UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

Centre for Biological Sciences

Mode of action studies on the nematicide fluensulfone

by

James Kearn

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ABSTRACT

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

SCHOOL OF BIOLOGICAL SCIENCES

Doctor of philosophy

MODE OF ACTION STUDIES ON THE NEMATICIDE FLUENSULFONE

James Kearn

Plant parasitic nematodes (PPNs) place a heavy burden on agriculture throughout the world. This burden is accentuated by a lack of effective and safe methods for the control of these crop pests, with many nematicides having been banned due to unacceptable non-target toxicity. Few alternatives have emerged to fill this gap. Fluensulfone is a newly registered nematicide that has a favourable toxicity profile relative to previously used nematicides. Fluensulfone has proven efficacy in the field against a range of nematode species. The mode of action of fluensulfone is however currently unknown. The aim of this thesis was to investigate the effects of fluensulfone on nematodes and to gain insight into its mechanism of action. *Caenorhabditis elegans* is a model organism with well characterised genetics, neurophysiology and behaviour. *C. elegans* has proven useful in previous mechanism of action studies on anthelmintics. Fluensulfone was found to have nematicidal activity against *C. elegans* but at concentrations ≥100-fold greater than those reported to be effective against the PPN *Meloidogyne javanica*. Fluensulfone affected a number of *C. elegans* behaviours, including locomotion, pharyngeal pumping, egg laying and development. A reverse genetic approach did not identify any probable targets but did indicate that fluensulfone has a novel mechanism of action relative to established anticholinesterase and macrocyclic lactone nematicides. Electrophysiological analysis found that fluensulfone has complex effects on the *C. elegans* pharyngeal system with lower concentrations exciting pharyngeal activity and higher concentrations inhibiting. The pharyngeal effects suggested that fluensulfone might influence feeding behaviour in PPNs. In PPNs, the behaviour of the stylet mouth spear is critical in feeding and host invasion. The action of fluensulfone on the stylet of the PPN *Globodera pallida* was therefore investigated, to validate observations made with *C. elegans*. The pharmacology of the stylet was also investigated, using knowledge of the *C. elegans* pharynx as a guide. The pharmacological regulation of the stylet was found to be similar to the *C. elegans* pharynx, with 5-HT an important regulator. Fluensulfone stimulated stylet activity and blocked 5-HT-stimulated activity. Studies with a 5-HT receptor antagonist on *G. pallida* and *C. elegans* indicated that fluensulfone interacts with 5-HT signalling to stimulate stylet and pharyngeal activity. The concentrations of fluensulfone that had acute effects on both *G. pallida* and *C. elegans* behaviour were still ≥50-fold greater than those reported to have nematicidal activity against PPN species. Immotility assays revealed that 1-30 µM fluensulfone elicited a progressive increase in paralysis over 14 days, leading to death. No such effect occurred in *C. elegans* adults or dauers. Staining with the metabolic marker MTT revealed that fluensulfone is nematicidal and that metabolic impairment may be the cause of this paralysis and death. Nile Red staining indicated that lipid consumption is reduced in the presence of fluensulfone. Fluensulfone also profoundly inhibited *G. pallida* hatching from cysts. The effects of fluensulfone on *G. pallida* physiology and metabolism require further investigation via rigorous measures such as oxygen consumption. If metabolic impairment is identified as the causative agent of fluensulfone nematicidal activity this must be further studied through metabolomics and other techniques.

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DECLARATION OF AUTHORSHIP

I, James Kearn, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
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3. Where I have consulted the published work of others, this is always clearly attributed;
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Signed: James Kearn

Date: 30/09/15

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Abbreviations

1, 3-D = 1, 3-dichloropropane

5-HT = 5-Hydroxytryptamine/ serotonin

5-MeO-DMT = 5-methoxy-N, N dimethyltryptamine

ACh = Acetylcholine

AChE = Acetylcholinesterase

ADP = Adenosine diphosphate

ATP = Adenosine triphosphate

ANOVA = Analysis of Variance

BSA = Bovine Serum Albumin

^{o}C = Degrees Celsius

cAMP = 3'-5'-cyclic adenosine monophosphate/ Cyclic AMP

CGC = *Caenorhabditis elegans* genetics centre

CN = Cyst Nematode

DCBP = Dibromochloropropane

DCMP = 2,5-dichloro-4-methoxyphenol

ddH_2O = Double distilled water

dH_2O = Distilled water

DiS-C_3(3) = 3,3'-dipropylthiocarbocyanineiodide

DMSO = Dimethyl sulfoxide

EDB = Ethylene dibromide

EMS = Ethyl methanesulfonate

EPG = Electropharyngeogram

EPSP = Excitatory post-synaptic potentials

FAD = Flavin adenine dinucleotide

Flu = Fluensulfone

FITC = Fluorescein isothiocyanate

FLP = FMRFamide-like peptide

Fluensulfone/ MCW-2 = 5-chloro-2-(3,4,4-trifluorobut-3-enylsulfonyl)-1,3-thiazole

G-protein = Guanine nucleotide-binding proteins

GABA = γ-aminobutyric acid

GFP = Green fluorescent protein

GluCl = Glutamate-gated chloride channel

GPCR = G-protein-coupled receptor

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

HPLC = High performance liquid chromatography

IPSP = Inhibitory post-synaptic potentials

IVM = Ivermectin

J1 = First-stage juvenile

J2 = Second-stage juvenile

J3 = Third-stage juvenile

J4 = Fourth-stage juvenile

L1 = Larval stage one

L2 = Larval stage two

L3 = Larval stage three

L4 = Larval stage four

LB = Luria Bertani (Broth/ Agar)

L4+1 = Larval stage four worms plus one day old

LD_{50} = Dose required for 50% lethality

M = Molar

mm = Millimetre

mM = Millimolar

MS = Mass spectrometry

MTT = (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide)

mV = Millivolt

nAChR = Nicotinic acetylcholine receptor

NAD = Nicotinamide adenine dinucleotide

NADP = Nicotinamide adenine dinucleotide phosphate

NGM = Nematode Growth Medium

NLP = Neuropeptide-like protein

nM = Nanomolar

NMJ = Neuromuscular junction

PCN = Potato cyst nematode

PPNs = Plant parasitic nematodes

RKN = Root Knot Nematode

RNAi = RNA interference

SAGE = Serial analysis of gene expression

s.e = Standard Error

Spp. = Species

SSRI = Selective serotonin reuptake inhibitor

TCA cycle = Tricarboxylic acid cycle

µm = Micrometre

µM = Micromolar

UV = Ultraviolet light

WHO = World Health Organisation

WT = Wild type

List of species names

A. duodenale = Ancyclostoma duodenale

A. besseyi = Aphelenchoides besseyi

A. fragariae = Aphelenchoides fragariae

A. thaliana = Arabidopsis thaliana

A. suum = Ascaris suum

A. lumbricoides = Ascaris lumbricoides

B. longicaudatus = Belonolaimus longicaudatus

B. xylophilus = Bursaphalenchus xylophilus

C. briggsae = Caenorhabditis briggsae

C. elegans = Caenorhabditis elegans

C. xenoplax = Criconemella xenoplax

D. dipsaci = Ditylenchus dipsaci

D. melanogaster = Drosophila melanogaster

E. coli = Escherichia coli

G. pallida = Globodera Pallida

G. rostochiensis = Globodera rostochiensis

H. glycines = Heterodera glycines

H. schachtii = Heterodera schachtii

M. hapla = Meloidogyne hapla

M. incognita = Meloidogyne incognita

M. javanica = Meloidogyne javanica

P. penetrans = Pratylenchus penetrans

P. thornei = Pratylenchus thornei

T. dubius = Tylenchorhynchus dubius

X. index = Xiphinema index

Chapter 1: General Introduction

1.1 The nematode burden on humanity

Nematoda is one of the most diverse of all the animal phyla, with nematode species having conquered almost every available ecological niche and climate on the planet, ranging from the sub-zero conditions of arctic sea ice, to the hostile sulphur-rich conditions in deep sea sediments (Tchesunov and Riemann, 1995, Nussbaumer et al., 2004). Phylum Nematoda is also the most successful in terms of animal numbers, with nematodes accounting for four in every five animals in the world. There are over 25,000 described nematode species (Zhang, 2013) although it is estimated that this could be nearer 1 million (Lambshead and Boucher, 2003). Of the identified 25,000 species, around 40% are parasites (Maggenti, 1981) and they are collectively able to parasitise almost every other species of animal and plant on the planet (Blaxter, 2011).

Animal parasitic nematodes, such as *Ascaris lumbricoides* and *Ancyclostoma duodenale*, are estimated to infect 1 billion people worldwide, predominately in tropical regions and the developing world (WHO, 2005). Infestation with parasitic nematodes is severely debilitating for the afflicted and can lead to severe morbidity, anaemia, blindness, disfigurement and retardation of mental and physical development (Bethony et al., 2006). Parasitic nematodes are prevalent in the world's poorest and are a significant barrier to economic development (WHO, 2005). Indeed, according to some estimates the collective disease burden of animal parasitic nematodes may be as great as malaria and tuberculosis (Chan, 1997, WHO, 2002, WHO, 2005). Animal parasitic nematodes are also prevalent in livestock and thus through reduction of productivity place further economic burdens on the human population (Jasmer et al., 2003).

Nematodes have further deleterious effects on agricultural productivity through infection of plants and crops. There are over 4000 nematode species that parasitise plants and every crop plant grown by mankind is vulnerable to infection by at least one species of plant parasitic nematode (PPN) (Bridge and Starr, 2007). There are few efficacious methods for PPN control (Fuller et al., 2008, Thomason, 1987). Furthermore, their biology permits the ability to enter a facultative diapause to survive harsh conditions (Blaxter and Bird, 1997), which adds to the difficulty of managing PPN infestations in the field. As a result, PPNs have a significant economic impact. Annual yield losses directly attributable to PPNs are estimated at $125 billion

(Chitwood, 2003b), resulting from a 12.3% yield reduction in the major human food staples (Sasser and Freckman, 1987). Such estimates fail to take into account the increased host plant susceptibility to other pathogens that results from PPN infection or the role of PPNs as direct vectors for plant viruses (Chen et al., 2004). Many of the chemical agents that have been used to control PPNs have been banned, are being phased out of use or are strictly regulated (Haydock et al., 2014). It is also possible that as the list of available nematicides grows smaller there is an increased potential for PPN strains arising that are resistant to the chemicals used against them (Meher et al., 2009). When these factors are considered with the rising global demand for food that accompanies population rise and economic development, it can be expected that crop losses resulting from PPNs will become a more significant problem in the future. It is therefore imperative that new methods of PPN control are found.

Fluensulfone (ADAMA agricultural solutions Ltd.) is a newly registered nematicide, which has a favourable toxicity profile relative to currently and previously used nematicides, acting more selectively against PPNs (see section 1.10). Fluensulfone has proven effectiveness as a nematicide, with activity against a number of the most damaging of PPNs (Oka et al., 2008, Oka et al., 2009, Oka et al., 2012, Oka et al., 2013, Oka, 2014). Currently, the molecular target of fluensulfone in nematodes is not known. It has been suggested that the mode of action of fluensulfone is likely to be distinct from previously used nematicides such as the organophosphates and carbamates, due to its distinctive effects on PPNs (Oka et al., 2009). Understanding the mechanism of action of fluensulfone is desirable due to its apparent selective toxicity. Identifying its molecular target would enable further research into other safer alternative chemicals for PPN control. The overall aim of this thesis was to investigate the effects of fluensulfone on nematodes and to attempt to uncover its molecular target(s).

This introduction will describe the biology and ecology of PPNs, including their interaction with plants and the damage that they can cause, with a particular focus on the potato cyst nematode *Globodera pallida*, used here for mode of action studies. The history and current state of PPN control through nematicides and other treatments will also be discussed. Fluensulfone will be introduced and the available literature describing its effects and effectiveness. Finally, the use of the model nematode *C. elegans* for mode of action studies will be discussed and the relevant aspects of PPN

and *C. elegans* biology will be introduced. As many anthelmintics and nematicides are known to target neural signalling pathways (see (Holden-Dye and Walker, 2014) for review), PPN and *C. elegans* neurobiology will be discussed. Some nematicides are also known to or thought to affect nematode metabolic pathways (Wade and Castro, 1973, Castro and Belser, 1978, Wright, 2011, Veloukas and Karaoglanidis, 2012, Hungenberg et al., 2013) and so what is known of nematode metabolism will also be addressed.

1.2 The phylum *Nematoda*

Nematodes (phylum *Nematoda*) (De Ley and Blaxter, 2002) are unsegmented worm-like animals that are described as pseudocoelomates, as they possess a fluid-filled body cavity that lies inside the body wall that bathes the internal organs (Bird and Bird, 1991). This pseudocoelom is distinct from the "true" coelomic cavity of vertebrates. Nematodes occupy many ecological niches. Many nematodes are free-living, feeding upon bacteria or other small organisms. Other nematodes parasitise animals and plants and have life cycles that utilise a diverse range of biological specialisations (Lee, 2002).

Although nematodes have diverse lifestyles, their basic body plan is relatively conserved (Figure 1.1). The nematode body consists of an internal cylinder, the digestive system, which is surrounded by the pseudocoelomic cavity that separates the inner cylinder from the outer cylinder and the body wall (Campbell and Reece, 2008). The body wall is made up of the musculature, the epidermis and the outer body cuticle. The majority of nematodes remain worm-like throughout their life cycle. Exceptions to this include sedentary endoparasitic PPNs, which swell into a ball-like shape on reaching adulthood (see section 1.6). The nematode life cycle consists of an egg, four larval stages (L1, L2, L3, L4 or J1, J2, J3, J4) and the adult stage, which is reproductively viable (Lee, 2002). The nematode undergoes moults between all the larval stages and the adult stage.

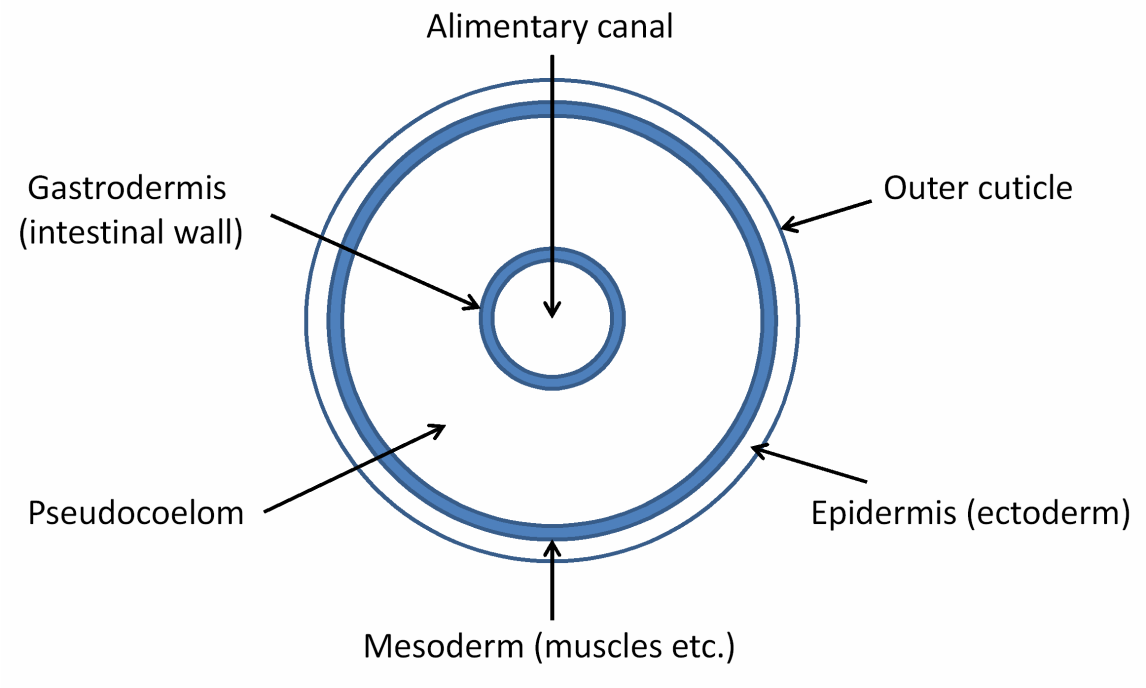

Figure 1.1 The body plan of the phylum nematoda. The alimentary canal is enclosed by the gastrodermis or intestinal wall. A fluid-filled cavity, the pseudocoelom, separates the intestines from the body wall, which consists of muscle, the epidermis and the outer cuticle.

1.3 Plant parasitic nematodes are an important agricultural pest around the world

Around 15% of all nematodes are obligate parasites of plants and are capable of feeding on most parts of vascular plants, including stems, leaves and roots (Fuller et al., 2008). It is those species that attack the roots of plants and other belowground structures, including tubers and bulbs that cause the greatest reduction in yield and place the highest burden on agriculture (Perry and Moens, 2013). This introduction will therefore focus on root parasitic nematodes.

PPNs require a host plant for their survival and propagation. Some species only require a host for feeding to allow growth, whereas other species must enter the host for survival and to allow reproduction to occur (see section 1.4). It is PPN feeding that harms the host plant, as nutrients are drawn from host and into the nematodes. Typically, many hundreds or thousands of PPNs will attack or infect one host plant and it is this sheer weight of numbers that has such an impact on crop yield (see section 1.9.1). Infection does not usually lead to the death of the host, unless another pathogen is introduced into the plant by the parasite. PPN infection can cause ulcerations which

may spread across an entire root section, possibly leading to root death and eventual host death (Decker, 1989). The main symptom of PPN infection however is stunted growth of the host plant through the disruption of osmotic transport and the diversion of nutrients to the nematode (Figure 1.2) (Fuller et al 2008). This diversion of nutrients can result in chlorosis. PPN infection can also impair CO_2 and nitrogen fixation in host plants and hence reduce photosynthetic ability (Bird and Loveys, 1975, Huang, 1987). Root infection is also associated with root galling, retardation of root growth and abnormal root growth (Decker, 1989).

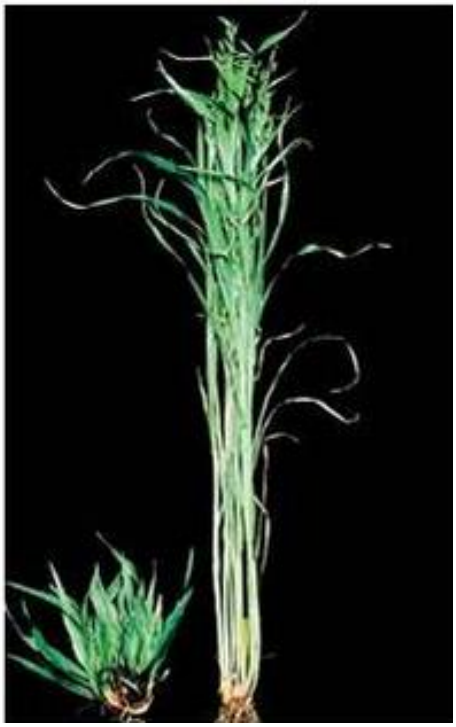

Figure 1.2. PPN infection causes stunted plant growth. The left oat plant is infested with the stem nematode *Ditylenchus dipsaci*, resulting in severe dwarfing, relative to the uninfected control on the right. From (Luc et al., 2005).

1.4 Plant parasitic nematode life cycle

The most distinguishing feature of the majority of plant parasitic nematodes (PPNs) is the stylet, a protractible, hollow mouth-spear that is crucial for the PPN life cycle, in particular hatching, host invasion and feeding (Decker, 1989). The length and type of stylet varies between species and stylet morphology is closely linked to the life strategy adopted by the nematode (see section 1.5).

PPNs can be divided into 3 groups based upon their mode of parasitism: ectoparasites, endoparasites and semi-endoparasites (Campbell and Reece, 2008). Ectoparasites feed from outside the host, remaining in the soil and never entering the host plants tissue (Jasmer et al., 2003). Endoparasites completely enter the host tissues, whilst semi-endoparasites only partially enter the root (Perry and Moens, 2011). Both ectoparasites and endoparasites can also be described as migratory or sedentary, with migratory nematodes feeding for short periods at multiple sites whereas sedentary nematodes remain at one site for extended periods (Perry and Moens, 2013).

1.4.1 Ectoparasites

Ectoparasitic PPNs such as *Longidorus* spp. feed by inserting their hollow stylet into the epidermal cells of the root or deeper into the tissue (Figure 1.3) (Wyss, 1997). Stylet length will vary between ectoparasitic PPN species and so different species will feed on different areas of the root (Bridge and Starr, 2007). Those bearing a shorter stylet feed predominately on the root epidermal cells and sometimes the outer cortical cells whereas those possessing a longer stylet feed on cells that are deeper within the root cortical tissue. The four juvenile stages and the adult stage of ectoparasitic PPNs are all vermiform and are all capable of feeding. Migratory ectoparasites such as *Belonolaimus spp*. and *Xiphinema spp*. feed for a relatively short time, usually at several sites on the root (Wyss, 1997, Tytgat et al., 2000), whereas sedentary ectoparasites such as *Paratylenchus spp*. feed on one cell for an extended period (Rhoades and Linford, 1961, Hussey et al., 1992). Some ectoparasitic PPNs, such as *Xiphinema spp.*, act as vectors for potentially devastating plant viruses such as tomato ringspot and can remain infective for months after acquiring the virus (Chen et al., 2004).

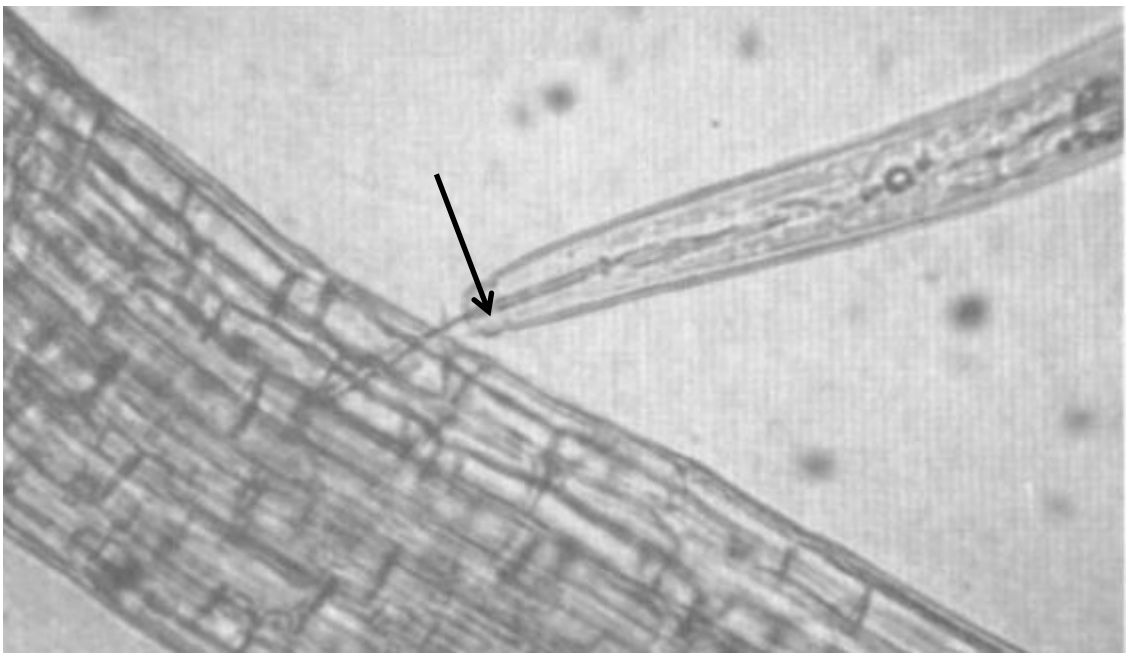

Figure 1.3. The ectoparasitic sting nematode, *Belonolaimus longicaudatus*, feeding on corn roots. The stylet (as indicated by the arrow) is inserted into the host root tissues to allow ingestion of host materials (From (Huang and Becker, 1997).

1.4.2 Migratory endoparasites

Migratory endoparasites feed within the root yet they can leave it at any point during their lifecycle (Tytgat et al., 2000). The juvenile stages and adult stage of most migratory endoparasites are capable of penetrating and feeding on roots. The movement of migratory endoparasites throughout the root system can cause extensive damage, killing a large number of cells (Bridge and Starr, 2007) and can increase host susceptibility to other pathogens (Zunke, 1990, Abawi and Chen, 1998). Examples of migratory endoparasites are the lance nematodes such as *Ditylenchus spp*., the lesion nematodes of the *Pratylenchus* genus (Bridge and Starr, 2007).

1.4.3 Sedentary endoparasites – Root-knot nematodes and cyst nematodes

Sedentary endoparasitic PPNs are reputed to be the most damaging of all PPNs and have the most evolutionarily sophisticated method of parasitism. The most well-known of these are root-knot nematodes (RKNs) of the *Meloidogyne* genus and the cyst nematodes (CNs) of the *Heterodera* and *Globodera* genera (Holden-Dye and Walker, 2011). Nematodes of the *Meloidogyne* genus are known as root-knot nematodes due to the characteristic root galls that form following infection (Figure 1.4). Root galling

results from hyperplasia and hypertrophy of tissues surrounding the nematode (Perry and Moens, 2011). RKNs lay their eggs into a gelatinous matrix that is secreted by the adult female, which shrinks and hardens when dry and prevents hatching in desiccated conditions (Bird and Soeffky, 1972). *Meloidogyne spp.* are perhaps the most damaging of all PPNs due to their polyphagous nature: the four most common *Meloidogyne spp.* are capable of parasitising nearly all crop plants across their combined host range (Abad et al., 2003).

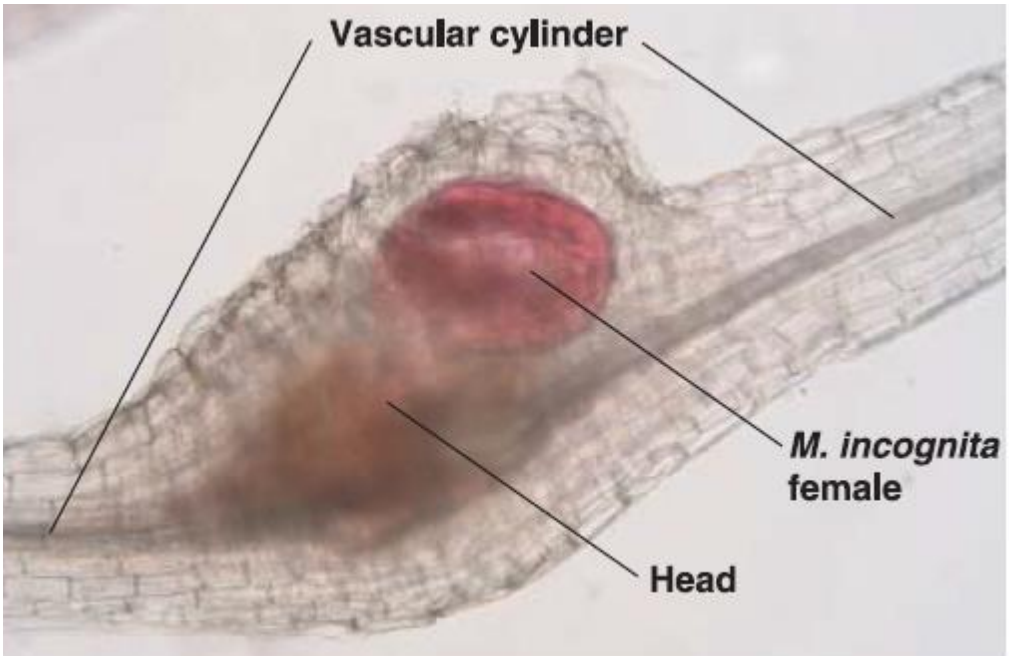

Figure 1.4. A female root-knot nematode, *Meloidogyne incognita*, parasitising an *Arabidopsis thaliana* root. The female has been stained with acid fuchsin. From (Fuller et al., 2008).

