergic effects could occur below the threshold for AChE inhibition has been fundamental to low-level OP research and was therefore a driving question in this project.

The results of this work present and support a mechanism by which low-level CPF exposure might interact with cholinergic signalling pathways in a manner that is specific to muscarinic signalling and does not involve nicotinic signalling. Specifically, low-level CPF treatment exerts its effect via highly sensitive muscarinic receptor signalling pathways, without reaching a threshold for cholinergic hyperexcitation mediated by synaptic nAChRs. This might explain 'below-threshold effects' reported at low-levels even if the primary mechanism of action, inhibition of AChE, remained the same.

AChE inhibition by OPs is well established, but whether this was the cause of the low-level effects described in this chapter is less clear. On one hand, knockout of class B AChE conferred resistance to the effect of CPF treatment, which supports the theory that AChE remains the primary mechanism of action. Conversely, inhibition of AChE measured during our enzyme activity assay was not statistically significant. However,

measurements of enzyme activity taken from whole-worms may not be sensitive to localised changes in extrasynaptic AChE activity, especially since most AChE is concentrated at synapses (Blotnick-Rubin and Anglister, 2018). It seems likely that subtle and localised changes in AChE activity can lead to significant changes in behaviour without causing nicotinic hyperexcitation. Therefore, future studies concerning the effects of low-level OP exposure should be sure to consider such nuances of AChE activity. Variations of the Ellman (1961) assay are standard for measuring AChE activity, including in *C. elegans* (Izquierdo, O'Connor, *et al.*, 2021). However, because of the volume of tissue required, these methods may not provide sufficient resolution to measure changes in activity caused by very low OP concentrations.

6. The effect of long-term low-level organophosphate exposure on mental health in a human population

6.1. Introduction

Organophosphates (OPs) are an important group of compounds used globally in a wide range of applications. Many domestic, industrial, and agricultural products contain OPs as active ingredients or functional additives, and consequently some level of human exposure to these compounds is often unavoidable. Exposure is particularly likely in agriculture, where workers may be required to work with OP-containing pesticides to protect crops from herbivorous pests and maintain livestock health against parasites. Following established guidelines for the proper use of OP products and using appropriate personal protective equipment (PPE) can help to protect against acutely toxic exposures. However, the potential for harm from the relatively low-level exposures associated with normal use has been a controversial topic (CoT, 1999; Mackenzie Ross et al., 2013).

Acute poisoning by OPs occurs via inhibition of AChE (AChE), which causes well-established nervous system dysfunction and clinical symptoms (Eddleston *et al.*, 2008). The severity of symptoms in acute OP poisoning usually relates directly to the amount of acetylcholine that builds at synaptic junctions as a result of AChE enzyme inhibition. Such cases usually present as medical emergencies with obvious symptoms, including muscle twitches, involuntary bladder and bowel activity, convulsions and breathing difficulty, which can be life threatening without effective treatment (Jokanović, 2009). Acute poisoning with OP pesticides would usually follow some noticeable accident, or intentional act in cases of self-poisoning (Slavica, Dubravko and Milan, 2018), which,

together with the recognisable and characteristic symptom set makes acute toxicity relatively simple to recognise. That OP poisoning is detrimental to human health is well documented and extensively researched, however the effects of low-level exposure are more controversial.

Previous research concerning the risks of low-level exposure to human health have produced mixed results (Freire and Koifman, 2013a; Mackenzie Ross *et al.*, 2013) Methodological inconsistencies and omissions are often acknowledged and may account for some of the discrepancies found throughout the literature (Mackenzie Ross *et al.*, 2013). For example, studies have often taken the confounding effects of stress into account. However, although participants' experiences of acutely stressful events have sometimes been accounted for, ongoing day-to-day stresses are not detected by those measures. Those day-to-day stresses may be more important drivers of mood, and so they will be specifically addressed in this study.

In addition, a range of physical health problems have been reported among agricultural workers. The effects of physical health on depression may be a principal factor in these groups, yet the relationship has not been thoroughly explored. To address this, the possible contribution of physical health problems to mental health impairments was also investigated in this study. Firstly, by exploring the differences in physical health issues between OP exposed agricultural workers and controls, and secondly, by testing for relationships between physical health, mood, and OP exposure in an exposed cohort.

Finding a control population with similar demographic characteristics, but without historic exposure to OPs presents another challenge (Harrison and Mackenzie Ross, 2016). Although organic farms are required to operate with fewer pesticides including

strict control of OP use, many organic farmers and contractors will have experienced 'traditional' farming practices, which include pesticide use, at some point during their careers (Fuhrimann *et al.*, 2019).

Different techniques and methodological flaws might be responsible for these discrepancies in the literature. We try to address some of these flaws in the following chapter. Specifically, selecting a control group with well-matched lifestyle and demographic characteristics may address some of the confounding factors such as education, or possible pesticide use, which have limited previous between group comparisons (Cole *et al.*, 1997; Fiedler *et al.*, 1997). The impact of life stress on other aspects of mental health, particularly depression, will be considered. Life stress has rarely been considered in previous studies, and when it has been considered, only acute stressors or major events have been included, with day-to-day stress factors being overlooked (Holmes and Rahe, 1967; Kessler, 1997; Harrison and Mackenzie Ross, 2016). This gap will be addressed by and accounted for by including a Daily Hassles scale to capture information that could be equally important to mental wellbeing (Kanner *et al.*, 1981; DeLongis *et al.*, 1982).

No biological measure of exposure will be used in this study, however, previous issues with exposure metrics will be addressed by collecting several different measures of exposure, including time spent working with pesticides, type of work performed, and whether any history of exposure or poisoning had occurred in both the exposed and control groups (Berent *et al.*, 2014).

Furthermore, although several studies have been concerned with aspects of physical health following exposure, studies concerning the potential effects of low-level exposure

have rarely considered the impact of poor physical health on mental health (Mackenzie Ross *et al.*, 2013). These deficits will be addressed as described below.

6.2. Chapter Objectives

There were three main objectives to this chapter:

1. To gather evidence regarding the prevalence of mood related symptoms among agricultural workers with a history of occupational exposure to organophosphates, and to compare this data to a control cohort with no known exposure to OPs.

2. To explore factors that might alter the severity of mood related symptoms in agricultural workers, other than OP exposure. For example, physical health status, stressful life events or day to day worries. Particularly, whether any of these factors are specific to the lifestyle associated with working in agriculture.

3. To establish whether any existing relationship between OP exposure and mood related symptoms occurs as a direct result of exposure, or whether the effect could be mediated by some other related outcome, such as poor physical health or environmental stress.

6.3. Study hypotheses

To meet the objectives laid out in this chapter the following hypotheses were tested:

Hypothesis 1: It was predicted that agricultural workers with a history of occupational OP exposure would report more severe psychiatric symptoms relating to mental disorder than controls with no known history of OP exposure.

Hypothesis 2: Accumulated exposure to low levels of organophosphates will be correlated with higher rates of mood related symptoms in agricultural workers.

Hypothesis 3: Ongoing 'day to day' stressors, such as financial, work or family related worries, will be more strongly related to mood and stress than one-off or acutely stressful 'major events' which have been considered in earlier studies (Mackenzie Ross *et al.*, 2013; Harrison and Mackenzie Ross, 2016).

Hypothesis 4: Finally, an exploratory hypothesis was that poor physical health may be a driver of negative mood symptoms in agricultural workers. This could be related to physical health impacts associated with farming, possibly including underreported pesticide poisoning.

6.4. Survey Results

6.4.1. Recruitment rates

Using the study recruitment methods outlined in Chapter 2, responses were received from 199 agricultural workers and 107 construction workers. The use of screener questions in online participant sourcing and survey software meant that most of the completed responses met the inclusion criteria. However, a total of 33 agricultural workers were excluded from the exposed group: 24 on the basis that they had not completed the questionnaire or given enough information, and 9 because they were uncertain whether they had worked with OPs and exposure could not be determined from their reported work history.

From the control group, a total of 12 participants were excluded, 5 of which had provided contradictory answers which suggested inaccurate or disingenuous responses. The remaining 7 construction workers were excluded because they answered "don't know" when asked whether they had been exposed to OPs, but they reported some previous work experience which suggested that exposure was very possible. These cases included reports of working in orchards, spraying pesticides for property maintenance, and administering treatment for woodworm.

After exclusions, 166 exposed and 95 controls were included for quantitative analysis.

6.4.2. Demographic information and group matching

Basic demographic and lifestyle information are presented in **Table 14**. Agricultural workers were successfully matched with controls on age ($t(232.52) = -1.912$, *ns*. 95% CI [-6.13, 0.28]) and educational attainment ($t(183.7) = 1.41$, *ns.* 95% CI [-0.99, 0.16]), but not gender. Within the exposed group, 3 individuals reported their gender as 'other' and

1 would rather not disclose theirs. All participants within the control group identified as either male or female, and even without including 3 non-binary agricultural workers and the individual who did not disclose, there was a significant male-bias among construction workers compared with agricultural workers ($\chi^2(1, N = 257) = 14.04$, $p < .001$).

A similar pattern of smoking behaviour was observed in both groups ($\chi^2(2, N = 261) = 2.78$, *ns*). Alcohol consumption was also similar, as measured by number of separate drinking sessions per week between groups ($t(260) = -1.11$, *ns*). Agricultural workers consumed slightly fewer drinks on average than construction workers, however this was not significant ($t(230.84) = 1.75$, *ns.* 95% CI [-0.04, 0.67]).

Table 14. Demographic and lifestyle information. Variables were successfully matched apart from gender. Non-binary (x3) and undisclosed gender (x1) were discounted from chi square test for gender matching because there were fewer than the required 5 responses in each of those categories. * indicates $p < 0.001$.

	Control group Mean (SD)	Exposed group Mean (SD)
Age	37.61 (10.81)	40.54 (13.58)
Gender*	73-M, 22-F	87-M, 75-F, 3-Other, 1-Prefer not to say
Education level	3.89 (2.36)	4.28 (2.19)
Smoking tobacco	28-Y, 43-N, 24-Ex smokers	34-Y, 82-N, 50-Ex smokers
Drinking (times per month)	5.67 (5.266)	5.78 (5.665)
Number of drinks per session	2.82 (1.24)	2.5 (1.62)
Hours slept per night	6.34 (1.17)	6.52 (1.23)

6.4.3. Between-group comparisons of common mental disorders and general health.

This section explores the differences between agricultural workers with previous exposure to organophosphates, and construction workers with no previous exposure.

The presence of symptoms related to common mental disorders was compared using log transformed DASS-21 scores for depression and anxiety. Self-reported general health, symptom questionnaire responses and self-rated bodily pain scores were used to assess physical health.

6.4.3.1. OP exposed agricultural workers score more highly for depression than controls, but not for anxiety.

First, to test whether overall mood was different between the exposed cohort and controls a multivariate analysis of variance (MANOVA) was performed using both depression and anxiety DASS-21 scores as outcome measures representing mood. Diagnostic scores for psychiatric disorders are naturally skewed in healthy populations (hence they are disorders) and so DASS-21 scores were transformed prior to analysis using natural log transformations ($\ln(x_i + 1)$).

Box's test of Equality of Covariance Matrices was used to check homogeneity of covariance, which was not significant (*Box's M* (0.69), $p = .87 > \alpha .001$). Using Wilk's Lambda test the MANOVA suggested a significant difference in common mental disorders symptoms between the exposed and non-exposed group (*Wilk's* $\lambda = .966$, $F(2, 258) = 4.54$, $p = .011$, multivariate $\eta^2 = .03$).

Univariate ANOVAs performed separately on each mood component revealed significantly higher depression scores in the exposed group $F(1, 261) = 8.86$, $p = .003$ (**Figure 59**), but anxiety scores were not significantly different between groups $F(1, 261) = 2.85$, $p = .217$ (**Figure 60**).

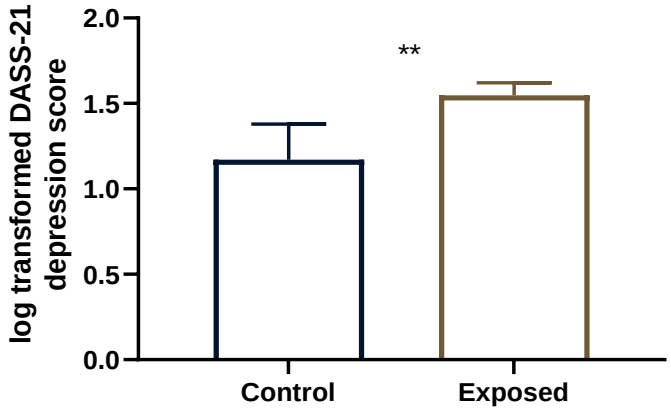

Figure 59. Depression scores were significantly higher in OP-exposed agricultural workers. Log transformed DASS-21 depression scores were significantly higher for the exposed group (n= 166) compared to control (n= 95). The bars in the figure represent the mean scores, with error bars indicating the 95% confidence intervals. Statistical significance is denoted by ** ($p < 0.01$).

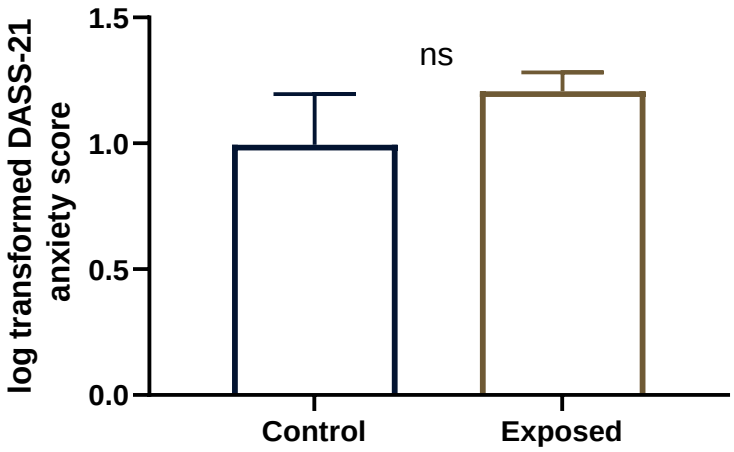

Figure 60. Anxiety scores were not significantly different between the exposed and control groups. Log transformed DASS-21 anxiety scores for the exposed (n= 166) and control (n= 95) were not significantly different (ns). The bars in the figure represent the mean scores, while the error bars indicate the 95% confidence intervals.

Mental health symptom scores were also investigated in terms of their categorisation into different levels of disorder. Fishers Exact Test revealed the number of scores reaching the threshold for disorder to be significantly different between groups for depression ($p < .05$), but not anxiety . A breakdown of scores shows a higher proportion of participants experiencing depression in the exposed compared to the control group (**Figure 61**). Scores for mild, moderate, severe, and extreme depression were generally higher in the exposed group (12%, 14.5%, 8.4% and 13.3% respectively) than the control group (7.4%, 14.7%, 5.3% and 7.4%) across the categories.

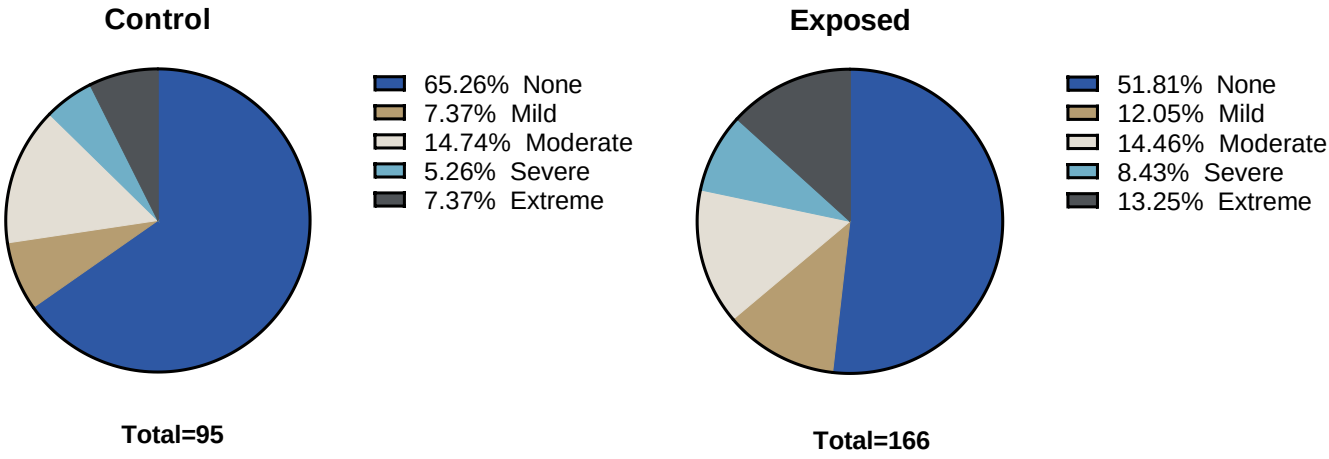

Figure 61. OP exposed agricultural workers are more likely to meet the threshold for depression. The figure displays the proportion of respondents in each exposure group whose DASS-21 depression scores meet the suggested cut-off values for depression severity. The exposed group showed a significantly different pattern of depression severity, with fewer scores meeting the threshold for 'none', and more meeting the threshold for 'mild', 'moderate' or 'severe' than controls (χ^2 = 11.548, df = 4, p = 0.021).

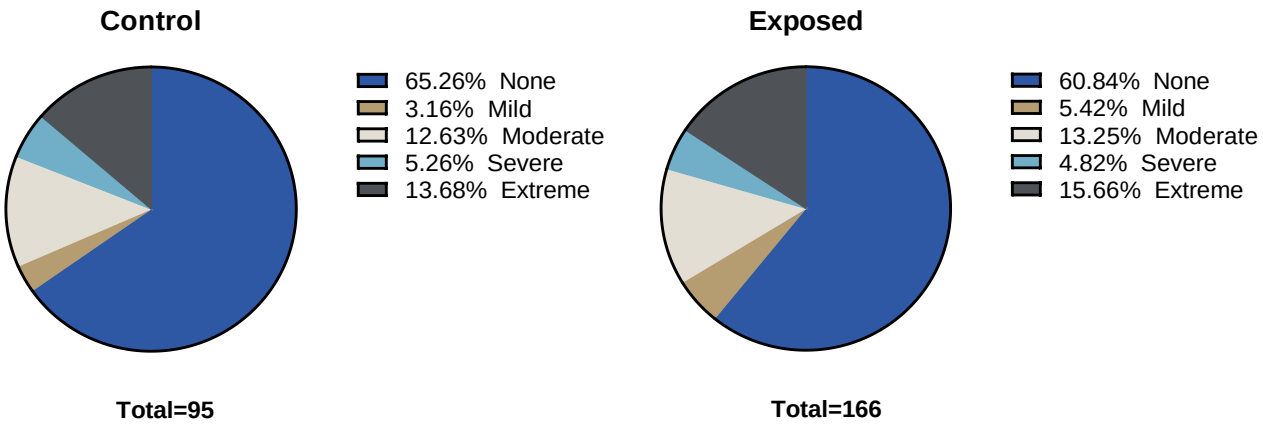

Figure 62. Exposed agricultural workers exhibit a similar pattern of anxiety severity compared to construction workers. The figure presents the proportion of respondents in each exposure group whose DASS-21 anxiety scores meet the suggested cutoff values for anxiety severity. There was no statistically significant difference in anxiety severity between the exposed group (agricultural workers) and the control group (construction workers) ($\chi^2 = 5.632$, df = 4, p = 0.229).

6.4.3.2. Agricultural workers experience poorer overall health than construction workers.

Since psychiatric health can be closely linked, and influenced by physical health, participants were asked to rate their own health in addition to answering questions about specific symptoms. This included questions about their own physical health and mood state and each response was measured on a Likert scale.

6.4.3.3. Agricultural workers reported poorer general health status than construction workers.

Participants were first asked how they would rate their own health 'in general', on a scale of 1 (terrible) to 5 (excellent).

Overall, construction workers reported better general health than agricultural workers (χ^2 (3, 261) = 174.1, p < 0.0001)(**Figure 63**). While around half of each group reported their general health to be 'good', a smaller proportion of agricultural workers considered their health to be 'excellent' (4.22% versus 11.58%) or 'very good' (23.49% versus 34.74%) than construction workers. Conversely, a greater proportion of agricultural workers reported 'poor' health (20.48%) compared to very few construction workers (3.16%). Finally, 1.81% of agricultural workers were suffering 'terrible' health status, compared with none of the construction workers.