The term cyst nematode describes the encysted adult females that erupt from the roots after reproduction has occurred. These cysts contain eggs, which on hatching will emerge from openings at the vulval and neck regions of the cyst. Cyst nematodes also invade the host at the J2 stage. Once the juvenile has developed into an adult female and the female is fertilised by a male, the female lays and retains eggs. After egg laying, the female dies and the outer cuticle hardens to form a protective cyst that encloses the eggs. Such cysts can remain in the soil for many years with the eggs remaining dormant until suitable conditions arise (see section 1.7). The general features of the RKN and CN life cycles are shown in Figure 1.5.

Most sedentary endoparasites are only infective as 2nd stage juveniles (J2) and this is the stage at which they hatch, locate the host plant and invade roots (Perry and Moens, 2011). The J2 stage of RKNs and CNs is non-feeding, resistant to environmental stresses and is specialised for dispersal. The J2 stage of RKNs and CNs has been compared to the dauer stage of free-living nematodes, a specialist survival stage that is induced during normal development by stressful environmental conditions (Bird and Opperman, 1998). The dauer stage of *C. elegans* for example, has a thicker, more hydrophobic cuticle and is non-feeding, with a closed mouth aperture and non-functional pharynx (Costa et al., 2007). In addition, the dauer stage has reduced overall metabolism relative to non-dauer *C. elegans* and utilises alternative pathways such as the glyoxylate cycle that allow generation of carbohydrates from lipids in the absence of any nutrient intake (O'Riordan and Burnell, 1990, Holt and Riddle, 2003). As such, dauers are reliant upon lipids reserves. J2 CN and RKNs are also reliant upon lipid stores and are also thought to employ pathways such as the glyoxylate cycle (see section 1.17).

Once within the root (see section 1.6.2 for host localisation), the J2 establishes a fixed feeding site and induces the formation of nurse cells or syncytia. The nematode's nutrient supplies comes from these specialised feeding sites and the worm then remains sedentary, developing to the rotund adult form that loses its vermiform shape (Figure 1.5). RKN gender is largely determined by environmental factors (Triantaphyllou, 1973). Under favourable conditions, RKNs will typically develop into egg-laying females, which reproduce via mitotic parthenogenesis (Bridge and Starr, 2007). When resources are limited or the host plant is stressed, more juveniles will enter the male developmental pathway (Triantaphyllou, 1973). It is known that damage to the host plant, high nematode density or the presence of host resistance genes can all increase the number of males developing (Davide and Triantaphyllou, 1968, Davide and Triantaphyllou, 1976, Moura et al., 1993). It is believed that sexual fate is determined around the time of giant cell induction by the feeding J2 (Triantaphyllou, 1973). At the J4-to-adult moult, the RKN will develop either to a sessile, bloated female or to a vermiform and motile male. The male will emigrate from the root to locate a female (Eisenback, 1985).

1.4.4 The cyst nematode life cycle

CNs reproduce sexually and will develop into both male and female. As with RKNs, gender is determined at the J2 stage. Once established within the root, the female CN cannot leave whereas males are capable of leaving and locating a partner with which to mate (Figure 1.5). Males are non-feeding and leave the root in search of a female although they can only survive in the soil for a short time.

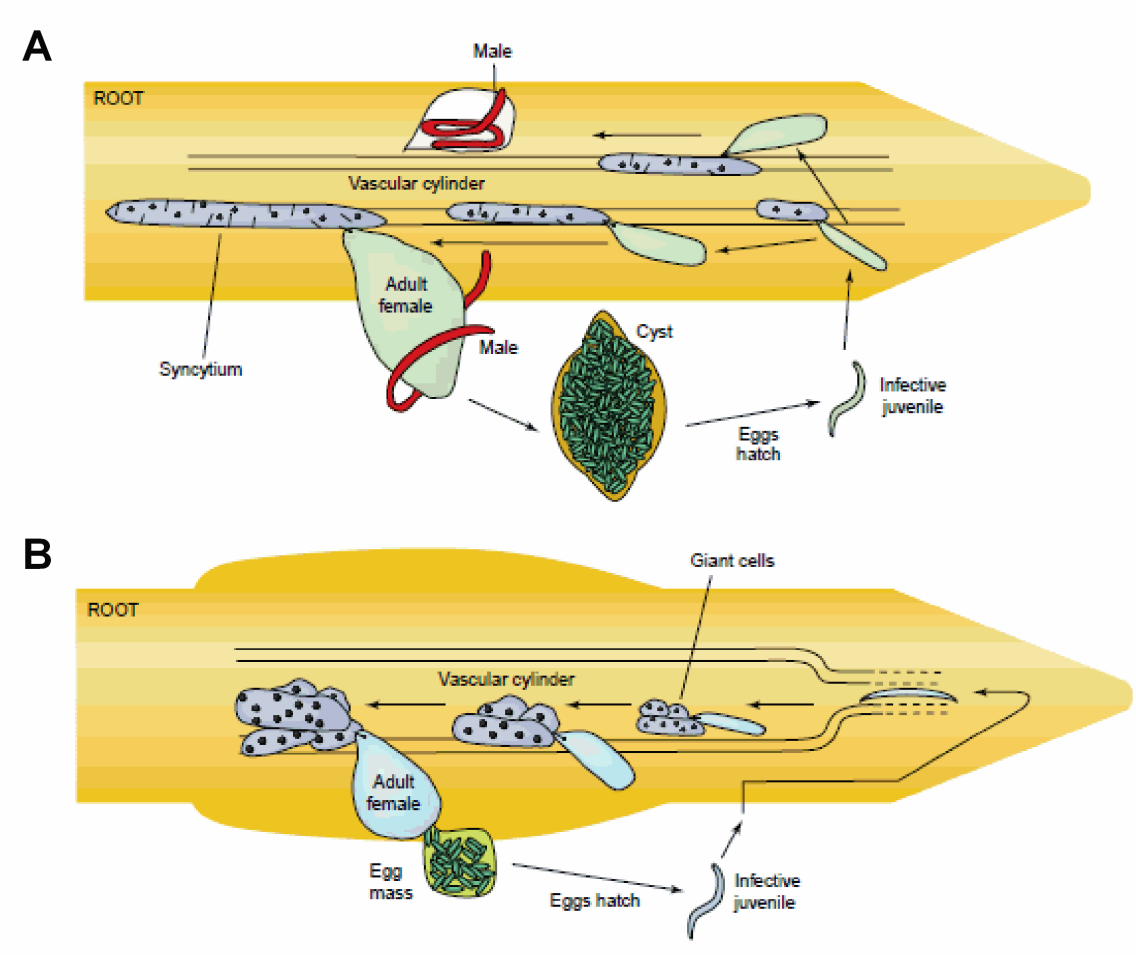

Figure 1.5. The life cycles of cyst nematodes and root-knot nematodes. **A**) J2 CNs establish a syncytium feeding site on entering the host. CNs differentiate into males and females and reproduce sexually. The male leaves the root to locate a female for mating. Once mating has occurred, the female retains the fertilised eggs, dies and becomes a cyst from which the eggs hatch. **B**) After invading the host, J2 RKNs enter the vascular cylinder near the root tip and establish giant cells from which to feed. RKN infection results in the swelling of the root tissues. The adult female reproduces by mitotic parthenogenesis and lays her eggs into an egg sac outside the root. Adapted from (Williamson and Gleason, 2003).

The pregnant female will retain its eggs and on dying, it metamorphoses into a cyst, as the outer cuticle thickens and tans to provide protection to the unhatched eggs from environmental stresses such as drought and temperature (Bridge and Starr, 2007). The J2s that hatch from the eggs will eventually rupture out of the protective cyst. Infection by CNs can be identified by the cysts that appear on the root surface once the females have laid their eggs.

1.5 The stylet and pharynx of plant parasitic nematodes

1.5.1 The structure and evolution of the stylet

As mentioned, the stylet is crucial to the PPN life cycle. The stylet is a cuticular specialisation that is in essence a mouth spear that can protract and retract and is crucial for hatching, host invasion and feeding. The basic stylet structure is a cylindrical shaft with a swelling at the posterior that attaches to the pharyngeal system. The stylet has a lumen through which materials can be ingested and through which secretions and enzymes can be ejected (Figure 1.8) (Perry and Moens, 2013).

The stylet has evolved independently on three occasions for each of the three major orders of plant parasitic nematodes (Figure 1.6) (Luc et al., 2005). In the order Tylenchida, which includes *Meloidogyne* and *Globodera*, the spear is known as the stomatostylet; in the order Dorylaimida, which includes *Longidorus*, the spear is known as the odontostylet ; in the order Triplonchida, which includes *Trichodorus*, the spear is known as the onchiostylet (Luc et al., 2005). For tylenchids, the stomatostylet is a cuticular tube which consists of an anterior conus with a ventral aperture attached at its posterior to a cylindrical shaft, the posterior of which typically swells to form three basal knobs (Baldwin et al., 2004a). The dorylaimid odontostyle consists of the anterior odontostyle, which has an anterior aperture, and a posterior odontophore, which supports the odontostyle and allows attachment of the stylet protractor muscles. The onchiostylet consists of an anterior onchium which is supported at the posterior by the onchiophore. The onchiostylet has a slight ventral curve, unlike the other stylet types. The stylet of all PPNs is constructed with a distinct type of cuticle (Ragsdale et al., 2008).

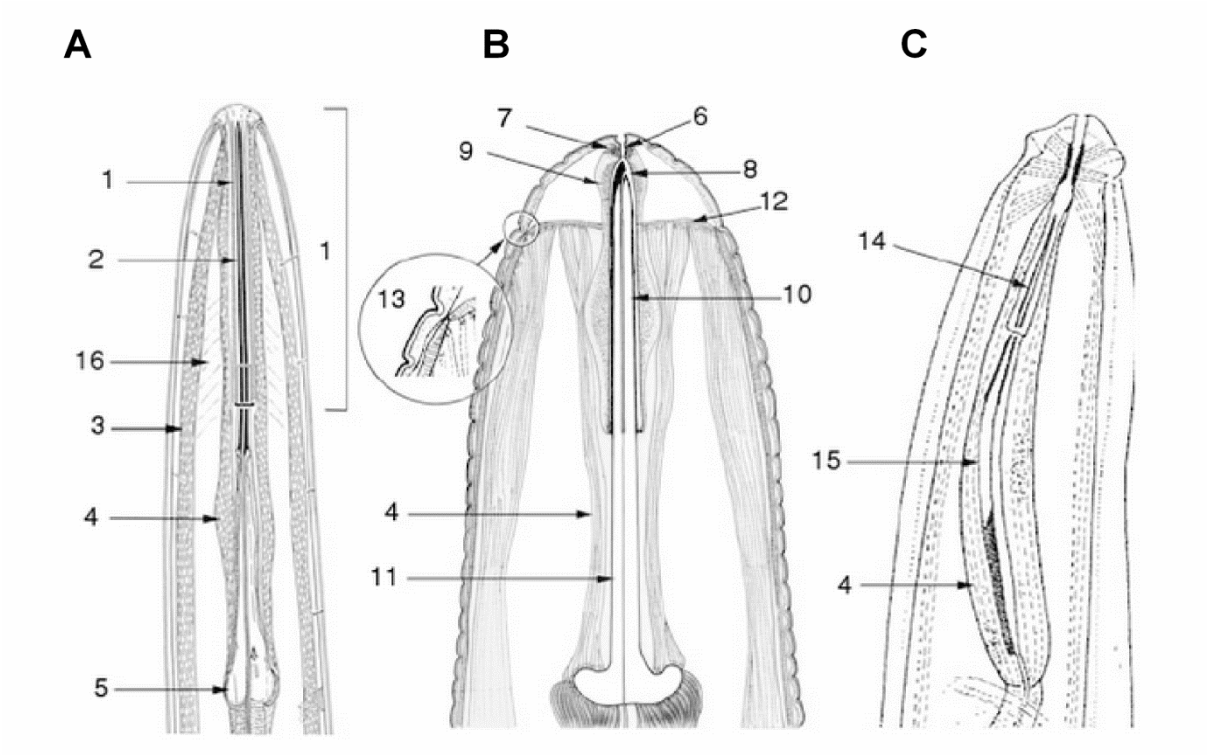

Figure 1.6. Morphological variation in the stylet and head regions of plant parasitic nematodes. Dorylaimid nematodes possess an odontostyle (**A**), tylenchid nematodes possess a stomatostylet (**B**) and triplonchid nematodes possess an onchiostylet (**C**). The numbers in the diagrams indicate the following morphological features: 1) cheilostome, 2) odontostyle, 3) somatic musculature, 4) stylet protractor muscles, 5) odontophore, 6) pre-stoma, 7) cuticular thickening around pre-stoma, 8) stylet opening, 9) stoma, 10) stylet conus, 11) stylet shaft and stylet knobs, 12) basal cephalic framework, 13) body cuticle, 14) onchium of the onchiostyle, 15) onchiphore of onchiostyle, 16) *dilatores buccae* muscles. Adapted from (Perry and Moens, 2013).

The different stylet types are moved through the activity of the stylet protractor muscles, which attach to the basal knobs of the stomatostylet, the odontophore of the odontostyle and the onchiophore of the onchiostylet (Figure 1.6) (Decraemer and Hunt, 2006, Perry and Moens, 2013). These muscles are required for the protrusion of the stylet outwards (Figure 1.7). Typically, the anterior of the protractor muscles are attached to the labial basal lamina. As the protractor muscles contract the stylet knobs are pulled towards the labial region and the stylet extends. In general, PPNs of the Dorylaimida and Triplonchida orders also possess stylet retractor muscles that pull the stylet back to withdraw it (Jairajpuri and Ahmad, 1992, Decraemer and Hunt, 2006). The presence and number of these retractor muscles can however vary between species. PPNs of the Tylenchida order on the other hand do not possess stylet retractor muscles, rather when the stylet protractor muscles relax the protruding stylet is withdrawn due to tension in the alimentary tract (Decraemer and Hunt, 2006).

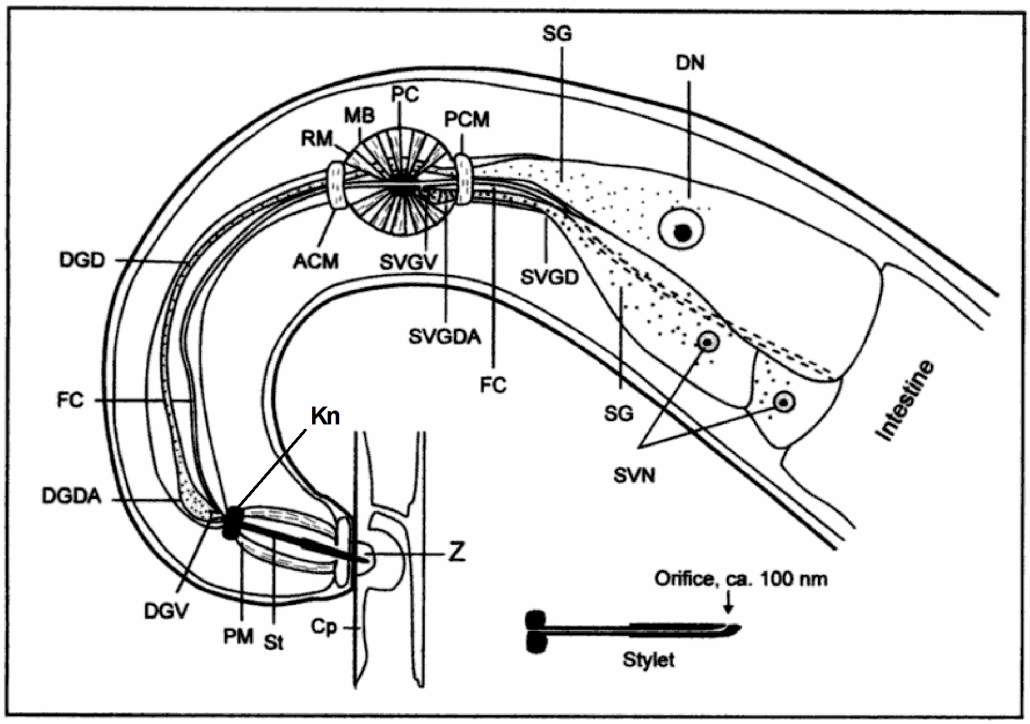

Figure 1.7. The stomatostylet and pharynx of a tylenchid nematode. (example shown is the migratory ectoparasite *Tylenchorhynchus dubius* feeding on a root hair). Abbreviations (from worm anterior to posterior): Z=Modified zone of the root cytoplasm surrounding the stylet tip, St=Stylet, PM=Stylet protractor muscles, Kn=Stylet Knob, DGV=Dorsal gland valve, DGDA=Dorsal gland duct ampulla, FC=Food canal, DGD=Dorsal gland duct, ACM=Anterior constraining muscles of median bulb, RM=Radial muscles, MB=Median/ metacorpal bulb, PC=Pump chamber, SVGV=Subventral gland valve, SVGDA=Subventral gland duct ampulla, PCM=Posterior constraining muscles, SVGD=Subventral gland duct, SG=Secretory granules, DN=Dorsal gland nucleus, SVN=Subventral gland nuclei. Picture taken from (Wyss, 1997).

In tylenchid nematodes like *Meloidogyne* and *Globodera spp*., the posterior of the stylet attaches to the pharynx. At its anterior, the pharynx is known as the procorpus, a narrow cylinder that expands out to form a muscular swelling, the median bulb, sometimes known as the metacorpal bulb (Figure 1.7, Figure 1.8). The median bulb narrows into the isthmus, which at its posterior expands into a glandular region, or a basal bulb (Siddiqi, 2000). Members of the Tylenchida order all possess 3 glands at this glandular region: two subventral glands and one dorsal (Tytgat et al., 2000). These glands typically overlap the intestine or are present in the basal bulb that adjoins to the intestine. These glands are single cells that are sometimes binucleate and each has a long cytoplasmic extension that is connected to the pharyngeal lumen (Tytgat et al., 2000, Robertson and Wyss, 1979, Endo and Wergin, 1988, Endo, 1984). From the base of the stylet, through the pharynx and to the pharyngeal-intestinal junction runs the pharyngeal or oesophageal lumen, through which glandular secretions pass, as does ingested food (Luc et al., 2005).

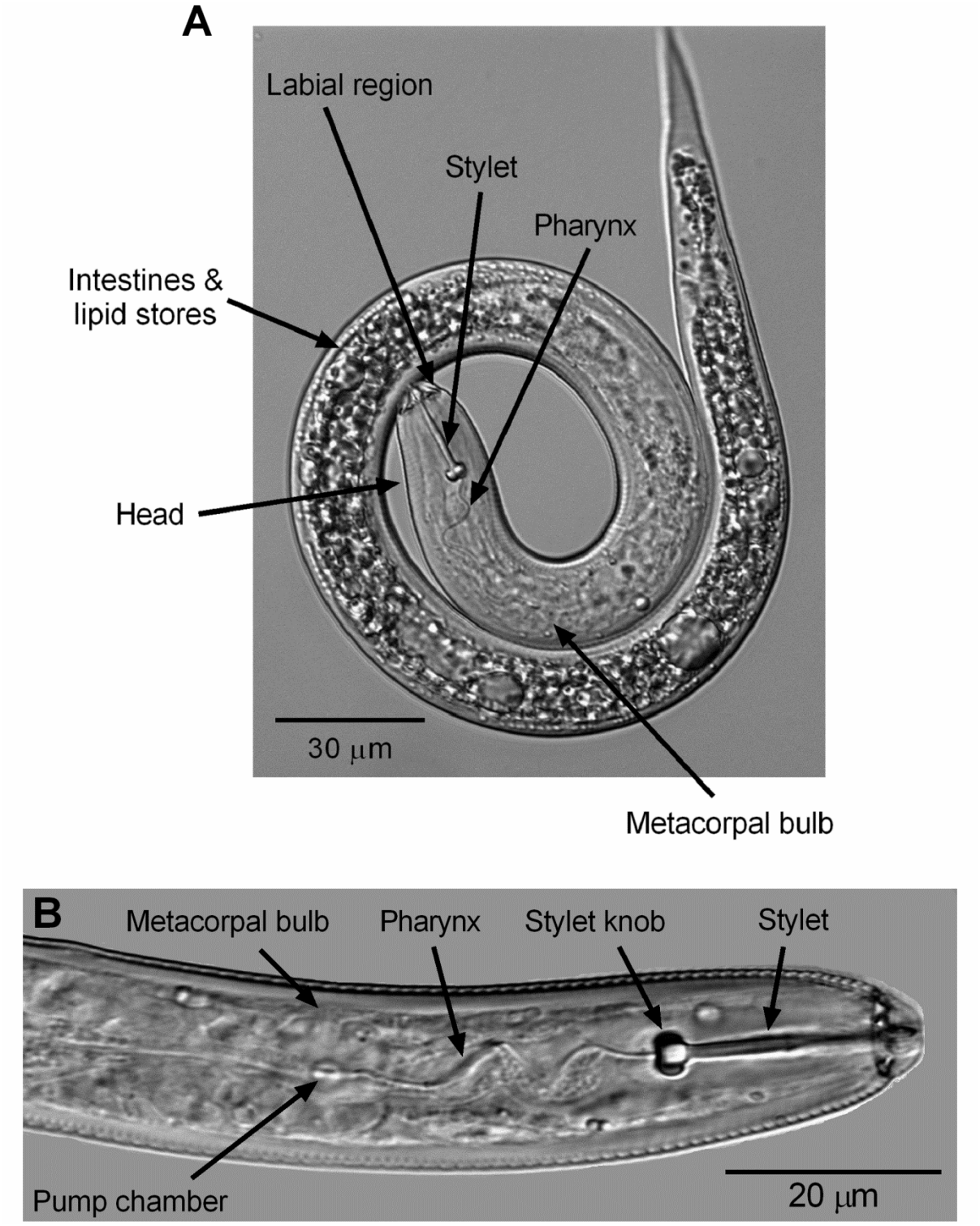

Figure 1.8. Representative images of a *Globodera pallida* J2 juvenile and its feeding apparatus. A) A second stage juvenile (J2) *G. pallida*. The head region can be identified by the stylet and other pharyngeal structures. The darker materials that are in the tail are lipid stores and the undeveloped intestines (as indicated with arrows). **B**) The stylet and associated feeding structures of *G. pallida*. The pharynx is attached to the stylet and pumping of the metacorpal bulb can allow both ingestion of plant materials and the secretion of various enzymes involved in host invasion and feeding.

1.5.2 The roles of the stylet in the parasitic life cycle

The stylet of PPNs is used for the mechanical piercing of host tissues during root invasion and the eggshell during hatching (Perry, 2002, Perry and Moens, 2013). The stylet also functions to expel nematode secretions that are released during parasitism and nematode hatching. Another role of the stylet is the ingestion of host plant materials during feeding. During feeding, the metacorpal pharyngeal bulb pulsates and pumps to suck host materials into the pharynx. The metacorpal bulb also exhibits activity during host invasion, migration through the host and whilst the nematode establishes a feeding site. This is believed to allow the secretion from the stylet of various enzymes that are critical in these steps. The subventral and dorsal glands generate these enzymatic secretions (see section 1.6).

1.6 The life cycle and behaviour of root-knot nematodes and cyst nematodes

1.6.1 Hatching and the pre-J2 stages

For both RKNs and CNs, after embryogenesis the first juvenile moult occurs within the egg to give the J2 stage (Perry and Moens, 2013). It is during this J1-J2 moult that the stylet is generated. The J2 is the infective stage of most sedentary endoparasitic nematodes (Figure 1.8). Most RKN J2s can hatch in the presence of water alone, although chemical cues from host plants (see section 1.7) have been reported to enhance hatching of species such as *M. hapla* (Perry, 2002). This reflects the polyphagous nature of most RKNs, as there is a high probability that a suitable host plant will be available for the hatched J2 to infect. Hatching of RKNs can however be highly dependent upon external temperature (Goodell and Ferris, 1989).

In contrast, many CNs are strongly reliant on the presence of host plants to induce their hatching, particularly *Globodera pallida*, and *G. rostochiensis* (Forrest and Perry, 1980, Perry, 2002). This requirement for cues from host plants to enable hatching is an important survival strategy that results from the narrow range of host plants that some CNs can successfully parasitise. Once hatched, the CN J2 leaves the cyst either via an

opening where the head of the encysted female has deteriorated or via an opening at the fenestral region (Castillo, 2010).

1.6.2 Locating suitable host plants

Once hatched, the J2 must locate a suitable host plant. As the infective J2 is non-feeding, it must rely on stored energy reserves, primarily neutral lipids and glycogen, until reaching a host plant (Cooper and Van Gundy, 1970, Cooper and Van Gundy, 1971b, Dropkin and Acedo, 1974). Lipid reserves are almost totally depleted at 3-4 weeks post-hatching and this depletion has been correlated with reduced motility and infectivity (Reversat, 1981). RKN J2s are believed to locate host plants via attractant and repellent cues (Reynolds et al., 2011). These include non-specific attractant cues such as CO_2 (Robinson, 2004, Robinson and Perry, 2011), which is released by respiring organisms and decaying matter. RKN J2s are repelled by ammonia, which can be released by decaying material (Castro et al., 1991) and it has been suggested that this may modulate J2 attraction to CO_2 and allow movement towards CO_2 released by living rather than decaying organisms (Perry et al., 2009). *In vitro* experiments have shown that *Meloidogyne spp*. are attracted to roots (Wang et al., 2009) and it has been postulated that plants may release allelochemicals that act as attractants to roots (Prot, 1980). There is however little direct evidence to support this hypothesis and no such chemicals have been identified (Perry et al., 2009). Of the cyst nematodes, only *Heterodera schachtii* (sugar beet nematode) has been found to show chemotaxis towards CO_2 (Johnson and Vigelierchio, 1961). It has been shown that *G. rostochiensis* is attracted to host root diffusates and exhibits increased movements in the presence of root diffusate (Devine and Jones, 2003), yet the chemicals that mediate these effects have not been identified. On nearing the root, it has been suggested that PPNs may respond to chemical cues emanating from the rhizosphere (Perry et al., 2009). The rhizosphere is the soil within millimetres of the root (Whipps, 2001). This areas is rich in other microorganisms (Ingham et al., 1985), which can be plant specific and may generate chemical cues that influence nematode behaviour (Perry et al., 2009). Despite the implied role for chemical cues in nematode host location, the chemical cues that allow nematode to locate their specific hosts are yet to be identified (Perry and Moens, 2013).

1.6.3 Behaviour at the root and root migration

The parasitic behaviour of the root knot nematode *Meloidogyne incognita* has been studied within the model plant *Arabidopsis thaliana*, due to the relative transparency of its roots (Wyss et al., 1992). On having located the root tip of a host, J2 *M. incognita* will begin searching for an invasion site, performing lip rubbing against the root epidermal cells as they explore (Wyss et al., 1992). During this exploratory behaviour, the stylet will move back and forth, and yet its tip will rarely extend beyond the oral aperture as it moves. The significance of this movement has yet to be ascertained. J2s will typically invade the root at the elongation zone of the root, where newly divided cells undergo elongation (Taiz and Zeiger, 2006). It has been found that the invasion ability of J2s is reduced at older regions of the root and there is also evidence that J2s cannot penetrate older root tissue (Wyss et al., 1992).

When invading the root, J2s destroy epidermal cells through lip rubbings and occasional stylet protrusions, which perforate the cell walls. The stylet perforates the host cells for around 30 seconds, during which the median bulb pulsates. The pulsation of the median bulb correlates temporally with the discharge of secretory granules from the subventral oesophageal glands (Figure 1.8). Once inside the host, root knot nematodes like *M. incognita* will use their stylet and head movements to separate host cells to facilitate intercellular migration through the middle lamella, a pectin layer adjoining neighbouring cells (Taiz and Zeiger, 2006). As a result of this intercellular migration the movement of RKNs through roots causes relatively little damage (Wyss et al., 1992). RKNs typically migrate down towards the meristematic zone near that root tip and then turn around to move up towards the vascular cylinder of the root. Whilst moving, as when invading the root, head movements constantly occur, as do stylet movements and occasional pumping of the median bulb (Wyss et al., 1992).

The movement of cyst nematodes (CNs) through the root is rather more destructive, as their migration is intracellular and so involves rupturing large number of cells by continual stylet thrusting (Wyss and Zunke, 1986). CNs also move directly towards the vascular cylinder and fail to show metacorpal bulb activity during migration, unlike RKNs.

Whilst the movement of the stylet and head movements appear to be important in the penetration of host tissues and subsequent migration through these tissues, enzymatic secretions seem to be equally crucial for effective parasitism. These secretions include several enzymes that are likely to be important in the process of root penetration and migration. For example β-1,4-endoglucanase or cellulase, an enzyme that can degrade polysaccharides containing cellulose or xyloglucan, is synthesised in the subventral oesophageal glands of several root knot nematode and cyst nematode species (Smant et al., 1998, de Muetter et al., 2001). Immunolocalisation studies have also shown that nematode-encoded cellulase is localised to the nematode migratory path within the root (Goellner et al., 2000). This suggests that nematode-secreted cellulase aids in root migration by degrading host cell walls. Other cell wall-degrading enzymes, including polygalacturonases and pectate lyases have also been identified in the subventral glands of some PPNs (Doyle and Lambert, 2002, De Boer et al., 2002, Popeijus et al., 2000).

1.6.4 Root-knot nematode and cyst nematode feeding site establishment

Once within the vascular cylinder, CNs and RKNs will select a parenchymous cell, typically one that is adjacent to xylem elements (Golinowski and Magnusson, 1991, Magnusson and Golinowski, 1991, Golinowski et al., 1996), and the stylet will be inserted into the cell and will remain protruded for hours (Wyss et al., 1992). For CNs, this initial cell will develop into a syncytium whereas for RKNs (Hussey and Grundler, 1998) it will develop into a multinucleate giant cell (Jones, 1981). Once a CN has inserted its stylet, the cell will begin to enlarge and organelles such as mitochondria will begin to proliferate rapidly (Golinowski et al., 1996). This will also begin in neighbouring parenchymous cells and cell wall dissolution will occur between them, resulting in the eventual incorporation of hundreds of nearby cells into one large syncytium (Figure 1.5). RKNs on the other hand will induce the formation of several multinucleate giant cells. Once a parenchyma cell has been selected it will undergo repeated nuclear divisions, or karyokinesis, without cell division occurring (Jones and Payne, 1978). As with syncytia, the organelles of giant cells proliferate (Bleve-Zacheo and Melillo, 1997). These feeding structures are highly metabolically active and develop a dense cytoplasm (Goverse et al., 2000) and also develop structural specialisations such as cell wall ingrowths adjacent to the xylem, which facilitate the extraction of nutrients and fluids from the plant (Bird, 1961). These feeding structures

are essential to the survival, growth and reproduction of CNs and RKNs as blocking feeding structure development with cell cycle inhibitors results in the arrest of sedentary endoparasites (Engler et al., 1999).

1.6.5 Secretions associated with plant parasitism

It is known that gene expression of infected cells changes extensively to produce these dramatic alterations in the morphology of giant cells and syncytia, including genes involved in cell cycle activation, cell wall modification and host defence responses (see (Quentin et al., 2013) for review). These changes are thought to be brought about by secretions that emanate from the dorsal oesophageal glands, which grow and exhibit increased activity during and after feeding site formation (Hussey, 1989). These secretions are thought to affect cellular metabolism, cell growth and plant innate immunity (Goverse and Smant, 2014) and are injected into the host cells via the stylet (Davis et al., 2008). Nematode secretions can also potentiate the function of host plant enzymes during parasitism. For example, *H. schachtii* possesses a cellulose-binding protein, which interacts with host pectin methylesterase, potentially promoting its activity and resulting in cell wall modifications (Hewezi et al., 2008).

RKNs and CNs secrete chorismate mutase, an enzyme that alters root growth (Doyle and Lambert, 2003) and can affect the levels of the plant hormone auxin within host cells. It is thought that auxin is important in giant cell and syncytium development as sedentary endoparasites fail to develop in an auxin-insensitive tomato mutant (Goverse et al., 1998). In addition, expression of an auxin-binding protein is upregulated in roots following infection by *G. rostochiensis*, and silencing this gene has detrimental effects on nematode feeding site establishment (Dabrowska-Bronk et al., 2015). Many other enzymatic secretions are also injected into the host to induce the formation of feeding structures. The CN *G. rostochiensis* secretes a protein CLE1 that is cleaved by plant proteases to an active form that mimics plant receptor ligands and subsequently alters root development to allow syncytia formation (Guo et al., 2011). There is also evidence that PPN secretions may directly affect host gene expression as RKN secretions have been found to be delivered directly to plant cell nuclei (Jaouannet et al., 2012, Lin et al., 2013).

Once the giant cells or syncytia have been generated, the juvenile will insert its stylet into them and begin to feed as the metacorpal bulb pumps (Wyss et al., 1992). It has been suggested that secretions from the stylet form the feeding tube, a tube that appears close to the stylet orifice just before feeding (Wyss et al., 1984, Wyss and Zunke, 1986). It appears that the feeding tube acts to prevent large particles such as whole organelles from being sucked into the stylet orifice, which might cause a blockage of the lumen (Razak and Evans, 1976). On initiating feeding, sexual fate is determined and after several days the J2-to-J3 larval moult will occur (Triantaphyllou, 1973).

It is clear that the stylet is essential to the parasitic behaviour of PPNs. Stylet behaviour therefore presents an attractive target for chemical protection of crops. Interfering with stylet activity could potentially prevent PPN feeding, entry into the host, migration through the host and the secretion of enzymes that facilitate parasitism. Inhibition of stylet thrusting could also prevent PPNs from breaking out of the egg. Utilising chemicals that do not act by direct toxicity could potentially limit effects on non-target organisms as well.

1.7 Nematode hatching

The exact process of juvenile hatching from eggs differs between different PPN species and species often have different requirements for the initiation of the hatching process. These requirements typically reflect the host specificity of the species in question. The majority of PPNs undergo the first larval moult within the egg and hatch at the 2nd juvenile stage (J2) (Figure 1.9). As mentioned, in sedentary endoparasitic nematodes, the J2 stage is the non-feeding infective stage. In some species, J2s also function as the survival stage.

The hatching of *Meloidogyne* J2s is dependent upon favourable environmental conditions, including suitable temperatures and soil moisture levels. In contrast, CNs such as *Globodera spp.*, which have a highly limited host range, are highly dependent on signals from the host to initiate the hatching process (Castillo, 2010). If *Globodera* J2s hatch and no appropriate host plant is present, the hatched J2 can only survive and remain infective for around two weeks, as they are reliant on their lipid reserves for energy (Robinson et al., 1987a). In contrast, unhatched *Globodera* J2s can remain

viable and infective for years and can hatch when a suitable host is present and other conditions are appropriate, for example temperature (Perry, 2002). Host signals can however increase the rate of hatching of polyphagous species as well (Perry, 2002).

Many PPNs are not only host specific but also are far more likely to successfully invade host plants of a certain age. As plants age their roots become tougher and the probability of successful nematode invasion decreases (Wyss et al., 1992). The life cycle of parasitic nematodes is therefore often synchronised with their host to increase the likelihood of successful invasion (Perry and Clarke, 1981). Potato cyst nematodes (PCNs) are also strongly reliant on host potato root diffusates (PRD) to stimulate the hatching process in a laboratory setting (Trudgill et al., 1996, Devine et al., 1999). PRD is generated by soaking the root systems of potato plants in distilled water (Rawsthorne and Brodie, 1986) for *in vitro* hatching and is believed to contain chemicals known as "hatching factors" which indicate the presence of a host plant and initiate the hatching process (Perry, 2002). There is some evidence that the glycoalkaloid α-solanine may be present in root diffusates and may be the factor that stimulates hatching (Byrne et al., 1998) although much remains unknown about the exact nature of these hatching factors. In the absence of any host stimulus, PCN J2s remain in a quiescent state within the egg (Perry, 2002). J2s in this quiescent state can remain viable within the egg for up to 30 years in the absence of a host plant (Spears, 1968). Quiescent J2s have reduced metabolic activity when compared to non-quiescent J2s and are resistant to nematicides (Spears, 1968, Cooper and Van Gundy, 1971a). Unhatched J2s are also protected by the eggshell, which consists of an inner lipid layer, a middle chitinous layer and the outer vitelline layer (Figure 1.9) (Perry and Wharton, 2011). The lipid layer is highly impermeable, protecting the unhatched nematode from desiccation and rendering them resistant to toxins such as nematicides (Perry and Wharton, 2011).

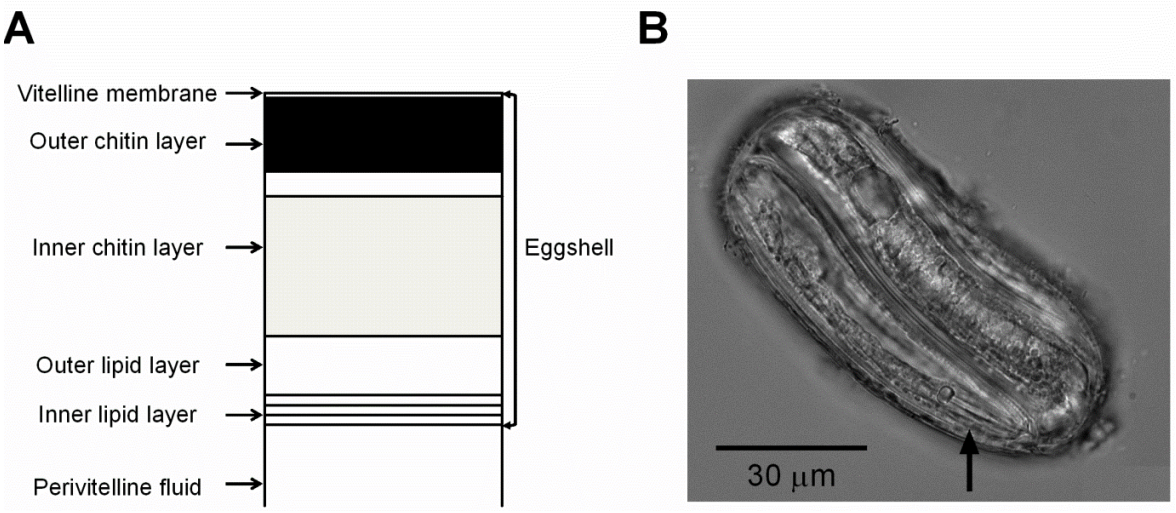

Figure 1.9. The plant parasitic nematode egg and eggshell. **A**) Diagrammatic representation of a cross-section of a tylenchid eggshell (modified from (Perry, 2002)). **B**) A *G. pallida* egg, containing the unhatched J2. Note that the J2 is fully formed, with structures such as the stylet and stylet knob (as indicated by the arrow) are clearly visible.

The physiological changes that occur during hatching remain incompletely understood, although there is some evidence that rehydration of the unhatched juvenile is important in this process. It has been reported that the perivitelline fluid, which surrounds the unhatched J2, has a high concentration of the disaccharide trehalose and it has been suggested that this aids in desiccation survival (Ellenby, 1968, Clarke and Hennessy, 1976, Perry et al., 1980, Perry, 1983). The high trehalose concentration is believed to place an osmotic stress on the J2, resulting in the partial dehydration of the juvenile and this is believed to limit metabolic activity and physical movement (Ellenby and Perry, 1976, Perry and Clarke, 1981, Behm, 1997, Perry, 2002). Ellenby & Perry (1975) used interference microscopy to estimate the water content of J2s of the CN *Heterodera rostochiensis*, and suggested that J2s may be rehydrated following exposure to PRD (Ellenby and Perry, 1976). It has been suggested that this uptake of water follows an increase in the permeability of the inner lipid layer of the eggshell (Perry, 2002). Following exposure to PRD, unhatched J2s are thought to exhibit increased oxygen uptake and an increase in cAMP content and to initiate consumption of endogenous lipid reserves (Atkinson and Ballantyne, 1977a, Atkinson and Ballantyne, 1977b, Robinson et al., 1985). It has been suggested that calcium flux may be involved in meditating changes in unhatched J2, although the evidence supporting this is conflicting or incomplete (Atkinson and Ballantyne, 1979, Clarke and Hennessy, 1981, Clarke and Hennessy, 1983, Clarke and Perry, 1985). Following exposure to PRD or other hatching stimuli, the juvenile begins moving within the egg and exhibits prolonged bouts of

stylet thrusting, typically directed at the narrower ends of the egg. The stylet perforates the eggshell and the juvenile can hatch. This process does vary between species however (see Perry (2002) for review). There is evidence for the involvement of enzymes in the hatching process, as lipase activity has been found in the hatching fluid of *M. incognita*. This appears to be species-dependent however, as no such activity was determined in the hatching fluid of *G. rostochiensis* (Perry et al., 1992).

1.8 Plant parasitic nematode physiology, neurobiology and metabolism

1.8.1 The neurobiology of PPN movement and behaviour

Relatively little is currently known about PPN neurobiology, relative to the model nematode *C. elegans* and animal parasitic nematodes such as *Ascaris suum*. As a result of this lack of insight, current views are based on inference from studies with free-living and animal parasitic nematodes (Perry et al., 2011). There are similarities between the neurotransmitters and neuromodulators involved in common behaviours such as locomotion in the nematodes *C. elegans* and *A. suum* (Lee, 2002). Feeding behaviour seems to be regulated by serotonergic signalling in *C. elegans*, animal parasitic nematodes, PPNs and other free-living worms such as *Pristionchus pacificus* (Avery and Horvitz, 1990, Brownlee et al., 1995, Masler, 2007, Song and Avery, 2012, Wilecki et al., 2015). This suggests that the neuropharmacological basis of "core" behaviours such as feeding and locomotion is likely to be conserved throughout the nematode phylum.

The sequencing of the genomes of the PPNs *M. incognita*, *M. hapla* and *G. pallida* has revealed that these species possess putative receptors, synthetic enzymes and transporters underpinning classical neurotransmitter and neuromodulator signalling (Abad et al., 2008, Opperman et al., 2008, Kikuchi et al., 2011, Cotton et al., 2014). This indicates that these species have the necessary molecular machinery for acetylcholine, GABA, glutamate, 5-hydroxytrptamine (serotonin, 5-HT), dopamine, octopamine, tyramine and neuropeptide signalling. When compared to *C. elegans* or animal parasitic nematodes little research has been conducted into the neuropharmacological or molecular basis of PPN behaviour but the sequencing of these

genomes could enable the use of techniques such as RNA interference that were previously unavailable (See (Fleming et al., 2007, Maule et al., 2011, Dalzell et al., 2012, Atkinson et al., 2013) for review).

1.8.2 Acetylcholine

In *C. elegans*, acetylcholine is the major excitatory transmitter at the neuromuscular junctions that control locomotion, pharyngeal pumping and egg laying (Richmond and Jorgensen, 1999, Kim et al., 2001, Bany et al., 2003, McKay et al., 2004, Song and Avery, 2012). The effects of cholinesterase inhibitors suggest that it is likely that acetylcholine is present as a transmitter in PPNs and may play a role in exciting the body wall muscle. Prior to inducing paralysis, the cholinesterase inhibitor oxamyl increases the movement of J2 *M. incognita*, supporting a role for acetylcholine in PPN movement (Evans and Wright, 1982, Wright et al., 1984). Cholinergic signalling may play a role in the upregulation of stylet thrusting as another cholinesterase inhibitor aldicarb stimulates stylet movements in *M. incognita* J2s (McClure and von Mende, 1987). Given the importance of acetylcholinesterase (AChE) as a target for chemical control of PPNs, much of the research on cholinergic signalling in PPNs has focussed on this enzyme. AChE has been identified in *G. pallida*, *H. glycines*, *M. incognita* and *M. arenaria* (Chang and Opperman, 1991, Chang and Opperman, 1992, Laffaire et al., 2003, Costa et al., 2009). The sequencing of the *G. pallida* genome indicates the presence of orthologues for of a number of enzymes and proteins that are present in *C. elegans* that are known to be important in cholinergic signalling (Cotton et al., 2014). These include choline acetyltransferase, the rate-limiting enzyme in acetylcholine synthesis (Alfonso et al., 1994), the synaptic acetylcholine transporter and several nicotinic acetylcholine receptors (Cotton et al., 2014).

1.8.3 GABA

In *C. elegans*, γ-aminobutyric acid (GABA) signalling inhibits body wall muscle contraction and acts with cholinergic signalling to generate a sinusoidal pattern of locomotion: as the muscles on one side of the worm contract, GABAergic signalling induces muscle relaxation on the opposing side (see section 1.14) (Mcintire et al., 1993a, McIntire et al., 1993b). GABA has been identified in *M. incognita* and *G.*

rostochiensis by indirect immunofluorescence and immunogold staining, respectively (Stewart et al., 1994). The GABA channel blocker picrotoxin has been reported to cause a reversible reduction in the movement of J2 *M. incognita* (Wright et al., 1984). In the same study, picrotoxin and the competitive GABA antagonist bicuculline were also reported to block the inhibitory action of avermectin on *M. incognita* J2s. However, the major target of ivermectin has been identified as glutamate-gated chloride channels in several species (Cully et al., 1994, Dent et al., 2000, Ikeda, 2003, Wolstenholme and Rogers, 2005). Indeed, both bicuculline and picrotoxin have been found to have no effect on the GABA receptor on *Ascaris* muscle cells and the reported effects are likely to be off-target on other transmitter systems (Wann, 1987, Holden-Dye et al., 1988). Thus, the role of GABA in PPNs remains unclear. Orthologues of *C. elegans* glutamate decarboxylase, the rate-limiting enzyme in GABA synthesis (Jin et al., 1999), the GABA transporter and several GABA receptors are indicated by the sequenced genome of *G. pallida*, indicating GABA is likely to be an important transmitter in the PPN nervous system (Cotton et al., 2014).

1.8.4 Glutamate

The functional inhibitory action of avermectin and abamectin on movement suggests that PPNs possess glutamate-gated chloride channels (Wright et al., 1984, Faske and Starr, 2006), although the macrocyclic lactones are known to interact with several targets at higher concentrations (Cully et al., 1994, Dent et al., 2000, Collins and Millar, 2010, Hernando and Bouzat, 2014). Glutamate signalling in *C. elegans* plays a role in regulating the duration of pharyngeal pumping (Dent et al., 1997, Niacaris and Avery, 2003) and may have a role in controlling PPN pharyngeal or stylet behaviour. Several putative glutamate-gated chloride channels and several metabotropic and ionotropic glutamate receptors have been identified in the *G. pallida* genome (Cotton et al., 2014). An orthologue of the vesicular glutamate transporter has also been identified in the *G. pallida* genome (Cotton et al., 2014).

1.8.5 Biogenic amine transmitters

Biogenic amines have also been tested for effects on body movement, head movement and stylet activity (Masler, 2007, Masler, 2008). Dopamine and octopamine were found to affect the movement frequency of *H. glycines*, with octopamine stimulating movement at 5 and 50 mM and dopamine stimulating movement at 5 mM and inhibiting at 50 mM (Masler, 2007). Dopamine signalling in *C. elegans* is important for modifying movement in response to food (Sawin et al., 2000) and could modify PPN locomotion in the presence of a host plant. Masler (2008) described how octopamine increased the frequency of head movements of *H. glycines* and *M. incognita* J2s. ≥500 μM 5-HT was reported to decrease the frequency of body and head movements (Masler, 2007, Masler, 2008). 5-HT was also found to have effects on stylet activity (see section 1.8.7). These effects suggest that biogenic amine signalling regulates PPN behaviour, as is the case with *C. elegans*. Sharpe and Atkinson (1980) have provided evidence that dopamine is present within the neurons of J2 *G. rostochiensis* using a glyoxylic acid fluorescence technique. One study has identified dopamine in homogenates of *M. incognita* by high-powered liquid chromatography (HPLC) although this study failed to identify dopamine in J2s by immunohistochemistry (Stewart et al., 2001). The rate-limiting enzymes in 5-HT, octopamine and tyramine synthesis, tryptophan hydroxylase, tyramine-β-hydroxylase and tyrosine decarboxylase, respectively, have all been identified in the *G. pallida* genome (Cotton et al., 2014). Reciprocal orthologues of the *C. elegans* vesicular monoamine transporter and the serotonin transporter have also been identified in *G. pallida* (Cotton et al., 2014). The presence of the dopamine reuptake transporter is also indicated by the *G. pallida* genome sequence but no reciprocal orthologue of the dopamine synthetic enzyme tyrosine hydroxylase has been identified (Cotton et al., 2014). Putative receptors for 5-HT, dopamine, octopamine and tyramine have all been identified in the sequenced *G. pallida* genome.

1.8.6 Neuropeptides

Neuropeptides are believed to be important in the control of most behaviours in nematodes (Li and Kim, 2008, Walker et al., 2009, Marks and Maule, 2010, Holden-Dye and Walker, 2011) and there is evidence that these peptides may play a role in feeding (Brownlee et al., 1995, Rogers et al., 2003, Kubiak et al., 2003, Papaioannou et al., 2005, Papaioannou et al., 2008, Nassel and Wegener, 2011), reproduction (Waggoner et al., 2000, Keating et al., 2003, Moffett et al., 2003, Marks and Maule,

2010) and locomotion (Holden-Dye et al., 1997, Fellowes et al., 1998, Moffett et al., 2003, Trailovic et al., 2005, Verma et al., 2007, Yew et al., 2007, Reinitz et al., 2011, Choi et al., 2013). As neuropeptides are believed to be important neuromodulators in *C. elegans* and animal parasitic nematodes (Walker et al., 2009), they may also be important in PPNs. The neuropeptide FLRFamide has been found to increase the frequency of body movement of *M. incognita* J2s (Masler, 2007). This is in contrast to the inhibitory action of FLRFamides on *Ascaris suum* muscle (Mousley et al., 2004), which suggests that neuropeptides can have distinct functions in different nematode species. Some neuropeptides have also been found to have effects on the frequency of head movements of *M. incognita* and *H. glycines* (Masler et al., 2012). A number of studies have identified neuropeptides in PPNs via immunocytochemistry (Atkinson et al., 1988, Masler et al., 1999a, Masler et al., 1999b, Kimber et al., 2001, Johnston et al., 2010). In these studies, positive immunoreactivity was seen in the central and peripheral nervous systems, which is consistent with the broad range of behaviours that neuropeptides are thought to regulate. Several FMRF-like peptide (FLP) genes have been identified and characterised in *G. pallida*, and silencing of these genes by RNAi affected J2 movement and migration, suggesting an important role for neuropeptides in the control of PPN movement (Kimber et al., 2001, Kimber et al., 2007). Knockdown of FLP-encoding genes has also been found to impair *M. incognita* development, suggesting that neuropeptide signalling is also of importance in developmental regulation (Dalzell et al., 2010). Several *flp* and *nlp* genes have been identified in *G. pallida*, *M. incognita* and *B. xylophilus*, including several not found in *C. elegans*, suggesting that neuropeptide signalling is also important in PPN neurobiology (Abad et al., 2008, Kikuchi et al., 2011, Cotton et al., 2014).

1.8.7 The neurobiology of stylet and pharyngeal behaviour

The roles of the pharyngeal nervous system, neurotransmitters and neuromodulators in regulating feeding behaviour in *C. elegans* are well understood. Less however, is known about the neural regulation of feeding activity in PPNs, including stylet behaviour and the activity of the pharynx.

Pharmacological studies have suggested a role for the biogenic amine 5-HT in the regulation of stylet behaviour (McClure and von Mende, 1987, Masler, 2007). This

suggests that 5-HT has a conserved role in nematodes as a regulator of feeding (Avery and Horvitz, 1990, Brownlee et al., 1995, Song and Avery, 2012). McClure and von Mende (1987) have shown that high concentrations of 5-HT stimulate stylet thrusting in *M. incognita* and that 5-HT induces stylet exudate to form. This result was confirmed by Masler (2007) in both *M. incognita* and in *Heterodera glycines*, where 5 mM 5-HT significantly increased stylet thrusting. Exogenous 5-HT has also been shown to stimulate stylet behaviour in *H. schachtii* (Jonz et al., 2001). Research by Robertson et al. (1999) has also suggested that presence of 5-HT receptors in PPNs, as 5-methoxy-N, N dimethyltryptamine (5-MeO-DMT), a hallucinogenic compound that is an agonist at mammalian 5-HT_{1A} and 5-HT_2 receptors (Krebs-Thomson et al., 2006), stimulates stylet thrusting and stylet exudation in *Globodera rostochiensis*. 5-MeO-DMT has also been found to induce stylet secretions in juvenile *H. glycines* (Goverse et al., 1994). This may indicate that 5-HT_1- and 5-HT_2 like receptors are involved in mediating stimulation of stylet thrusting by 5-HT, although there is evidence that 5-MeO-DMT may also inhibit 5-HT re-uptake (Nagai et al., 2007) which could also account for its effects of stylet thrusting. Whilst 5-MeO-DMT is a known 5-HT receptor agonist at mammalian 5-HT receptors it may act differently in invertebrates, as the pharmacology of 5-HT receptors can differ significantly between vertebrates and invertebrates (Tierney, 2001). The other biogenic amines dopamine and octopamine have not been found to have any stimulatory effect on stylet thrusting (Masler, 2007). Tyramine has not been tested for effects on PPN stylet thrusting. In *C. elegans*, octopamine has inhibitory effects on pharyngeal pumping (Horvitz et al., 1982, Niacaris and Avery, 2003, Packham et al., 2010), with antagonistic effects to 5-HT and this may be similar in PPNs. Exogenous tyramine is also known to antagonise that activity of 5-HT in *C. elegans* (Rex et al., 2004, Alkema et al., 2005, Packham et al., 2010). If tested in the presence of 5-HT it may be found that octopamine, tyramine or dopamine have inhibitory effects on PPN stylet behaviour. Dopamine, octopamine and tyramine may have differing roles in different PPN species.