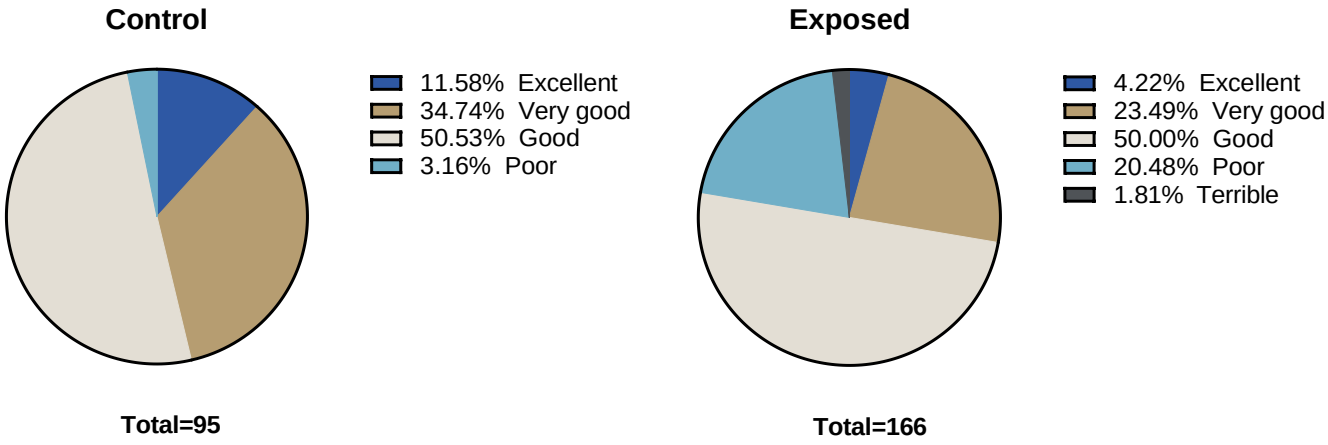

Figure 63. OP exposed agricultural workers report poorer general health than construction workers. The pie charts represent the proportion of respondents in each exposure group whose general health scores fall into different categories. Fisher's exact test revealed a statistically significant association between the exposed group and general health scores (p = 0.0036). Notably, the exposed group exhibited a significantly higher proportion of individuals in the 'poor/terrible' category compared to the control group. Furthermore, the proportions in the 'excellent,' 'very good,' and 'good' categories were similar between the groups.

6.4.3.4. Agricultural workers reported more specific symptoms overall than construction workers.

To gain a more objective insight into participants' physical health they were asked a series of questions in relation to various specific physical symptoms. While some symptoms could conceivably result from perturbation of the cholinergic system, such as muscular twitches, others are considered less likely to be related to cholinergic function, such as toothache. The relationships between cholinergic and non-cholinergic symptoms are explored later in this chapter.

Participants were asked to provide information on the frequency of experiencing specific symptoms over the preceding 4 weeks. They were presented with response options that ranged from 'never-0' to 'always-3', indicating how often they encountered each symptom. To examine potential patterns in symptom occurrence, individual scores for each symptom were calculated and presented in **Table 15**.

Additionally, to obtain an overall measure of participants' physical health, we recorded a sum of symptom occurrences. This sum was derived by adding up the scores for each symptom reported by the participants. The possible range of scores for this measure ranged from 0 to a maximum value of 72, representing the highest potential symptom burden experienced by an individual within the given time frame.

The overall symptom score differed significantly between groups ($t(253.6) = -5.75$, $p < 0.001$. 95% CI [-16.32, -7.99]). (**Figure 64**).

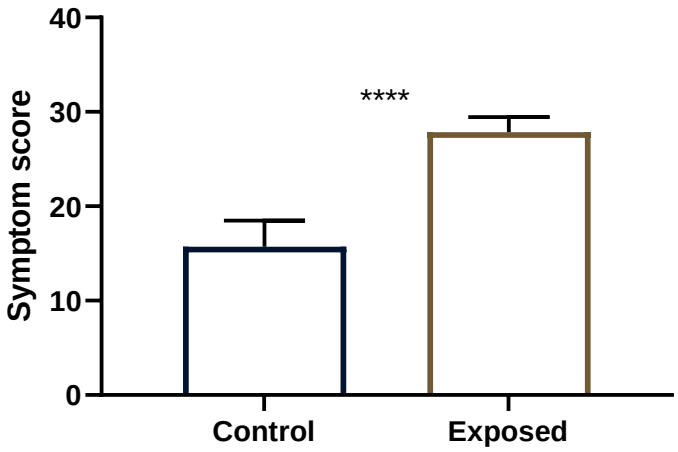

Figure 64. Overall physical symptom scores by exposure group. Participants were asked to report the frequency of experiencing symptoms over the previous 4 weeks, using a scale ranging from 'never-0' to 'always-3'. The overall symptom score exhibited a significant difference between the exposed group (n = 166) and the control group (n = 95), with notably higher scores in the exposed group. The bars in the figure represent the mean scores, while the error bars indicate the 95% confidence intervals. Statistical significance is denoted by **** ($p < 0.0001$).

6.4.3.5. Most, but not all symptoms were reported more often by agricultural workers than construction workers.

Knowing which symptoms affected agricultural workers more or less-frequently than construction workers, could help to determine any different health pressures that might affect each group. This information could be of use in identifying any links to changes in mood, or OP exposure.

To test for differences in specific symptoms between the two groups, a series of one-way ANOVAs were performed with the severity rating scores for 24 symptoms listed in the

health questionnaire included. In line with the higher overall symptom scores in agricultural workers (**Figure 64**) the results from the ANOVAs showed that most, but not all symptoms were suffered more severely in agricultural workers than construction workers.

Total reported impact of headaches did not differ significantly between the two groups ($F(1, 259) = .131$, $p = 0.72$). There were no significant differences in indigestion ($F(1, 259) = 2.244$, $p = .135$), or 'numbness or tingling in any part of the body' either ($F(1, 259) = 1.484$, $p = .224$). However, all other symptoms on the questionnaire were suffered to a greater extent by agricultural workers (**Table 15**).

Many symptoms which are classically related to acute OP poisoning were significantly more prevalent in agricultural workers, including excessive sweating ($F(1, 259) = 16.799$, $p < 0.001$), blurred vision ($F(1, 259) = 13.065$, $p < 0.001$), dizziness ($F(1, 259) = 10.949$, $p = 0.001$), muscular pain/cramps ($F(1, 259) = 10.020$, $p = 0.002$), muscle tremors or twitches ($F(1, 259) = 8.559$, $p = 0.004$), nausea ($F(1, 259) = 4.836$, $p = 0.029$), shortness of breath ($F(1, 259) = 11.334$, $p = 0.001$), muscle weakness ($F(1, 259) = 7.396$, $p = 0.007$), diarrhoea ($F(1, 259) = 6.054$, $p = 0.015$), and urinary problems ($F91, 259) = 8.477$, $p = 0.004$), than in construction workers.

Other symptoms which are not classically associated with acute poisoning but could be linked to altered cholinergic signalling, such as fatigue ($F(1, 259) = 15.160$, $p < 0.001$), poor balance/coordination ($F(1, 259) = 14.9$, $p < 0.001$), difficulty remembering things/concentrating ($F(1, 259) = 19.372$, $p < 0.001$), constipation ($F(1, 259) = 9.092$, $p = 0.003$), were also more common in agricultural workers than construction workers.

Alcohol and chemical intolerance are associated with chronic organophosphate induced psychiatric disorder (COPIND; Davies et al., 1999) and was also more highly reported ($F(1, 259) = 9.194$, $p = 0.003$) in agricultural workers. Other symptoms which were more common in agricultural workers which are unlikely to be related to OP exposure, were toothache ($F(1, 259) = 6.362$, $p = 0.012$), hearing problems ($F(1, 259) = 5.475$, $p = 0.02$), skin problems ($F(1, 259) = 23.396$, $p < 0.001$), joint stiffness or pain ($F(1, 259)$, unintended weight loss or gain ($F(1, 259) = 7.470$, $p = 0.007$), and hay fever or allergies ($F(1, 259) = 11.264$, $p = 0.001$).

6.4.3.6. The frequency of reported symptoms did not always correspond to their impact on everyday life.

Although adverse physical health symptoms can contribute towards poor mental health in people, symptoms do not necessarily affect everyone to the same extent. The results described so far describe the presence and frequency of symptoms reported by each group, but do not account for the severity of symptoms. Furthermore, the same symptom experienced with similar severity might impact individuals differently depending on their own work demands or daily activities performed. For example, muscular weakness might make life more difficult for a person with a more physically demanding job than a more sedentary occupation, and gastrointestinal and urinary problems could be more stressful when working in remote locations. It was therefore considered likely that the subjective impact of physical symptoms on daily life would be more influential to an individual's psychiatric health than the presence or frequency of symptoms alone.

To account for the severity and subjective impact of physical health symptoms, participants were asked to rate the extent to which each specific symptom affects their

daily lives. Possible answers ranged from '0 - not at all', '1 - a little', '2 - moderately' to '3 - severely'.

To test the difference in subjective life impact of each symptom between agricultural workers and construction workers, a series of one-way ANOVAs were performed with the impact score for every symptom as the outcome variable. In most cases, symptoms that were more severe in agricultural workers than construction workers also had a higher impact on the daily lives of agricultural workers (**Table 15**). However, this was not always the case. While headaches seemed to occur at a similar frequency in both groups, on average they had a significantly greater impact on agricultural worker's daily lives ($F(1, 259) = 5.958$, $p = 0.015$). Conversely, although hearing problems were more highly scored by agricultural workers, the impact on the daily lives of construction workers caused by hearing problems was similar ($F(1, 259) = 1.477$, $p = 0.225$).

There was no significant difference in the frequency of symptoms or life impact together, for numbness or tingling in any part of the body ($F(1, 259) = 0.437$, $p = 0.509$), or indigestion ($F(1, 259) = 0.787$, $p = 0.376$).

Table 15. Significant differences in the rated severity and life impact of individual health symptoms between agricultural workers and construction workers.

Symptom	Total difference in rated impact of specific symptoms	Difference in perceived impact on everyday life
Excessive sweating	***	**
Headaches	ns	*
Toothache	*	*
Numbness or tingling in any part of the body	ns	ns
Blurred vision	***	**
Fatigue (feeling constantly tired)	***	**
Loss of balance/coordination	***	***
Dizziness	***	*
Ringing in the ears/hearing problems	*	ns
Skin problems	***	***
Joint stiffness/pain	**	**
Muscular pain/cramps	**	**
Muscle tremors/twitches	**	**
Nausea	*	ns
Difficulty remembering things/concentrating	***	***
Chest pain/tightness/shortness of breath	**	**
Muscle weakness	**	**
Unintended weight-loss or gain	**	*
Indigestion	ns	ns
Constipation	**	**
Diarrhea	*	ns
Hay fever or allergies	**	**
Alcohol/chemical intolerance	**	*
Urinary problems	**	**

* = p<.05, ** = p<.01, ***= p<.001

6.4.3.7. Bodily pain

The levels of bodily pain experienced by an individual can contribute to both physical and psychiatric health and can also be modulated by the cholinergic system (Naser and Kuner, 2018). To account for this, participants were asked how much bodily pain they had experienced during the previous 4 weeks. They were asked to rate their bodily pain on a 6-point Likert scale, ranging from 'none' to 'very severe'.

Overall, self-reported bodily pain scores were significantly higher in the exposed group than controls ($t(259) = -2.17$, $p < 0.05$: 95% CI [-.694, -.034])(**Figure 65**).

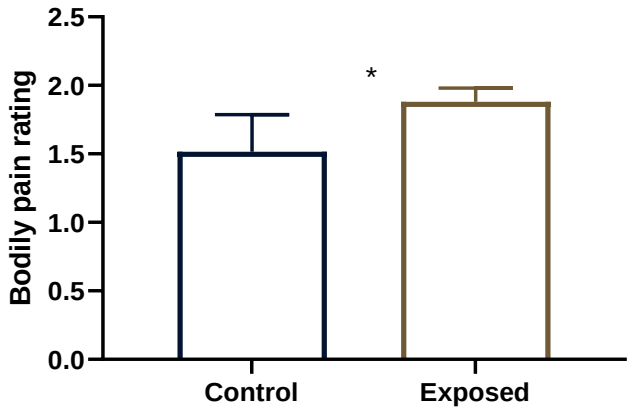

Figure 65. Bodily pain was higher in OP exposed agricultural workers. Participants were surveyed regarding their level of bodily pain experienced within the preceding 4 weeks. They rated their bodily pain on a 6-point Likert scale, ranging from 'none' to 'very severe'. Self-reported bodily pain scores were significantly higher in the exposed group (n = 166) compared to the control group (n = 95). The bars in the figure represent the mean scores, and the error bars represent the 95% confidence intervals. Statistical significance is denoted by * ($p < 0.05$).

6.4.4. Factor analyses reveals differences in patterns of health symptoms between groups.

As presented above, agricultural workers who had been exposed to organophosphates reported poorer physical health and experienced greater life stress than construction workers. In order to make use of this information it was necessary to determine whether some of these differences could be linked to OP exposure in some way, or whether the main between-group differences were due to differences in lifestyle between the two groups. To facilitate this, multiple exploratory factor analyses were performed, using the results of the symptomatic health questionnaire and the Daily Hassles scale. The purpose of which was to identify any common themes among sets of symptoms or stressors, and whether or why these might differ between groups. Factors were also saved for further analysis within the exposed cohort described later.

6.4.4.1. Health Factors: physical symptoms grouped by factor analysis.

To investigate the contribution of physical health to mood, and further facilitate comparisons of health status between cohorts, the frequency of physical symptoms scores was analysed by factor analysis. Physical symptoms can be complex and the underlying causes less simple to define for the purpose of grouping items within factors. Therefore, instead of extracting a set number of factors from the data, a more exploratory approach was taken and all factors with a minimum eigenvalue of 1 were considered. Factor extraction was performed using orthogonal (varimax) rotation. Items with a correlation coefficient greater than .3 were included in each factor. DASS scores were not included in in the factor analysis for health scores.

The factor analysis extracted 5 separate groups of symptoms which are displayed in **Table 16**. Interestingly, the first four factors contain groups of typically cholinergic-linked symptoms, some of which could be theorised as being mediated via nicotinic receptor activation, and others that seem to be a more typically muscarinic receptor mediated response.

Differences between groups were explored using regression factor scores for each factor and analysed using multiple t-tests. A two-stage linear step-up procedure was used to control the false discovery rate using Q = 1% (Benjamini, Krieger and Yekutieli, 2006).

6.4.4.2. Physical symptoms: factor 1

Factor 1 seemed to be composed of specifically neuromuscular related symptoms **(Table 16**), which could possibly be related to nicotinic receptor mediated effects within the cholinergic system. Although Factor 1 was represented in agricultural workers to a greater extent than in construction workers, this was not significant at the 95% confidence interval ($t(259) = 1.871$, ns).

6.4.4.3. Physical symptoms: factor 2

The second factor contained symptoms which could be linked to the autonomic nervous system, including respiratory, urinary problems and thermoregulation (**Table 16**). There was a striking difference between groups for factor 2, with significantly more of these symptoms affecting agricultural workers ($t(259) = 4.526$, FDR adjusted $p < 0.001$).

6.4.4.4. Physical symptoms: factor 3

The third factor extracted contained a mixture of symptoms relating to cognitive function, motor coordination, muscular tremors, and blurred vision (**Table 16**). This set of

symptoms also seemed to affect agricultural workers significantly more than controls ($t(259) = 3.247$, $p < 0.01$).

6.4.4.5. Physical symptoms: factor 4

Factor 4 was composed of headaches, nausea, and gastrointestinal related problems (**Table 16**). This set of symptoms were not collectively different between the two groups ($t(259) = -1.225$, *ns.*).

6.4.4.6. Physical symptoms: factor 5

The final collective factor extracted from the physical symptom questionnaire contained only 'unintended weight-loss or gain' and toothache. Factor 5 did differ significantly between groups and was more present in the exposed group ($t(239.267) = -3.048$, $p < 0.01$.).

Table 16. Factors extracted using physical health symptoms.

Factor	Components
Physical symptoms factor 1	• Joint stiffness/pain • Muscular pain/cramps • Numbness or tingling in any part of the body • Muscle weakness • Fatigue
Physical symptoms factor 2	• Skin problems • Alcohol/chemical intolerance • Hay fever or allergies • Chest pain/tightness/shortness of breath • Problems controlling temperature/sweating • Urinary problems
Physical symptoms factor 3	• Difficulty remembering things/concentrating • Loss of balance/coordination • Muscle tremors/twitches • Blurred vision
Physical symptoms factor 4	• Headaches • Nausea • Indigestion • Constipation
Physical symptoms factor 5	• Unintended weight-loss or gain • Toothache

6.4.4.7. Physical health symptoms influence between-group differences in depression symptomology.

Poor physical health is a significant risk factor for depression. It was therefore possible that the increased depression symptoms in the exposed group was influenced by the different physical symptoms reported. To test this, a one-way ANCOVA was conducted using log transformed DASS-21 depression scores as a dependent variable, and the

physical symptom factors that were different between groups (Factor 2, 3, and 5:**Table 16**) as covariates.

The between group difference in depression scores was not significant after considering the different rates of physical symptoms reported (F(1, 256) = 3.66, p=0.057, 95% CI [-.007, .487].

The implication of physical symptom factors in depression score differences support the suggestion that physical health is an important factor in mental health status in these groups. However, it does not directly address whether OP exposure plays a role. To investigate this further, within-group analyses were carried out in 6.4.6 using the exposed agricultural worker group only. This would allow pesticide exposure to be considered in the context of increased depression symptomology and other contributing factors within this group.

6.4.5. Between group comparisons of environmental and experienced stress

To account for the effects of internal and external stress on psychiatric health, participants were assessed for symptoms of stress using the third component of the DASS-21 scale (Lovibond and Lovibond, 1996), and for their experiences of major stressful events, and ongoing daily stressors, using the SRRS and Daily Hassles scale, respectively (Holmes and Rahe, 1967; Kanner *et al.*, 1981).

6.4.5.1. Internal stress

Internal stress levels were measured using the DASS-21 scores for stress. The results suggested that agricultural workers in this sample experience higher levels of stress than the construction workers (**Figure 66**; **Figure 67**). Following a significant multivariate difference in log transformed DASS-21 anxiety and depression scores (described in

section 2.3.5.3), follow-up univariate analysis confirmed that there was also a significant difference in stress levels between the two groups ($F(1, 261) = 4.77$, $p < 0.05$)(**Figure 66**).

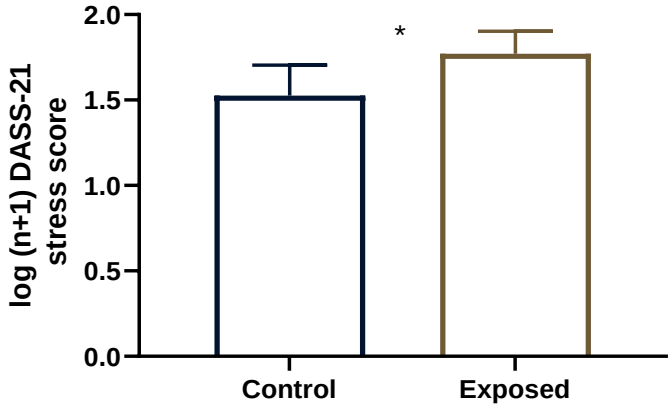

Figure 66. Overall stress scores were higher in OP-exposed agricultural workers. Dass-21 stress scores were found to be higher in the exposed group (n = 166) compared to the control group (n = 95). The bars in the figure represent the mean scores, and the error bars represent the 95% confidence intervals. Statistical significance is denoted by * ($p < 0.05$).

Further analysis of DASS-21 stress scores, when compared against established cut-off figures for degree of stress severity (**Figure 67**), showed that differences between groups were slightly apparent in the top and bottom end of the score counts; more specifically, a greater proportion of construction workers scored below the threshold for mild stress than agricultural workers (69.47% versus 64.46%), and at the opposite end of the scale, more agricultural workers were suffering from severe stress than construction workers (16.87% versus 10.53%). Whereas similar proportions of mild and moderate stress were seen in both groups. Taken altogether however, the overall difference between groups did not reach the threshold for significance when comparing the percentage of exposed

individuals within each cut-off against the corresponding control numbers as observed versus expected values, respectively ($\chi^2(3, 261) = 7.100$, $p = 0.068$).