Electrophysiological recordings of stylet thrusting have been conducted on *G. pallida* (Hu et al., 2014) and *G. rostochiensis* (Rolfe and Perry, 2001) using the same technique as employed when conducting electropharyngeogram (EPG) recordings on *C. elegans*, with extracellular recording of the electrical flow associated with the pharyngeal contraction-relaxation cycle (see section 1.15.9 for the principles of EPG recordings) (Raizen and Avery, 1994). Hu et al. (2014) showed that 5-HT-stimulated stylet thrusting

by J2 *G. pallida* generated an electrical signal that correlated with the motions of the stylet. These recordings were made in a microfluidic device that is designed to conduct EPG recordings. Three types of waveform of distinct durations were observed in these recordings, which indicates that the movements of the stylet can vary and this may reflect the different actions and functions that the stylet performs during plant parasitism. Small transient potentials were observed during these stylet-associated waveforms, and this may reflect neuromuscular activity that occurs during stylet thrusting. This technique provides a means of further investigating the biology of stylet behaviour (Hu et al., 2014).

The stimulation of stylet exudation or stylet secretions by 5-HT and 5-MeO-DMT suggests that 5-HT signalling upregulates PPN pharyngeal activity (Robertson et al., 1999). In one study, the presence of 50 mM 5-HT was found to induce the uptake of fluorescein isothiocyanate (FITC), a fluorescent dye, into the stylet lumen and the pharyngeal lumen of *M. incognita*, suggesting serotonergic regulation of pharyngeal activity (Rosso et al., 2005). These observations in PPNs are consistent with *C. elegans,* in which 5-HT is essential in regulating the rate of pharyngeal pumping (Sze et al., 2000, Avery and Horvitz, 1990). Interestingly, whilst octopamine does not affect stylet activity (Masler, 2007) evidence suggests that it does increase pharyngeal activity, as 50 mM octopamine induces FITC uptake into the stylet lumen of *G. pallida* (Urwin et al., 2002). This is in stark contrast to *C. elegans*, in which octopamine inhibits pharyngeal activity and acts as a physiological antagonist to 5-HT (Horvitz et al., 1982). This observation may however reflect non-specific effects at the high concentration of octopamine used.

Neuropeptides have been shown to have effects on the pharyngeal activity of *C. elegans* (Papaioannou et al., 2005, Papaioannou et al., 2008, Cheong et al., 2015). It is possible that neuropeptides may also play a role in regulating stylet behaviour and pharyngeal activity in PPNs, as a study using immunocytochemistry has found FMRFamide-like peptide (FLP) immunoreactivity in neurons innervating the pharynx and the stylet protractor muscles of *H. glycines*, *G. pallida* and *G. rostochiensis* (Atkinson et al., 1988, Kimber et al., 2001, Kimber et al., 2002). FLP immunoreactivity has also been observed in neurons innervating the median bulb of *M. incognita* (Johnston et al., 2010). Whilst some FLPs have been shown to increase head movement in *M. incognita* and *H.*

glycines (Masler et al., 2012), no effects have yet been observed on PPN stylet activity or pharyngeal activity. Many other neuropeptides have not been tested for effects on stylet or pharyngeal activity. Neuropeptide signalling in PPNs could have both excitatory and inhibitory effects on stylet activity, as neuropeptides have been reported to have both inhibitory and excitatory effects on *C. elegans* pharyngeal pumping (Rogers et al., 2001). Indeed, the expression patterns of genes encoding FMRFamide-like peptides (FLP) are distinct between *G. pallida* and *C. elegans*, which may suggest they have different functions in different species. Furthermore, FLP-6, which has an excitatory effect on the pharynx (Papaioannou et al., 2005) actually inhibits *Ascaris* pharyngeal activity (Brownlee et al., 1995). There is also the possibility that cholinergic signalling is involved in mediating stylet thrusting as the cholinesterase inhibitor aldicarb has been reported to induce stylet movements (McClure and von Mende, 1987), possibly due to the build-up of extra-synaptic acetylcholine.

1.9 Control of plant parasitic nematodes

1.9.1 An evaluation of the damage caused by plant parasitic nematodes

In a study by Sasser and Freckman (1987) it was estimated that average yield losses in a number of crops in fields infested with different PPN species were 12.3%, with a range of 3.3% to 20.6%. Such figures can however be misleading as some species can cause yield reductions of more than 30% (Sikora and Fernandez, 2005) and in some circumstances, for example where long term monoculture has been employed, yield losses can exceed 70% (Zawislak and Tyburski, 1992). Whilst surveys have been conducted to assess the damage caused by particular species of nematode or the damage in certain crops (Trudgill et al., 2003, Hussain et al., 2011), no other such comprehensive studies have been conducted in more recent years. In 1986, it was estimated that crop production losses due to nematodes were $121 billion globally (Sasser and Freckman, 1987). It is likely that this figure has grown in the intervening years, as many nematicides that were used in the 1980's have now been phased out or are more strictly regulated. This figure also does not include the yield losses that are caused by the plant viruses that nematodes can directly introduce to the host, or the infection of the host by other pathogens that are able to establish themselves due to the weakening of the host immune response (see (Goverse and Smant, 2014) for review).

The *Globodera* genus is of particular concern for agriculture in the U.K. *Globodera* is a quarantine organism yet is present in all EU countries (EFSA, 2012). In some countries, such as the Netherlands, *Globodera* are widespread and must therefore be constantly managed to maintain low population densities (EFSA, 2012). The presence of *Globodera spp*. in potato fields can reduce yield by as much as 22 tonnes/ha (Brown and Sykes, 1983), and yield losses can potentially reach 50% (Trudgill et al., 2003). As a result of these high yield losses *G. pallida* is believed to cost the U.K. potato industry £50 million per annum (Wale et al., 2011).

1.9.2 A history of PPN control – Fumigant nematicides

Chemical control agents have been the predominant means of reducing PPN number and increasing yield in infested fields (Perry and Moens, 2013). Chemical control agents that are effective against PPNs are broadly defined as "nematicides". A true nematicide is a compound that is lethal to nematodes. Some compounds, including the organophosphates and the carbamates can be described as "nematostatics" as they do not kill nematodes but cause a paralysis that can reverse on removal of the compound (Opperman and Chang, 1991, Mcleod and Khair, 1975). Throughout this thesis, the term "nematicide" will be used to describe any compound that is or has been used for nematode control, whether or not it is a true nematicide or a nematostatic.

Nematicides achieve crop protection by reducing the numbers of nematodes feeding on or in the crop plant and thus reducing damage to roots (Eisenhauer et al., 2010). Nematicides can also be used to prevent/ impair PPN reproduction to further control nematode numbers.

Nematicides can be separated based upon their mode of application into either fumigant nematicides or non-fumigant nematicides. Fumigant nematicides are typically directly injected into the soil, although irrigation is a possible application method for some. Once applied fumigant nematicides show gaseous dispersion throughout the soil (Chen et al., 2004). These fumigants are incorporated into the soil at the time of crop planting. The active substance then dissolves in the soil moisture, which reduces the number of

eggs that hatch and the ability of hatched J2 to invade the host, hence reducing crop damage. Fumigants are highly effective at removing PPNs (Haydock et al., 2014).

Fumigant nematicides are compounds that are based on halogenated hydrocarbons or those that release methyl isothiocyanate. Examples of halogenated hydrocarbons include methyl bromide and 1,3-dichloropropene. Little research has been conducted concerning the mode of action of fumigant nematicides. They are thought to work by directly impairing a number of biochemical pathways, including those involved in protein synthesis and respiration (Chitwood, 2003a). One study with nematodes found that ethylene dibromide blocked respiration by oxidisation of iron centres in the cytochrome-mediated electron transport chain and caused alkylation of proteins (Wade and Castro, 1973, Castro and Belser, 1978, Wright, 2011).

The first fumigant nematicide that came into use was carbon disulphide, which was introduced in the late 19th century (Haydock et al., 2014). In the first half of the 20th century, other fumigants were developed, including methyl bromide and 1,3-dichloropropene. Fumigant nematicides were typically highly effective at controlling PPN numbers and most were appropriate for use against a wide range of species. Due to their volatility, such chemicals spread from the site of application (see Table 1.1). Methyl bromide was the primary fumigant chemical control for PPN infestation throughout the 20th century. The use of methyl bromide in agriculture is now banned as it was found to be a major factor in the depletion of the ozone layer (Fuller et al., 2008). Methyl bromide also has a broad, non-selective spectrum of activity, acting against insects, weeds, rodents and fungi (Duniway, 2002). High levels of exposure can also cause serious harm to humans, with severe disability and epilepsy resulting from inhalation in documented cases (Rathus and Landy, 1961). The only nematicidal fumigant that is still widely used in agriculture is 1, 3-dichloropropane (1, 3-D), although it has been banned in the EU. 1, 3-D is effective against PPNs but it also shows phytotoxicity, especially towards seeds and seedlings and so can only be used as a pre-plant treatment to prevent crop damage (Chen et al., 2004). Two other soil fumigants, dibromochloropropane (DCBP) and ethylene dibromide (EDB) have also been withdrawn from the market due to concerns over potential human and environmental impacts (Oka et al., 2000). Another, DCMP (2,5-dichloro-4-

methoxyphenol) was found to cause male sterility and so was de-registered in the 1970's.

Finding replacements for fumigant nematicides has been problematic and no alternatives have emerged with the same broad-spectrum activity against PPNs. In the control of PPNs, expensive mixtures of non-fumigant nematicides are often required to achieve the same level of control.

Active substance	Chemical group	Usage (EU)	Side-effects
Methyl bromide	Halogenated hydrocarbon	Banned in 2010 (Regulation 1005/2009)	Ozone depletion Severe non-target toxicity
1,3-dichloropropene	Halogenated hydrocarbon	Banned in 2011 (Regulation 1107/2009)	Phytotoxicity. Carcinogenicity. Non-target toxicity.
Ethylene dibromide	Organobromine	Banned 1988 (79/117/EEC)	Carcinogenicity. Non-target toxicity.

Table 1.1. Examples of fumigant nematicides, their side-effects and the usage status.

1.9.3 A history of non-fumigant nematicides

Non-fumigant nematicides are non-volatile and disperse in the solid water phase (Chen et al., 2004). These are either applied through foliar spray onto the crop leaves or through soil drenching (Oka et al., 2012), making application easier and reducing the likelihood of environmental damage, relative to fumigants. Granular formulations can also be directly added to the soil. Some non-fumigant nematicides can have systemic effects, where the chemical is able to translocate through the plant tissues to further reduce PPN infestation and protect the whole root system (Oka et al., 2012). This is a desirable characteristic, if the chemical does not remain in the crop after harvest and is not phytotoxic (Haydock et al., 2014).

Up until recent years, non-fumigant nematicides almost exclusively consisted of two chemical groups, the carbamates and the organophosphates. Both the organophosphates and the carbamates are nematostatics as they cause paralysis of nematodes through inhibition of cholinesterases (Husain et al., 2010). The organophosphates phosphorylate cholinesterases and thus cause an irreversible inhibition of their enzymatic activity

(Costa, 2006) whilst carbamates cause a reversible inhibition of the enzyme (Meher et al., 2009). This results in the build-up of acetylcholine at the synaptic cleft (Alfonso et al., 1993) which then leads to hyper-stimulation of acetylcholine receptors, hypercontraction and the associated nematode spastic paralysis (Husain et al., 2010, Opperman and Chang, 1991).

At the concentrations of carbamates and organophosphates that nematodes are exposed to in the field, the resulting paralysis is reversible once the drug concentration has reduced sufficiently (Oka et al., 2009). This means that the timing of application relative to planting and crop growth is critical to ensure maximal crop protection. There is also evidence that carbamates and organophosphates can impair chemoreception and thus reduce the ability of the nematode to locate the host at concentrations that do not elicit paralysis (Winter et al., 2002).

As acetylcholine is conserved as a neurotransmitter across the animal kingdom (Holden-Dye and Walker, 2011) these nematicides lack selectivity and non-target organisms may be harmed by their use. In humans, exposure to high levels of organophosphates leads to cholinergic crisis, which can be followed by seizures, respiratory failure and ultimately death (Husain et al., 2010). Many carbamates and organophosphates are highly toxic to other non-target organisms. Aldicarb and carbofuran, for example, are both highly toxic to birds and fish (van Straalen and van Rijn, 1998, Stenersen, 1979). There is also some evidence that long-term exposure to organophosphates can lead to long-term health defects with exposure linked to delayed neuropathy in non-target organisms (Costa, 2006) and long-term behavioural defects in humans such as impaired cognition and speed of information processing (Steenland, 1996).

Along with their effects on non-target organisms there are other practical issues with current chemical controls. As mentioned, organophosphate and carbamate nematicides are nematostatics and their application at recommended doses does not necessarily result in nematode death (Oka et al., 2012). Therefore, on withdrawal of the treatment surviving PPN populations may recover. Indeed, it has been found that the paralysis that results from treatment with cholinesterase inhibitors can actually reduce energy consumption by nematodes, which reduces lipid consumption and can therefore prolong their infectivity (Perry and Moens, 2013). Non-fumigant nematicides must therefore be

applied regularly if they are to be effective, which requires greater expenditure and increases the likelihood of damage to nearby ecosystems.

Another disadvantage of many non-fumigant nematicides is their ability to leach away from the site of application and cause environmental damage. Several non-fumigants, including aldicarb, oxamyl and fenamiphos are known to be highly mobile in soil and leaching is a considerable issue with these compounds (Zaki et al., 1982, Bilkert and Rao, 1985). Many non-fumigants are also systemic and are taken up by the plant. This can mean that chemical residues remain in the plant after harvest and can pose a health risk to humans, as is the case with aldicarb (Chen et al., 2004). Biodegradation of chemical treatments is another problem. Extended use of a single chemical at the same site can allow for more rapid degradation of the compound by soil dwelling bacteria (Smelt et al., 1996) which again results in the need for more frequent treatments. One example is fenamiphos, which is easily degraded in alkaline soils (Singh et al., 2003). Extended singular use of a nematicide may also lead to problems with PPN resistance. One study (Meher et al., 2009) has shown that the virulence of *M. incognita* can increase on continuous exposure to sub-lethal concentrations of certain chemicals, including the carbamate-based pesticide carbofuran.

Many of the non-fumigant nematicides have been or are being phased out of use. Aldicarb, carbofuran and parathion for example have all been banned in most countries (Table 1.2). Despite this wide-scale prohibition, nematicides are still the most effective method of controlling PPNs and are still used, often as a part of integrated pest management strategies (Sikora et al., 2005, Westphal, 2011). Nevertheless, alternative, safer nematicides are in demand, as are non-chemical approaches to PPN management.

Active substance	Chemical group	Usage (EU)	Side-effects
Aldicarb	Carbamate	Banned 2003 (Regulation 689/2008)	Non-target toxicity. Leaching. Crop residues.
Carbofuran	Carbamate	Banned 2009 (Regulation 1107/2009)	Non-target toxicity.
Cadusafos	Organophosphate	Banned 2007 (Regulation 1107/2009))	Non-target toxicity.
Fosthiazate	Organophosphate	Authorised for use	Non-target toxicity.

Table 1.2. Examples of non-fumigant nematicides, their side-effects and their usage status.

1.9.4 Alternative methods for nematode control

Alternatives to chemical control of PPNs include biological control, host resistance, and agricultural techniques such as crop rotation. There are benefits and drawbacks to each of these alternative approaches that will be touched upon below.

1.9.5 Biological control

Biological control is the management of plant diseases or pathogens, including PPNs, using other living organisms (Stirling, 2011). Potential biological control agents include any organism that negatively affects the pest organism, including predators, parasites, parasitoids and microbial pathogens. Biological control of pests is believed to be far less likely to adversely affect the environment or non-target organisms than chemical control (Stirling, 2011). Unfortunately, few organisms have been effectively developed to fulfil the role of a biological control agent for PPNs and none have yet been widely used (Viaene et al., 2013) (see Stirling (2011) for review). For some control agents, such as predatory nematodes there are difficulties in cultivating sufficient numbers to manage PPN infestations (see Khan and Kim (2007) for review) (Salinas and Kotcon, 2005). The most promising potential biological control agents are fungi and bacteria (Stirling, 2011). The fungus *Purpureocillium lilacinus* has proven, though variable, nematode control activity and has been developed for control of both cyst and root knot nematodes (Wilson and Jackson, 2013, Parajuli et al., 2014). The efficacy of fungal and microbial biological agents in nematode management can however be affected by uncontrollable abiotic factors such as moisture and soil pH (Roberts et al., 1981, Cayrol,

1983). Fungal and microbial agents can also be consumed by other soil organisms (Jaffee et al., 1997). Due to these factors, it is widely considered that most forms of biological control are likely to be insufficient alone to successfully manage PPNs yet may form an important part of integrated pest management strategies (Viaene et al., 2013).

1.9.6 Host resistance

Host resistance involves the use of cultivars that support little or no nematode growth or have increased tolerance to PPNs and can grow more effectively in the presence of damaging nematodes (Fuller et al., 2008, Starr et al., 2013). Host resistance can be exploited for nematode management through breeding and selecting for resistant traits and through genetic engineering of resistance. The mechanisms of innate host resistance are poorly understood (Starr et al., 2013). There is evidence that plant-produced phytoalexins may be involved in host resistance in some cases and may act as repellents to some nematode species (Harborne, 1990, Mazid et al., 2011). Host resistance is an attractive prospect in nematode management, with economic and environmental advantages over both chemical and biological control methods. Host resistance is likely to have reduced efficacy in nematode management when compared to chemical control. There is also a consistent issue with resistance breakdown, as virulent strains of nematode are selected for that are able to overcome host resistance (Ornat et al., 2001, Aubertot et al., 2006). This can also an issue in crops genetically engineered for resistance, although specific steps in the infection cycle can be targeted to lessen this possibility (Cottage and Urwin, 2013).

Research suggests that all methods of nematode control have drawbacks, with environmental concerns over chemical control agents and issues with limited efficacy or the potential for limited efficacy when using biological control, host resistance and genetically engineered resistance. It is widely accepted that the route to effective nematode control is likely to be through integrated pest management strategies, using a combination of the strategies described above and other cultural strategies such as crop rotation (Hillocks and Cooper, 2012). Such strategies are tailored for specific pests and/or crops and reduce the concerns over each individual strategy whilst providing greater protection against PPNs.

1.10 An introduction to the new nematicide fluensulfone

Fluensulfone (full name: (5-chloro-2-(3,4,4-trifluorobut-3-enylsulfonyl)-1,3-thiazole) (also known as MCW-2 and Nimitz) is a new nematicide belonging to the heterocyclic fluoroalkenyl sulfones (see Figure 1.10 for chemical structure). Fluensulfone is a contact nematicide that is applied via direct soil application and it is used for protecting a range of crop plants from PPN damage and infestation.

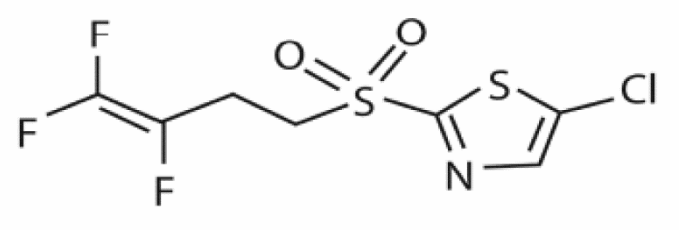

Figure 1.10. The chemical structure of the heterocyclic fluoroalkenyl sulphone, fluensulfone.

Studies have shown that fluensulfone has direct nematicidal activity against a number of PPNs, including *Meloidogyne spp*., *Xiphinema index*, *Pratylenchus penetrans* and *P. thornei* (Oka et al., 2009, Oka et al., 2012, Oka, 2014). Fluensulfone was found to be nematicidal against these species at 3.4-54.4 µM (1-16 mg.L). In these studies, worms were soaked in fluensulfone for up to 48 hours and recovery was assessed. It was observed in these species that fluensulfone exposure resulted in a paralysis from which the worms failed to recover and this paralysis was characterised by a rod-shaped body posture. In another experiment, *M. javanica* were exposed to fluensulfone for just 12 hours, at which stage they were still moving, and then washed. The worms became immobile with time, despite being washed whilst still active. This suggests that *M. javanica* cannot recover after absorbing a lethal dose of fluensulfone and that there is delay between absorbing a lethal dose and the death of the worm. The rod-shaped posture in the presence of fluensulfone is distinctive from that seen in the presence of organophosphate and carbamate nematicides, which cause a shrunken, "wavy" posture (Oka et al., 2009). This indicates a novel mode of action for fluensulfone relative to other currently used nematicides. PPNs can also recover after treatment with organophosphates and carbamates yet do not recover from fluensulfone treatment, which further implicates a novel mode of action relative to current nematicides. This

lack of recovery also indicates that fluensulfone is a true nematicide and not a nematostatic like the organophosphates and carbamates.

In pot studies with *M. javanica,* fluensulfone has been shown to prevent root galling. Root galls are only formed when *Meloidogyne spp*. have entered the host and induced the formation of nurse cells (Oka et al., 2009). This indicates that fluensulfone either prevents the juveniles from invading the host plant, prevents the induction of nurse cells or prevents the maturation and development of PPN inside the host. Furthermore, fluensulfone was shown to prevent the production of eggs in tomato roots exposed to *M. javanica* (Oka et al., 2009, Oka et al., 2012).

Fluensulfone has been applied to pepper plants after inoculation with *M. incognita* to assess whether fluensulfone can act systemically and reduce PPN damage after root invasion (Oka et al., 2012). The prevention of root damage by soil drenching with fluensulfone declined with time after the initial inoculation with *M. incognita*. This suggests that fluensulfone has some activity against *Meloidogyne spp*. both pre- and post-invasion. As the ability of fluensulfone to reduce damage declines with time after root invasion this suggests that earlier developmental stages are more susceptible to fluensulfone or that fluensulfone inhibits juvenile motility in the root, feeding site formation or stylet behaviour. In this study, foliar spraying of fluensulfone was also found to reduce damage by *M. incognita,* which indicates that fluensulfone may be symplastic and can translocate from the foliage to the roots. Foliar spraying before inoculation with *M. incognita* was more effective at reducing damage than post-inoculation spraying. This lends weight to the hypothesis that fluensulfone is more effective against earlier developmental stages, or that these stages exhibit behaviours, such as locomotion and stylet activity, that are more sensitive to fluensulfone (Oka et al., 2012).

In another study, fluensulfone was found to reduce the penetration ability of *M. javanica* at sub-lethal concentrations and exposure times (Oka et al., 2013). *M. javanica* were pre-treated with 1 mg.L (3.4 µM) fluensulfone for 24 hours and then washed. At this concentration, only 12% of the juveniles were immobile yet root penetration by the juveniles was reduced by 90%. In contrast, treatment with the organophosphate fenamiphos did not reduce penetration relative to the control. This implies that sub-

lethal fluensulfone exposure impairs the ability of some PPNs to invade the host plant. This may be due to diminished host-finding capability. These observations also serve to highlight the difference between the nematostatic organophosphates, from which PPNs can rapidly recover, and fluensulfone, which has longer lasting effects.

In another study, the effects of fluensulfone on the parasitic capability of migratory PPNs were established (Oka, 2014). The nematodes tested included *X. index*, *P. penetrans* and *Ditylenchus dipsaci*. All of the migratory nematodes were less sensitive to fluensulfone than *Meloidogyne* spp. and some species, including *D. dipsaci*, *Bursaphelenchus xylophilus* and *Aphelenchoides besseyi*, were unaffected by 48 hour fluensulfone treatment at the highest concentration tested, 16 mg.L (54.4 µM). This shows that fluensulfone susceptibility differs between nematode genera. These species have also been shown to be less susceptible to other nematicides (Lee et al., 1972, Voss and Speich, 1976, Oka, 2014). The lower susceptibility of these species may reflect biological differences that relate to their differing life styles, relative to sedentary endoparasites such as *Meloidogyne spp*. In these experiments, mixtures of different developmental stages were used, whereas for experiments with *Meloidogyne spp*. only J2s were used and therefore some of this difference may be accounted for by varying susceptibility between developmental stages. *X. index*, *P. penetrans* and *P. thornei* on the other hand were immobilised by fluensulfone treatment. Fluensulfone treatment was also found to reduce the penetration of *Pratylenchus spp*. into roots, albeit at higher concentrations than those required to prevent root invasion by *M. javanica*.

1.10.1 Fluensulfone has a favourable toxicity profile relative to organophosphate and carbamate nematicides

Throughout the history of nematicide use, a major issue is non-specific toxicity and the potential for harming non-target organisms (see section 1.9). Fluensulfone however has a favourable toxicity profile when compared to the organophosphates and carbamates (Table 1.3, Table 1.4) (Dewhurst and Tasheva, 2013). It has been demonstrated that fluensulfone has a toxicity to vertebrates that is around 1000-fold lower than the toxicity of organophosphates. The acute LD_{50} value for oral administration in rats is 671 mg.kg for fluensulfone, compared to 0.8 mg.kg for the carbamate aldicarb and 2.7 mg.kg for the organophosphate fenamiphos (Oka et al., 2009, Everich, 2012). In one study, an 18-

month fluensulfone treatment caused increased incidence of benign adenomas in the lungs of mice (Strupp et al., 2012). No increase in the incidence of carcinomas occurred however and the authors concluded that with the different biology of humans and mice, increased tumour occurrence is unlikely to be a risk in humans (Strupp et al., 2012).

Fluensulfone has also been found to have low toxicity towards non-target invertebrates such as honeybees, relative to current nematicides (Table 1.4) (Oka et al., 2008). Other tested invertebrates, such as *Drosophila melanogaster* are also less susceptible to fluensulfone than PPNs. This greater efficacy and speed of action toward nematodes should limit toxicity towards insects at the concentrations applied in the field. Furthermore, in another study it was shown that fluensulfone treatment does not significantly affect the soil microbial community (Rousidou et al., 2013) and should therefore have fewer negative impacts on the soil ecosystem. Despite the generally favourable toxicity profile of fluensulfone relative to other nematicides, it still has non-target toxicity, for example against algae (Table 1.4).

1.10.2 The field dynamics of fluensulfone

Fluensulfone and other fluoroalkenyl compounds are non-polar (Phillion et al., 1999) and so it exhibits low leaching in soil due to its relatively low solubility, a factor that increases effective periods against PPNs and that will limit unwanted damage to nearby ecosystems (Oka et al., 2008, Everich, 2012). Leaching has been an issue with some carbamate and organophosphate nematicides (Zaki et al., 1982, Bilkert and Rao, 1985). Fluensulfone also has a half-life of around 11-22 days in the soil (Norshie, 2014), which also reduces the chances of leaching and causing damage away from the site of application. Other nematicides, such as fenamiphos, have a longer half-life of around 50 days, an attribute which can exacerbate problems with leaching (Oka et al., 2009).

Another drawback is biodegradation of pesticides by soil microorganisms (Smelt et al., 1996). If a nematicide is used frequently in the same field, its nematicidal efficacy can begin to be impaired due to enhanced biodegradation (Smelt et al., 1996, Smelt et al., 1987, Suett and Jukes, 1988). In a study by Oka et al. (2013), it was found that the nematicidal efficacy of fluensulfone did not become impaired after repeated applications at the same site, whereas the nematicidal efficacy of fenamiphos did. This

suggests that fluensulfone is less affected by biodegradation than other available nematicides.

Chemical class	**Fluoroalkenyl**	**Organophosphate**		**Carbamate**	
Active ingredient	**Fluensulfone**	**Fosthiazate**	**Fenamiphos**	**Aldicarb**	**Oxamyl**
Acute oral LD_{50} (mg/kg)	671	73	2.7	0.8	5.4
Acute dermal LD_{50} (mg/kg)	>2000	2396	225	20 mg/kg	2960
Acute inhalation LC_{50} (mg/L)	> 5.1	0.83	$\geq$0.1	< 0.007	0.12-0.17
Eye Irritation	No	Mild	Mild	N/A	Mild
Skin irritation	No	No	No	N/A	No

Table 1.3. A comparison of the toxicity of fluensulfone, organophosphates and carbamates towards rats. The data shown are the LD_{50} values (Dewhurst and Tasheva, 2013).

Chemical class	Fluoroalkenyl	Organophosphate		Carbamate	
Active ingredient	**Fluensulfone**	**Fosthiazate**	**Fenamiphos**	**Aldicarb**	**Oxamyl**
Birds LC50 (mg/kg)	> 1102	10	0.8	3.4	3.16
Fish LC50 (mg/L)	13	114	0.0093	0.56	3.13
Bioaccumulation	Low	Low	Moderate	Low	Low
Daphnia LC50 (mg/L)	29	0.282	0.0019	0.42	0.319
Algae EC50 (mg/L)	0.018	> 4.51	3.8	50	0.93
Bees LD50 (µg/bee)	170	0.256	0.28	0.09	0.38
Earthworms LC50 (mg/kg soil)	153	209	444	65	112

Table 1.4. A comparison of the toxicity of fluensulfone, organophosphates and carbamates towards non-mammalian species. The values shown are the most sensitive LD_{50} values for the particular animal group (Dewhurst and Tasheva, 2013).

1.10.3 Mode of action studies on fluensulfone

The majority of the work carried out with fluensulfone has focussed on its effectiveness as a nematicide in the field. No studies have focussed specifically on determining its mode of action but observations have been made regarding its effects on PPNs.

As mentioned, mobility assays with *M. incognita* have found that fluensulfone elicits a rod-shaped paralysis that is distinctive from the short, "wavy" paralysis that results from exposure to cholinesterase inhibitors such as fenamiphos (Oka et al., 2009). This supports the hypothesis that fluensulfone is unlikely to act as a cholinesterase inhibitor, like the organophosphates and carbamates. It was also observed that after several days in a control solution, the body contents of J2 *M. incognita* appeared depleted, relative to pre-soaking. In contrast, the body contents of J2s soaked in fluensulfone do not become depleted (Oka et al., 2009).

Whilst fluensulfone clearly paralyses some PPN species and this may account for its effectiveness at reducing crop damage, other experiments have shown that root

penetration can be reduced by exposure to sub-lethal concentrations that do not cause complete immobility (Oka et al., 2012).

1.11 Using *C. elegans* for nematicide mode of action studies

In this study, the free-living nematode *Caenorhabditis elegans* (Figure 1.11) will be used for mechanism of action studies on fluensulfone. *C. elegans* is a small nematode of the Rhabditina order that was first described as a species in 1900 (Maupas, 1900) and has since become a key model organism (Brenner, 1974) and the first multi-cellular organism to have its entire genome sequenced (The *C. elegans* sequencing consortium, 1998). *C. elegans* was selected as a model organism due to a number of useful traits, including its ease of maintenance in the laboratory, its relatively small genome, its amenability to genetic manipulation and chiefly, its anatomical simplicity, including its translucent body (Brenner, 1974).

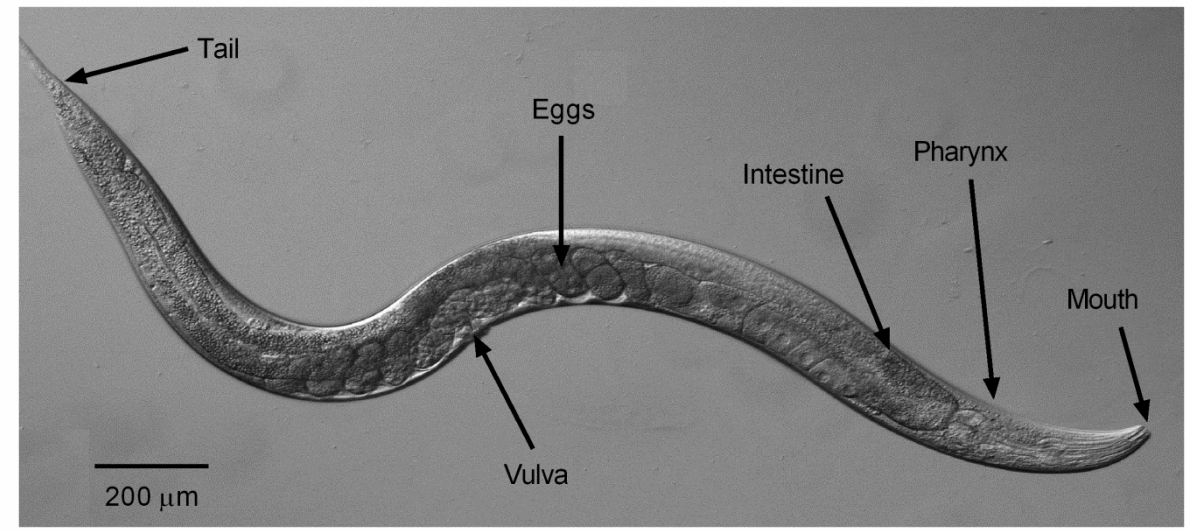

Figure 1.11. A *C. elegans* adult hermaphrodite.

C. elegans is now one of the most well understood multi-cellular organisms on the planet and is certainly the most studied nematode (Riddle, 1997). *C. elegans* is also more easily cultured than parasitic nematodes and is more amenable to experimentation, as most animal and plant parasites require a host for their survival and propagation (Lee, 2002). It has been suggested that because of the strong evolutionary pressures on parasitic nematodes that result from interactions with their specific hosts, parasites are

likely to be more distinct from one another than from free-living nematodes (Blaxter, 2011). These advantages have led to many studies into anthelmintic mechanisms of action adopting *C. elegans* as a model. *C. elegans* has been used as the primary study organism or to perform further research into a number of anthelmintics including ivermectin (Dent et al., 2000, Pemberton et al., 2001, Ardelli et al., 2009), emodepside (Bull et al., 2007, Guest et al., 2007, Crisford et al., 2011), tribendimidine (Hu et al., 2009, Schaeffer et al., 1992), levamisole (Qian et al., 2006, Qian et al., 2008), moxidectin (Ardelli et al., 2009) and apigenin (Kawasaki et al., 2010). *C. elegans* is well established for studies into chemicals for the treatment of animal parasitic nematodes but has not been used as extensively for studies into nematicides for PPN treatment. In recent years, *C. elegans* has been used for some studies into the organophosphates and carbamates, nematicides with a known mode of action (Nguyen et al., 1995, Lewis et al., 2013). It has also been suggested that *C. elegans* can be useful as a model for studies into PPN biology and for screening potential nematicidal and anthelmintic compounds (Costa et al., 2007, Jones et al., 2011, Burns et al., 2015).

Many techniques have been used in the study of *C. elegans* that have not been used in PPNs or are not viable for use in PPNs. In mechanism of action studies for example, forward and reverse genetic approaches can be utilised for *C. elegans*, including mutagenesis (Kutscher and Shaham, 2014). Other genetic techniques, such as RNA interference and DNA microarrays are available for use in *C. elegans* (Portman, 2006, Ohkumo et al., 2008). Techniques like RNA interference are becoming more widely used in PPN research (Fleming et al., 2007) yet many techniques remain unexplored in PPNs. Electrophysiological techniques have also been used in *C. elegans* relatively extensively yet have only been sparsely used in PPN studies (Avery et al., 1995, Goodman et al., 2012).

Many nematicides and anthelmintics achieve nematode control through interaction with and manipulation of the signalling pathways involved in neurotransmission (Holden-Dye and Walker, 2014). The sections below will therefore discuss the neural regulation of several important *C. elegans* behaviours that are known to be affected by anthelmintics, nematicides and other toxins. Some other nematicides are known to affect nematode metabolism or are thought to have metabolic effects (section 1.17)

(Wade and Castro, 1973, Castro and Belser, 1978, Wright, 2011, Veloukas and Karaoglanidis, 2012). Nematode metabolism will therefore also be discussed.

1.12 *C. elegans* culture and maintenance

In this thesis, the N2 laboratory strain is used as the wild-type reference. The N2 strain was originally isolated by Sydney Brenner (Brenner, 1974) and it has been suggested that the native habitat for this free-living nematode is decomposing fruit in temperate climates (Cutter et al., 2006). The information in this introduction is based upon research using the N2 strain, unless stated. The N2 laboratory strain is not strictly considered a "true" wild-type strain due to its prolonged domestication under strictly controlled laboratory conditions (Barriere and Felix, 2005, Felix and Braendle, 2010, Frezal and Felix, 2015). Any references to "wild type" in this thesis will however refer to the N2 strain.

1.12.1 The *C. elegans* life cycle

At 20^{o}C the *C. elegans* life cycle takes roughly 3.5 days and lasts for 3 days at 25^{o}C and 6 days at 15^{o}C (Figure 1.12) (Brenner, 1973). An adult *C. elegans* hermaphrodite can survive for around 20 days, although beyond 5 days most stop laying eggs (Byerly et al., 1976, Wood, 1988). It has an egg stage and four larval stages that precede the adult stage and the larva undergoes ecdysis between these stages (Wood, 1988). The L4 larval stage is easily recognisable due to the developing vulva, and thus *C. elegans* can be simply age synchronised for experimentation. All of the *C. elegans* larval stages and the adult stage are free-living bacterivores and so all stages are available for experimentation and study.

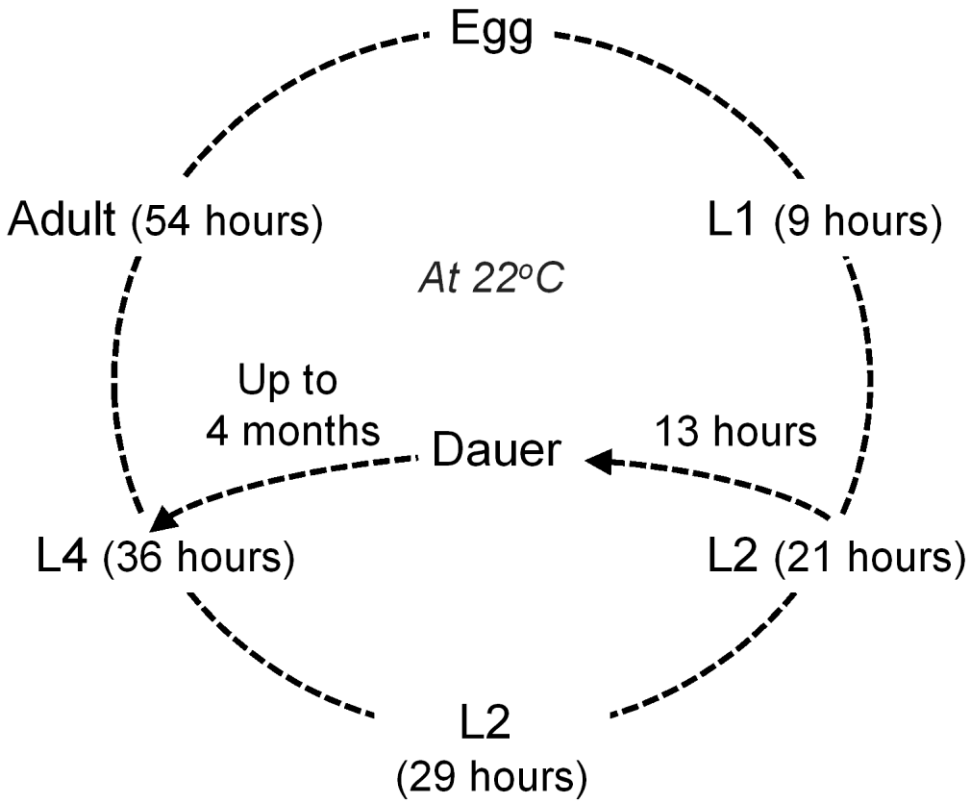

Figure 1.12. The *C. elegans* life cycle. The shift to dauer occurs at the L1-to-L2 moult under sub-optimal conditions. The dauer can survive for up to 4 months in the absence of food and will develop to L4 when food becomes available.

C. elegans are grown on petri dishes containing a modified agar known as nematode growth medium (NGM) that has been inoculated with OP50 *E. coli*. Under normal laboratory conditions, 99.9% of *C. elegans* eggs will develop into adult hermaphrodites that self-fertilise, with very few males developing except under stressful conditions (Brenner, 1974, Ward and Carrel, 1979). An adult hermaphrodite has 959 cells and males have 1031 cells, despite the smaller body size of the males. The higher cell number of males reflects a greater number of sexually specialised cells relative to hermaphrodites (Sulston and Horvitz, 1977, Sulston et al., 1980). The cell lineage of both hermaphrodites and males does not vary between individuals (Sulston and Horvitz, 1977, Sulston et al., 1980). *C. elegans* hermaphrodites are protandrous as at the late L4 stage sperm production begins and this switches to oocyte production at the adult stage (Riddle, 1997) at which point the adult is essentially female and the sperm are stored in its spermatheca. Each hermaphrodite alone can produce 300-400 offspring over 4 days through self-fertilisation. Alternatively, when a male is mated with, a hermaphrodite can

generate up to 1000 offspring. Due to its hermaphroditic nature, a particular strain or genotype can be selfed or can be crossed with males of a different genotype, facilitating genetic study (Wood, 1988). *C. elegans* is also capable of surviving freezing at -80^{o}C or in liquid nitrogen and so can be stored for years.

Whilst developing, *C. elegans* can enter a survival dauer stage when exposed to adverse conditions, such as high temperatures, poor food availability or overcrowding (Golden and Riddle, 1984). This transition to the dauer stage occurs following the moult from the L1 stage to L2. Under stressful conditions, *C. elegans* L2 larvae release dauer pheromone, which is composed of a mixture of ascarosides, and this initiates the transition to the dauer developmental pathways (Jeong et al., 2005, Butcher et al., 2007). *C. elegans* dauers have limited movement relative to other larval stages and are incapable of reproduction (Cassada and Russell, 1975). *C. elegans* dauers have morphological distinctions such as a specialised cuticle, a constricted pharynx that does not exhibit pharyngeal pumping, and an internal plug that seals their oral orifice (Cassada and Russell, 1975, Riddle et al., 1981, Vowels and Thomas, 1992). When developing from the L1 to L2 stage, *C. elegans* undergo a metabolic shift from principally employing the glyoxylate cycle, which generates carbohydrates from lipids, to aerobic respiration via the tricarboxylic acid cycle (TCA) (Wadsworth and Riddle, 1989). This shift does not occur in dauer larvae and they are relatively metabolically inactive in comparison to the L3 stage. Dauer larvae can survive for 70 days in the absence of any sustenance and are resistant to chemicals (Cassada and Russell, 1975, Klass and Hirsh, 1976). A dauer larva will come out of the dauer state and undergo the third and fourth larval moults when food becomes available (Klass and Hirsh, 1976).

1.13 The *C. elegans* nervous system and behaviour

The *C. elegans* hermaphrodite nervous system comprises 302 neurons, with 282 in the somatic nervous system and 20 in the largely autonomous pharyngeal nervous system (White et al., 1982, White et al., 1986). Despite the small size and relative simplicity of this nervous system when compared to other organisms, *C. elegans* exhibit a number of behaviours, including feeding, locomotion, egg-laying, learning and memory, and these behaviours are largely under neural control (de Bono and Maricq, 2005, Giles et al., 2006, Avery and You, 2012, Gjorgjieva et al., 2014). In addition, *C. elegans* can adapt

these behaviours in response to other organisms and changes in external conditions. For example, *C. elegans* reduce locomotion in the presence of food, and this reduction is dependent upon the nutritional status of the worm (Sawin et al., 2000). *C. elegans* can also alter their pattern of locomotion under crowded conditions and will exhibit increased roaming and dispersal to locate other, less heavily populated food sources (Avery and You, 2012). The effects of chemicals on these behaviours can be robustly measured when investigating drug action. In the sections below, the neural basis of locomotion, pharyngeal pumping and egg laying will be described as these are key behaviours that can be measured when studying chemicals and that are known to be affected by several anthelmintics and nematicides (Holden-Dye and Walker, 2014).

1.13.1 The anatomy of the *C. elegans* nervous system

The connectivity and overall structure of the nervous system of *C. elegans* has been deduced by reconstructing electron micrographs (White et al., 1982, White et al., 1986). These reconstructions were used to group *C. elegans* neurons into 118 classes, based on morphological features and connectivity (White et al., 1986). Each of these neurons has been assigned a name of two or three letters, for example, NSM, the neurosecretory motor neuron (Hall and Altun, 2008). An additional "L" or "R" in the name signifies if the neuron is positioned dorsally or ventrally of a radially symmetrical pair. The positioning of each of these neurons is relatively invariant between individual worms, as is their morphology. Connectivity, for example through gap junctions, can however vary between individual worms (Altun et al., 2009).

In the hermaphrodite nervous system, there are estimated to be around 5000 chemical synapses, 600 gap junctions and 2000 neuromuscular junctions (White et al., 1986). There are also 56 glial cells in hermaphrodites that are thought to play a role in neuronal morphology and plasticity (Oikonomou and Shaham, 2011). Half of all neuronal cell bodies reside within the nerve ring, the name given to a concentration of neurons that encircles the pharynx (Figure 1.13). The nerve ring is connected to the pharyngeal nervous system by a pair of RIP interneurons. The nerve ring integrates information received from sensory neurons in the head and tail and has connections to motor neurons in the ventral and dorsal nerve cords, which are longitudinal concentration of neurons (Riddle, 1997). Motor neurons project from the ventral or dorsal nerve cords to

the ventral or dorsal body wall muscles to form neuromuscular junctions (Jorgensen and Nonet, 1995). At *C. elegans* chemical synapses, signalling is achieved via the classical neurotransmitters acetylcholine, γ-aminobutyric acid (GABA), glutamate and the biogenic amines 5-hydroxytryptamine (5-HT), dopamine, octopamine and tyramine (Riddle, 1997). Neurotransmission is also modulated through neuropeptide signalling (Li and Kim, 2008).

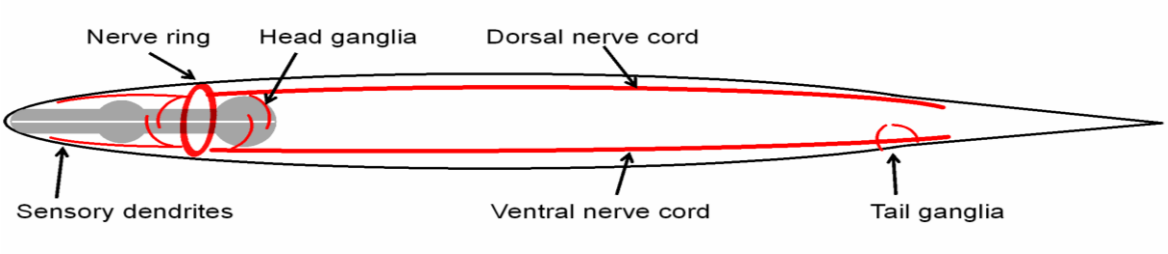

Figure 1.13. The schematised organisation of the *C. elegans* nervous system. The neurons are red and the pharynx is in grey. Adapted from (Hobert, 2010) .

1.14 *C. elegans* locomotion

C. elegans locomotion is undulatory (Gray and Lissmann, 1964, Riddle, 1997). On a solid substrate, *C. elegans* moves via "body bends", which are characterised by sinusoidal propulsions in the direction of movement. In liquids of similar viscosity to water, *C. elegans* swim, also known as "thrashing" (Pierce-Shimomura et al., 2008). Thrashing is characterised by C-shaped bends around the worm's midpoint (Figure 1.14). The adoption of either of these forms of locomotion is dependent upon the mechanical load placed on worm and proprioceptive feedback from the environment (Vidal-Gadea et al., 2012). 5-HT and dopamine signalling modulate proprioception and the locomotory program, with dopamine controlling crawling or body bends and 5-HT controlling thrashing (Vidal-Gadea et al., 2012).

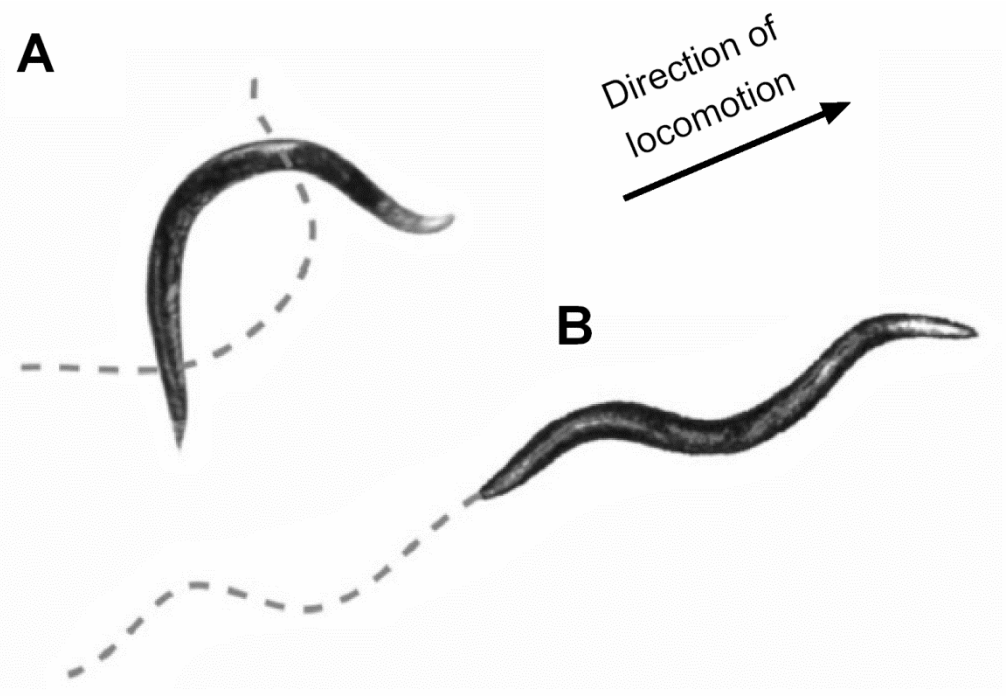

Figure 1.14. *C. elegans* movement in liquid and on a solid substrate. When in liquid, *C. elegans* swim or "thrash" (**A**). Thrashing is characterised by C-shaped flexes of the body around the midpoint. When on a solid surface, *C. elegans* perform body bends or crawling (**B**). Body bends are sinusoidal undulatory movements. The dashed line denotes the position of the worm at the preceding locomotion cycle. From (Gjorgjieva et al., 2014).

Despite the nuances of body bends and thrashing, these locomotory behaviours both occur through the out of phase contraction and relaxation of the 95 muscles cells of ventral and dorsal body wall muscle (Sulston and Horvitz, 1977, Riddle, 1997). For example, a ventral body bend is generated by the contraction of the ventral body wall muscle and the simultaneous relaxation of the dorsal body wall muscle. The head and neck muscles of the worm are innervated by projections from nerve ring motor neurons, whilst the body wall muscle along the rest of the body projects processes called muscle arms to the ventral and dorsal nerve cords (White et al., 1976, Von Stetina et al., 2005). Gap junctions are present between these muscle arms at the nerve cords, allowing the electrical-coupling of these cells that is required for the propagation of dorso-ventral bends (Liu et al., 2011). The contraction of both the dorsal and ventral body wall muscle is controlled by cholinergic signalling at the neuromuscular junctions, whilst relaxation is controlled by GABAergic signalling (Figure 1.15) (Chalfie and White, 1988, Riddle, 1997, Liewald et al., 2008). When either the ventral or dorsal muscles contract, it is thought that the D neurons cross-inhibit simultaneous contraction of muscles on the opposing side of the worm via GABAergic signalling (White et al., 1986, Chalfie and White, 1988). GABA signals through the UNC-49 receptor in the

control of locomotion (Richmond and Jorgensen, 1999). Cholinergic control of locomotion is regulated by nicotinic acetylcholine receptors containing the UNC-38, UNC-29 and UNC-63 subunits (Fleming et al., 1997, Richmond and Jorgensen, 1999, Culetto et al., 2004).

The head and neck muscles of *C. elegans* are innervated by 28 motor neurons and the body muscles are innervated by 75 motor neurons (White et al., 1986, Chen et al., 2006). Multiple neurons innervate each of the muscle cells of the body. These neurons are separated into eight classes: VA, VB, VC and VD innervate the ventral body wall muscle and AS, DA, DB and DD innervate the dorsal body wall muscle. Of these motor neurons, the A- and B-types are cholinergic and excitatory, whilst the D-type motor neurons are GABAergic and inhibitory (McIntire et al., 1993b, Riddle, 1997, White et al., 1986, White et al., 1976). Forward locomotion is controlled by DB, VB, DD and VD. Ablation of B-type motor neurons has been found to impair forward locomotion and calcium epifluorescence has shown that DB and VB are active during forward locomotion (Chalfie et al., 1985, Haspel et al., 2010). DB and VB are both excitatory, whilst DD and VD are inhibitory. Backward locomotion is controlled by DA, DD, VA and VD, with DA and VA excitatory and DD and VD inhibitory. Ablation of DA has been shown to prevent backward locomotion and these neurons exhibit higher activity during backward locomotion (Chalfie et al., 1985, Haspel et al., 2010, Kawano et al., 2011). DA and VA are excitatory, whilst DD and VD are inhibitory. The role of the AS and VC motor neurons in modulating locomotion is unclear (Haspel et al., 2010, Faumont et al., 2011). The connectivity of the VC neurons suggests that they may modulate the activity of the VD and DD motor neurons (White et al., 1986, Haspel and O'Donovan, 2011).

The motor neurons described above are innervated by five classes of interneurons, AVA, AVB, AVD, AVE and PVC, which are commonly referred to as “command” interneurons (Chalfie et al., 1985). AVB and PVC ablation affects forward locomotion forward locomotion and these neurons are active during forward locomotion, which suggests that these command interneurons modulate the signalling controlling forward locomotion (Chalfie et al., 1985, Faumont et al., 2011, Kawano et al., 2011). In contrast, AVA, AVD and AVE exhibit activity during backward locomotion and their ablation affects backward locomotion (Chalfie et al., 1985, Faumont et al., 2011, Kawano et al.,

2011). The ablation of these neurons does not completely abolish locomotion but results in slow, uncoordinated and aberrant movement (Zheng et al., 1999, Kawano et al., 2011). Some sensory neurons and other interneurons have connections with these command interneurons and affect locomotion (Tsalik and Hobert, 2003, Piggott et al., 2011). For example, the AIY interneuron modulates reversal frequency through interaction with command interneurons (Tsalik and Hobert, 2003).