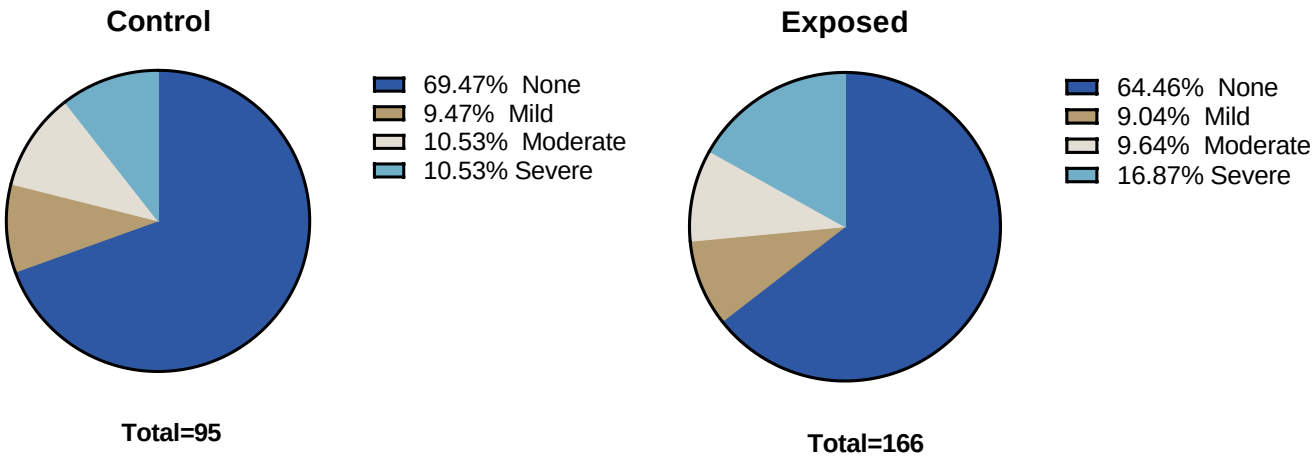

Figure 67. Proportion of participants experiencing different levels of stress based on DASS-21 stress scores and established cut-off values Stress scores, when compared against established cut-off figures for degree of stress severity were similar between the exposed and control groups ($\chi^2(3, 261) = 7.100$, $p = 0.068$). (Lovibond and Lovibond, 1996).

6.4.5.2. External stressors

To investigate and better account for possible reasons why agricultural workers appeared to be more stressed than construction workers, each group's responses to the SRRS and Daily Hassles scale were compared against each other.

6.4.5.3. Major stressful events measured using the Social Readjustment Ratings Scale

The SRRS was used to capture information about participants' experiences of 40 different events which could have caused higher levels of stress in the previous 12 months. Firstly, a basic comparison between the exposed and control groups was made using the total score from each group (**Figure 68**).

Overall, agricultural workers reported more stress-inducing events in the previous 12 months than construction workers ($t(259) = -2.53$, $p < 0.05$. 95% CI [-109.99, -13.76]).

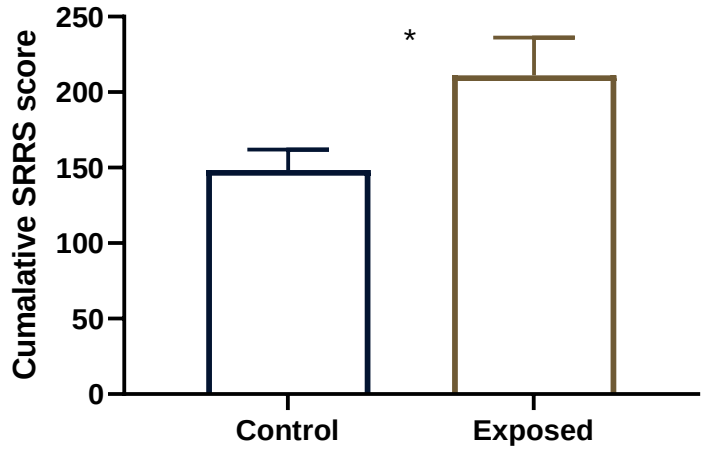

Figure 68. Agricultural workers reported a higher number of stress-inducing events compared to construction workers. The impact of major stressful events was measured using the Social Readjustment Ratings Scale (Holmes and Rahe, 1967). The scores obtained were significantly higher in the exposed group (n = 166) compared to the control group (n = 95). The bars in the figure represent the mean scores, with error bars indicating the 95% confidence intervals. Statistical significance is denoted by * ($p < 0.05$).

6.4.5.4. Ongoing daily stressors measured using the Daily Hassles scale.

In addition to the stressful events explored above, stress can also be caused by factors which may exist in the absence of individual, identifiable or recent events. To account for this, participants were asked to report whether and to what extent they had been affected by 46 of such items on the Daily Hassles scale (Kanner *et al.*, 1981).

Scores from the Daily Hassles scale suggested that agricultural workers also suffer more stress from day-to-day issues (**Figure 69**). Overall Daily Hassles scale scores were significantly different between groups ($t(259) = -2.96$, $p < 0.01$. 95% CI [-11.89, -2.39]).

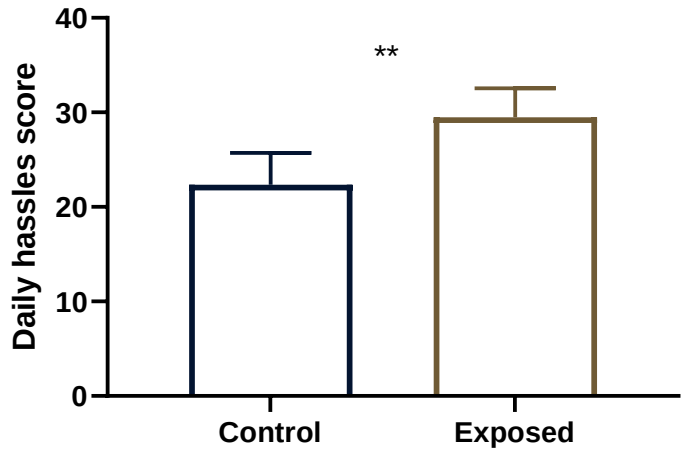

Figure 69. Ongoing daily stressors were found to be more prevalent among agricultural workers exposed to OP compounds.The impact of day-to-day stressors was assessed using the Daily Hassles scale (Kanner et al., 1981). The scores obtained were significantly higher in the exposed group (n = 166) compared to the control group (n = 95). The bars in the figure represent the mean scores, with error bars indicating the 95% confidence intervals. Statistical significance is denoted by ** ($p < 0.01$).

6.4.5.5. Day-to-day stress factors

The individual items listed in the Daily Hassles scale could be perceived as following eight main life aspects as potential sources of stress. More specifically, the questions address issues relating to social, family, domestic and health issues, as well as work, financial, legal, and environmental concerns. Based on this assessment an exploratory factor analysis was performed using the Daily Hassles results and the results distilled into eight factors. The results of the factor analyses using the Daily Hassles scores are shown in **Table 17.** A regression factor score for each was extracted and used to compare differences between groups for each resulting factor. Factor extraction was performed using orthogonal (varimax) rotation. Items with a correlation coefficient greater than .3 were included in each factor.

6.4.5.6. Work-related stressors: factor 1

Exploratory factor analysis revealed a coherent theme of work-related issues as the most prominent factor (**Table 17**: factor 1). The impact of factor 1 as a work-related construct appeared to affect agricultural workers and construction workers to a similar extent ($t(259) = -0.555$, *ns*. 95% CI [-0.298, 0.167]).

6.4.5.7. Financial issues: factor 2

The second factor extracted identified a group of items relating to financial and economic issues (**Table 17;** factor 2), and also 'parents or in-laws' which could represent some level of financial dependency or responsibility within the familial relationship. The regression factor scores for this finance-related construct suggested that these issues were also important to a similar extent for both groups ($t(223.39) = -1.237$, *ns*. 95% CI [-0.346, 0.079]).

6.4.5.8. Isolation and social interaction: factor 3

The third factor extracted was made up of items relating to geographic or social isolation, and other social factors (**Table 17**: factor 3). Taken as a combined construct factor 3 did not differ significantly between the exposed and control group ($t(259) = -0.941$, *ns*. 95% CI [-0.325, 0.115]). However, since loneliness is a known issue in rural and farming communities the specific items from factor 3 were investigated further (**Figure 70**). To control the false discovery rate multiple t-tests were performed using the two-stage linear step-up procedure (Benjamini, Krieger and Yekutieli, 2006) with individual items from Factor 3 (**Table 17**) as dependent variables. This approach revealed only 'feeling lonely' to be significantly different, with loneliness being higher in agricultural workers ($t(259) = 3.281$, FDR adjusted p (Q) = 0.007).

Table 17. Factors extracted using Daily Hassles scores.

Factor	Components
Daily Hassles factor 1	• The type of work you do • The amount of work you do • Your supervisor or employer • Customers or clients • Your co-workers • Deadlines or goals at work • How much free time you have, or lack of • Job security
Daily Hassles factor 2	• Parents or in-laws • Having money for socialising, holidays, or hobbies • Having money for the future (pensions or emergencies) • Vehicle maintenance • Saving water, gas, electricity, or fuel • Financial investments • Paying money for/to someone who does not live with you
Daily Hassles factor 3	• Feeling isolated/far away from things or people • Not socialising • Feeling lonely • Own physical appearance • Relationships with other relatives • Socialising • Friendships
Daily Hassles factor 4	• Personal health • Own physical abilities • Personal medical care
Daily Hassles factor 5	• Your children • Housework • Building repairs/DIY • Health or wellbeing of family members • Spouse or partner
Daily Hassles factor 6	• Politics • Things you hear in the news • Where you live • Neighbours • Your environment (clean air, noise, greenery) • Sexual relationships
Daily Hassles factor 7	• Mood altering drugs • Legal matters • Family commitments • Time spent with family • Other relatives • Paperwork
Daily Hassles factor 8	• Your drinking • Your smoking

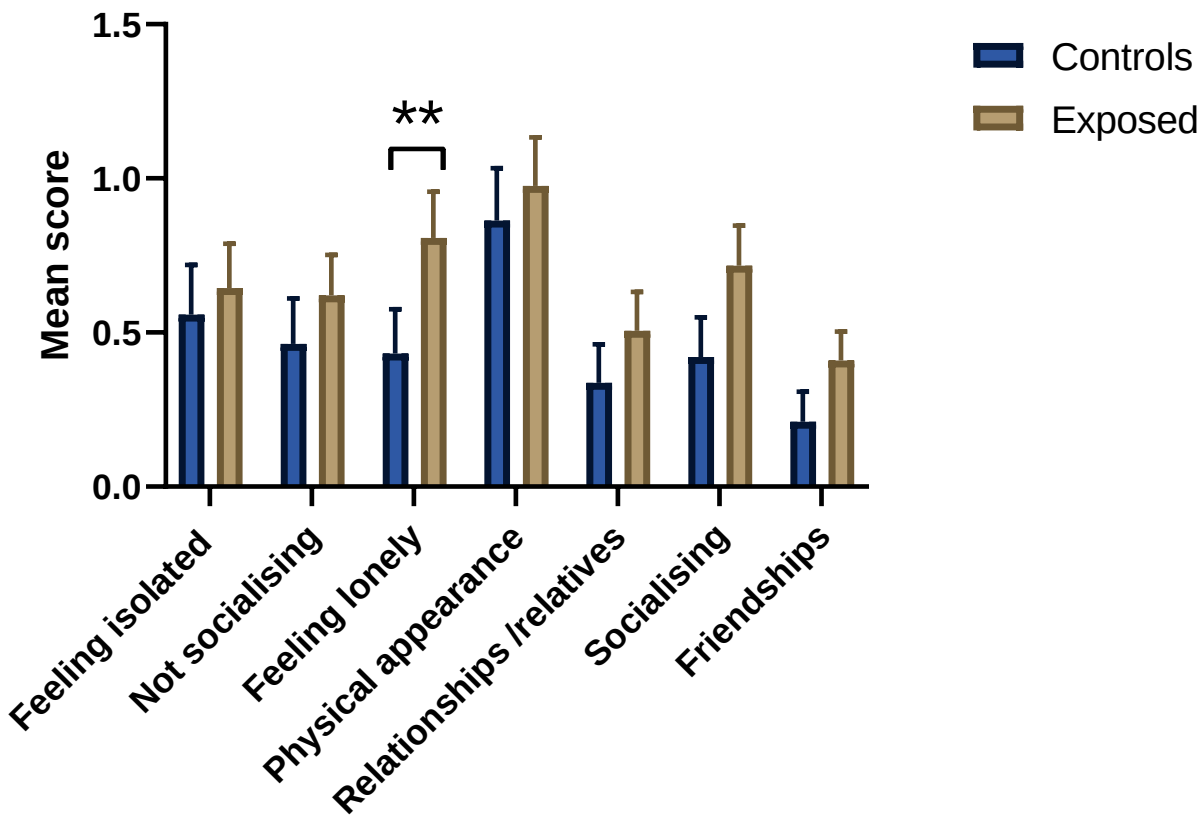

Figure 70. OP-exposed agricultural workers report more feelings of loneliness than construction workers. Participants rated how much stress was caused by several social-related environmental stressors: 0 (none), 1 (a little stress), 2 (quite a bit of stress) or 3 (very much stress). The exposed group (n = 166) scored significantly higher for feeling lonely than the control group (n = 95). The bars in the figure represent the mean scores, with error bars indicating the 95% confidence intervals. Statistical significance is denoted by ** ($p < 0.01$).

6.4.5.9. Physical wellbeing: factor 4

The fourth factor was the only full factor which differed significantly, with the exposed cohort suffering worse than the control group ($t(253.512) = -4.33$, $p < 0.001$. 95% CI [-

0.623, -0.233]). Factor 4 comprised only 3 items: 'personal health', 'own physical abilities' and 'personal medical care', which all centred around individual physical wellbeing (**Table 17**).

6.4.5.10. Home life/domestic responsibilities: factor 5

The fifth factor seemed to involve items relating to immediate family such as spouse and children, and domestic responsibilities such as home repairs and housework (**Table 17**). This factor did not differ significantly between the two groups ($t(259) = -0.181$, *ns*. 95% CI [-0.235, 0.195]).

6.4.5.11. Environmental factors outside the home: factor 6

Items in factor 6 shared a theme relating to the local or wider environment outside the home. These include current affairs, environmental issues, the locality where participants lived and their neighbours (**Table 17**). Sexual relationships were also included in factor 6. Together, factor 6 was not significantly different between the two groups ($t(259) = -1.030$, *ns.* 95% CI [-0.316, 0.099]).

6.4.5.12. Miscellaneous: Factor 7

The seventh factor followed a less distinct theme, including a collection of family related issues, but also: 'mood altering drugs', legal issues, and 'paperwork' (**Table 17**). This factor was not significantly different between the exposed and control groups ($t(259) = -1.222$, *ns.* 95% CI [-0.345, 0.081]).

6.4.5.13. Drinking and smoking: factor 8

The last factor containing only smoking and drinking which, in agreement with demographic information collected in the survey, was also not significantly different between the two groups ($t(259) = 1.155$, *ns*. 95% CI [-0.09, 0.348]).

6.4.6. Within group analysis of factors affecting mood

Comparison between agricultural and construction workers showed that they were mostly well matched on basic demographic and lifestyle criteria but seemed to experience some differences in life-stress and physical health issues. The difference in DASS-21 stress scores between groups was likely a direct result of the different stressors they each experienced, however, although anxiety scores were not different, depression scores did differ significantly. It is still unclear whether these differences could be responsible for higher levels of depression in agricultural workers, or whether occupational exposure to OPs is more likely to be responsible. Testing for differences between the groups revealed several factors that could have influenced the observed difference in depression scores. To investigate this further those factors were used to explore relationships within the exposed cohort alone with the addition of variables related to OP exposure.

6.4.6.1. Basic relationships between factors

To check for any unexpected relationships within the data, correlation matrices were created using Spearman's rank correlations for selected variables of interest. All matrices included DASS-21 scores for mood and stress, along with measures relating to OP exposure. Different aspects of health and lifestyle were added to provide information on which variables might warrant further analysis. Likewise, since the reliability of OP exposure metrics is unknown, a range of measures were added, including total lifetime exposure in hours, days, and years, as well as the number of years elapsed since participants had been exposed. Frequency of personal protective equipment (PPE) use was also included.

6.4.6.2. General health, age, and stress

Firstly, general health and lifestyle items were added, along with aggregate scores for stressors and health symptoms (**Figure 71**). Unsurprisingly, mood and stress scores were strongly positively correlated with stressors, as shown by aggregate Daily Hassles and SRRS scores. Mood and stress were also positively correlated with overall physical symptom and bodily pain scores, and negatively related to both general health and the amount of sleep participants reported having each night.

Age was associated with lower general health (r_s = -.20, 95% CI [-.35, -.041], p < .05, N 160) and increased bodily pain (r_s = .2, 95% CI [.041, .35], p < .05, N = 160) but not physical symptom score (r_s = .028, 95% CI [-.13, .19], p = .72, N = 160). Interestingly, increased age was associated with lower scores for depression (r_s = -.232, 95% CI [-.38, -.075], p < .01, N = 160) and stress (r_s = -.211, 95% CI [-.36, -.053], p < .01, N = 160). That stress scores were lower with increased age is likely linked to the fact that Daily Hassles scores (r_s = -.284, 95% CI [-.42, -.13], p < .001, N = 160) and SRRS/major stressful events scores (r_s = -.375, 95% CI [-.50, -.23], p < .00001, N = 160) were also negatively correlated with age.

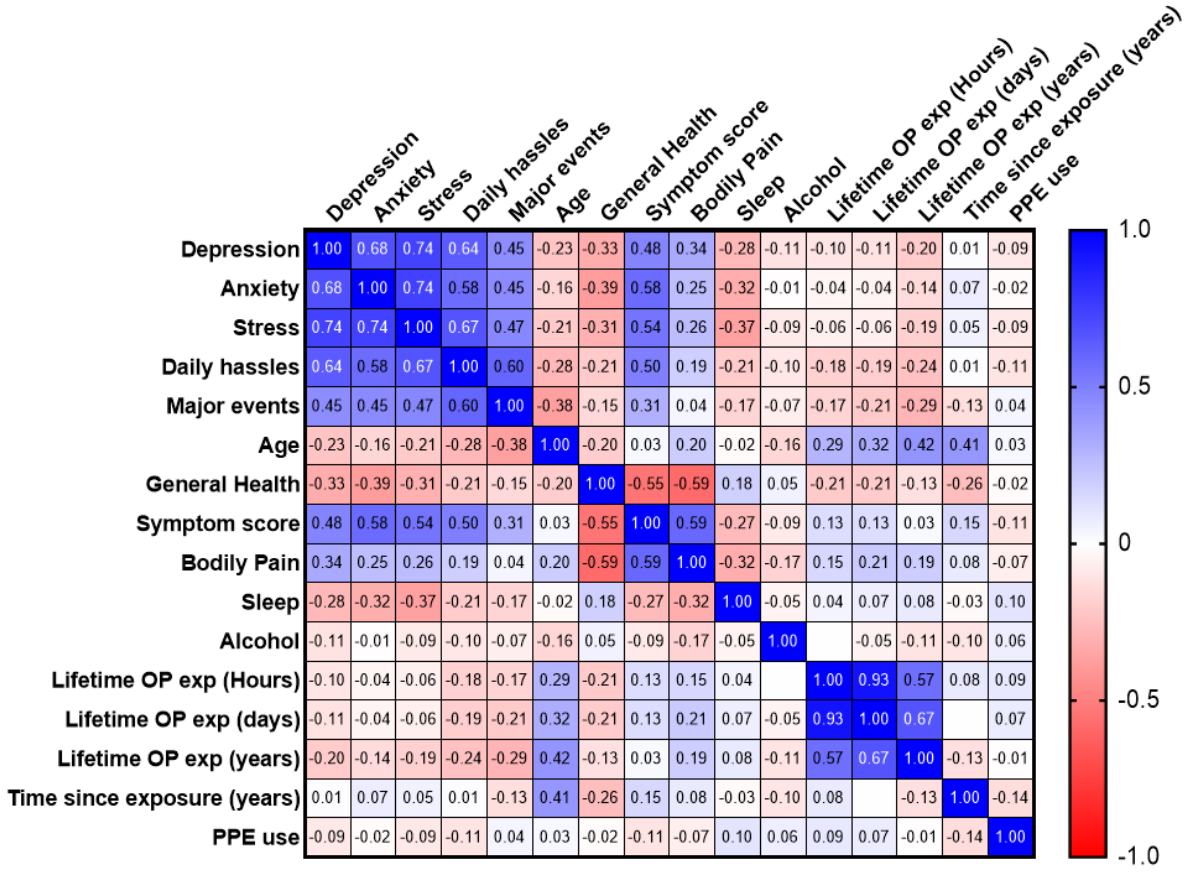

Figure 71. Correlation matrix showing relationships between a general set of variables in the exposed group using Spearman's rank correlation. Spearman r values are shown to 2 decimal places within each cell. Blue shading denotes a positive relationship and red denotes a negative relationship as shown on the scale to the right.