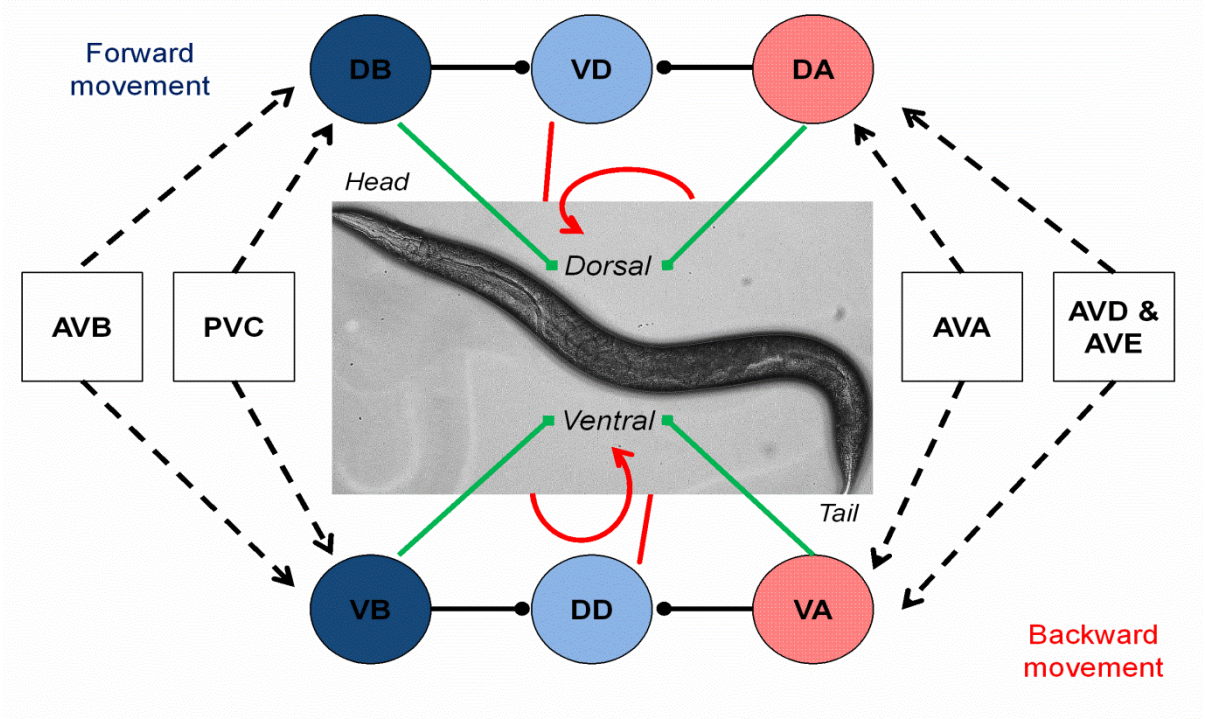

Figure 1.15. The network of motor neurons and interneurons that control *C. elegans* sinusoidal locomotion. The dorsal and ventral body wall muscle receives excitatory cholinergic signals (green lines) from VB and DB, which mediate forward locomotion. Excitatory signalling from DA and VA mediate backward locomotion. Inhibitory GABAergic signalling from DD, VD, VB and DC also mediates forwards movement. DD and VD synapse onto the body wall muscle on the opposite side of the worm relative to their presynaptic partners, allowing the reciprocal inhibition that coordinates sinusoidal locomotion (red lines). The interneurons AVD and PVC regulate forward locomotion, whilst AVA, AVD and AVE regulate backward locomotion (Adapted from (Chalfie and White, 1988) and (Riddle, 1997)).

Neuromodulators such as the monoamines and neuropeptides can also affect the neural pathways controlling locomotion (Bargmann, 2012, Flavell et al., 2013). 5-HT is known to be a key regulator of the enhanced slowing response, in which starved worms drastically reduce movement following contact with a food source to maximise feeding (Sawin et al., 2000). 5-HT reduces the release of acetylcholine at the neuromuscular junction to achieve this reduction in movement (Nurrish et al., 1999). Dopaminergic signalling regulates the basal slowing response that occurs when well-fed worms

encounter a food source and is thought to be involved in fine-tuning the rate of locomotion (Sawin et al., 2000, Omura et al., 2012). Exogenous octopamine causes uncoordinated locomotion and thus octopaminergic signalling may have a role in locomotory modulation (Horvitz et al., 1982). Tyramine reduces GABAergic signalling to the ventral body wall muscle, allowing the ventral turn that occurs during the escape response (Donnelly et al., 2013).

1.15 *C. elegans* pharyngeal pumping

C. elegans is a filter feeder, as it takes in a mixture of liquid and food particles (bacteria) through the mouth. The food particles are retained yet the liquid is expelled again through the mouth (Avery and Shtonda, 2003). The pharynx is the neuromuscular organ that is crucial for proper and efficient feeding, transporting food particles from the mouth and through to the intestines. It acts as a pump, generating a negative pressure that forces food into the intestine. This is necessary because the pressure inside the nematode is greater than that of the surrounding environment (Harris and Crofton, 1957). The pharynx also plays an important role in the filtering of the food particles from the liquid. This filtering allows the food particles to be more concentrated as they enter the intestines, permitting more time within the intestines, more effective absorption and reduced dilution of the gut digestive enzymes.

1.15.1 The anatomy of the pharynx

The anatomy of the pharynx has been deduced from reconstructed serial sections electron micrographs (Albertson and Thomson, 1976). The pharynx is divided into 3 distinct regions: the corpus, which is connected to the mouth, the isthmus which is flanked by the corpus and the posterior terminal bulb, which is connected to the intestine (Figure 1.16). The terminal bulb possesses a cuticular specialisation called the grinder, which mechanically disrupts food before it passes to the intestine (Avery and Shtonda, 2003, Albertson and Thomson, 1976, Raizen and Avery, 1994). The corpus can be further subdivided into a cylindrical anterior section, the procorpus, and a posterior bulb known as the metacorpus. The terminal bulb, after grinding up the food,

pushes it through the pharyngeal-intestinal valve and into the intestine (Doncaster, 1962).

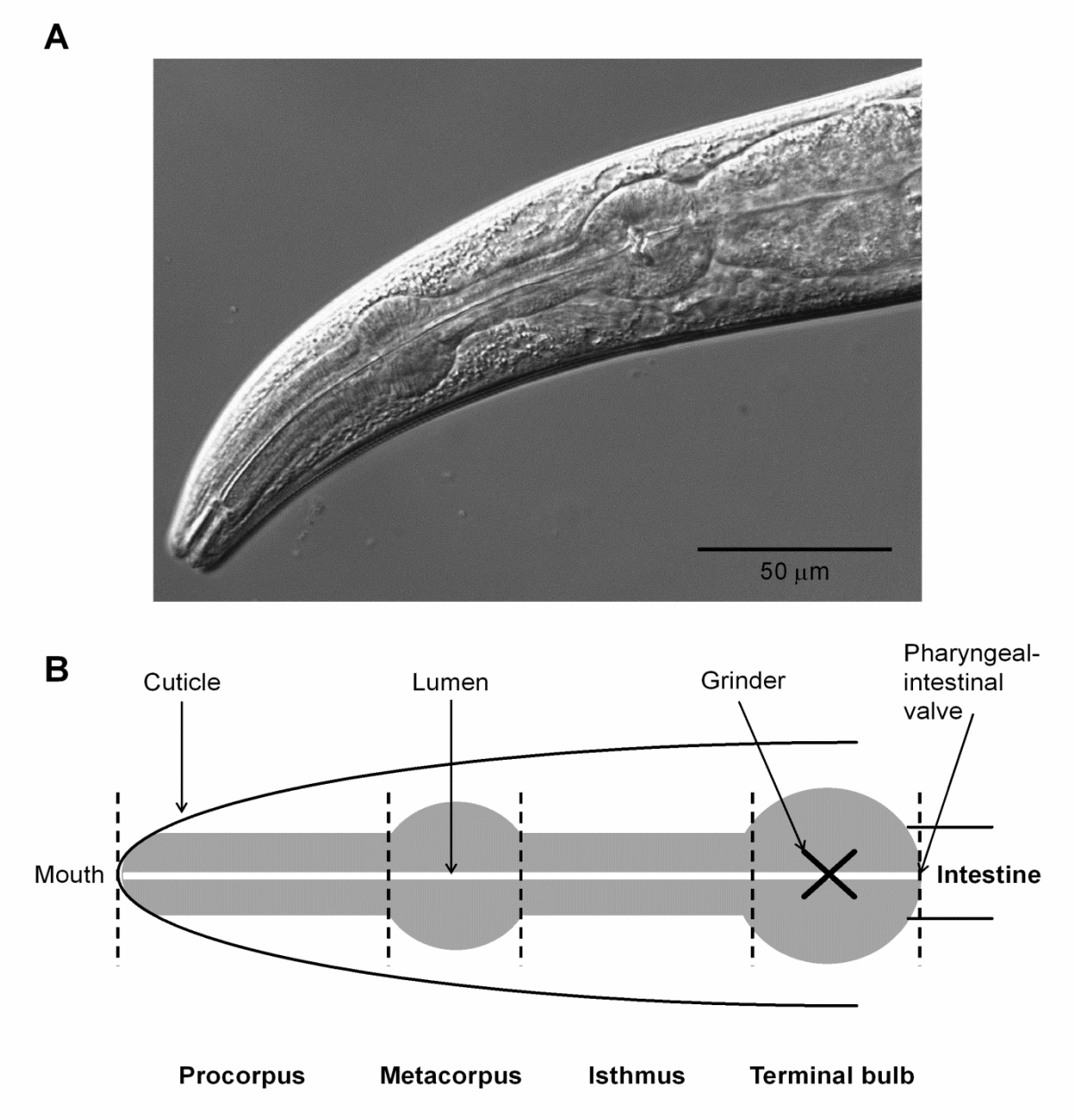

Figure 1.16. The *C. elegans* pharynx. **A**) The *C. elegans* pharynx. **B**) A diagrammatic representation of the morphology of the *C. elegans* pharynx. The 20 cells of the pharyngeal muscle form the procorpus, the metacorpus, the isthmus and the terminal bulb. The procorpus and metacorpus collectively are known as the corpus. During pharyngeal pumping, the corpus and terminal bulb contract near-simultaneously to draw in liquid through the mouth and into the pharyngeal lumen. The isthmus undergoes peristalsis every four pumps, passing food materials through to the terminal bulb, where each contraction rotates the grinder to smash food particles. The food is then passed through the pharyngeal-intestinal valve and into the intestine.

The pharynx is composed of 20 muscle cells, 20 neurons, 9 epithelial cells, 9 marginal cells and 5 gland cells (Albertson and Thomson, 1976). The muscle cells are divided

into eight classes; pm1-pm8 (Albertson and Thomson, 1976). The corpus contains pm1-pm4 cells, the isthmus pm5 and the terminal bulb pm6-pm8. The actin-myosin filaments of the pharyngeal muscle cells are of a radial orientation so that when they contract the lumen opens (Avery and Shtonda, 2003). The orientation of these filaments means that when they contract the muscle cells become longer in the circumferential dimension yet they become thinner in the radial dimension, which then opens the lumen.

1.15.2 The motions of the pharynx

Pharyngeal motion consists of two actions: pumping and isthmus peristalsis (Raizen and Avery, 1994, Avery and Horvitz, 1989). Pumping takes food into the mouth and the corpus from the external environment and isthmus peristalsis transports these particles towards the posterior terminal bulb. Isthmus peristalsis is a travelling wave of contraction that occurs once every 4 or so pumps (Shimozono et al., 2004). Pharyngeal pumping occurs through a contraction-relaxation cycle (Avery and Horvitz, 1989). The whole contraction-relaxation cycle that occurs during pharyngeal pumping is normally less than 0.2 seconds long (Avery and Shtonda, 2003). Firstly, the corpus, the anterior half of the isthmus and the terminal bulb contract near-simultaneously. This causes the lumen to open and food and liquid is sucked into this opening. The posterior isthmus does not contract and thus the isthmus lumen remains closed. As the terminal bulb contracts the plates of the grinder rotate, mechanically disrupting the bacteria which allows the debris to be passed through to the intestine (Doncaster, 1962). This is followed by near-simultaneous relaxation of these muscles, closing the lumen of the corpus and the anterior half of the isthmus, which forces the fluid back out though the mouth. The food particles however remain trapped and are transported through to the terminal bulb by isthmus peristalsis which occurs exclusively in the posterior isthmus (Avery and Horvitz, 1987). The isthmus is believed to act as a kind of double valve by separating the lumen of the corpus, which is at a low pressure, from the higher pressure regions that are posterior to the isthmus (Riddle, 1997).

The majority of filter feeders possess some form of mesh or other structure, which is able to trap food particles, yet *C. elegans* does not appear to possess any such filter. Instead it is thought that the motions of the pharynx and the precise timing of the contraction-relaxation cycle cause differential motion of food particles and the fluid in

the lumen (Avery and Shtonda, 2003). There is some evidence for this in the corpus. When the corpus is relaxed, food particles are scattered throughout. When the pharyngeal muscles contract these food particles are forced towards the posterior isthmus by the rush of fluid. When the corpus relaxes again, the fluid is forced out yet the food particles remain at their posterior position within the pharynx. This may be due to the slight delay between the motions of the corpus and those of the anterior isthmus. It has been suggested that these differential motions may be down to the varied properties of gap junctions in the pharynx (Li et al., 2003).

Gap junctions composed of innexins (Phelan and Starich, 2001) are present between the muscle cells, allowing the correct timing of pharyngeal muscle contraction that is so crucial to efficient feeding (Li et al., 2003). These gap junctions allow electrical connectivity between the different muscle cells of the pharynx and it has been suggested that they allow the propagation of the waves of myogenic excitation (see below) (Franks et al., 2006). Gap junctions between the muscle cells are also thought to allow a coordinated response to neuronal signalling. It is believed that the gap junction protein EAT-5 ensures the propagation of the electrical signal to the isthmus, while another gap junction protein INX-6 maintains propagation to the anterior procorpus. *inx-6* and *eat-5* null mutants are starved in appearance, which is probably due to an inability to properly time muscle contraction, which prevents efficient feeding (Avery, 1993a, Starich et al., 1996, Li et al., 2003). *inx-6* mutants also exhibit asynchronous contraction of the procorpus and metacorpus (Li et al., 2003), whilst *eat-5* mutants exhibit asynchronous contraction of the terminal bulb and corpus (Starich et al., 2003).

As mentioned, the *C. elegans* pharynx also possesses nine marginal cells (Albertson and Thomson, 1976). The marginal cells have been divided into 3 classes, mc1, mc2 and mc3 (Albertson and Thomson, 1976). Of these, the mc2 cells are innervated by the MC neurons and the mc3 cells are thought to be innervated by M5. The function of marginal cells in pharyngeal pumping is not currently fully understood. It is known that the marginal cells share gap junctions with the pharyngeal muscle cells (Riddle, 1997). The presence of gap junctions between marginal cells and muscle cells has led to speculation that they may function to generate the rhythmic, myogenic activity of the pharyngeal muscle, much like the Purkinje fibres that allow the synchronised contraction of the

heart muscle in mammals (Camborova et al., 2003). It has also been suggested that the marginal cells anchor the pharyngeal lumen in place (Raizen and Avery, 1994).

Two sets of gland cells are also part of the *C. elegans* pharynx (Albertson and Thomson, 1976). The role of these gland cells in pharyngeal physiology is also currently not understood but it has been postulated that secretions from these cells may contain mucoids, which function as lubricants (Franks et al., 2006).

1.15.3 Neural control of pharyngeal motions

The 20 neurons of the pharyngeal nervous system can be split into 14 anatomical types (Albertson and Thomson, 1976), including six classes of interneuron, Il-I6, and five classes of motor neuron, M1-M5. In the pharynx there are also the neurosecretory motor (NSM) neurons, that are believed to function as sensory-motor neurons and may be involved in signalling the presence of food, (Riddle, 1997, Albertson and Thomson, 1976), and the motor and interneuron (MI), the function of which is unknown. The final neuronal type is the marginal cell (MC) motor neurons that are believed to be important during rapid pharyngeal pumping (Avery and Horvitz, 1989, Raizen et al., 1995). All of the pharyngeal neurons lie between the pharyngeal muscle and the basal lamina, in the folds of the pharyngeal basal membrane (Albertson and Thomson, 1976). In contrast, the neurons of the somatic nervous system are separated from the body wall muscle by a basal lamina.

The pharyngeal nervous system is nearly entirely autonomous from somatic nervous system, to which it makes a connection with through a single pair of bilaterally symmetrical extrapharyngeal interneurons, the RIP neurons (White et al., 1986). Four pairs of extrapharyngeal neurons provide inputs to the RIP neurons (White et al., 1986): IL1, a mechanosensory neuron mediating the head withdrawal reflex (Hart et al., 1995), the chemosensory IL2 neurons (Riddle, 1997) and RME and URA, motor neurons that innervate the head muscle (White et al., 1986). Such connectivity allows the modulation of pharyngeal pumping in response to sensory stimuli, as for example the stimulation of IL1 can cause inhibition of pumping (Chalfie et al., 1985).

There is evidence that pharyngeal pumping can occur in the absence of any neural input and that pumping is myogenic, as laser ablation of the entire pharyngeal nervous system aside from the M4 motor neuron still allows maturation to adulthood and pumping still occurs (Avery and Horvitz, 1989). Myogenic means that the pharyngeal muscle can contract rhythmically in the absence of any neural input, although neural signalling may be necessary for the modulation of this intrinsic activity. M4 is required for isthmus peristalsis and so must be present to allow the transport of food particles to the terminal bulb and the intestine. In experiments by Avery and Horvitz (1987) ablation of M4 in newly hatched larvae prevented isthmus peristalsis. Whilst pharyngeal pumping continued, the animals starved to death as food could not be passed through the isthmus and into the intestine. The rest of the pharyngeal neurons are required for modulating parameters of pumping such as pump rate and duration.

Other than MC and M4, 12 of the 14 types of neurons in the pharynx have been termed the GREEN neurons by Avery and Horvitz (1989) and Avery (1993b). These neurons seem to be necessary for the efficient trapping of bacteria in the pharynx as their ablation results in a "slippery pharynx" in which the bacteria are poorly retained. Pumping can however still occur when these neurons are removed and maturation to adulthood is unaffected. It has been suggested that these neurons modulate the precise timing of pharyngeal muscle relaxation that may be important in allowing the trapping of bacteria in the pharyngeal lumen (Avery and Horvitz, 1989, Avery, 1993a). This slippery pharynx phenomenon is only seen when all the GREEN neurons are ablated, which suggests redundancy between these neurons (Avery and Horvitz, 1989).

Whilst pumping can occur in the absence of neural input, two types of pharyngeal neuron, the MC and M3 motor neurons, are essential for the modulation of the rate and duration of pharyngeal pumping, allowing the upregulation of pumping that occurs when bacteria are present (Niacaris and Avery, 2003). The M3s are inhibitory glutamatergic neurons (Avery, 1993b) that modulate the timing of relaxation in the pharyngeal muscle. Ablation of the M3s results in an increased pump duration due to the lack of this inhibition. The M3s fire during the pharyngeal action potential and release glutamate to act on a glutamate-gated chloride channel. The activation of this channel then causes inhibitory post-synaptic potentials, which promote repolarisation and the end of the pharyngeal muscle action potential (Avery, 1993b, Dent et al., 1997,

Li et al., 1997). During rapid pumping such as when on food or when exposed to exogenous 5-HT, M3 activity is necessary to reduce the length of each pump of the pharynx and allow for a faster rate of pumping. If M3 is removed, pump duration does not decrease and so the pharynx cannot pump as rapidly. AVR-15 is a subunit of the glutamate receptor and it is required for the pharyngeal muscle to respond to M3 activity (Dent et al., 1997). In the absence of AVR-15, the P-phase spikes, which represent M3 activity, are absent from electropharyngeogram recordings. It has been previously been suggested that M3 may constitute a single-neuron proprioceptive loop as it has putative proprioceptive free endings (Albertson and Thomson, 1976) in the metacorpus that may be capable of sensing muscle contraction, which then triggers glutamate release, thus ending the pharyngeal action potential and muscle contraction (Raizen and Avery, 1994).

The MCs on the other hand are excitatory cholinergic motorneurons (Raizen et al., 1995), the signalling of which depolarises and excites the corpus through a synapse onto the mc2 marginal cells (Albertson and Thomson, 1976) and possibly the pm4 muscle cells (Avery and Shtonda, 2003, Shtonda and Avery, 2006). This excitatory activity is then transmitted to the terminal bulb, through the isthmus by electrical coupling between the muscle cells (Avery and Horvitz, 1989, Starich et al., 1996). There are gap junctions between the marginal cells and the muscle cells and so the marginal cells may serve as a conduction pathway to excite the muscle (Riddle, 1997). There is also some evidence that MC may act as a sensorimotor neuron as it has a putative mechanosensory ending at the procorpus-metacorpus boundary and so may be stimulated by the presence of bacteria in the pharynx and muscle motions (Raizen et al., 1995). The MC neuron excites the pharyngeal muscle through the EAT-2/EAT-18 acetylcholine receptor which results in an increased rate of pumping (McKay et al., 2004, Raizen et al., 1995), through the opening of voltage-gated calcium channels (Lee et al., 1997).

1.15.4 The pharyngeal action potential

Each contraction-relaxation cycle that occurs during pharyngeal pumping is maintained by muscle action potentials (Franks et al., 2002). Calcium influx and potassium efflux are the agents for the membrane depolarisation and repolarisation, respectively, that occurs during each action potential (Shtonda and Avery, 2005). The MC-mediated

EPSPs depolarise the membrane to -30 mV, which triggers the activity of CCA-1, a T-type calcium channel. *cca-1* null mutants do not elicit pharyngeal muscle action potentials in response to MC excitatory neurotransmission (Steger et al., 2005). As calcium influx occurs, membrane potential reaches around -10 mV and an L-type calcium channel, EGL-19, is activated (Lee et al., 1997). Calcium influx through this channel stimulates muscle contraction and maintains membrane depolarisation.

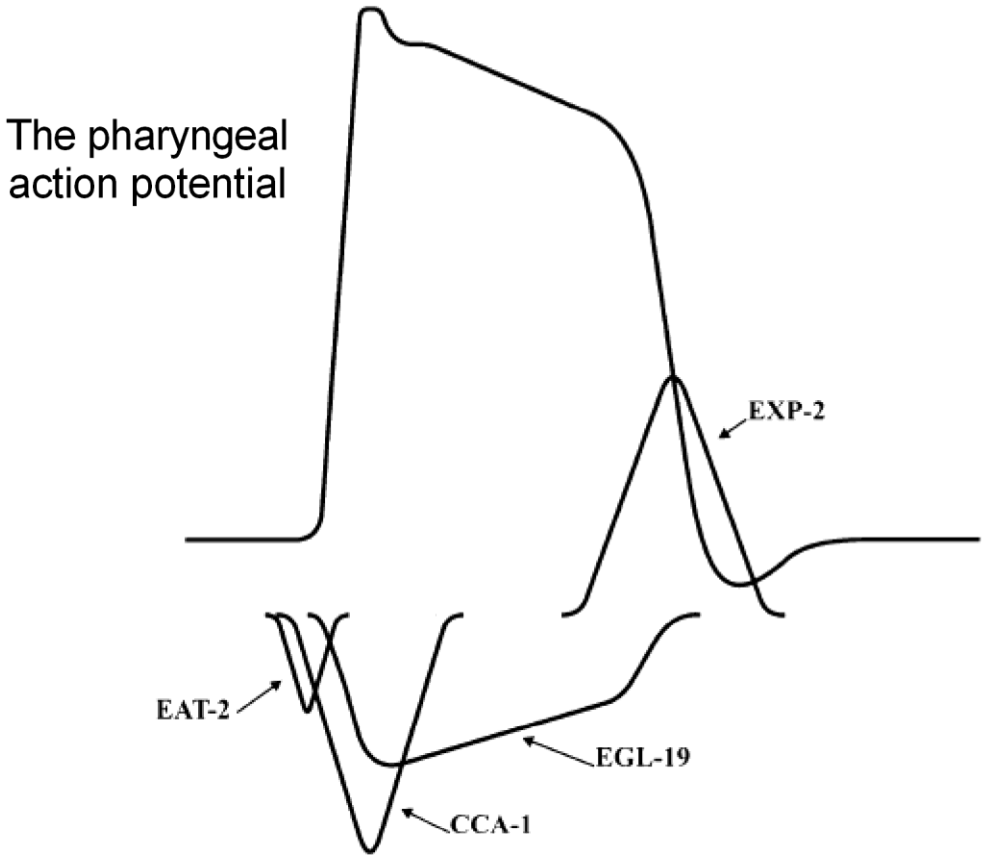

Figure 1.17. A diagrammatic representation of the currents that underpin the pharyngeal muscle action potential in *C. elegans*. The downward shapes indicate events that cause muscle depolarisation and upward shapes indicate events that lead to muscle repolarisation. The larger upward shape represents the membrane potential change that occur during a pharyngeal action potential, the smaller triangles indicated by arrows represent the flow of current through channels that mediate the action potential. Activation of nicotinic acetylcholine receptors containing EAT-2 causes a slight depolarisation, which activates the calcium channel CCA-1. This leads to a rapid depolarisation, activating EGL-19, which deactivates more slowly and thus maintains the action potential. EXP-2 is activated during this maintained depolarisation, allowing potassium efflux and repolarisation of the muscle (Lee et al., 1997, Davis et al., 1999, Steger et al., 2005, Shtonda and Avery, 2005). From (Franks et al., 2006).

The EGL-19 channel slowly inactivates, possibly in a calcium-dependent manner, which causes membrane repolarisation and when this reaches a particular threshold, the

potassium channel EXP-2 reactivates. As this repolarisation occurs, inhibitory neurotransmission via M3 through glutamate-gated chloride channels speeds up the process. Potassium efflux through EXP-2 generates a large outward current, which repolarises the membrane and results in the termination of the pharyngeal muscle action potential (Figure 1.17).

1.15.5 The neuropharmacology of pharyngeal pumping and feeding – 5-HT

5-HT is thought to be a critical neurotransmitter in the regulation of the rate of pharyngeal pumping (Figure 1.18). The application of exogenous 5-HT has been demonstrated to increase the rate of *C. elegans* pharyngeal pumping in the absence of any food (Horvitz et al., 1982, Avery and Horvitz, 1990, Niacaris and Avery, 2003). The rate of pumping observed in the presence of serotonin is comparable to that seen in the presence of food. The *tph-1* mutant provides further evidence for an important role for serotonin in the regulation of the rate of pharyngeal pumping (Sze et al., 2000). This mutation is deficient in tryptophan hydroxylase activity, the rate-limiting enzyme in the synthesis of serotonin. When off food, the basal pumping rate of these mutants does not differ from that of the wild type. When on food however whilst wild type worms upregulate pharyngeal pumping, *tph-1* mutants are unable to maintain fast pumping, indicating a critical role for 5-HT (Sze et al., 2000).

5-HT is also able to modulate pump duration. Application of the 5-HT antagonist gramine has been found to increase pump duration to up to 1 second (Niacaris and Avery, 2003) and to block the 5-HT-stimulated increase in the rate of pharyngeal pumping (Avery and Horvitz, 1990). Application of exogenous 5-HT has been reported to decrease pump duration (Niacaris and Avery, 2003). This effect is dependent on the MC and M3 neurons, as their ablation prevents the 5-HT-mediated decrease in action potential duration that usually occurs (Niacaris and Avery, 2003).

5-HT has been reported within the pharyngeal motorneurons (Horvitz et al., 1982) and the 5-HT receptors SER-1, SER-4 and SER-7b are present within the pharynx (Hamdan et al., 1999, Olde and McCombie, 1997, Hobson et al., 2003). *tph-1* is only expressed in the NSMs in the pharyngeal nervous system (Axang et al., 2008). *tph-1* is also expressed in the ADFs, sensory neurons that may contribute to modulation of pump rate

in response to food signals (Jafari et al., 2011). In the pharynx *ser-1* is expressed on the pharyngeal muscle (Tsalik et al., 2003), raising the possibility that 5-HT may act directly on the muscle cells. It has been demonstrated however that *ser-1* null mutants can still upregulate pumping when on food and so it is unlikely that this is the major receptor mediating the effects of serotonin in the pharynx (Hobson et al., 2006). Pumping in these mutants is more variable than in wild type worms and this indicates that this receptor allows for effective maintenance of fast pumping (Hobson et al., 2006).