Importantly, lifetime OP exposure was not significantly correlated with mood or stress scores when measured in hours or days. However, the overall trend between OP exposure and DASS-21 scores appeared negative (**Figure 71**), and when measured in years the negative relationship was significant with both depression (r_s = -.205, 95% CI [-.35, -.050], p < .01, N = 166) and stress (r_s = -.185, 95% CI [-.33, -.029], p < .05, N = 166). This is directly the reverse to what would be expected if OPs were the cause. The overall pattern of relationships with each of the other factors in the matrix also appeared very similar

between age and depression (**Figure 71**). This relationship therefore needed to be explored further.

6.4.7. Mediation analyses

6.4.7.1. The negative relationship between depression and years of OP exposure is mainly mediated by age.

Lifetime OP exposure was of course related to age, and so it was possible that the observed relationship between years of pesticide exposure and depression was caused by participants' age rather than exposure to specific chemicals. To test this theory a simple mediation analysis was performed using the PROCESS tool in SPSS (model 4). Log transformed DASS-21 depression score was used as the outcome variable. The predictor variable was 'lifetime exposure (years)' and age was added as the mediator. This model confirmed that years of OP exposure was strongly predicted by age, and that the total effect of the model was mediated by the indirect effects of age (**Figure 72**). There was no direct effect of OP exposure on depression in this model.

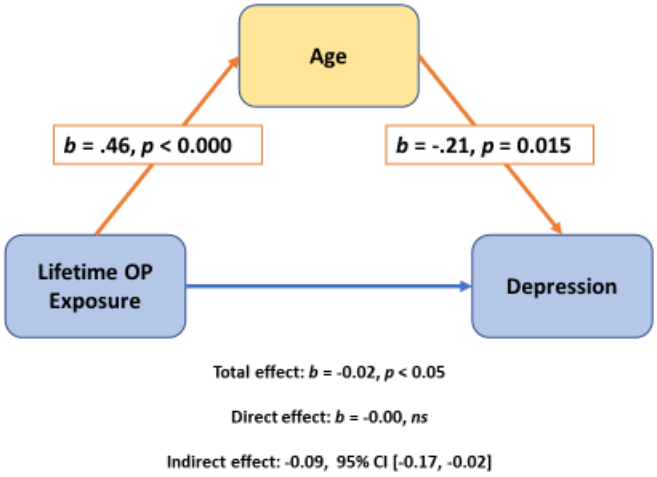

Figure 72. OP exposure negatively predicts depression scores, but the effect is mediated by age. Although DASS-21 depression scores seemed to be significantly reduced along with lifetime OP use, there was no direct relationship between exposure and depression in this model. Rather, lifetime exposure was positively associated with increased age, and increased age negatively predicted depression scores in this group. Therefore, the overall effect of this model was mediated by age. Beta values are standardised coefficients and the confidence interval for the indirect effect is a BCa bootstrapped CI based on 5000 samples. Ns = not significant.

6.4.7.2. Relationships between Daily Hassles factors, age, and depression

Scores from the Daily Hassles and major events (SRRS) scales were closely related to mood and stress scores, but OP exposure was not (**Figure 71**). However, since the scores for both stressor scales were aggregate scores comprising potentially different combinations of items, the individual components of these scales warranted further

investigation. In section 6.4.5.5 exploratory factor analysis was used to extract 8 separate factors from the results of the Daily Hassles scale (**Table 17**). The factors from the Daily Hassles scale were added to a correlation matrix alongside depression score, age, and OP exposure measurements (**Figure 73.**)

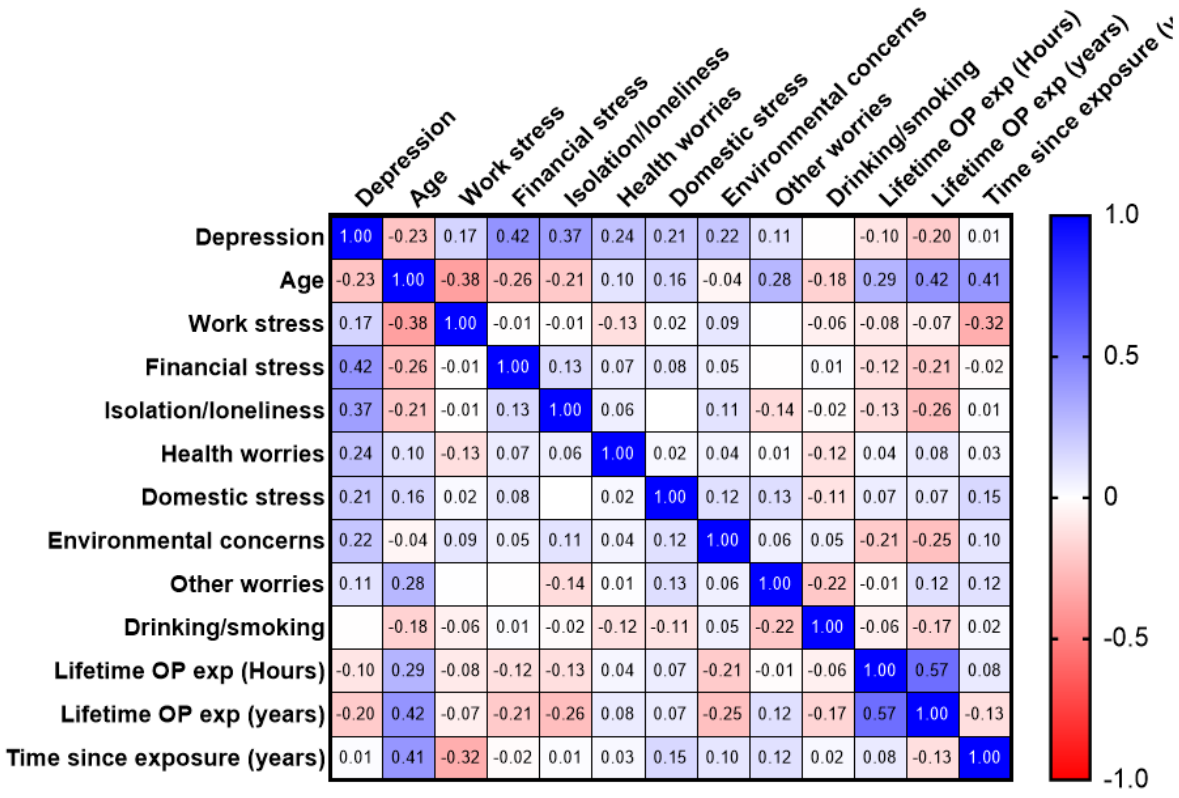

Figure 73. A matrix showing chronic stress related factors and their correlation with OP exposure. Blue shading represents a positive relationship. Red shading represents a negative relationship. Cell values represent the correlation coefficient. All stress-related factors were positively correlated with depression score. Age was negatively correlated with depression score, and also with a subset of stress factors, including work stress, financial stress, and isolation/loneliness.

Adding the Daily Hassles factors to the correlation matrix (**Figure 73**) illustrates that some specific stress factors appeared to correlate negatively with age, but positively with DASS-21 depression scores. In particular, financial-related problems (r_s = -.256, 95% CI [-.40, -.10], p = 0.001, N = 166), and social isolation and loneliness (r_s = -.213, 95% CI [-.36, -.05], p = .007, N = 166) were negatively correlated with age, but positively with depression (financial stress: r_s = .420, 95% CI [.28, .54], p <.001, N = 166) and (social isolation and loneliness: r_s = .347, , 95% CI [.23, .50], p < .001, N = 166). The most noticeable relationship with increased age was a reduction in work-related stresses (r_s = -.375, 95% CI -.50, -0.23], p <.001, N = 166), albeit with a smaller but still significant positive correlation with depression (r_s = .167, 95% CI [.010, .32], p < .05, N = 166). Worries related to drinking and smoking also decreased with age (r_s = -.177, 95% CI [-0.33, -0.018], p < .05, N = 166), but were not related to depression scores in this group.

The remainder of Daily Hassles factors had positive relationships with both age and depression scores. The strongest of which for age was the factor termed 'other worries' (factor 7 **Table 17**), which included concerns about legal matters, family commitments, and paperwork (r_s = 0.279, , 95% CI [0.12, 0.42], p < .001, N = 166), however the relationship with depression was not significant (r_s = 0.107, 95% CI [-0.051 to 0.26], *ns*, N = 166). The impact of domestic stressors increased slightly with age (r_s = 0.156, 95% CI -0.0035, 0.31], p < .05, N = 166) and were more strongly associated with depression scores (r_s = 0.210, 95% CI [0.055 to 0.35], p < .01, N = 166). Interestingly, although health worries were related to depression scores (r_s = 0.242, 95% CI [0.088 to 0.38], p < .001, N = 166) they did not increase significantly with age in this cohort (r_s = 0.104, 95% CI [-0.057 to 0.26], *ns*, N = 166).

The changes in day-to-day stressors related to age were likely to be associated with the age-mediated effect on depression observed in this cohort (**Figure 72**), which in turn could help to separate any effect of OP exposure. Therefore, the relationship between age and depression in this group was explored further.

6.4.7.3. Age-associated relief from social isolation, work and financial worries drives lower depression scores in agricultural workers.

Associations between aging and reduced depression risk have been the topic of much debate (Jorm, 2000), and the relationship observed here was unexpected. However, correlations observed between day-to-day stress factors produced in the factor analysis (6.4.5.5), indicated a change in specific types of stressors with increased age.

Based on those observations it was hypothesised that financial stress becomes less burdensome in agricultural workers as they become more financially secure with age. Also, that work stress is reduced as individuals become more settled, or senior in their role. It was also predicted that effects of social isolation and loneliness would decrease as family and social networks are built, and requirements for social interactions change with age (Blakemore and Mills, 2014).

To test this, a mediation model was created using the PROCESS tool (model 4) in SPSS (**Figure 74**). Age was added as the independent variable, with log transformed DASS-21 depression scores as the outcome variable. From the factors extracted from the Daily Hassles scale, three were correlated both negatively with age, and positively with depression, factor 1: work related stressors, factor 2: financial worries, and factor 3: social isolation and loneliness. These three factors were added to the model as potential mediators and standardised coefficients were produced for 5000 bootstrapped samples.

The null hypothesis was rejected, because increased age significantly predicted a reduction in each of the three stress factors (work stress: -.381, 95% BCa CI [-.309, -.139]; financial worries: -.249, 95% BCa CI [-.248, -.060], and social isolation: -.239, 95% BCa CI [-.240, -.053]) (**Figure 74**). Each of the three stress factors were shown to increase depression scores (work stress: .171, 95% BCa CI [.029, .341]; financial worries: .349, 95% BCa CI [.219, .500], and social isolation: .374, 95% BCa CI [.247, .529). Taking all of these factors into account, there was no direct effect of age on depression score, but a total indirect reduction in depression score of 24% by age, mediated by reductions in work stress (-.065, 95% BCa CI [-.127, -.009]), financial worries (-.087, 95% BCa CI [-.141, -.035]), and social isolation and loneliness (-.089, 95% BCa CI [-.149, -.031]) (**Figure 74**).

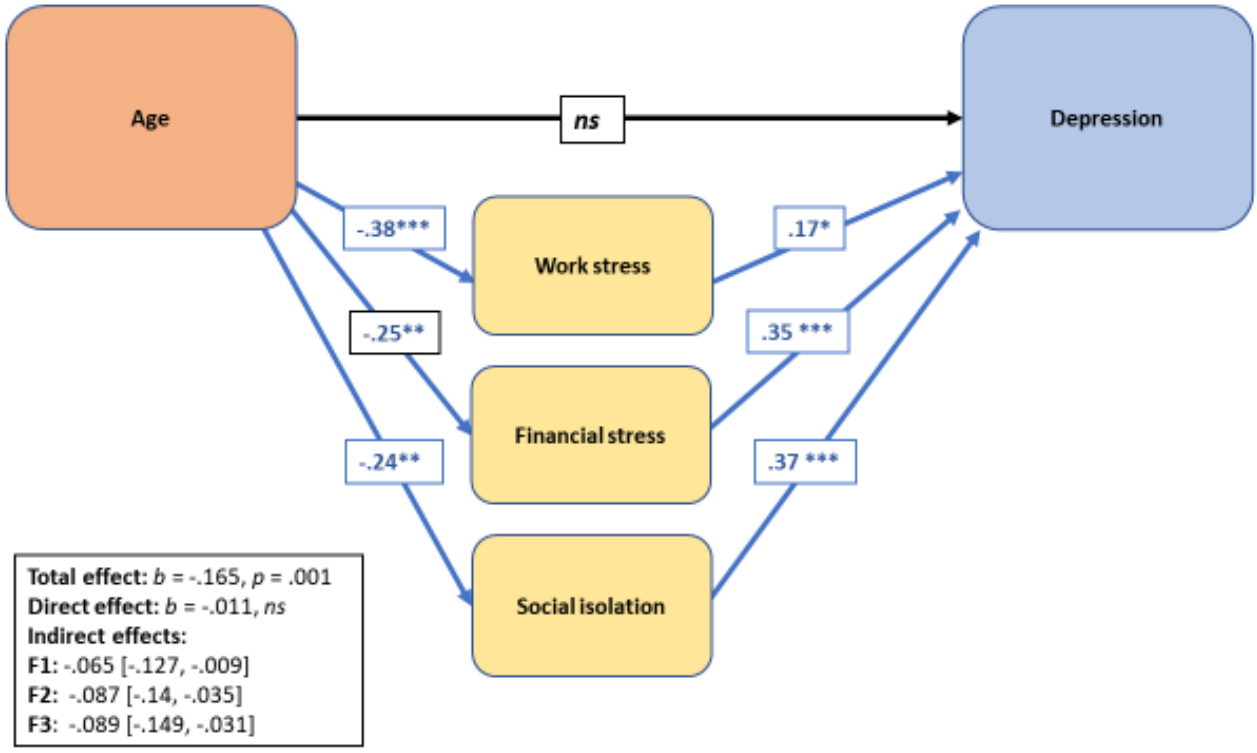

Figure 74. Increased age is associated with reduced stress relating to work, finances, or social isolation, which indirectly impacts depression scores. Mediation analysis shows that age did not affect depression scores directly in this group. However, work stress, financial stress, and social isolation are each reduced with increased age. The decrease in these stress factors predicted lower depression scores. It could be possible that this indirect effect of age on depression scores, mediated by reduced stress, could be a confounding factor in the effects of lifetime OP exposure (Hayes and Rockwood, 2017). *** = $p < 0.001$, ** = $p < 0.01$, * = $P < 0.05$, ns = not significant.

6.4.7.4. Depression symptoms are inconsistently mediated by a single physical symptom factor.

After clarifying the indirect relationship between age-linked factors and depression, no direct relationship between depression and OP exposure had yet been identified. It seemed unlikely that OP exposure directly caused a reduction in depression symptoms, because most previous studies looking at OP exposure in humans have shown either an increase in evidence for depression in exposed groups, or no relationship at all. However, some rodent studies have reported decreased anxiety-like behaviour following OP treatment (Savy *et al.*, 2015), and so the possibility of relief from mental health related symptoms may be possible.

To test for interactions between physical health, pesticide exposure, and depression, Mediation analysis was performed using PROCESS (model 4). Log transformed DASS-21 depression score was used as the outcome variable. The predictor variable was 'lifetime exposure (years)', and all five physical Symptom Factors were tested as possible mediators. Since we had established that the effect of years OP exposure was largely a function of age-related factors, the age variable was added as a covariate to control for its effect.

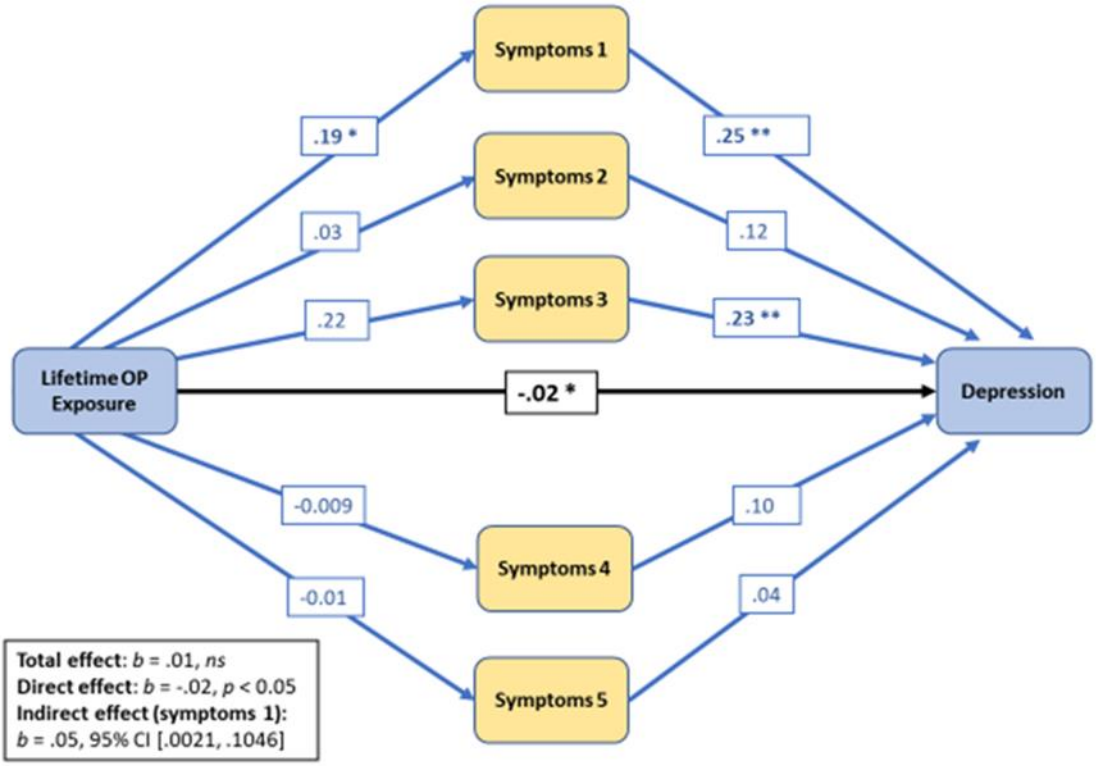

Figure 75 Lifetime OP exposure predicts an increase in neuromuscular-related health symptoms that predict higher depression scores in UK agricultural workers. This mediation model does not show a net overall effect of Lifetime OP Exposure on Depression (inset); Greater lifetime exposure predicted lower depression scores (-0.02*; black arrow). Conversely, symptom factor 1 and factor 3 each predicted an increase in depression score (0.25** and 0.23 **, respectively). These opposing relationships result in a 'suppressor effect', in which the effect of physical symptoms causes the effect of lifetime exposure to be no longer significant, and vice-versa (Hayes and Rockwood, 2017). Symptom factor 1 was positively associated with lifetime exposure (0.19*) and depression (0.25**) suggesting an increase in depression scores was indirectly mediated by neuromuscular symptoms in factor 1. ** = p < 0.01, * = P < 0.05, ns = not significant.

The model confirmed that OP exposure (years) significantly predicted symptom factor 1, which includes a collection of neuromuscular-related items, such as joint stiffness and

muscular pain (b = 0.19, p = 0.034, 95% BCa CI [.0009, .027]). This was true even when considering the effects of age as a covariate (b = 0.11, p = 0.156, 95% BCa CI [0.001, 0.027]). None of the other symptom factors were predicted by OP exposure in this model when controlling for age (**Figure 75**). Depression was positively predicted by both symptom factor 1 (b = 0.25, p = 0.002, 95% BCa CI [0.103, 0.446]), and symptom factor 3 (b = 0.23, p = 0.002, 95% BCa CI [0.097, 0.428]). However, although lifetime OP exposure was associated with increased muscular symptoms, the positive-directional effect of physical symptoms on depression was in opposition to the negative-directional effect of years of OP exposure, which was shown earlier in this chapter to be driven by age-related lifestyle factors (b = -0.211, p = 0.01, 95% BCa CI [-0.237, -0.032]). Such opposing relationships are known as suppressor effects, in which one factor reduces the strength of another factor, rather than showing a net effect of its own (Hayes and Rockwood, 2017). In this case, the suppressor effect was caused by a significantly-positive effect on depression mediated by Symptom Factor 1 (indirect effect: b = 0.046, 95% BCa CI [0.002, 0.106]), being suppressed by the apparent negative effect of lifetime exposure on depression.

These results describe an important route for depression in agricultural workers, in which time spent working with pesticides is associated with an increased risk of developing physical symptoms which in turn increases the risk of depression (**Figure 75**). Symptom factor 1 is the most strongly correlated set of symptoms with lifetime exposure (**Figure 76**) and is predicted by the number of years a person has spent working with pesticides (**Figure 75**). That specific set of symptoms is also the strongest predictors of depression in the model tested (**Figure 75**).

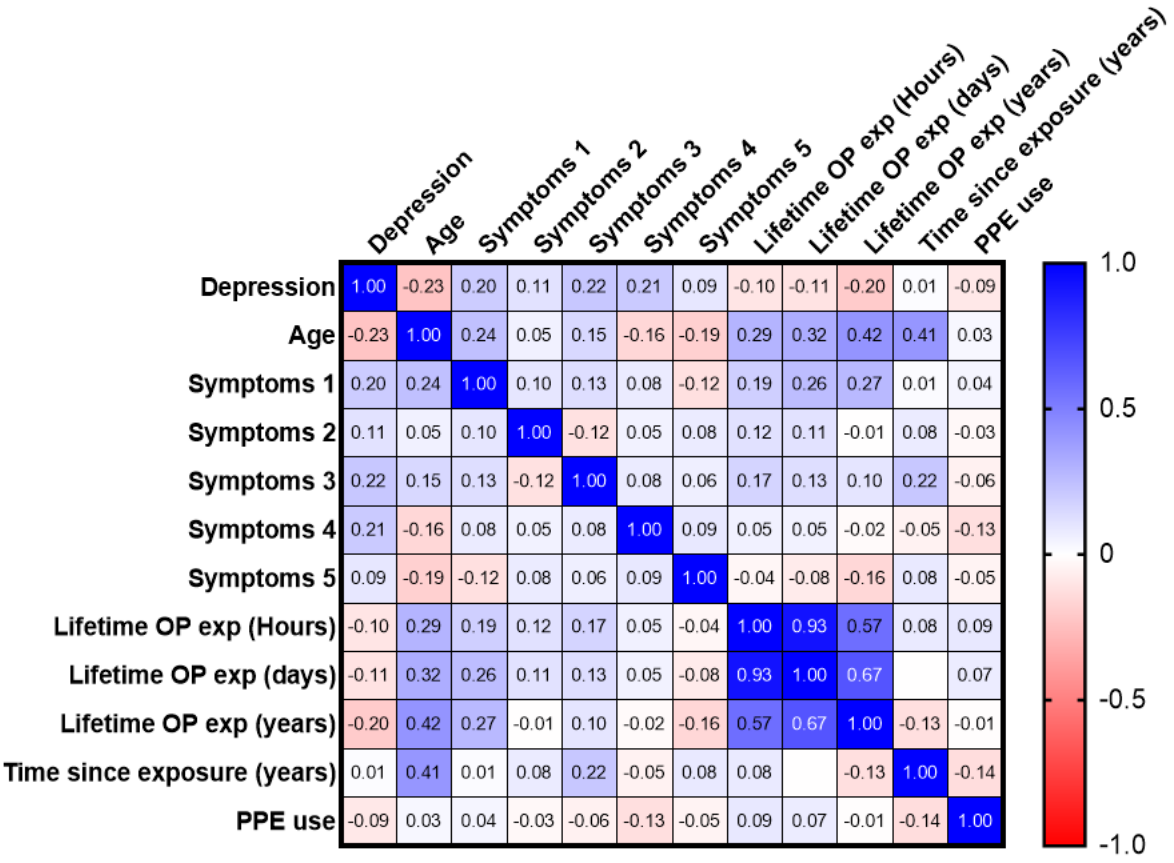

Figure 76. A correlation matrix shows relationships between exposed and control groups of physical symptoms, depression, age, and exposure metrics. Symptoms 1: joint stiffness/pain, muscular pain/cramps, Numbness or tingling, muscle weakness, fatigue. Symptoms 2: skin problems, chemical intolerance, hay fever/allergies, chest pain/shortness of breath, thermoregulation/sweating, urinary problems. Symptoms 3: poor memory, poor coordination, tremors/twitches, blurred vision. Symptoms 4: headaches, nausea, indigestion, constipation. Symptoms 5: unintended weight-loss or gain, toothache. Blue shading represents a positive relationship. Red shading represents a negative relationship. Cell values represent the correlation coefficient.

It can therefore be concluded from this model, that increased time spent working in roles involving the use of pesticides can increase the risk of depressive symptoms via specific

physical health problems. However, using such a simple exposure metric does not allow us to conclude that exposure to OP compounds is the causal factor.

6.5. Discussion

6.5.1. Study objectives

This study aimed to investigate the prevalence of symptoms of common mental disorders among agricultural workers with occupational exposure to organophosphates (OPs). The study also compared the data from the exposed group to a matched control cohort with no known exposure to OPs. We sought to assess factors known to affect mental health independently of pesticide exposure to control for confounding effects and identify specific factors contributing to poor mental health in this group. In addition, the study aimed to explore the possible relationships between OP exposure, mental health, lifetime pesticide exposure, and confounding lifestyle factors specific to working in agriculture. The overall goal was to determine whether a relationship exists between OP exposure and symptoms of common mental disorders, either as a direct result of exposure or mediated by other related outcomes such as poor physical health or environmental stress.

6.5.2. Evidence for depression and anxiety between groups

These findings indicated that symptoms of depression were more frequently experienced by exposed agricultural workers compared to controls, which aligns with previous research reporting associations between exposure and depression in farming cohorts (Beseler et al., 2006; Beseler and Stallones, 2008; Beard et al., 2014). However, in contrast to at least one other study (Harrison and Mackenzie Ross, 2016), anxiety symptoms were not significantly different between the exposed and control groups in this study. The discrepancy could be attributed to the different scales used and the more specific inclusion of sheep farmers in that previous study. It is possible that specific factors related to sheep farming contribute to the risk of experiencing anxiety. This could relate

to type of chemicals sheep farmers are exposed to, or something else entirely. Additionally, the control group in this study (construction workers) might have been better matched to the exposed group than the rural police used as controls in the previous study. However, the incidence of anxiety in the police control group was relatively common, making it unlikely that their lower anxiety rates could explain the higher incidence observed in sheep farmers.

Depression scores were higher in the group of agricultural workers compared to controls, consistent with other studies (1.5.2). This study aimed to explore the factors contributing to these scores in this population.

6.5.3. Acute and chronic stress

One notable finding of this study was the importance of daily hassles or chronic stress in relation to depression scores. Daily hassles and major events ratings were highly correlated with depression scores and self-reported stress scores, while OP exposure did not show a significant correlation. This suggests that lifestyle factors and stress experienced by farmers may play a significant role in reporting poor mental health in this group. This finding provides an alternative explanation for the existence of poor mental health, beyond pesticide exposure.

We hypothesised that day-to-day stress would be as influential as acutely stressful major events on mental health in these groups, and the results confirmed this hypothesis. Financial stress and loneliness were particularly important contributors, with the effects diminishing with age. Younger farmers may be more vulnerable to loneliness and financial difficulties, which could become less common as they develop their careers, achieve financial stability, or establish social connections. It is worth noting that younger

adults are generally more susceptible to loneliness compared to older adults (Beam and Kim, 2020).

Another study by Povey et al. (2014) found that farmers who had carried out lambing were less likely to screen positively for depression. This suggests that certain farming activities might have a beneficial effect in reducing depression. However, this contradicts the finding that a higher number of years working with sheep was associated with higher rates of depression. Further investigation is needed to understand these relationships fully (Povey et al. 2014).

6.5.4. Physical health

General health was reported to be better in construction workers compared to agricultural workers, both in terms of subjective scoring and overall symptom frequency. Previous studies have associated self-reported poor health with depression in farm residents (Stallones and Beseler, 2002). The relationship between physical health and depression is well-established (NICE, 2010; Ohrnberger, Fichera and Sutton, 2017), and the data from this study also demonstrated a strong association between physical health and increased reports of depression symptoms in the agricultural workers. However, it remains unclear whether OP exposure contributes to the health issues leading to depression, and this could not be fully investigated through comparisons with the control group.

The relationship between physical health and depression scores within the exposed group in this study interestingly seemed to be driven largely by a single set of symptoms. Factor 1 contained a set of neuromuscular-related symptoms, such as joint stiffness and muscle discomfort. It is possible, but difficult to prove that these could be a consequence

of some pesticide exposure, whether to OPs or otherwise. This suggestion is interesting, because links between low-level pesticide exposure and depression are generally thought to be associated with altered neurotransmission. It is established that severe acute OP poisoning can lead to longer-term poor physical health (Müller-Vahl, Kolbe and Dengler, 1999; Kazi and Oommen, 2012). The risk that poor mental health may accompany poor physical health in such circumstances seems obvious, however similar explanations have not been as often suggested for low-level exposure, presumably because physical symptoms caused by pesticide exposure would be considered to be evidence of a history of poisoning, and therefore lead to exclusion of the individual from a study. Some possible explanations for this may be important to consider. Firstly, the reported physical symptoms could be unrelated to pesticide exposure, perhaps relating to some other aspect of farming life, or unrelated to farming entirely. Alternatively, the physical health symptoms may be related to undetected, or unreported acute poisoning incidents, in which case they should be discounted from the effect of low-level exposure. Another possibility, however, is that the physical symptoms are linked to low-level exposure, perhaps as a consequence of longer-term exposure. Any of these suggestions would fit the results of our mediation analysis, in which this particular set of symptoms seemed to contribute to higher depression scores through inconsistent mediation (MacKinnon, 2011).

6.6. Study limitations

6.6.1. The sample

Lifestyle factors such as smoking, drinking, and sleeping habits are associated with mood (Riemann *et al.*, 2020), and so matching these factors was important. Similarly, education level, which is a known protective factor against depression, was also well matched. Age was not significantly different between groups. This was important since age had an unexpectedly important effect on the relationships found between lifetime exposure, chronic stress, and symptoms of depression in agricultural workers.

The control group was generally well matched, apart from gender, for which there was a significant male bias in construction workers. There were also only three non-binary respondents, all of which were in the exposed group. Symptoms of depression and anxiety have been shown to present more frequently in gender minority groups than in cisgender adults, and more frequently in females than males (Reisner *et al.*, 2016). This is likely partly linked to social factors and societal pressures, however recent work suggests AChE inhibition may be more strongly associated with depression in females than in males (Suarez-Lopez *et al.*, 2021).

Some limitations may have been imposed by the quality of the sources from which participants were recruited. Firstly, some participants were recruited through the Sheep Dip Sufferers Association. Many of the members of this group have suffered acute poisoning and may be involved in litigation regarding injury or health issues alleged to be caused by OP exposure. It was felt that excluding this group on the basis of membership alone could have biased the sample by excluding individuals unfairly. Therefore, the invitation to participate was extended to the group and individuals were

assessed based on their reported exposure and any poisoning history before being included or excluded. The possibility that some conflict of interest might lead to some response bias is acknowledged.

Some participants were sourced from an online participant sourcing platform. Such platforms pay a small financial reward for completing surveys online, and despite using stringent selection criteria, there remains a possibility that not all respondents are honest or genuine. To mitigate this, each response was screened for unrealistic completion times, or nonsensical answers. The addition of free text responses was also intended to prevent users from quickly clicking through check boxes to earn financial reward. These measures reduce the relative value of the reward for non-genuine respondents, however the possibility that not all responses were genuine cannot be entirely prevented. Therefore, future work should consider the suitability of using such platforms.

6.6.2. Mood/Self-report

This study used self-report scales, which are subject to bias, and participants were not asked whether they had ever been diagnosed with depression or anxiety. Future work could include more objective measures, such as structured clinical interviews, as performed by Harrison and Mackenzie Ross (2016). More detailed information about participants' psychological history could also be collected, to explore differences between persistent, recurring and incident anxiety.

6.6.3. Measuring exposure

The effective measurement of OP exposure is a limitation of most studies in this field. We collected several items of information, including the use of PPE, and the type of activities performed, with the intention of finding the best possible metric for exposure. However,

of all the measures recorded time spent working with pesticides was used as the main estimate of OP exposure. This is a relatively crude measure and may not closely represent biologically relevant exposure to OPs. The exposed group in this study was selected deliberately from agricultural workers with experience working with pesticides in the United Kingdom. This was intended to exclude potential confounding factors such as different workplace safety standards, availability of PPE or product training, or other cultural differences that may exist between different nations. Nevertheless, some participants in this study will have seen considerable changes in UK working practices during careers spanning several decades. It is therefore possible that for example, a modern professional pesticide applicator working in a highly controlled environment, would be exposed to a different amount of pesticide during an hour's work, than a casually employed farm hand dipping sheep in the 1970s, over the same unit of time. We asked participants to report on their use of protective equipment in an attempt to account for this, however the reliability of self-reporting on PPE use can be impaired by human memory, or conflict between mandatory workplace policy and actual cultural practice. In any case, the reported use of PPE in this study had no impact on any of the outcome variables tested. Likewise, we asked participants about the amount of time that had elapsed since they last worked with pesticides, and that also showed no discernible relationship with anything other than the participants' age, which was a common confound when using time to measure exposure.

It is clear from the literature that the lack of a reliable measure of human exposure is a common limitation in studies concerning the health effects of low-level OP exposure. The same was true here, as the reliability of our exposure metric is a major limitation of this study. The decision to use a basic measure of lifetime exposure was guided by concern

that arbitrary weightings given to multiple variables in a more complex exposure metric might produce unreliable, or even misleading calculations of exposure. A preferable alternative would be to use physical measurement of biological samples, such as AChE, BuChE, or urinary metabolites. However, even studies that have employed these measures have produced mixed results, with some showing no effect in exposed groups (Cole *et al.*, 1997; Berent *et al.*, 2014), but others reporting higher levels of anxiety and depression associated with recent, or higher levels of OP exposure (Salvi *et al.*, 2003; Mackenzie Ross *et al.*, 2010). Interestingly, Salvi and colleagues (2003) found that exposure to OPs was linked to mental disorder specifically when the exposure was recent. This could potentially explain some of the variation between conflicting study results, if like the results of this study, many participants have ceased working with pesticides. It might also offer some hope that any mental impairment that may have been caused by low-level OP exposure may be reversible after exposure has ceased. However, it should be noted that the collection of biological samples and their measurement can add their own limitations. At low levels, OPs are metabolised relatively quickly, and so samples must be collected fairly soon after exposure to produce meaningful results. Moreover, samples such as urine or serum need to be processed within hours for the most reliable results.

Another limitation that is shared between this and other studies, is the difficulty involved in separating exposure to OPs from that of other compounds. This is particularly true in agriculture, where workers may be involved with, or in proximity to the treatment of crops and livestock with various combinations of insecticides, nematicides, herbicides, fungicides, molluscicides, rodenticides, or other biocidal products, as well as fuels, solvents, cleaning products or other potentially harmful chemicals. Our results cannot

account for these factors, and it is likely that for some of these participants, our measure of lifetime exposure would apply to any or several of these listed substances, to a similar extent as it would to OP exposure. Nevertheless, this limitation could equally apply to studies that have measured AChE and BuChE activity or metabolites in biological samples, without testing for all possible chemicals at once. This would likely be prohibitive in terms of time and cost, and highlights the value of experimental laboratory studies, in which these variables can be controlled.

6.6.4. Physical symptoms

The physical symptom set used in this study was somewhat limited, and slightly biased toward symptoms that could be linked to cholinergic poisoning, although other symptoms (such as toothache) were included in an attempt to balance this. The intention was to avoid an overly lengthy survey in order to encourage better completion rates (Sahlqvist *et al.*, 2011). That decision remains valid, although the inclusion of a more comprehensive set of symptoms in chronically exposed individuals may have offered more insight into any physiological effects occurring below the threshold for significant AChE inhibition. On the other hand, such effects are difficult to detect, even in relatively simple experimental models, therefore maintaining an optimal response rate may be of more benefit unless a specific hypothesis is to be tested.

7. General discussion

7.1. General Overview

In this project, two primary aspects were investigated: the toxicodynamic impact of low-level organophosphate exposure using the model organism *C. elegans* to experimentally explore the molecular mechanisms or targets involved, and a survey-based investigation into the mental health outcomes in OP exposed UK agricultural workers. In this chapter, a summary of the main findings and limitations of both parts, and their implications are discussed, along with some suggestions for future work.

The primary objective of this project was to investigate the molecular targets of low-level Chlorpyrifos (CPF), exposure and to explore the possibility that low-level exposure to OP pesticides might worsen symptoms linked to common mental disorders. While the deleterious impacts of acute OP exposure on the AChE and the consequent increase in cholinergic signalling are well-documented (Jokanović, 2009; Slavica, Dubravko and Milan, 2018), a growing body of evidence suggests that even comparatively minimal exposure to OPs may impact human health. This happens without evidence of overt poisoning symptoms and potentially through a mechanism distinct from AChE inhibition. Notably, a relationship has been reported between deteriorating mental health in agricultural workers and occupational exposure to OP pesticides (Stallones and Beseler, 2016). However, existing research regarding low-level OP exposure in these groups has yielded inconsistent results, and no certain relationship with mental health or a biological justification for such an association, has yet been established.

To address this, this investigation adopted an interdisciplinary approach. Initially, we analysed the toxicodynamic impacts of low-level treatment with the OP chlorpyrifos on the nematode model organism *C. elegans.* Given that many essential genes, proteins, and signalling pathways are conserved between *C. elegans* and higher organisms (Hunt, 2017), this enabled us to explore the biological targets of low-level chlorpyrifos treatment and any mechanisms that could potentially influence psychiatric function in humans. We evaluated the behaviour of CPF-exposed worms in several assays, known to depend on potentially relevant neurotransmitter pathways. Then, we designed a custom assay with the aid of WormLab behavioural tracking software, to detect the subtle effects of exposure to 0.05 mg/L CPF, a dosage level that had so far not been associated with any behavioural impact in existing literature. Finally, genetic tools were used to investigate potential molecular targets of treatment with 0.05 mg/L CPF that mediate the observed behavioural effects.

The secondary component of our interdisciplinary investigation aimed to determine whether occupational exposure to OPs leads to an increase in symptoms of common mental disorders among a cohort of UK agricultural workers. We conducted a survey employing a series of validated questionnaires to evaluate participants' experience with OP pesticides, and their physical and mental well-being. For comparison, we included a group of UK construction workers with no known exposure to OPs, as a control group. This group was identified as a more suitable match to our exposed group than the control groups used in earlier studies. We attempted to address some shortcomings of earlier studies by recording participants' experiences of chronic life stress, a factor previously overlooked in OP research, using an adaptation of the Daily Hassles Scale (DeLongis et al., 1982). Previously, only acutely stressful life events had been considered, which were also

incorporated in this study (Holmes and Rahe, 1967). Additionally, we considered specific clusters of physical health symptoms, complementing the general health questions usually asked. Finally, we sought to untangle the significant factors contributing to poor mental health outcomes in UK agricultural workers by applying mediation analyses (Hayes and Rockwood, 2017) to examine potential causal relationships between these and other potentially relevant demographic and lifestyle factors.

7.2. Key findings from the experimental work using *C. elegans*

7.2.1. Impacts of chlorpyrifos exposure on behaviour at 0.05 mg/L and implications for future studies

One of the first and most important challenges in using *C. elegans* to investigate the effects of CPF treatment, was to find an appropriate concentration to represent low-level exposure. Generally, low-level exposure is considered as an exposure or dosage that is insufficient to cause overt toxicity. In the case of OP exposure, overt toxicity is mediated by the inhibition of AChE, and it has been suggested that the effects of low-level exposure on mental health could be mediated by mechanism other than AChE. Therefore, testing CPF-treated animals for effects on known neurotransmitter pathways at concentrations that do not inhibit AChE, was an early goal in the project.

Based on results of previous studies in the literature initial experiments were carried out using a concentration of 0.5 mg/L CPF, which had been described as a low-level exposure concentration for *C. elegans* using similar exposure methodology to those used in this study (Viñuela et al., 2010). However, our findings questioned the appropriateness of 0.5 mg/L CPF as an optimal treatment concentration to model low-level exposure in *C. elegans*. Behavioural assays using automated tracking software and measurement of AChE enzyme activity showed the lower threshold for significant AChE inhibition by CPF to be around 10-fold lower than the initially chosen concentration suggest that this concentration may be unsuitable for further study as a low-level exposure.