Another 5-HT receptor that has been identified in *C. elegans* is the 5-HT_7-like SER-7 (Hobson et al., 2003). SER-7 has been identified in several neurons of the pharyngeal nervous system including MC, M3, M4, M2 and M5 (Hobson et al., 2006). *ser-7* null mutants do not upregulate pharyngeal pumping in response to 5-HT application yet pumping is upregulated when on food (Hobson et al., 2006). This also suggests that there are other signalling pathways involved in the upregulation of pumping and that the serotonergic pathway shows some redundancy. The pump rate of *ser-7* null mutants is lower however when on food and they have a reduced ability to maintain fast-pumping behaviour, much like *tph-1* mutants. It appears therefore that 5-HT is important in maintaining and modifying pumping yet is not utterly essential for upregulation of pumping. Interestingly, EPGs recorded from *ser-7* null mutants lack the e spike of the EPG waveform (Hobson et al., 2006), which is believed to represent cholinergic signalling by MC (McKay et al., 2004, Dillon et al., 2009).

1.15.6 5-HT and acetylcholine signalling interact in modulation of pump rate

Cholinergic signalling is necessary for the 5-HT-mediated increase in pharyngeal pumping (Figure 1.18). Exogenous application of nicotine has been found to stimulate pumping (Avery and Horvitz, 1990, Raizen et al., 1995), which suggests that signalling via nicotinic acetylcholine receptors mediates an increase in pump rate. Furthermore, the nicotinic receptor antagonist d-tubocurarine has been found to decrease the rate of pumping in the presence of 5-HT (Raizen et al., 1995, Chiang et al., 2006, Ruiz-Lancheros et al., 2011). This indicates that signalling through nicotinic acetylcholine receptors occurs downstream of 5-HT. The nicotinic acetylcholine receptors EAT-2 and EAT-18 have been identified in the pharynx (McKay et al., 2004) and mutations in

either of these receptors causes a reduction in the rate of pharyngeal pumping in the presence of food (Raizen et al., 1995). EAT-2 and EAT-18 form a nicotinic acetylcholine receptor complex on the pharyngeal muscle at the MC synapse (McKay et al., 2004).

It has been demonstrated that 5-HT can only stimulate pumping in animals capable of cholinergic signalling, as a *cha-1* mutation suppresses pumping in the presence of 5-HT (Raizen et al., 1995, Song and Avery, 2012). *cha-1* mutants have a 99% reduction in the activity of choline acetyltransferase, which is required for acetylcholine synthesis (Rand and Russell, 1984). A mutation in *eat-2* also prevents 5-HT stimulated pumping (Raizen et al., 1995, McKay et al., 2004, Song and Avery, 2012). The same mutation also causes defects in MC neurotransmission (Raizen et al., 1995, McKay et al., 2004). This indicates that 5-HT signalling via SER-7 induces acetylcholine release from MC, which signals through the EAT-2/ EAT-18 receptor to depolarise the pharyngeal muscle and stimulate pumping (Song and Avery, 2012). It has also been shown by Song and Avery (2012) that the same signalling pathway through M4 stimulates isthmus peristalsis. 5-HT signalling via SER-7 also increases glutamate signalling from M3, shortening pump duration to allow more rapid pumping (Niacaris and Avery, 2003).

In the pharynx, the NSMs are known to contain 5-HT (Albertson and Thomson, 1976, Ranganathan et al., 2000) and may have a neurohumoral function, with 5-HT acting as a neurohormone to activate the signalling pathways that increase pump frequency (Komuniecki et al., 2004). The NSMs have putative sensory endings near the pharyngeal lumen, at the point between the metacorpus and the isthmus (Albertson and Thomson, 1976). As this is the point where food accumulates, it has been postulated that the presence of food in the pharynx increases 5-HT release from the NSMs to increase pump rate. Ablation of the NSMs has little effect on pump rate however (Avery et al., 1993). Other neurons are also thought to have sensory endings that may detect the presence of food in the pharynx, and so the NSMs may be redundant with these neurons in signalling to increase pumping (Riddle, 1997).

1.15.7 Muscarinic acetylcholine signalling

Acetylcholine signalling through muscarinic receptors is also thought to play a key role in the regulation of pharyngeal muscle activity (Steger and Avery, 2004). A loss-of-

function mutation in *gar-3*, which encodes a muscarinic acetycholine receptor (Hwang et al., 1999), results in more rapid pumping and a reduction in pump duration relative to N2 (Steger and Avery, 2004). It has been suggested that this may be due to heightened excitability of the pharyngeal muscle (Steger and Avery, 2004, Steger et al., 2005). The effects of GAR-3 on pharyngeal pumping appear to be dependent upon the activity of EGL-19, a voltage-gated calcium channel (Steger and Avery, 2004). This has led to the proposition that acetylcholine signalling through GAR-3 alters calcium levels to regulate muscle activity and optimise pumping (Steger and Avery, 2004).

1.15.8 Additional modulators of pharyngeal pumping

Whilst 5-HT and acetylcholine appear to be the key regulators of pump rate, additional biogenic amines affect the pharyngeal system. Octopamine has antagonistic effects to those of 5-HT on *C. elegans* behaviour, including pharyngeal pumping (Chase and Koelle, 2007). Exogenous octopamine has been shown to block the stimulatory effects of 5-HT on pharyngeal pumping and this block is prevented by the octopamine antagonist epinastine (Horvitz et al., 1982, Rogers et al., 2001, Packham et al., 2010). Niacaris and Avery (2003) have also shown in a wild type and *eat-18* mutant background that octopamine reduces the activity of M3 and increases pump duration, with 5-HT having opposing effects. These observations support the hypothesis that octopamine acts as a physiological antagonist to 5-HT in *C. elegans* (Horvitz et al., 1982). It has also been proposed that the antagonistic action of 5-HT and octopamine allow adaptation to food availability (Franks et al., 2006, Avery and You, 2012). When food is absent octopamine levels may rise, reducing food-associated behaviours such as rapid pumping. 5-HT also stimulates egg laying and reduces locomotion, whilst octopamine has the opposte effects (Horvitz et al., 1982, Segalat et al., 1995, Sawin et al., 2000). This supports this hypothesis, as egg laying increases in the presence of food whilst locomotion is reduced. Octopamine has not yet been identified in the pharyngeal system but could act as a neurohormormone (Figure 1.18). Tyramine may also have a similar function in *C. elegans* as it also inhibits 5-HT-stimulated pumping (Packham et al., 2010). In addition, the tyramine receptor SER-2 is expressed on the pharyngeal muscle (Tsalik et al., 2003). There is currently no published evidence that dopamine is directly involved in mediating pharyngeal pumping.

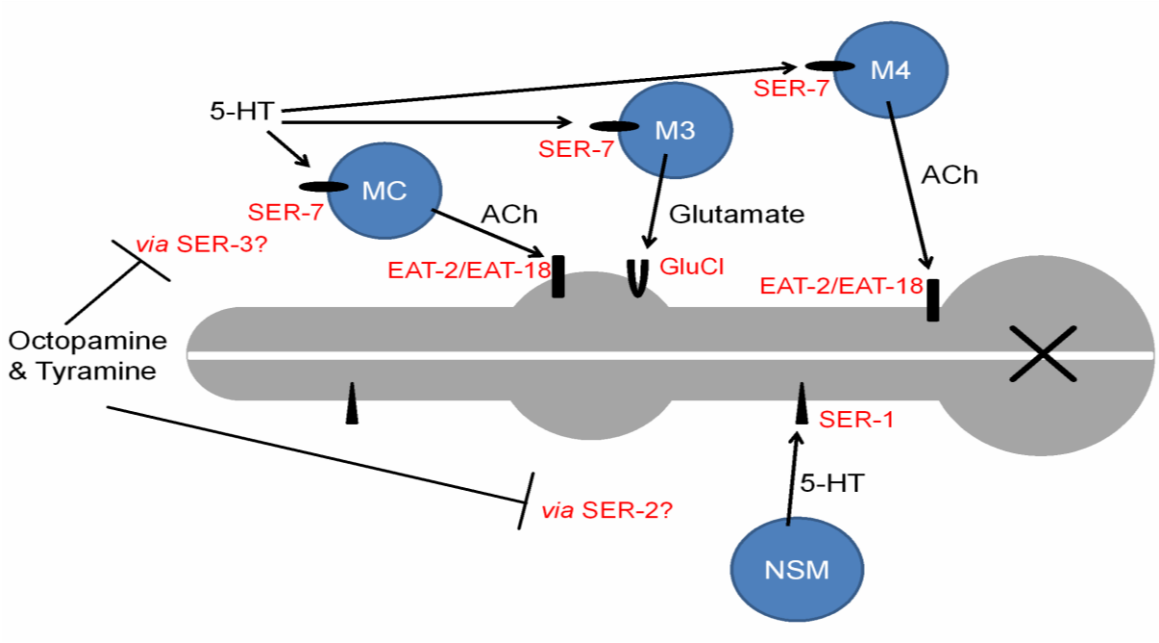

Figure 1.18. Cartoon highlighting the major points of transmitter regulation of *C. elegans* pharyngeal pumping and isthmus peristalsis. (ACh=Acetylcholine, GluCl=Glutamate-gated chloride channel). 5-HT interacts with SER-7 on neurons and SER-1 on the pharyngeal muscle to upregulate pumping (Niacaris and Avery, 2003, Hobson et al., 2003, Hobson et al., 2006). Activation of SER-7 results in acetylcholine signalling from MC and M4 to upregulate pump rate and the rate of isthmus peristalsis, respectively (Avery and Horvitz, 1989, Raizen et al., 1995, Franks et al., 2006). Acetylcholine released from MC interacts with the EAT-2/EAT-18 receptor to enhance pump rate (McKay et al., 2004). Activation of SER-7 also increases glutamate release from M3, to shorten pump duration through interaction with glutamate-gated chloride channels (Avery, 1993b, Dent et al., 1997). Octopamine and tyramine can inhibit 5-HT-mediated effects, possibly through the SER-3 and SER-2 receptors, respectively (Horvitz et al., 1982, Tsalik et al., 2003, Suo et al., 2006).

Studies into neuropeptides indicate that these neuromodulators may also regulate pharygeal behaviour in *C. elegans*. A number of neuropeptides are known to be expressed in the pharyngeal neurons (Nathoo et al., 2001, Kim and Li, 2004, Li, 2005) and several have been shown to have excitatory and inhibitory effects on pumping (Rogers et al., 2001, Papaioannou et al., 2005, Papaioannou et al., 2008, Cheong et al., 2015). Cheong et al. (2015) have shown that some neuropeptides may play a role in regulating feeding rate in response to starvation conditions.

1.15.9 The electropharyngeogram

The electropharyngeogram (EPG) is an extracellular electrophysiological technique that can be used to visualise electrical activity in the pharynx. This technique was developed by Raizen and Avery (1994) and allows recording of the electrical activity in the pharyngeal muscle. In the EPG, a depolarisation resulting in a positive alteration in

membrane potential gives a positive spike, whereas repolarisation gives a negative spike (Riddle, 1997).

To record an EPG the anterior, or nose, of the worm is sucked onto a recording electrode (see chapter 2, section 2.3.20). A reference electrode is present within the recording chamber and the worm is immersed in saline solution. Each pharyngeal pump generates a stereotypical waveform and evidence suggests that the various components of the waveform correspond to the neural and muscular activity that are involved in and regulate each pump (Figure 1.19) (Raizen and Avery, 1994, Avery, 1993b, Raizen et al., 1995, Avery et al., 1995, Niacaris and Avery, 2003).

The EPG can be divided into three phases: the E-phase, the P-phase and the R-phase (Figure 1.19). During the E (excitation)-phase, there are two upward spikes, the small e spike and the larger E spike. Ablation of MC prevents the e spike, which suggests that this indicates MC activity (Raizen et al., 1995). The E spike still occurs following ablation of the pharyngeal neurons and is correlated with the contraction of the corpus and terminal bulb, which suggests that this spike represents the excitation and contraction of the muscle (Raizen and Avery, 1994). The P (plateau) phase correlates with the sustained contraction of the muscle (Raizen and Avery, 1994). During the P-phase, small negative P spikes are sometimes recorded. These spikes are likely to be due to inhibitory glutamatergic signalling by M3, as these spikes are missing in M3-ablated worms or in an *avr-15* mutant background (Dent et al., 1997). Pump duration is increased by M3 ablation or in *avr-15* worms, which indicates that this glutamatergic signalling acts to shorten pump duration (section 1.15.3). The P-phase is followed by the R (relaxation)-phase in which there is the large negative R spike and the smaller negative r spike. The R and r spikes correlate with the relaxation of the corpus and the terminal bulb, respectively (Raizen and Avery, 1994). Between pumps, small positive I (interpump) spikes can occur (Raizen et al., 1995). These spikes are absent in MC-ablated animals, which suggests that they may represent MC activity.

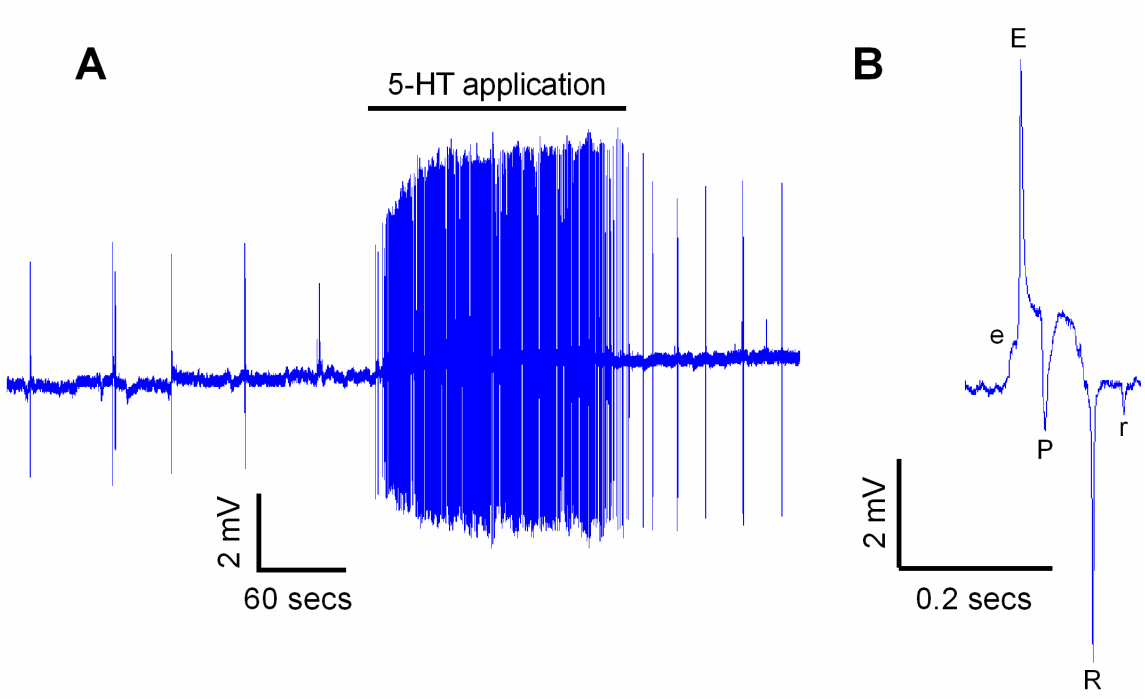

Figure 1.19. The EPG waveform. A) Cut head *C. elegans* pump infrequently in saline alone. When 500 nM 5-HT is applied (as indicated), the rate of pharyngeal pumping increases. **B**) The components of the EPG waveform. The E and R spikes correlate with contraction and relaxation muscle of the corpus, respectively. The r spike correlates with terminal bulb relaxation. The e spike is thought to represent MC cholinergic signalling, whilst the P spikes are thought to represent M3 glutamatergic signalling (Raizen and Avery, 1994, Raizen et al., 1995, Dent et al., 1997, Niacaris and Avery, 2003, McKay et al., 2004).

1.16 *C. elegans* egg laying

C. elegans hermaphrodites generate sperm at the L4 stage, which are stored in the spermatheca. Following the L4-to-adult moult, oocytes are generated and are fertilised following ovulation (Pazdernik and Schedl, 2013), after which embryogenesis occurs. At the early adult stage, these fertilised eggs will accumulate in the uterus. Egg laying occurs when the hermaphrodite-specific vulval muscles contract.

It is believed that 16 muscle cells are involved in the egg laying process (Schafer, 2005). Of these muscle cells, the four vm2 muscles are crucial in the opening of the vulva, as laser ablation of these cells eliminates egg laying behaviour. The vm2 cells are electrically-coupled by gap junctions and are the only vulval muscles cells that are innervated by neurons (White et al., 1986). These neurons are the two HSN (hermaphrodite-specific neurons) and the six VC neurons. The HSNs utilise 5-HT, acetylcholine and neuropeptides as transmitters (Desai et al., 1988, Schinkmann and Li,

1992, Duerr et al., 2001). Signalling by the HSNs is critical in the egg laying process as ablation greatly reduces the rate of egg laying (Desai and Horvitz, 1989). The role of the VC neurons in egg laying is less clear. In an *egl-1* mutant background, which results in the loss of the HSNs, reduced egg laying is further impaired by ablation of VC4 and VC5 (Waggoner et al., 1998). Several mutants that constitutively lay eggs have however been found to have defective VC morphology or have diminished transmitter release from the VCs (Bany et al., 2003). These observations suggest the VCs can stimulate and inhibit egg laying. Whilst the role of the VCs in egg laying is not fully understood, it is known that these neurons are cholinergic and may use 5-HT and neuropeptides as transmitters (Schinkmann and Li, 1992, Duerr et al., 1999, Duerr et al., 2001).

An important function of 5-HT signalling is regulating the timing of egg laying events. Exogenous 5-HT stimulates egg laying and rescues egg laying defects in HSN ablated worms (Trent et al., 1983). Exogenous 5-HT has also been shown to increase the frequency of calcium transients in the vulval muscles (Shyn et al., 2003). It is thought that the HSNs release 5-HT to increase vulval muscle activity (Shyn et al., 2003). *tph-1* mutants, which have greatly reduced 5-HT levels (Sze et al., 2000) have a less profound phenotype than HSN-ablated worms, which suggests that other neurotransmitters are also likely to be involved (Weinshenker et al., 1995, Kim et al., 2001). The 5-HT receptors SER-1 and SER-7 are both expressed in the vulval muscle and are required for the 5-HT-mediated increase in egg laying, as loss-of-function mutations in the genes encoding these receptors reduces 5-HT-induced egg laying (Dempsey et al., 2005, Hobson et al., 2006, Hapiak et al., 2009). Interestingly, 5-HT actually inhibits egg laying in *ser-1; ser-7* mutants and this inhibition is dependent on the 5-HT receptors MOD-1 and SER-4 (Hapiak et al., 2009). This suggests that 5-HT signalling through distinct receptor types can have opposing effects on egg laying behaviour. Hapiak et al. (2009) also suggest a role for the putative 5-HT receptor SER-5 in mediating 5-HT-stimulated egg laying.

Cholinergic signalling is also thought to be important in the regulation of egg laying, as nicotinic acetylcholine receptor agonists stimulate egg laying (Weinshenker et al., 1995). Biogenic amines are also likely to function as regulators of egg laying, as exogenous dopamine, octopamine and tyramine all inhibit egg laying (Schafer and

Kenyon, 1995, Weinshenker et al., 1995, Rex et al., 2004). Neuropeptides are also modulators of egg laying (Ringstad and Horvitz, 2008).

1.17 Energy metabolism – a new target for chemical control of nematodes?

Many anthelmintics interact with the nematode nervous system to elicit paralysis or death (see (Holden-Dye and Walker, 2014) for review). The anticholinesterase nematicides the carbamates and the organophosphates achieve nematode control through impairment of acetylcholine recycling at the neuromuscular junction (Doctor et al., 1998, Selkirk et al., 2005, Jadhav and Rajini, 2009). The nematode nervous system is therefore an established target for chemical control agents. In the search for new nematicides and anthelmintics, nematode metabolism has more recently become a focus of interest for potential targets (Rana and Misra-Bhattacharya, 2013, Taylor et al., 2013). A recurring issue with nematicides and anthelmintics that target the nematode nervous system is a lack of selective toxicity (Chitwood, 2003a, Haydock et al., 2014). Many aspects of neurobiology are conserved and shared amongst animals from different phyla and thus selectivity can be difficult to achieve (Ghysen, 2003). Whilst the fundamental aspects of energy metabolism are also highly conserved amongst most eukaryotes (Smith and Morowitz, 2004), the life cycle and specific biology of parasitic nematodes may render them particularly sensitive to selective metabolic insults (Rana and Misra-Bhattacharya, 2013, Taylor et al., 2013). For example, many animal parasites are reliant upon anaerobic metabolic pathways in their life cycle, due to the anoxic environments in which they reside (Tielens, 1994, Komuniecki and Harris, 1995, Kita et al., 1997). Plant parasites, specifically the sedentary endoparasites, have life cycle stages that are non-feeding (Perry and Moens, 2013) and therefore potentially susceptible to specific metabolic insults that target pathways critical for metabolism of their energy stores.

Of the nematicides that have been used for PPN control in the field, it is thought that some fumigants are nematicidal as a result of inhibition of nematode metabolism, although little research has been conducted regarding the mechanism of action of fumigant nematicides (Chitwood and Wright, 1981, Chitwood, 2003a). This suggests that metabolism may be an effective target for nematode control. More recently, the

compound fluopyram has been launched for nematode control in the field (Hungenberg et al., 2013). Fluopyram is a member of a group of succinate dehydrogenase inhibitors (SDHIs), which have been used in the past for control of fungi in the field (Veloukas and Karaoglanidis, 2012). There are 15 SDHI compounds, which come from seven different chemical classes. All share the same mechanism of action by inhibiting succinate dehydrogenase (also known as succinate: quinone-oxidoreductase), which is complex II of the mitochondrial electron transport chain (Cecchini, 2003). Inhibition of succinate dehydrogenase prevents the function of the electron transport chain and thus impedes cellular respiration (Matsson and Hederstedt, 2001). Whilst no other nematicides with a known metabolic target have been launched, it is known that various metabolic inhibitors have effects on PPNs (Butterworth et al., 1989). More recently, lactone and aldehyde compounds have been demonstrated to have nematicidal activity against *M. incognita* and *M. arenaria*, and it was suggested that this occurred through inhibition of ATPase activity (Caboni et al., 2014). ATPases couple the hydrolysis of ATP to ADP the energy released from this reaction is used to drive a number of processes (Pedersen, 2005, Beyenbach and Wieczorek, 2006). For example, the Na^+/K^+-ATPase is essential for maintenance of cell membrane potential (Kaplan, 2002).

1.17.1 The fundamental aspects of intermediary metabolism

Metabolism is the set of chemical reactions that occur within the cells of living organisms that allow the fundamental aspects of life to occur (Berg et al., 2002, Campbell and Reece, 2008). These chemical reactions form components of a series of pathways and cycles that form a linked network that is controlled by a variety of regulatory processes and mechanisms. Catabolism is the breakdown of larger, complex molecules into their smaller components and anabolism is the synthesis of large molecules from smaller constituents (Berg et al., 2002).

Intermediary metabolism is a term that describes the pathways of metabolism that are involved with the utilisation, generation and storage of metabolic energy (Berg et al., 2002). The networks and pathways of intermediary metabolism are thought to be highly conserved amongst eukaryotic organisms (Figure 1.20) (Smith and Morowitz, 2004). In eukaryotes, the majority of catabolic reactions function to generate adenosine triphosphate (ATP) from larger precursor molecules such as lipid, carbohydrates and

proteins. ATP acts as a free energy donor that drives most energy-requiring processes in living organisms. In aerobic respiration, electron carriers are generated, including nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), flavin adenine dinucleotide (FAD) and succinate.

Energy generation occurs through the catabolism of the energy storage molecules, carbohydrates, lipids and proteins (Berg et al., 2002). Carbohydrates such as glucose can be directly catabolised or can undergo glycogenesis to produce the carbohydrate storage molecule glycogen. When required, glucose can be generated from glycogen by glycogenolysis. Lipids are stored as triglycerides, which consist of three fatty acids and one glycerol molecule. Lipids can be generated from excessive carbohydrates for energy storage in the process of lipogenesis (Owen et al., 1979, Berg et al., 2002). Excessive amino acids can be catabolised to generate energy, as can proteins (Brosnan, 2003). Proteins will typically only be used as an energy source under starvation conditions when carbohydrate and lipid reserves have been depleted (Berg et al., 2002).

The major pathways in the generation of energy from carbohydrates, lipids and proteins are glycolysis, β-oxidation, proteolysis and gluconeogenesis (Berg et al., 2002). Glycolysis allows the anaerobic generation of pyruvate from glucose (Berg et al., 2002). In an aerobic environment, the pyruvate that is generated by glycolysis can be decarboxylated by the enzyme pyruvate dehydrogenase to generate acetyl-CoA. Alternatively, in an anaerobic environment pyruvate is disposed of through fermentation into lactate and ethanol. Triglycerides are broken down into fatty acids in the process of lipolysis (Berg et al., 2002). Fatty acids are then catabolised in the process of β-oxidation, which generates acetyl-CoA (Houten and Wanders, 2010). Proteolysis of proteins generates smaller peptides or amino acids, which can be converted to carbohydrates by gluconeogenesis (Berg et al., 2002).

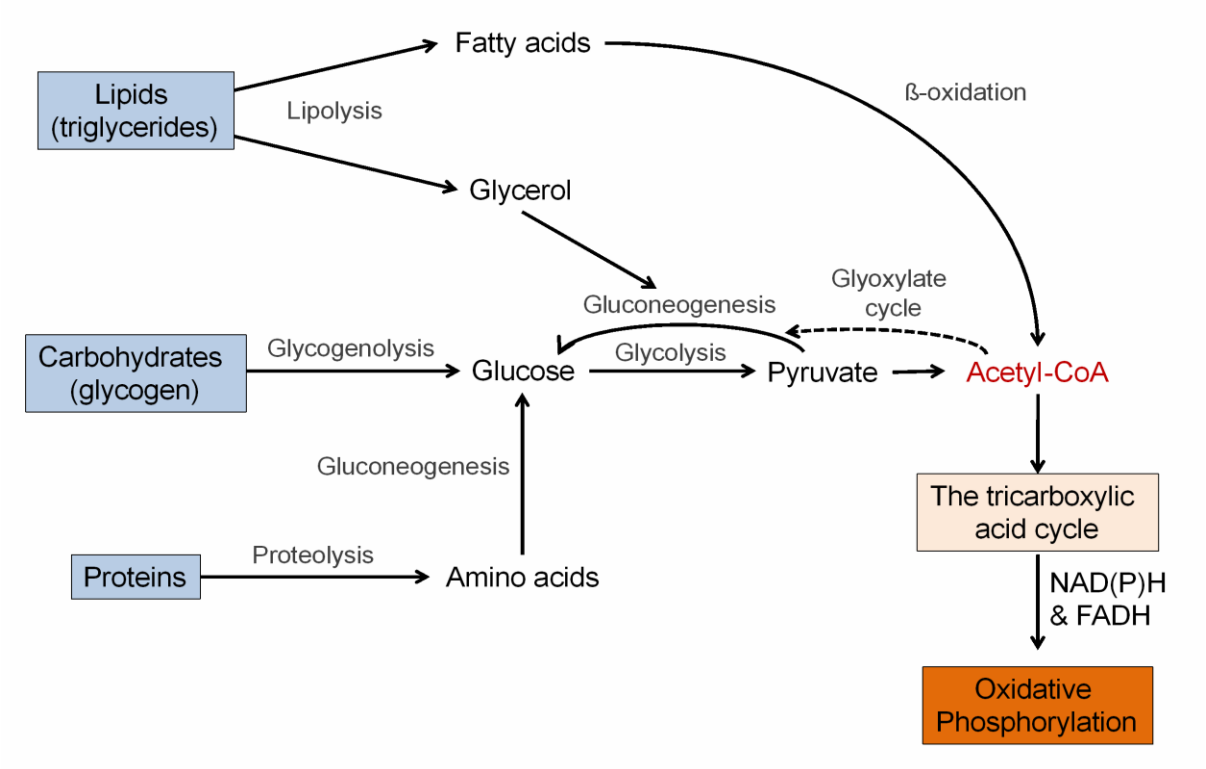

Figure 1.20. A diagrammatic summary of the fundamental pathways of intermediary metabolism. The basic energy storage molecules, lipids, carbohydrates and proteins are catabolised to smaller components. Glycolysis of glucose and β-oxidation of fatty acids generates acetyl-CoA. Gluconeogenesis can generate glucose from amino acids and other non-carbohydrate precursors such as pyruvate. Plants, fungi, bacteria and nematodes possess a functional glyoxylate cycle, which converts acetyl-CoA to malate and succinate, which can then enter the gluconeogenesis pathway. Acetyl-CoA enters the tricarboxylic acid cycle, which generates NAD(P)H and FADH. NAD(P)H and FADH are then utilised in the electron transport chain and oxidative phosphorylation to generate ATP.