From the perspective of CPF research in *C. elegans*, the identification of behavioural effects at the much lower concentration of 0.05 mg/L was one of the most important findings in this study. To the best of our knowledge, this is the lowest treatment

concentration at which behavioural effects have been reported (Roh, Lee and Kwon, 2016). This is certainly true for experiments using solvent spiking in NGM media as a delivery method at the time of writing. This is important because concentrations greater than 0.05 mg/L were shown to inhibit AChE, and yet overt behavioural effects were difficult to find without the aid of computer tracking tools. It is therefore possible that other studies could underestimate the threshold at which low-level effects occur. Indeed, even though our chosen concentration of 0.05 mg/L CPF did not inhibit AChE to a statistically significant extent, further experiments (discussed later in this chapter) support the hypothesis that some small, biologically-significant, perhaps very localised change in AChE, may have been involved in its effect. Taken together, these results highlight the importance, and some limitations, of biochemical measurement of AChE where non-cholinergic effects are of interest, or suspected. Our results also support the use of body-length measurement as an indicator of possible AChE inhibition, although not as replacement for biochemical methods such as the Ellman assay (1961).

7.2.2. Muscarinic, but not nicotinic signalling is required for the effects of low-level CPF treatment on C. elegans foraging behaviour.

It was originally hypothesised that low-level CPF treatment might interact with some target other than AChE to mediate the effect of CPF on *C. elegans* foraging behaviour. To test this hypothesis candidates such as DA, and 5-HT signalling were explored because of their know interactions with human mental wellbeing. However, taken together, the experimental results described in this thesis do not directly implicate any non-cholinergic signalling pathways, including DA and 5-HT, in the causal mechanism of CPF treatment, even at very low treatment concentrations of CPF at which overt effects are not observed. While these results alone cannot exclude the possibility of non-cholinergic

effects, the most likely explanation for effects described here, is that very low levels of CPF cause very small fluctuations in AChE inhibition, specifically in this case, of ACE-2 which affect activation of the muscarinic ACh receptor, GAR-3. This explanation seems to be contradicted by our statistical analysis of the total AChE enzyme activity measured using the Ellman assay (1961), in which inhibition of AChE by 0.05 mg/L CPF was not found to be statistically significant. However, it is possible that whole body AChE measurements are too crude a measure, and that very small or localised fluctuations of AChE occur undetected by these methods. By their nature, AChE activity and the resulting levels of synaptic ACh are not static, and studies rarely report absolute zero values for AChE inhibition. Our dose response curve estimated an IC02 value for AChE inhibition of 0.044 mg/L CPF, which is almost the same as our final working concentration of 0.05 mg/L. Such a difference would likely be below the limit of quantification for CPF in biological samples, even using modern analytical techniques such as gas chromatography coupled mass spectrometry (Dai et al., 2017). This estimate indicates that around 2% of baseline AChE may have been inhibited in our 0.05 mg/L CPF-treated groups. Such a difference could presumably show as statistically non-significant with a p-value cut-off of 0.05 but carry biological significance through the activation of highly sensitive muscarinic receptors such as GAR-3 (Dittman and Kaplan, 2008). Muscarinic receptors have approximately 1000-fold higher affinity than nicotinic receptors for the ACh ligand (Hille, 1992; Dittman and Kaplan, 2008), which could explain why the effect of treatment with 0.05 mg/L CPF was affected by knockout of the GAR-3 muscarinic receptor but not the ACR-16 nicotinic receptor, as described in chapter 5. This is further supported by the fact that both AChE and nicotinic receptors tend to be enriched at synaptic terminals (Chan et al., 2013; Blotnick-Rubin and Anglister, 2018). AChE is highly efficient in its role of

hydrolysing ACh, and so the relatively low-affinity nAChRs would be less sensitive to such small ACh fluctuations caused by tiny changes in AChE activity concentrated at the synapse (**Figure 58**). Conversely, small fluctuations of ACh spillover from the synapse could have a considerably more potent effect on high-affinity metabotropic receptors, such as GAR-3, located extrasynaptically (Dittman and Kaplan, 2008).

7.2.3. The broader significance of these findings

The findings described in these chapters offer evidence that even at very low concentrations, previously not known to cause observable effects in *C. elegans*, biological effects can be detected using computer-assisted behaviour tracking. The discovery of these effects is particularly interesting given that the lowest observable concentration for CPF effect delivered via NGM plates was ten times lower than the concentration formerly identified as a low-level exposure (Viñuela et al., 2010). Consequently, this newly identified effect concentration and the specific conditions necessary for its detection provide useful guidance for future research involving *C. elegans*, particularly in the investigation of low-level CPF and other OP exposures.

Beyond the discovery of CPF's impact at low treatment concentrations, the observation that this effect continues to occur through the cholinergic signalling pathway adds significant implications for several reasons. Firstly, the genetic conservation between *C. elegans* and higher organisms suggests numerous similarities in toxicodynamic interactions involving receptors, ligands, and other molecular elements (Hunt, 2017). This conservation implies that the findings presented here might contribute significantly to the ongoing debate over the potential effects of low-level OP exposures on targets other than cholinergic pathways, especially in the absence of overt AChE inhibition. Despite efforts to identify non-cholinergic impacts, no such effects were evident here.

Hence, based on this evidence, we conclude that the primary effects of low-level CPF treatment likely act through the cholinergic pathway. This finding agrees with the general understanding of AChE inhibition as the primary target of CPF and aligns with the hypothesis that non-cholinergic effects of low-level CPF exposure may be secondary to AChE inhibition. This was shown to be true in adult rats in response to acute CPF treatment and has been proposed as a possible mechanism for increased depression and anxiety following OP exposure (Judge et al., 2016). Conversely, a study by the same group did report reduced dopamine levels in CPF-exposed rats, in the absence of detectable changes in AChE activity, and this was accompanied by increased anxiety-like behaviour (Savy et al., 2015). Savy et al. (2015) measured AChE activity in blood, as well as several brain regions, including the cerebellum, caudate putamen, hippocampus, and prefrontal cortex, but could not rule out transient fluctuations of AChE, or changes in AChE activity in other brain regions. This draws another parallel with our own findings, and highlights some of the challenges with determining very small or localised changes in response to very low exposure concentrations.

The novel finding in this study, that treatment with 0.05 mg/L CPF resulted in an effect via the muscarinic signalling pathway, but not through fast-ionotropic/nicotinic-receptor signalling, suggests a specific and nuanced response mechanism within the cholinergic system (**Figure 58** & **Figure 59**). It is conceivable that relatively minor changes in cholinergic signalling, brought about by exposure to low levels of CPF, might pose a lesser risk of long-term neurological effects than acute cholinergic hyperexcitation. This is based on the presumption that small changes in AChE activity that increase ACh levels sufficiently to activate mAChRs, but not elicit a nicotinic signal, would at least remain in the physiologically acceptable concentration range for ACh. However, this does not

consider the possibility of direct effects by CPF on mAChRs, which also cannot be excluded. We did not test for direct effects of CPF on the GAR-3 mAChR, and while it is likely that activation of GAR-3 by ACh would be responsible if AChE inhibition was indeed the primary mechanism, direct effects on the GAR-3 could be another possibility.

7.2.4. Linking these results to mental health in humans

A primary focus of this project was to investigate possible links between low-level OP exposure and mental health outcomes in humans. A limitation of the *C. elegans* model is that it does not exhibit 'depression-like' behaviour that can be used to test for similar symptoms in humans. However, we did test for effects of CPF on several neurotransmitter pathways that are known to be relevant to mood regulation in humans, including DA and 5-HT signalling, in which no significant effect was found. Nevertheless, even though our results suggest a primarily cholinergic mechanism for the effect of low-level CPF treatment, such a mechanism could affect mental health in humans in several ways.

Firstly, the effect of low-level CPF treatment we observed required the GAR-3 muscarinic receptor. We took this to suggest that mAChR activation may be a result of low-level CPF exposure, potentially due to inhibition of AChE. In mammals, overstimulation of mAChRs caused by reduced AChE activity can lead to a compensatory downregulation of mAChRs (Li et al. 2003). Human studies have reported reduced mAChR expression in the brains of depressed patients (Cannon et al. 2006; Gibbons et al. 2016). Some mAChRs function as autoreceptors, inhibiting ACh release, which is prevented following mAChR downregulation, leading to an increase in ACh which it is suggested could contribute to the symptoms of depression (Dulawa and Janowsky, 2019).

Alternatively, activation of mammalian M2/M4 muscarinic autoreceptors can suppress ACh release, which in turn reduces β2-nAChR activation on dopaminergic terminals and reduces DA release (Threlfell *et al.*, 2010). Moreover, these mAChRs can also function as heteroreceptors, meaning that they can modulate signalling via several different neurotransmitter systems (Picciotto, Higley and Mineur, 2012). These present examples of circuit-level effects that may not be generalisable from *C. elegans* but present testable mechanistic theories having already established the requirement for mAChR signalling in the effect of low-level CPF treatment.

Regarding the requirement food deprivation in the expression of the CPF-induced effect, it is interesting to consider potential parallels between the observed reduction in foraging behaviour caused by low-level OP treatment in food-deprived nematodes and the anhedonia exhibited by mammals, including humans with depression. Anhedonia, characterised by diminished internal drives, often results in disrupted feeding behaviour across various species (Lim et al., 2012; Figueroa et al., 2015; Fureix et al., 2015; Han et al., 2017; Milton, Oldfield, and Foldi, 2018). However, while it is tempting to draw such comparisons, it is crucial to bear in mind the considerable difference in complexity and anatomy between the human brain and the nematode's nervous system. Therefore, despite certain useful similarities in toxicodynamic function at the molecular level, direct connections between nematode behavioural states and complex human conditions, like depression, are at best tenuous and should be approached with caution.

7.2.5. Practical implications

The other methodologies developed in this thesis, such as the generation of a heat map from computer tracking results, may also be useful, such as for screening large compound libraries for effects against multiple genetic targets. Initially used to investigate potential

targets of CPF relevant to human health in this study, this method could prove useful in a variety of applications, including screening compounds for mechanisms of action like plant protection products, or in drug discovery.

Moreover, this approach could be a useful addition for read-across methods aiming to reduce the reliance on higher animal testing in chemical hazard assessment. Read-across is a strategy used to extrapolate toxicological outcomes among groups of substances sharing commonalities such as structural or physicochemical properties (Escher et al., 2019). When effective, this approach can serve as an alternative to new animal testing and is usually based on quantitative structure-activity relationships (QSARs). These are computational models which anticipate the biological effects of compounds based on their structure but are currently limited to predicting relatively simple biological effects and their accuracy depends on the quality of their training data (Huang et al., 2021; ECHA, 2022). There could be an opportunity to integrate the sensitive, objective, and comprehensive data derived from programs like WormLab, to create rich training datasets. This data could be utilised to test and validate read-across justifications by assessing substances using methods similar to those developed in this study. As a result, large sets of whole-organism toxicology data could be generated at a relatively low time and financial cost. This data can then be used to support existing models, or even contribute to the development and enhancement of new QSAR models. Such a strategy could improve the predictive accuracy of QSAR models. As a result, it could serve as a useful tool for understanding complex toxicological effects, reducing the need for animal testing, and ultimately contributing to a more sustainable and ethical approach to chemical hazard assessment.

Furthermore, the applicability of these methods extends beyond toxicology. For example, in the field of drug discovery, where high-throughput screening of large compound libraries for effects against multiple genetic targets is a common practice, these methodologies could enhance efficiency and accuracy. In conclusion, the methods described in this thesis have broad potential for improving both toxicological and pharmacological research, demonstrating potential value from this project beyond its primary findings and objectives.

7.2.6. Limitations of the C. elegans model for investigating the effects of low-level CFP exposure

The *C. elegans* model has been extensively used in this, and other studies to investigate issues related to human activities and health conditions. However, it is important to acknowledge some limitations inherent to this model system, some of which have been discussed in this and previous chapters.

Several differences between humans and *C. elegans*, such as size, anatomy, lifespan, and behavioural range, contribute significantly to the model's experimental convenience and tractability. However, these differences also pose challenges when extrapolating findings to humans. For example, the anatomical simplicity of *C. elegans*, while convenient for certain types of studies, may limit the applicability of findings to complex mammalian systems. Additionally, the comparatively short lifespan of *C. elegans*, which is allows for higher experimental throughput, may not be representative of chronic effects or longer-term adaptive changes in humans.

7.2.7. Dose extrapolation to humans: challenges and considerations.

While the *C. elegans* model offers unique advantages for testing OP exposure, including its small size, short generation time, and relatively simplistic nervous system, these features also pose challenges for accurate dose extrapolation to humans. Potentially relevant factors such as dosage, cumulative exposure periods, or complex circuit-level signals may be missed due to these differences. Certain physiological aspects, such as an adaptive immune system, cholesterol or heme synthesis capability, or specific organs, are entirely absent in *C. elegans*. Additionally, there are differences in shared systems, for instance, the endocrine system, where no specific homology exists between human oestrogen receptors and those of *C. elegans* (Gill, 2006).

In some respects, *C. elegans* physiology may be more complex, such as in the cholinergic system. Humans have one gene encoding for acetylcholinesterase (AChE) and an additional pseudocholinesterase (BuChE). Whereas *C. elegans* possesses four genes encoding AChE, an important benefit of which is that it is possible to knock out individual ACE genes without causing lethality(Combes et al. 2000). However, our data suggest that knocking out each of these individual ACE genes results in distinct interactions with CPF. Therefore, attention should be given to differences in sequential structure and localised expression of these enzymes. In this study, ACE-2 appeared to be the most important to CPF's effect. ACE-2 is known to be predominantly expressed in *C. elegans* neurons rather than muscle (Combes et al., 2003; Chan et al., 2013). While the structural and functional differences and similarities between human AChE and *C. elegans* ACE-2 were not explored in this project, they present an avenue for future research. If structural differences underlie ACE-2's sensitivity to CPF compared to ACE-1 or ACE-3, it is plausible that differences between ACE-2 and human AChE might impact the generalisability of our

findings. Understanding the relative sensitivity of different nematode AChE enzymes and human AChE, potentially based on their protein structure, could enhance our understanding of specific molecular interactions involved. Such insights could inform the development of novel treatments for OP toxicity or safer plant protection products.

Our methodology also presented a limitation in how AChE activity was measured. With hindsight, a range-finding test might have helped establish a suitable range of exposure concentrations and discern a no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) for AChE inhibition.

Furthermore, the method for testing AChE activity required around 20 mg of tissue, or approximately 1-5 million cells per sample (Abcam). Given that an adult *C. elegans* hermaphrodite contains only 959 somatic cells and weighs around 660 nanograms (Wormbase), testing each replicate of each treatment group required several densely populated plates of worms. While such large-scale collection was necessary for detecting any difference in AChE activity, it might be too crude a measure to capture small, local fluctuations relevant to the effects discussed in this thesis. Hence, this represents another limitation to consider in future research and interpretations.

7.2.8. Limitations of C. elegans in Predicting Secondary Steps in Humans

While *C. elegans* has proven invaluable for uncovering fundamental biological mechanisms, it is important to recognise its limitations when it comes to predicting the secondary steps involved in chemical effects on the human brain. Differences in neuronal complexity and signalling pathways limit the direct extrapolation of findings from *C. elegans* to the human brain, particularly concerning secondary steps and downstream connections. For example, a compound that is metabolised to a toxic form in humans may

not be metabolised to a toxic form in the same way in *C. elegans*. We demonstrated that acute CPF treatment exerts clear toxic effects and inhibits AChE in *C. elegans*. This suggests metabolism of CPF to its toxic oxon metabolite occurs in *C. elegans*, and this is supported in the literature (Roh et al. 2016). However, some metabolic differences mean that *C. elegans* cannot metabolise all compounds in the same way (Harlow et al. 2018).

7.2.9. Bioactivation of OPs by cytochrome P450

In the case of OPs, many of these compounds require bioactivation by CYP enzymes to form the active metabolites that exert their toxic effects. *C. elegans* lacks a complex CYP system that is directly comparable to humans. As a result, the bioactivation pathways for OPs and the subsequent toxic effects they induce in *C. elegans* may not fully mirror those in humans. Therefore, caution should be exercised when extrapolating findings from *C. elegans* to predict OP toxicity in humans because the metabolic processes and bioactivation pathways involved in human CYP-mediated metabolism are more complex and diverse compared to those in *C. elegans*.

To overcome these limitations and improve the predictive value of using *C. elegans* in studying OP toxicity, it may be necessary to consider complementary approaches. These could include *in vitro* human cellular models that express relevant CYP enzymes involved in the bioactivation processes or employing animal models, such as rodents, that possess a more closely related CYP enzymes to humans. Nevertheless, as we and others have demonstrated, *C. elegans* is capable of metabolising many compounds in similar ways and producing the same metabolites as in mammalian pathways (Roh et al. 2016; Harlow et al. 2018) and therefore offers value as a model as long as the nuances of toxic metabolism between species are acknowledged.

7.2.10.Assessment of C. elegans as a model to predict human neurotoxicity.

C. elegans is a useful model organism for studying basic biological processes and has provided valuable insights into neurobiology. However, its ability to predict human neurotoxicity has limitations which should be considered. *C. elegans* has a simpler nervous system compared to humans, with fewer neurons and lacks specialised brain regions and complex neural circuits. Although some neurotransmitter systems and receptors are conserved, they are not identical between *C. elegans* and humans. These differences restrict the direct applicability of *C. elegans* findings to the human brain.

Predicting neurotoxicity requires understanding complex secondary steps and effects in the human brain, which *C. elegans* may not fully capture due to its simplified nervous system. To achieve a more comprehensive understanding of human neurotoxicity, it can often be more appropriate to incorporate additional model systems such as rodents, and *in vitro* human cellular models. These models possess more complex nervous systems and better reflect human neurobiology, providing more reliable predictions of neurotoxicity.

However, despite these limitations, *C. elegans* is a relatively inexpensive and easy to maintain making it a valuable tool for high-throughput screening of potential neurotoxicants. Moreover, its short lifespan allows for rapid assessment of toxicity which could be valuable during the development of drugs, plant protection products, or industrial chemicals. The *C. elegans* nervous system also includes many conserved molecular components, and is well characterised, meaning that potential neurotoxicants can be assessed relatively easily compared to more complex models. Furthermore, since it was first introduced as a model a great number of mutant strains have been developed and can procured, or new mutants created. This presents the opportunity to test

alternative mechanistic theories and hypotheses with relative ease. This was demonstrated through the use of several different mutant strains relating to separate mechanistic hypotheses, with the results presented in a heatmap in the final *C. elegans* results chapter.

7.3. Key findings from the survey-based human study

7.3.1. Evidence for mental health issues in an OP exposed cohort

This study aimed to investigate the prevalence of symptoms of common mental disorders among agricultural workers with occupational exposure to OPs compared to a control group without exposure. The main findings indicated that exposed agricultural workers had a higher frequency of symptoms of depression compared to the control group, consistent with previous research (Harrison and Mackenzie Ross, 2016; Phillips and Deshpande, 2016). However, there was no significant difference in anxiety symptoms between the two groups, contrary to some previous studies (Harrison and Mackenzie Ross, 2016). The discrepancy may be attributed to variations in study samples and measurement scales.

The study also explored the role of acute and chronic stress in mental health outcomes. We found that daily hassles and major stressful events were highly correlated with depression scores and self-reported stress, while OP exposure did not show a significant correlation. This suggests that lifestyle factors and chronic stress experienced by agricultural workers may be important drivers of poor mental health in this population. The results highlight the significance of day-to-day stressors, such as financial stress and loneliness, in contributing to symptoms of depression. These effects were found to reduce with age, potentially due to changes in social and financial circumstances over time.

Furthermore, the study examined the relationship between physical health and mental health outcomes. General health was reported to be better in the control group (construction workers) compared to agricultural workers, and physical health was strongly associated with increased reports of depression symptoms in the agricultural

workers. Interestingly, a specific set of neuromuscular-related symptoms was found to contribute to higher depression scores within the exposed group. This suggests a potential link between physical symptoms and low-level OP exposure. Therefore, while we did not find a direct link between OP exposure and mental health in this cohort, it is possible that OP exposure may contribute to physical health symptoms, thus indirectly impacting mental health. These results of our mediation analysis support such a hypothesis. However, the symptoms included in Symptom factor 1 are theoretically more closely associated to nicotinic rather than muscarinic effects. Moreover, the symptoms listed could also result from other factors associated with working in agriculture, such as physically demanding work, injuries, or exposure to extreme weather conditions, and may not be specific to altered cholinergic signalling. Therefore, future research could explore the relationship between low-level OP exposure and physical health symptoms further.

7.3.2. Limitations of the survey-based human study

Despite these findings, there are several limitations to consider. The sample may have been influenced by selection biases, particularly among participants recruited through the Sheep Dip Sufferers Association, who may have been involved in litigation related to OP exposure. The control group also had a significant male bias. Additionally, there were only three non-binary respondents, all of which were in the exposed group. This could have skewed the results, as symptoms of depression and anxiety have been shown to present more frequently in gender minority groups and in females than males.

Additionally, the study used self-report scales and not structured clinical interviews to diagnose psychiatric disorders introduce potential measurement biases. Participants were not asked whether they had ever been diagnosed with depression or anxiety, and

more detailed information about participants' psychological history could have been collected.

The measurement of OP exposure also poses challenges, with this study relying on a relatively crude measure of lifetime exposure. The likely possibility that some of these participants have been exposed to other potentially harmful chemicals, and the difficulty of separating exposure to OPs from other compounds further complicate the assessment of any effects of OP exposure.

The physical symptom set used in this study was somewhat limited, and slightly biased toward symptoms that could be linked to cholinergic poisoning. The inclusion of a more comprehensive set of symptoms may have offered more insight into any physiological effects occurring below the threshold for significant AChE inhibition.

7.3.3. The broader significance of these findings

The broader relevance of this work concerns the mental health of agricultural workers with a history of occupational OP exposure and the findings support previous research showing a higher prevalence of depression symptoms in this population. Agricultural workers who are exposed to OPs may be at increased risk of developing symptoms of depression. Even though no causal link between OP exposure and depression was found, this remains a significant finding, as depression is a major public health problem, affecting millions of people worldwide. The findings of this study could be help to raise awareness of the potential contributing factors to depression in these groups, and to develop targeted interventions to improve the mental health of agricultural workers.

The identification of lifestyle factors, chronic stress, and physical health as important contributors to mental health outcomes adds some insight into the complexities of these

relationships. These findings suggest a need to consider not only pesticide exposure but also the broader context of agricultural work, including stress management, financial support, and social connections, particularly for younger farmers.

The findings of this study also have implications for research. They suggest that further research is needed to investigate the link between OP exposure and mental health. This could include studies that use more objective measures of exposure, such as biological markers. It would also be important to study the long-term effects of OP exposure on mental health, which were not specifically addressed in this cross-sectional study.

7.4. Suggestions for future work

There are several possible avenues and additions that could compliment and build upon the experimental work described in this thesis.

7.4.1. Future directions for experimental work

Firstly, it would be interesting to explore the interaction between CPF and ACE-2 in more detail, possibly through genetic rescue experiments to confirm whether restoring ACE-2 restores foraging behaviour to wild-type levels. This would help to determine the requirement for ACE-2 in the low-level effect of CPF by examining if treating the ACE-2 rescue strain with CPF produces a similar reduction in foraging range as seen in treated wild-type animals.

It would also be interesting to investigate why knockout of ACE-2 resulted in resistance to CPF, while knockout of ACE-1 or ACE-3 did not. This might be through structural or sequential differences, or differences in the localisation of ACE-2. These possibilities could be tested by inserting ACE-1 or ACE-3 copies under the ACE-2 promoter and observing if the transgenic rescue animals show similar resistance to CPF as the *ace-2* strains, indicating a dependence on structure, or if the successful rescue indicates differences in ACE-2 localization.

The identification of GAR-3 as a key player in mediating the effects of CPF on foraging behaviour suggests that further exploration of muscarinic signalling is warranted. Future studies could investigate the specific downstream signalling pathways and neural circuits associated with muscarinic receptors to gain a deeper understanding of how CPF affects neuronal function in *C. elegans.*. Muscarinic signalling is an important pathway in the cholinergic system, and its dysregulation has been implicated in various neurological

disorders in humans. The observation that low-level CPF exposure primarily affects muscarinic signalling in *C. elegans* suggests that similar mechanisms may be at play in higher organisms, including mammals. Further investigations in mammalian model systems, such as rodents, would be valuable in validating and extending the findings from *C. elegans* to more complex neurobiological systems. These studies could involve assessing the effects of low-level CPF exposure on muscarinic receptors, downstream signalling pathways, and related behaviours in mammalian models. Such research would contribute to our understanding of the neurotoxic effects of CPF and its potential implications for human health.

In particular, it would be interesting to test whether short, or longer-term exposure to low-levels of OPs leads to any upregulation or downregulation of muscarinic receptors. If this were indeed the case then this could indicate a potential link between low-level exposure and mental health in humans (Picciotto, Higley and Mineur, 2012; Dulawa and Janowsky, 2019). In combination with this, the interactions of low-level OP exposure and behaviour would be interesting to test alongside drugs that affect muscarinic signalling, some of which have been shown to have therapeutic potential in the treatment of depression (Drevets and Furey, 2010; Jaffe, Novakovic and Peselow, 2013).

Another natural direction to follow from this project would be to test whether similar results could be observed following treatment with different OP compounds, or similar substances. The differences in chemical structure could provide valuable insight into the specific interactions with protein structures and neurotoxicological outcomes.

The use of *C. elegans* helped to determine a muscarinic-signalling-specific effect of low-level of CPF treatment, however some limitations of the model, including the

generalisability of these results to humans has already been discussed. It might therefore be worth considering the use of alternative models to extrapolate the findings and investigate them further in a more relevant physiology, and where a more realistic dose comparison could be made. That being said, ethically we must consider the value of conducting more animal testing using higher organisms, especially since the use of OP pesticides has been heavily restricted in developed countries, reducing the societal and environmental benefits of such research.

7.4.2. Future directions for human studies

Despite a reduction in OP-pesticide use in some parts of the world, developing countries continue to use OPs extensively, and so for future research into the effects of occupational exposure in humans, it would be useful to incorporate more objective measures, such as structured clinical interviews, to diagnose psychiatric disorders and obtain a more accurate assessment of mental health outcomes, rather than self-reporting screening questionnaires. Moreover, longitudinal studies might provide a better understanding of the causal relationships between OP exposure, chronic stress, and mental health. Further investigation is needed to explore the mechanisms linking physical symptoms to low-level OP exposure and to differentiate the effects of OPs from other compounds commonly encountered in agricultural settings. The use of a more homogeneous sample with known exposure to specific compounds, such as practicing pesticide applicators or contractors, would also help to reduce the variability involved with exposure assessment in this sample. This could be complemented with some objective biological measure, such as the measurement of AChE activity, or OP metabolites, in blood or urine. However, such measures are often impractical, and such a perfect sample difficult to find. An alternative

approach may find value in repeating this study in other countries where OP pesticide use is still common. Such an approach may help to produce a larger sample and could address an issue in this study in which many participants had not worked with OPs for some time. Finally, studies that assess the effectiveness of interventions targeting mental health in agricultural workers, taking into account the complexities highlighted in this study, would be of value to help to ensure the wellbeing of farming communities.

7.5. Concluding summary

In conclusion, this research project investigated the effects of low-level CPF exposure through a combination of experimental and human studies.

The experimental findings in *C. elegans* demonstrated the involvement of a class B AChE isoform and muscarinic signalling via the GAR-3 receptor, in mediating the effects of low-level CPF on behaviour in certain conditions. These findings provide novel insights into the molecular mechanisms underpinning the effects of low-level OP exposure which merit further investigation.

The human study revealed a higher prevalence of depression symptoms in agricultural workers with CPF exposure and emphasizes the impact of chronic stress and lifestyle factors on mental health outcomes in this group. These findings highlight the importance of considering broader contextual factors when studying the effects of OP exposure in human populations and suggest that any effect of low-level OP exposure on mental health conditions could be mediated via physical health.

The studies presented in this thesis both reveal important insights into the neurotoxic effects of low-level OP exposure. The experimental study, conducted using the model organism *C. elegans*, revealed important insights into the neurobehavioral effects of CPF and the underlying mechanisms. The human study focused on agricultural workers with occupational CPF exposure, providing valuable information on the mental health outcomes in this population. Currently it is not possible to directly relate the findings from these studies. While *C. elegans* provided valuable insights into the basic mechanisms and specific aspects of CPF toxicity, its ability to predict secondary steps in humans and accurately model human neurotoxicity is limited due to differences in neuronal

complexity and signalling pathways. Therefore, efforts should be made to enhance the applicability of the *C. elegans* model for toxicology studies by developing methods for assessing complex neurotoxicological effects and by incorporating additional model systems, such as rodents, non-human primates, and *in vitro* human cellular models, to gain a more comprehensive understanding of the effects of CPF and other organophosphate pesticides on the human brain. The reduction of the use of animal models should also be given consideration, especially considering the reduction in OP pesticide use globally and its impact on the relevance of such research.

Overall, this research project contributes to the understanding of the neurobehavioral and mental health effects of low-level CPF exposure and underscores the importance of considering both experimental and human studies to comprehensively assess the impact of chemical exposures on human health. By integrating findings from different model systems and approaches, we can advance our knowledge of neurotoxicity, inform risk assessments, and work towards the development of safer practices and policies in pesticide use.

8. References

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Appendix

8.1. Survey questionnaire

Start of Block: Information about study and consent form

Q1

Invitation to Participate in Open University Research

Study aims and information

You are being invited to take part in a study investigating the health and well-being of UK farmers and agricultural workers. In particular, we are interested in factors that might cause ill health in farming groups. These factors range from stressful life- or job-related events, to unusual patterns of sleep, through to exposure to certain substances which may be harmful (in this case, organophosphates). This work is being carried out by Alex Dawson at the Open University and has been approved by the OU's Human Research Ethics Committee.

What does it involve?

As part of this study, you will be asked a series of questions covering the range of factors

described above. Just answer them as honestly as you can. The whole survey should take no longer than 15 minutes.

Compensation

As compensation for completing the survey you will receive a £5 gift voucher from Amazon. Information about how to collect this voucher can be found at the end of the questionnaire.

Privacy policy

All information collected by the questionnaire will be anonymous and you will not be identified in any report or publication resulting from this study. Your contribution will be used for research purposes only and no personal information will be passed to anyone outside the research team. Your participation will be treated in strict confidence in accordance with the Data Protection Act.

How will the information collected by the survey be used?

Data collected by the survey will be stored on a secured, encrypted server. All data collected will be anonymous, and only aggregated summaries of the results will be reported.

Survey feedback

An anonymised summary of the overall research findings will be available to you at the end of the study. If you would like a copy of this, please email Alex Dawson at alex.dawson@open.ac.uk.

Voluntary participation

Taking part in this survey is entirely voluntary. If you decide to take part, you are free to withdraw at any time (during the study) without having to give a reason and without negative consequence. Once you have completed the survey, you also have the right to ask that any data you have supplied be withdrawn/destroyed, up until the point of data analysis. If you wish to have your data withdrawn/deleted, please notify alex.dawson@open.ac.uk by 1st August 2018.

Questions, comments or complaints

If you have any questions or comments about this study, we would be very happy to discuss these with you. Please contact Alex Dawson on alex.dawson@open.ac.uk or 01908 652244.

If you have any concerns or complaints about any aspect of your participation in this study, please contact Dr Gini Harrison (research supervisor at The Open University) at gini.harrison@open.ac.uk or 01908 654437.

If you think you have been affected by any of the issues that are covered in this questionnaire, we advise you to contact your GP to discuss any health concerns you may have.

Q2, Do you consent to take part in this survey?

- ○ Yes
- ○ No

Q3

Thank you for your interest in our study.

If you have any queries about this research, please contact Alex Dawson at alex.dawson@open.ac.uk

Q70 Please tell us where you heard about this study.

_____

Q4, Do you think that you might have come into contact with, or worked around organophosphate pesticides?

- ○ No
- ○ Yes

Q5, Are you based in the UK?

- ○ No
- ○ Yes
- ○ Not currently, but I have worked in the UK where I might have been exposed to organophosphates

End of Block: Information about study and consent form

Start of Block: Demographics

Q6 Please tell us a little bit about yourself.
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Q7 What gender do you identify as?

- ○ Male
- ○ Female
- ○ Other
- ○ Prefer not to say

Q8 What is your age group?

- ○ Under 18
- ○ 18 - 24 years old
- ○ 25 - 34 years old
- ○ 35 - 44 years old
- ○ 45 - 54 years old
- ○ 55 - 64 years old

- ○ 65 - 74 years old
- ○ 75 years or older

Q9 What is your ethnic group? Choose one option below that best describes your ethnic group or background

- ○ English / Welsh / Scottish / Northern Irish / British
- ○ Irish
- ○ Gypsy or Irish Traveler
- ○ Any Other White background
- ○ White and Black Caribbean
- ○ White and Black African
- ○ White and Asian
- ○ Any other Mixed / Multiple ethnic background
- ○ Indian
- ○ Pakistani
- ○ Bangladeshi

- ○ Chinese
- ○ Any Other Asian background
- ○ African
- ○ Caribbean
- ○ Any other Black / African / Caribbean background
- ○ Arab
- ○ Any other ethnic group

Q10 What is the highest qualification you currently hold?

- ○ No formal qualifications
- ○ GCSE/O Levels
- ○ NVQ Levels 1 or 2
- ○ A Levels/BTEC National Diploma/IB
- ○ NVQ Level 3
- ○ NVQ Level 4-5
- ○ Bachelor's Degree (e.g. BSc, BA)

- ○ Master's Degree (e.g. MSc, MA, MRes)
- ○ Doctorate
- ○ Professional qualifications (e.g., teaching, nursing, accountancy etc.)
- ○ Foreign qualifications (i.e. a qualification not from the UK)
- ○ Other (please describe) _____

Q11 What is your occupation?

_____

Q12, Do you smoke?

- ○ Yes, I am a smoker
- ○ I used to smoke but I gave up
- ○ I have never smoked

Q13 How often do you drink alcohol?

- ○ Never
- ○ Less than monthly
- ○ A few times a month
- ○ 1-3 times a week
- ○ 4 or more times a week
- ○ Every day

Q14 How many drinks containing alcohol do you have on a typical day when you are drinking?

- ○ I do not drink alcohol
- ○ 1-2
- ○ 3-4
- ○ 5-6

- ○ 7 or more

Q15 Do you regularly take any other drugs or medicines?

- ○ Yes
- ○ No
- ○ I would rather not say

Q16

- ○ What medicine/drug to you take? _____
- ○ What condition do you take it for? _____
- ○ Roughly, when did you start taking it? _____

End of Block: Demographics

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Start of Block: Sleep habits

Q18 Please tell us about your sleeping habits within the last month.

Q19 How many hours do you usually sleep at night?

	3 or less	4	5	6	7	8	9	10	11 or more
	○	○	○	○	○	○	○	○	○

Q20 Was this amount:

- ○ Not enough
- ○ About right
- ○ Too much

Q21 Roughly, how many days per week do you have a problem with feeling sleepy during the day?

	Never	1	2	3	4	5	6	Every day
	○	○	○	○	○	○	○	○

Q22 How many times (each night) do you usually wake up during night?

	Usually none	1	2	3	4	5 or more
	○	○	○	○	○	○

Q23 On how many nights per week does it take you more than 30 minutes to fall asleep?

	Never	1	2	3	4	5	6	Every night
	○	○	○	○	○	○	○	○

End of Block: Sleep habits

Start of Block: Physical Health

Q24 Please answer some questions relating to your general health and wellbeing.

Q25 In general, would you describe your health as: (please tick **one**)

- ○ Excellent
- ○ Very good
- ○ Good
- ○ Poor
- ○ Terrible

Q26 During the **past 4 weeks,** to what extent has your physical health interfered with your work or daily household activities?

- ○ Not at all
- ○ Slightly
- ○ Moderately
- ○ Quite a bit
- ○ Extremely

Q27 During the **past 4 weeks**, to what extent has your physical health interfered with your normal social activities with family, friends, neighbours, groups etc.?

- ○ Not at all
- ○ Slightly
- ○ Moderately

- ○ Quite a bit
- ○ Extremely

Q28 How much **bodily pain** have you had in the **last 4 weeks?**

- ○ None
- ○ Very mild
- ○ Mild
- ○ Moderate
- ○ Severe
- ○ Very severe

Q29 **Physical symptoms:** During the last **4 weeks** to what extent has your physical health affected your daily life?

Please select the appropriate responses from **both** the 'How often' and 'How much does this affect your daily life' columns.

	How often?	How much does this affect your daily life?
⊗ Problems controlling your temperature/sweating	▼ Never ... Always	▼ Not at all ... Extremely
⊗ Headaches	▼ Never ... Always	▼ Not at all ... Extremely
⊗ Toothache	▼ Never ... Always	▼ Not at all ... Extremely
Numbness or tingling in any part of the body	▼ Never ... Always	▼ Not at all ... Extremely
Blurred vision	▼ Never ... Always	▼ Not at all ... Extremely
Fatigue (feeling constantly tired)	▼ Never ... Always	▼ Not at all ... Extremely
Loss of balance/coordination	▼ Never ... Always	▼ Not at all ... Extremely
Dizziness	▼ Never ... Always	▼ Not at all ... Extremely
Ringing in the ears/hearing problems	▼ Never ... Always	▼ Not at all ... Extremely
Skin problems	▼ Never ... Always	▼ Not at all ... Extremely
Joint stiffness/pain	▼ Never ... Always	▼ Not at all ... Extremely
Muscular pain/cramps	▼ Never ... Always	▼ Not at all ... Extremely
Muscle tremors/twitches	▼ Never ... Always	▼ Not at all ... Extremely
Nausea	▼ Never ... Always	▼ Not at all ... Extremely
Difficulty remembering things/concentrating	▼ Never ... Always	▼ Not at all ... Extremely
Chest pain/tightness/shortness of breath	▼ Never ... Always	▼ Not at all ... Extremely
Muscle weakness	▼ Never ... Always	▼ Not at all ... Extremely
Unintended weight-loss or gain	▼ Never ... Always	▼ Not at all ... Extremely

Indigestion	▼ Never ... Always	▼ Not at all ... Extremely
Constipation	▼ Never ... Always	▼ Not at all ... Extremely
Diarrhea	▼ Never ... Always	▼ Not at all ... Extremely
Hay fever or allergies	▼ Never ... Always	▼ Not at all ... Extremely
Alcohol/chemical intolerance	▼ Never ... Always	▼ Not at all ... Extremely
Urinary problems	▼ Never ... Always	▼ Not at all ... Extremely

Q30 If you suffer from any other **physical symptoms** that you would like to tell us about then please use this space:

_____

End of Block: Physical Health

Start of Block: Pesticide Exposure

Q31 We now have some questions relating to your exposure to organophosphate pesticides.

Q32 Have you ever worked with organophosphate pesticides (e.g., sheep dip or other insecticides), or maybe worked or lived in a place where you might have come into contact with them?

☐ Yes, I have worked with them - (please describe briefly) _____

☐ I have not worked with them, but I have been exposed in another way - (please describe briefly) _____

☐ No

☐ Do not know

Q33 Have you ever been involved in the following? (Please tick all that apply):

☐ Crop/weed spraying

☐ Sheep dipping

☐ Animal handling

- [ ] Crop harvesting (cutting, picking, or packing)
- [ ] Mixing/preparing pesticides
- [ ] Working in orchards
- [ ] Working with treated grain
- [ ] Sheep shearing
- [ ] Treating cattle for warble fly
- [ ] Other pesticide-related activity (please specify) _____

Q34, Can you name any of the pesticides that you have worked with? (Mention any that you can remember and don't worry about spelling the name right)

_____

Q35 For how many **years** have you worked around pesticides throughout your life? (If you are not sure then just give your best guess)

- ○ Up to one year
- ○ 2 years
- ○ 3 years
- ○ 4 years
- ○ 5 years
- ○ 6 years
- ○ 7 years
- ○ 8 years
- ○ 9 years
- ○ 10 years
- ○ 11 years
- ○ 12 years
- ○ 13 years

- ○ 14 years
- ○ 15 years
- ○ More (please specify) _____

Q36 Usually, how many **months** each year have you worked around pesticides?

- ○ Up to 1 month
- ○ 2 months
- ○ 3 months
- ○ 4 months
- ○ 5 months
- ○ 6 months
- ○ 7 months
- ○ 8 months
- ○ 9 months
- ○ 10 months
- ○ 11 months

○ 12 months/all year round

Q37 How many days per week have you usually worked around pesticides?

	1 day per week	2 days per week	3 days per week	4 days per week	5 days per week	6 days per week	7 days per week
I usually worked around pesticides for:	○	○	○	○	○	○	○

Q38 When you worked around pesticides, how many hours per day did you usually work (during a normal working day)?

○ 1 hour

○ 2 hours

○ 3 hours

○ 4 hours

- ○ 5 hours
- ○ 6 hours
- ○ 7 hours
- ○ 8 hours
- ○ 9 hours
- ○ 10 hours
- ○ 12 hours
- ○ 13 hours
- ○ 14 hours
- ○ 15 hours
- ○ more (please specify) _____

Q39 When working with pesticides, did you wear any protective equipment (PPE)?

- ○ Never
- ○ Sometimes
- ○ About half the time

- ○ Most of the time
- ○ Always

Q40, Do you think working with pesticides poses any risks to your health?

- ○ No
- ○ A slight risk
- ○ A medium risk
- ○ A large risk
- ○ A very high risk

Q41 Have you ever been worried about working with pesticides for any reason?

- ○ Not at all
- ○ A little worried
- ○ Somewhat worried

○ Very worried

Q42 Very briefly, what worried you about working with pesticides?

_____

Q43 Do you still work around pesticides?

○ Yes

○ No

Q44 Roughly, what date did you last work with pesticides (i.e., month/year)

_____

Q45 Have you ever been diagnosed with an illness that the Doctor specifically stated was a result of pesticide exposure?

- ○ No
- ○ I'd rather not say
- ○ Yes - (please state what illness and how long ago this happened) _____

Q46 Have you ever suffered from 'Dipper's Flu'?

- ○ Don't know
- ○ No
- ○ Yes, once
- ○ More than once
- ○ Many times

Q47 Did you notice any changes in your health **while** you were working with pesticides?

- ○ Yes - (please describe) _____
- ○ No
- ○ Don't know

Q48 After you **stopped** working with pesticides, did you notice any changes in your health?

- ○ No
- ○ Don't know
- ○ Yes - (please describe) _____

Q49, Do you want to add anything else about your experience with pesticides?

○ No

○ Yes - (please explain) _____

End of Block: Pesticide Exposure

Start of Block: Major events

Q50 Below is a list of life events that are known to cause some level of stress in some people.

In the last 12 months, have any of these things happened to you? If you don't know or you think something doesn't apply to you then just select 'no'.

Death of a Spouse or long-term partner	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Divorce	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Separated from Spouse or long-term partner	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Spent time in prison	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Death of a close family member	▼ No ... Yes, this has happened to me more than once (in the last 12 months)

Personal injury or illness	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Become married	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Fired from work	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Marital reconciliation (got back together)	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Retired	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change in health of a family member	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Expecting a baby	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Sexual problems	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Gained a new family member	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
A change to business arrangements	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change in financial situation	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Death of a close friend	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change to a different line of work	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Increase in arguments at home	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Taken out a mortgage	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Had contact from debt collectors	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change in responsibilities at work	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Son or daughter leaving home	▼ No ... Yes, this has happened to me more than once (in the last 12 months)

Trouble with in-laws	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Outstanding personal achievement	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Spouse or partner begins or stops work	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Begin or finish studying/school	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change in living conditions	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Changing your own personal habits	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Trouble with boss	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change in work hours or conditions	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Moved home	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Changed school/college	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change in hobbies/recreation	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change in church activities	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change in social activities	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Taken out a loan	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change in sleeping habits	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change in number of family get-togethers	▼ No ... Yes, this has happened to me more than once (in the last 12 months)
Change in eating habits	▼ No ... Yes, this has happened to me more than once (in the last 12 months)

End of Block: Major events

Start of Block: Day to day

Q51 Tell us about things that might cause some level of stress from day to day. Please give answers based on how you feel over the longer term rather than any one single event.

How much stress do the following issues cause in your day-to-day life?

If you feel that any of these things don't apply to you then just select 'None at all'.

The weather	▼ None at all ... Very much stress
Things you hear in the news	▼ None at all ... Very much stress
Politics	▼ None at all ... Very much stress
Your environment (clean air, noise level, greenery)	▼ None at all ... Very much stress
Getting enough exercise (or not)	▼ None at all ... Very much stress
Your physical appearance	▼ None at all ... Very much stress
Socialising	▼ None at all ... Very much stress
Not socialising	▼ None at all ... Very much stress
Your smoking	▼ None at all ... Very much stress
Your drinking	▼ None at all ... Very much stress
Mood-altering drugs	▼ None at all ... Very much stress
Your medical care	▼ None at all ... Very much stress

Your health	▼ None at all ... Very much stress
Your physical abilities	▼ None at all ... Very much stress
Your neighbours	▼ None at all ... Very much stress
The place where you live	▼ None at all ... Very much stress
Feeling isolated/far away from things or people	▼ None at all ... Very much stress
Saving water, gas, electricity, or fuel	▼ None at all ... Very much stress
Vehicle maintenance	▼ None at all ... Very much stress
Housework	▼ None at all ... Very much stress
Building repairs/DIY	▼ None at all ... Very much stress
Doing paperwork (bills, forms, tax returns etc.)	▼ None at all ... Very much stress
How much free time you have, or the lack of	▼ None at all ... Very much stress
Feeling lonely	▼ None at all ... Very much stress
Any legal matters	▼ None at all ... Very much stress
Your children	▼ None at all ... Very much stress
Your parents or in-laws	▼ None at all ... Very much stress
Your spouse or partner	▼ None at all ... Very much stress
Other relative(s)	▼ None at all ... Very much stress
Time spent with family	▼ None at all ... Very much stress
Health or well-being of family member(s)	▼ None at all ... Very much stress
Sexual relationships	▼ None at all ... Very much stress
Family commitments	▼ None at all ... Very much stress
Your friendships	▼ None at all ... Very much stress
Your co-workers	▼ None at all ... Very much stress
Customers or clients	▼ None at all ... Very much stress
Your supervisor or employer	▼ None at all ... Very much stress
The type of work you do	▼ None at all ... Very much stress
The amount of work you do	▼ None at all ... Very much stress
Your job security	▼ None at all ... Very much stress

Deadlines or goals at work	▼ None at all ... Very much stress
Having enough money for food, clothing, and housing	▼ None at all ... Very much stress
Having enough money for hobbies, socialising, or holidays	▼ None at all ... Very much stress
Paying money to/for someone who doesn't live with you	▼ None at all ... Very much stress
Having money for the future (pensions or emergencies)	▼ None at all ... Very much stress
Financial investments	▼ None at all ... Very much stress

Q52 Is there anything else that causes you stress from day to day?

- ○ Yes - (please describe) _____
- ○ No
- ○ Not sure

End of Block: Day to day

Start of Block: Block 8

Q53 How do you rate yourself on the following:

Feeling low (Depression)	▼ I do not have a problem with this ... Extremely Severe
Feeling worried (Anxiety)	▼ I do not have a problem with this ... Extremely Severe
Stress	▼ I do not have a problem with this ... Extremely Severe

Q54 To what extent does your stress, worry or feeling low cause you distress?

- ○ Not at all
- ○ Mildly
- ○ Moderately
- ○ Severely
- ○ Extremely Severely
- ○ Not applicable

Q55, It looks like you might be going through a hard time at the moment. If you haven't already, we advise you to contact your doctor or regular health care professional to discuss how you are feeling. If you are feeling particularly low, and need immediate help, you can find the contact details for several helplines here: https://www.nhs.uk/Conditions/stress-anxiety-depression/Pages/mental-health-helplines.aspx

Q56

We would now like to ask you how any feelings of stress, anxiety, or depression that you are experiencing are impacting on your work and home life.

For each statement, please select the number that best describes your situation.

	0 not at all	1 .	2 slightly	3 .	4 definitely	5 .	6 markedly	7 .	8 severely
Because of my problems, my **ability to work** is impaired.	○		○		○		○		○
Because of my problems, my **home management**	○		○		○		○		○

(cleaning, tidying, shopping, cooking, looking after home or children, paying bills) is impaired.					
Because of my problems, my **social leisure activities** (with other people, e.g., parties, pubs, clubs, outings, visits, dating, home entertainment) are impaired.	○	○	○	○	○
Because of my problems, my **private leisure activities** (done alone, e.g., reading, gardening, collecting, sewing, walking alone) are impaired.	○	○	○	○	○
Because of my problems, my ability to **form and maintain close relationships** with others, including those I live with, is impaired.	○	○	○	○	○
Because of my problems, my	○	○	○	○	○

overall ability to **lead a normal life** is impaired.

Q57

How have you been feeling over the past week?

Please read each statement and check the number 0, 1, 2 or 3 which indicates how much the statement applied to you over the past week. There are no right or wrong answers. Do not spend too much time on any statement.

The rating scale is as follows:

0 Did not apply to me at all

1 Applied to me to some degree, or some of the time

2 Applied to me to a considerable degree, or a good part of time

3 Applied to me very much, or most of the time

	0	1	2	3
I found it hard to wind down	○	○	○	○
I was aware of dryness of my mouth	○	○	○	○
I couldn't seem to experience any positive feeling at all	○	○	○	○
I experienced breathing difficulty (e.g., excessively rapid breathing, breathlessness in the absence of physical exertion)	○	○	○	○
I found it difficult to work up the initiative to do things	○	○	○	○
I tended to over-react to situations	○	○	○	○
I experienced trembling (egg, in the hands)	○	○	○	○
I felt that I was using a lot of nervous energy	○	○	○	○
I was worried about situations in which I might panic and make a fool of myself	○	○	○	○
I felt that I had nothing to look forward to	○	○	○	○

I found myself getting agitated	○	○	○	○
I found it difficult to relax	○	○	○	○
I felt down-hearted and blue	○	○	○	○
I was intolerant of anything that kept me from getting on with what I was doing	○	○	○	○
I felt I was close to panic	○	○	○	○
I was unable to become enthusiastic about anything	○	○	○	○
I felt I wasn't worth much as a person	○	○	○	○
I felt that I was rather touchy	○	○	○	○
I was aware of the action of my heart in the absence of physical exertion (e.g., sense of heart rate increase, heart missing a beat)	○	○	○	○
I felt scared without any good reason	○	○	○	○
I felt that life was meaningless	○	○	○	○

Q58 In the last 6 months, have you seen or used any of the following to help you manage your depression, anxiety and/or stress?

Select any/all that apply

- ☐ GP
- ☐ Psychologist
- ☐ Psychiatrist
- ☐ Social worker
- ☐ Mental health or other nurse
- ☐ Religious counsellor
- ☐ Alternative medical professional
- ☐ Support group
- ☐ Meditation or exercise class
- ☐ Online counsellor
- ☐ Online support group, forum, or chat room

- [ ] Internet therapy program
- [ ] Self-help book(s)
- [ ] Mobile app
- [ ] Other

End of Block: Block 8

Start of Block: Block 9

Q59 Thank you for completing our questionnaire.

In order to comply with data protection regulations, all of the information you have provided will be kept anonymous. However, in order to allow us to locate your data (in the event that you wish to withdraw from the study in the future, or if you take part in future related research), we need you to create a unique personal identifier, that only you will know.

To do this, please enter all of the numbers (not the letters) found in your postcode,

followed by the last 4 digits of your mobile phone number (e.g., MK**11 9**PG 078*647**3263** would become: **1193263**).

Q60 Please enter your unique personal identifier below:

_____

End of Block: Block 9

8.2. Reagents used.

Table 18. Reagents used.

NAME	SUPPLIER	SERIAL NUMBER
Agarose	Sigma	A9539
Bactoagar	Stratech	A0930
Bactopeptone	BD Biosciences	211677
Sodium chloride (NaCl)	Sigma	S3014
Potassium phosphate monobasic (KH_2PO_4)	Sigma	P0662
Potassium phosphate dibasic (K_2HPO_4)	Sigma	P3786
Calcium chloride ($CaCl_2$)	Sigma	C3881
Magnesium sulphate (heptahydride) ($MgSO_4 \cdot 7H_2O$)	Sigma	M2773
Ethanol	Sigma	51976
Sodium dodecyl sulphate (SDS)	Sigma	L4390
Glycine	Sigma	47627
Acetic acid	Sigma	A6283
Cholesterol	Sigma	C8503
Bromophenol blue	Sigma	B0126
Xylene cyanol FF	Sigma	X4126
Ficoll	Sigma	F2637
Tween 20	Sigma	P7949
Sodium hydroxide (NaOH)	Sigma	S5881
Deoxyribonucleotide triphosphate (dNTPs)	Fisher	PCR-345-010P
Proteinase K	Sigma	P5568
Potassium chloride (KCl)	Sigma	P9541
Sodium hypochlorite	Sigma	239305
Phusion high fidelity DNA polymerase 2µ/µl	NEB	F530S
SYBR Safe DNA Gel Stain	ThermoFisher	S33102
1Kb plus DNA Ladder - Invitrogen	ThermoFisher	10787018
Tris	Sigma	T1350
Boric acid	Sigma	B6768
Ethylenediaminetetraacetic acid (EDTA)	Sigma	E5134
dithiobis (2-nitrobenzoic acid) – (DTNB)	Abcam	ab138871

Acetylthiocholine	Abcam	ab138871
Acetylcholine	Abcam	ab138871
AChE enzyme assay buffer	Abcam	ab138871
Chlorpyrifos	Sigma	45395

8.2.1. Buffers and Solutions

Nematode growth media (NGM)

17 g/L Bactoagar
2.5 g/L Bactopeptone
3 g/L NaCl
Mix, autoclave then add:
1 mM $CaCl_2$
1 mM $MgSO_4$
0.25 M K_3PO_4
5 µg/ml cholesterol (in ethanol)

Low Peptone NGM

20 g/L Bactoagar
0.13 g/L Bactopeptone
3 g/L NaCl
Mix, autoclave then add:
1 mM CaCl2
1 mM $MgSO_4$
0.25 M K_3PO_4
5 µg/ml cholesterol (in ethanol)

M9 Buffer

0.02 M KH_2PO_4
0.04 M Na_2HPO_4
0.09 M NaCl
0.01 M $MgSO_4$

S. Basal

0.1 M NaCl
0.05 M K_3PO_4 (pH6)
5 mg/L cholesterol

TE Buffer

10 mM Tris (pH8)
1 mM EDTA

TBE Buffer

0.089 M Tris Base
0.089 M Boric acid
2 mM EDTA

Worm Lysis Buffer

130 mM Tris (pH8)
SDS 1% (w/v)
50 mM EDTA
0.1 M NaCl

Single Worm PCR Lysis Buffer

10 mM Tris (pH8)
50 mM KCl
2.5 mM $MgCl_2$
0.45 % (w/v) Nonidet-P40
0.45 % (w/v) Tween 20
0.01 % (w/v) Gelatin
(Autoclaved and stored at 4°C)

Worm Freezing Solution

0.1 M NaCl
0.05 M NaOH
0.05 M KH_2PO_4
300 g/L glycerol
Autoclave then add:
3 mM $MgSO_4$
4 g/L Bacto-agar (dissolved into solution using a microwave)

8.3. Preliminary work on exposure duration

8.3.1. Testing a drug-sensitive, cuticle defective mutant to improve CPF sensitivity.

An initial concern during preliminary development of CPF exposure methodology, was that CPF may not pass through the cuticle, hindering uptake of the compound. To address this proactively, a mutant known for its defective cuticle and heightened drug sensitivity (*bus-8(e2698)*, Partridge et al., 2008) was tested as a potential main test strain, but was rejected due to it's unreliable locomotory behaviour.

At 24 hours after L4 stage, control worms on NGM showed no change in body bend frequency (**Figure 77**). A slight decrease was seen in both solvent control and 0.5 mg/L CPF groups at 48 and 72 hours but this was not significant ($F(2, 171) = 1.902$, ns). A 2-way ANOVA showed that the number of exposure days was the only applied factor that had any significant effect on body bend frequency ($F(2, 171) = 3.131$, $P = 0.0462$), with no significant interaction with treatment ($F(4, 171) = 0.5935$, ns). Importantly, no effect demonstrated here could be specifically attributed to CPF.

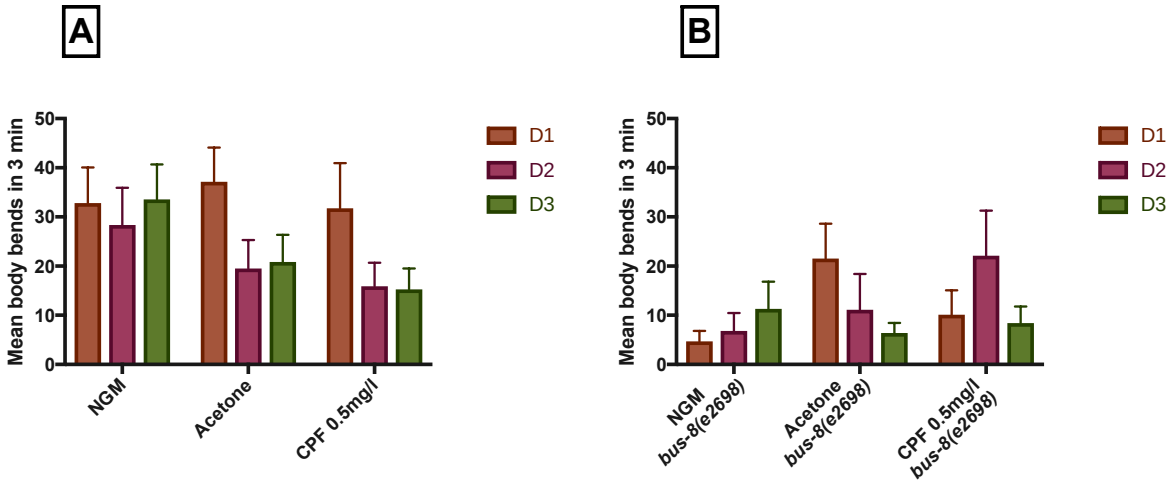

Figure 77. The bus-8 mutant was assessed but excluded as a main test strain. This figure presents preliminary trials of the *bus-8(e2698)* mutant known for its defective cuticle and heightened drug sensitivity (Partridge et al., 2008). Despite these potentially useful traits, its altered locomotory behaviour and the sufficient response of the *n2* wild-type strain to CPF treatment deemed the inclusion of *bus-8* mutant unnecessary for the project.

8.4. The effect of freezing and thawing of CPF stock solution

During the course of the project, it was observed that more consistent results were obtained using CPF stock solution stored at room temperature rather than repeated freeze-thaw cycles. Aliquots were therefore kept in the dark at room temperature; however, this effect was not investigated any further. A representative set of data is shown in **Figure 78**.

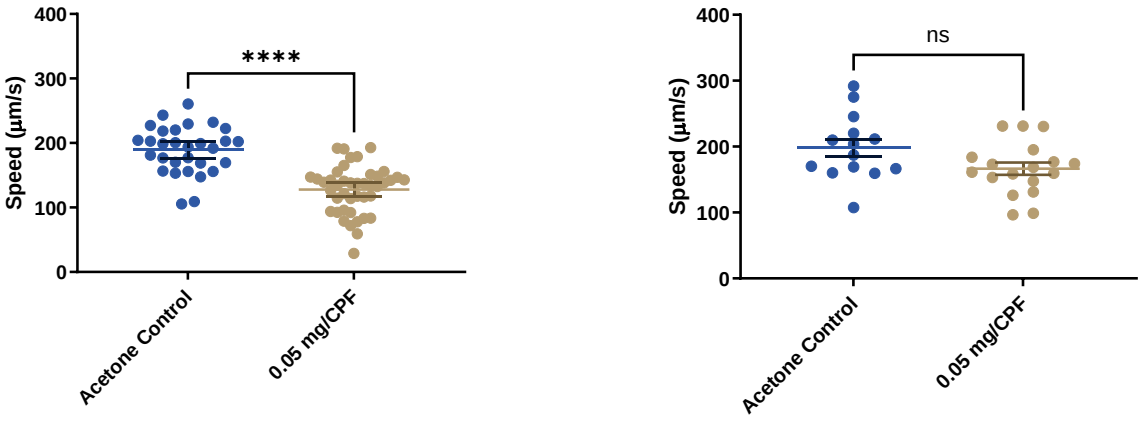

Figure 78. Results were more consistent using CPF stock solution stored at room temperature. Locomotory speed was generally reduced by treatment with 0.05 mg/L CPF in animals that were deprived of food for 30 mins (left-hand-graph) unpaired t test ($t(71) = 7.114$, **** = $P < 0.0001$. $n = 73$). However, some results obtained using stock solution that had been repeatedly freeze-thawed (right-hand graph) did not show a significant effect of CPF treatment ($t(30) = 2.030$, ns = not significant. $n = 32$). Blue circles represent the speed score of individual control animals and tan circles individual CPF treated animals. Bars represent mean with 95% confidence intervals.