Acetyl-CoA generated from these processes then enters the aerobic pathway known as the tricarboxylic acid (TCA) cycle. The TCA cycle is the final common pathway in the oxidation of all fuel molecules, including carbohydrates, lipids and amino acids. In the TCA cycle, a series of oxidation reactions occur, resulting in the generation of NADH, $FADH_2$ and carbon dioxide (Figure 1.21). The NADH and $FADH_2$ generated through glycolysis, β-oxidation and the TCA cycle is then utilised in the process of oxidative phosphorylation. In oxidative phosphorylation, electron donors such as NAD(P)H, $FADH_2$, and succinate are oxidised, with molecules such as oxygen acting as electron acceptors. These electrons are transferred from donor molecules to oxygen by the protein complexes NADH-Q oxidoreductase, Q-cytochrome c oxidoreductase and cytochrome c oxidase. Along with succinate-Q-reductase, these complexes are collectively known as the electron transport chain. This transfer of electrons out of the

mitochondrial matrix generates a proton gradient. As protons flow back into the mitochondrial matrix through ATP synthase, ATP is generated.

1.17.2 The glyoxylate pathway

Plants, fungi, bacteria, protists and nematodes possess another metabolic pathway known as the glyoxylate cycle (Kondrashov et al., 2006). There is conflicting evidence as to whether this cycle may be present in some vertebrates too (Davis et al., 1990, Davis and Goodman, 1992, Jones et al., 1999). The glyoxylate cycle is in essence a variation of the TCA cycle and allows the synthesis of succinate, and subsequently carbohydrates from simple carbon compounds such as acetyl-CoA (Figure 1.21) (Berg et al., 2002). The glyoxylate cycle effectively allows the generation of glucose from acetyl-CoA that is produced via β-oxidation of fatty acids. The glyoxylate cycle therefore bypasses the requirement for uptake of carbohydrates from external sources. During the glyoxylate cycle, isocitrate lyase and malate synthase catalyse the production of succinate and malate from isocitrate and acetyl-CoA. Malate then enters the gluconeogenesis pathway to generate glucose (Berg et al., 2002).

This ability to synthesise carbohydrates in the absence of any nutrient uptake is important in plants, fungi and bacteria, which possess cell walls and thus have a high requirement for carbohydrates during growth (Lorenz and Fink, 2001, Berg et al., 2002, Lorenz and Fink, 2002). For example, during seed germination, the young plant is reliant upon lipid stores and so the glyoxylate cycle is crucial to allow growth and maturation. This pathway is also thought to be important in nematode biology (see section 1.17.5).

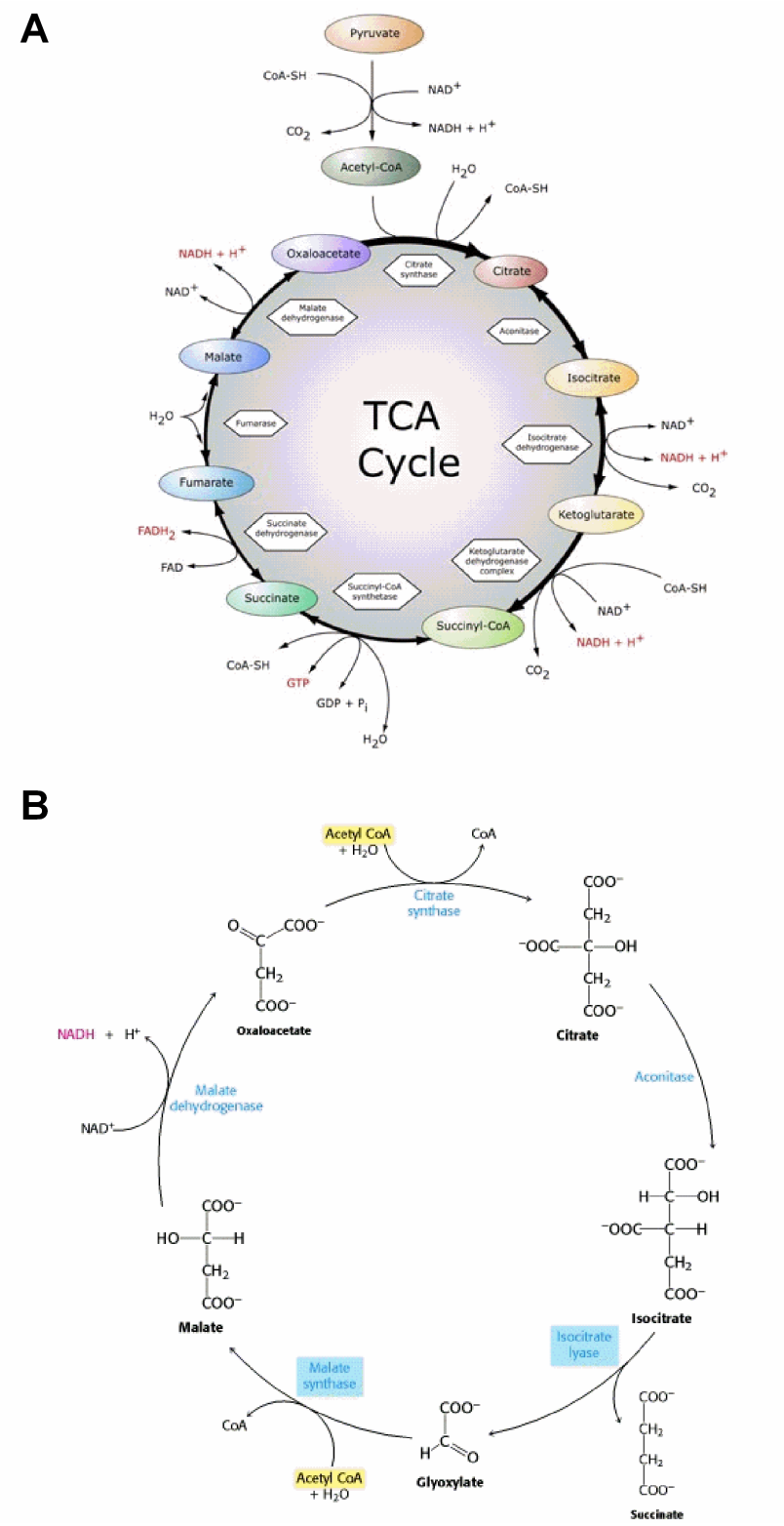

Figure 1.21. The tricarboxylic acid cycle and the glyoxylate cycle. **A**) Acetyl-CoA generated through glycolysis and β-oxidation enters the TCA cycle. Through a series of reactions, NADH and $FADH_2$ are generated, which are then used in oxidative phosphorylation to generate ATP. From (https://biochembayern.wordpress.com). **B**) The glyoxylate cycle centres on the conversion of acetyl-CoA into succinate, malate and oxaloacetate. Succinate and malate can enter the tricarboxylic acid cycle or can be converted to oxaloacetate, which can then enter into gluconeogenesis. The enzymes that catalyse the steps in the pathway are in blue. From (Berg et al., 2002).

1.17.3 Metabolism in nematodes

C. elegans possesses orthologues for most of the enzymes and proteins that are key components of the machinery of intermediary metabolism in other organism, which suggests that *C. elegans* utilises the same metabolic pathways as other eukaryotes (Figure 1.22) (O'Riordan and Burnell, 1989, O'Riordan and Burnell, 1990, Holt and Riddle, 2003, Wang and Kim, 2003, Ashrafi, 2007, Braeckman et al., 2009). Serial analysis of gene expression (SAGE) has indicated the presence of various key enzymes involved in metabolism in *C. elegans*, including enzymes that mediate glycolysis, gluconeogenesis and the TCA cycle (Holt and Riddle, 2003). Profiles of gene expression in dauers and non-dauers by DNA microarrays have shown that *C. elegans* expresses genes involved in glycolysis, gluconeogenesis, the Kreb's cycle, lipid metabolism and β-oxidation and the metabolism of amino acids, proteins and carbohydrates (Wang and Kim, 2003). The mitochondrial genome of *C. elegans* indicates the presence of genes encoding proteins of the electron transport chain and those involved in oxidative phosphorylation (Okimoto et al., 1992). Proteomic analysis of *C. elegans* has also indicated that mitochondrial proteins are highly conserved relative to mammals (Li et al., 2009). It is therefore thought that *C. elegans* metabolism is comparable to metabolism in other eukaryotic organisms. The genome sequences of *G. pallida*, *M. incognita*, *M. hapla* and *Bursaphalenchus xylophilus* suggest that PPNs are also likely to possess similar metabolic machinery to other eukaryotes (Abad et al., 2008, Opperman et al., 2008, Kikuchi et al., 2011, Cotton et al., 2014).

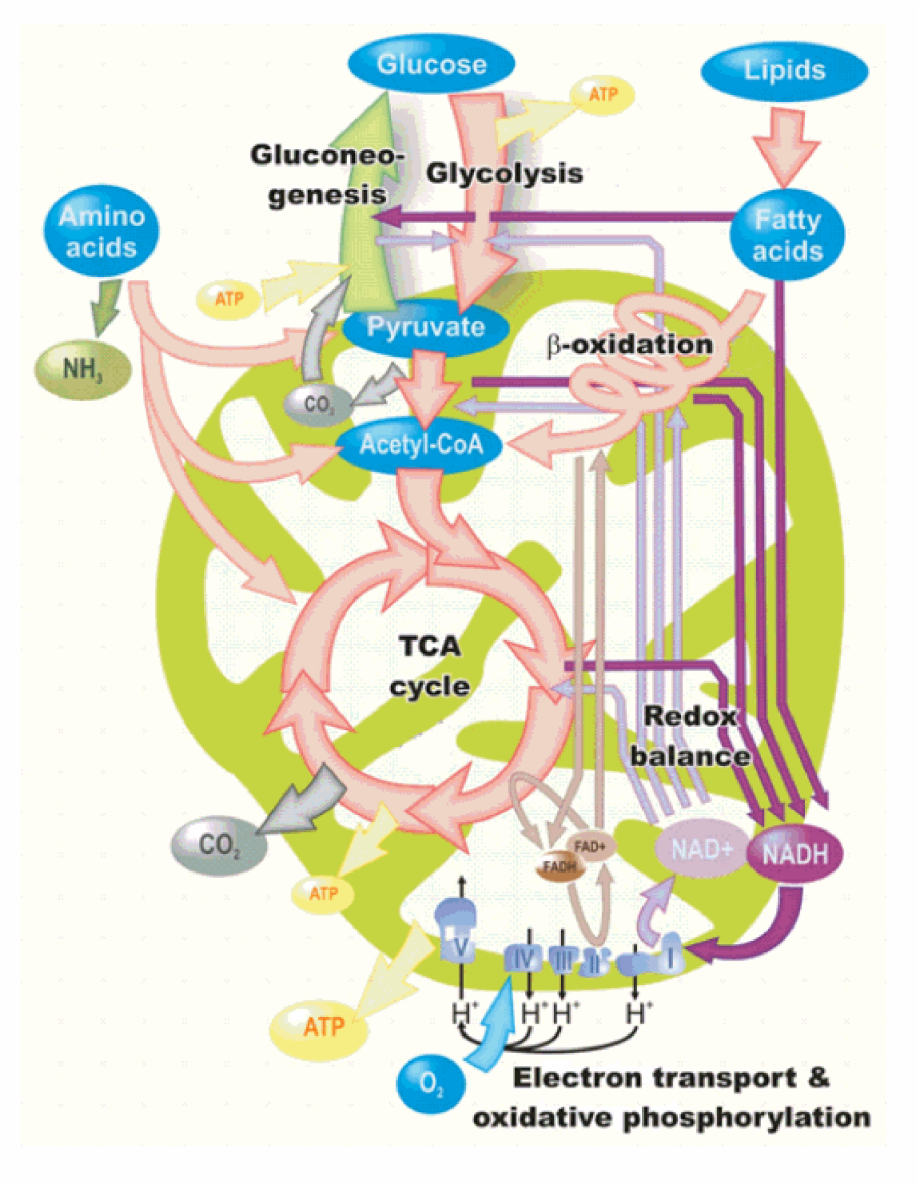

Figure 1.22. An overview of the metabolic pathways thought to be present in a *C. elegans* adult. Lipids, carbohydrates and amino acids are catabolised to acetyl-CoA, which enters the TCA cycle. NAD(P)H and $FADH_2$ are predominately generated by the TCA cycle and act as reducing agents in oxidative phosphorylation, allowing the generation of ATP. From (Braeckman et al., 2009).

1.17.4 Energy storage in nematodes

Lipids and glycogen are the principle forms of energy storage in nematodes, although the levels of each varies greatly between animal parasitic, plant parasitic and free living nematodes. Around 20-35% of the dry body mass of *C. elegans* is lipid, whilst PPN lipid content varies from 11-67% of dry weight (Cooper and Van Gundy, 1970, Hutzell and Krusberg, 1982, Barrett and Wright, 1998, Braeckman et al., 2009). *M. incognita*

and *M. arenaria* second stage juveniles contain 46% and 40% lipid, respectively (Krusberg et al., 1973).

Around 3.3% of the dry weight of *C. elegans* is glycogen and in the absence of food glycogen stores are rapidly depleted (Cooper and Van Gundy, 1970). Glycogen reserves in PPNs vary from 3-20% dry weight (Perry et al., 2011). In contrast, animal parasitic nematodes rely on glycogen reserves more so than lipids, with up to 80% dry weight glycogen (Geovannola, 1936, Elliot, 1954, Jones, 1955, Fairbairn, 1960, von Brand, 1966). This reflects the low oxygen levels that animal parasites are often exposed to, as lipid metabolism requires oxygen (Cooper and Van Gundy, 1970). Dauer *C. elegans* are reliant upon their lipid reserves for prolonged survival in the absence of any food source (O'Riordan and Burnell, 1990). Second stage juveniles of sedentary endoparasitic PPNs such as *Globodera* and *Meloidogyne* are non-feeding and are reliant upon their lipid reserves until they establish a feeding site in a suitable host plant (Cooper and Van Gundy, 1970, Cooper and Van Gundy, 1971b, Dropkin and Acedo, 1974, Reversat, 1981). Electron micrograph analysis of the lipid and glycogen stores of *M. incognita* suggests that the balance between lipid and glycogen usage shifts throughout the life cycle (Dropkin and Acedo, 1974). J2s store the majority of energy as lipids in the intestine prior to host invasion. On invading the host, these vast lipid reserves are utilised and glycogen deposits accumulate, which suggests that lipid may be converted to glycogen. Lipid then accumulates again at the adult female stage.

C. elegans lipids are thought to be stored in intestinal cells and epidermal cells and in the hypodermis (Hellerer et al., 2007, Mullaney and Ashrafi, 2009). Oil RedO staining indicates that PPN lipid stores are predominately in the posterior of the worms and not the head region and electron microscopy indicates that these stores are in the intestines (Dropkin and Acedo, 1974, Stamps and Linit, 1995).

1.17.5 Dauer *C. elegans* and some PPNs utilise the glyoxylate cycle

As mentioned, *C. elegans* can enter a dauer survival stage on transition from the L2 stage to L3 (see section 1.12). The dauer stage is non-feeding and is metabolically distinct from feeding L4 and adult worms, with reduced oxygen consumption (O'Riordan and Burnell, 1990, Vanfleteren and DeVreese, 1996, Houthoofd et al., 2002,

Braeckman et al., 2009). It is thought that this results from reduced oxidative phosphorylation and TCA cycle activity relative to other developmental stages (O'Riordan and Burnell, 1989). As dauers are non-feeding and must rely on internal lipid reserves, these pathways have reduced activity. SAGE analysis and microarray studies indicate that dauer larvae have increased transcription of enzymes involved in β-oxidation, glycolysis, gluconeogenesis and the glyoxylate cycle (Jones et al., 2001, Holt and Riddle, 2003, Wang and Kim, 2003, McElwee et al., 2004, McElwee et al., 2003, McElwee et al., 2006). This suggests consumption of lipid reserves and their conversion into sugars as an energy source.

The glyoxylate cycle is active in the L1 stage and dauer stage, whereas the TCA cycle predominates in the other developmental stages (Wadsworth and Riddle, 1989). This allows the generation of carbohydrates from lipid stores (see section 1.17.2), which is of particular importance in the non-feeding dauer (Berg et al., 2002). The glyoxylate cycle has also been proposed to act as a response to starvation conditions in larvae. PPNs are also known to possess glyoxylate cycle enzymes, including J2 *M. incognita* and the glyoxylate cycle may be important for energy generation in the non-feeding J2 (McCarter et al., 2003, Kondrashov et al., 2006). It has been shown that the carbohydrate stores of J2 *M. javanica* remain steady after storage for several days, whilst lipid stores decrease (Reversat, 1981). This may indicate that as lipid reserves are consumed, the glyoxylate cycle is used to replenish carbohydrate stores.

1.17.6 Metabolism as a target for chemical control of PPNs

As described above, the fundamental aspects of metabolism are highly conserved amongst eukaryotic organisms (Smith and Morowitz, 2004). This calls into question the safety and selectivity that any nematicide would have that targets nematode metabolism. Despite such concerns, some aspects of the PPN life cycle and the specific metabolisms required for the life cycle may permit selective toxicity of metabolic inhibitors towards PPNs. For example, the reliance of the non-feeding J2 stage of sedentary endoparasitic nematode on lipid stores may render them more sensitive to specific metabolic insults. At the J2 stage, β-oxidation is presumably a crucial pathway to allow energy generation from lipid stores. Therefore, it is reasonable to assume that inhibition of this pathway is a potential route for chemical control of PPNs. If the glyoxylate cycle is also important

for the generation of energy in J2 PPNs, inhibition of this pathway may be another route to selective toxicity. Inhibition of other pathways that link in with β-oxidation and the glyoxylate cycle, for example gluconeogenesis, may also be an effective means of PPN control.

1.18 Project aims

The mode of action of fluensulfone is not currently known. The toxicity of fluensulfone is relatively selective when compared to other currently and previously used fumigant and non-fumigant nematicides. Discovering the target(s) of fluensulfone is therefore desirable, as this will enable further research into the target itself and potentially aid the development of new nematicides and anthelmintics with selective toxicity against PPNs. Understanding the mechanisms of pesticide toxicity are also useful for the safe and appropriate application of such chemicals in the field.

The principle aim of this study is to investigate the action of fluensulfone on nematodes and to use this knowledge to determine the target(s) that mediate fluensulfone toxicity against nematodes.

1. To characterise the effects of fluensulfone on the model genetic organism *C. elegans*, with a view to gaining an insight into potential targets by electrophysiological and genetic investigation.
2. To investigate the acute effects of fluensulfone on the PPN *G. pallida*, with a particular focus on the stylet system.
3. To study the pharmacology of stylet behaviour, a potential system for studying chemicals in PPNs.
4. To determine the effects of chronic exposure to fluensulfone on nematodes, as such conditions are likely to be encountered in the field.

Chapter 2: Materials and Methods

2.1 *C. elegans* techniques

2.1.1 *C. elegans* culture

C. elegans were cultured on Nematode Growth Medium (NGM) plates, a modified agar that also contains peptone and sodium chloride. A plate pouring machine (Jencons Scientific Ltd.) was used to pour the plates to a volume of approximately 10 mls per 5 cm petri dish. These NGM plates were inoculated with 50 µl *E. coli* OP50 as a food source for *C. elegans* and the OP50 were allowed two nights to grow and dry at approximately 20^{o}C before worms were added. Plates were used up to 14 days after pouring.

NGM plates were sealed using parafilm to prevent any contamination and stored in an incubator at 20^{o}C. *C. elegans* L4 stage larva were picked onto fresh food NGM plates the day before (20-24 hours) experimentation to give a synchronised population of L4+1 day adult worms. For generating synchronised populations of L1, L2/3 and L4 larvae populations of eggs were collected and allowed to grow for 12 hours, 30 hours, 36 hours or 45 hours post-egg laying, respectively, to reach the required developmental stage.

2.1.2 *C. elegans* strains

The N2 Bristol strain was used as the reference strain to which all other were compared. All strains were obtained from the CGC (*Caenorhabditis elegans* genetics centre; Table 2.1).

Strains used were:

Strain	Gene	Allele
DA1316	*avr-14; avr-15; glc-1*	ad1302, ad1051, pk54
MT6318	*eat-4*	n2472
VC671	*egl-3*	ok979
DA2109	*ser-1; ser-7*	tm1325, ok345
NM1968	*slo-1*	js379
RB1161	*tbh-1*	ok1196
GR1321	*tph-1*	mg280
CB113	*unc-17*	e113

Table 2.1. *C. elegans* mutant strains used in this thesis.

2.1.3 Bleaching to remove contamination

The process of bleaching was used to remove contaminants, such as bacteria, fungi or yeast, from strains. Gravid adults were transferred to a seeded plate where they were picked into a small volume (approximately 2 µl) of bleaching solution, containing one part bleach to one part 4M sodium hydroxide. Worms were removed from this solution after roughly 30 seconds and physically broken apart with a worm pick to release their eggs.

2.1.4 Maintenance of *OP50 E. coli*

E. coli OP50 was stored on LB agar plates at 5^{o}C and periodically streaked to fresh LB plates to produce multiple colonies. To inoculate NGM plates, an individual OP50 colony was picked to inoculate LB broth which would then be grown up at 37^{o}C overnight.

2.2 Preparation of drugs

2.2.1 Fluensulfone

Fluensulfone was provided by ADAMA agricultural solutions Ltd. Fluensulfone was stored at 5^{o}C in the presence of desiccant silica granules. Gloves were always worn when handling fluensulfone and a face mask was used when weighing out.

2.2.2 Preparation of fluensulfone plates

NGM bottles were microwaved and their temperature equilibrated in a water bath at 50^{o}C. Appropriate amounts of phosphate buffer, cholesterol, magnesium sulphate and calcium chloride were added (section 2.6). Fluensulfone was dissolved in 100% acetone and then added to the NGM mixture so that the final acetone concentration was 0.5% of the total NGM volume. The NGM was then dispensed into 50 mm petri dishes. Plates were wrapped with parafilm once set after pouring and re-wrapped after seeding with OP50, unless otherwise stated. All fluensulfone plates were poured three days before use and, where applicable, were seeded with OP50 *E. coli* one day before use.

When making serotonin creatinine sulphate monohydrate (5-HT) plates, the drug was directly added to the molten NGM as a powder and thoroughly mixed to ensure it was dissolved before the plates were poured.

2.3 *C. elegans* behavioural assays

2.3.1 Mortality assays

50-100 staged-synchronised worms, at L1, L2/3, L4 or adult were soaked in M9 buffer in fluensulfone for 24 hours at 100 μM, 300 μM, 1 mM or in vehicle. The worms were then washed several times and placed on unmodified, OP50 *E. coli*-seeded NGM plates and lethality was scored. Worms that were completely immotile with a granular appearance, suggesting disintegration of internal structures, were defined as dead (Figure 2.1).

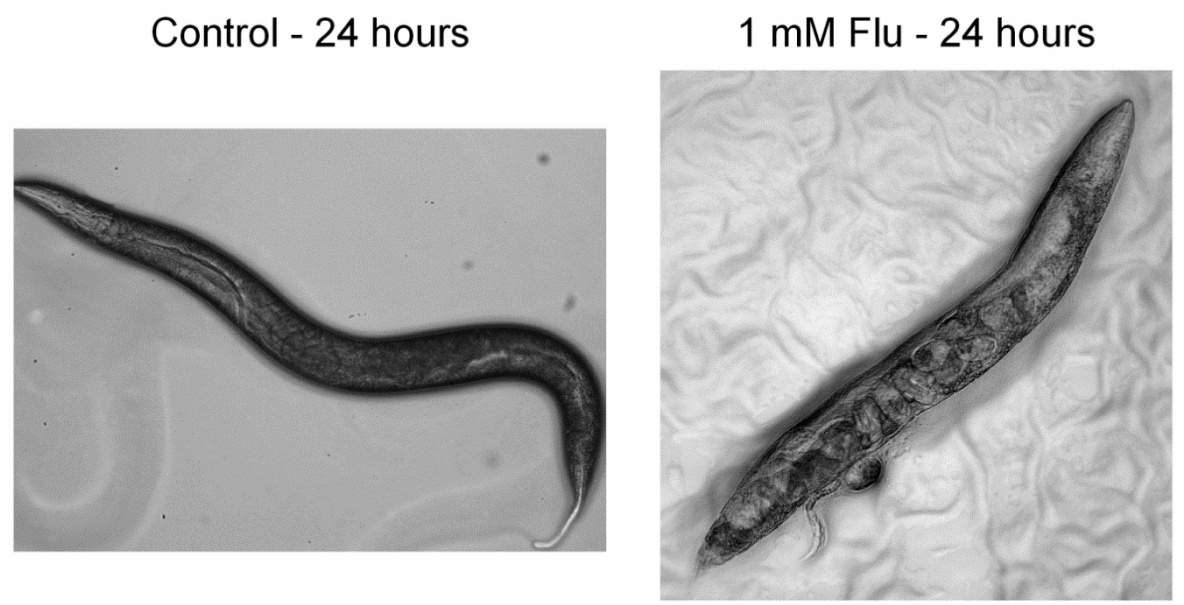

Figure 2.1. Representative images of alive and dead adult *C. elegans* on agar in the absence of food. A) A live worm. **B**) A dead worm that is immotile and unresponsive following 24 hour exposure to 1 mM fluensulfone. The swollen appearance is due to retention of eggs, often referred to as bagging.

2.3.2 Egg laying and egg hatching assays

10 N2 L4+1 day old adult worms were individually transferred to OP50 *E. coli*-seeded NGM plates that were modified with either vehicle or 1 mM fluensulfone. After 1 hour the adult worms were removed and the number of eggs laid was counted per plate. The same eggs were then scored for hatching 24 hours later and percentage hatching was calculated.

Worms underwent the same treatment for the experiments examining embryo viability following *in utero* exposure to 1 mM fluensulfone (see above). After removal from the fluensulfone- or vehicle-modified plates however the worms were transferred to an

unmodified OP50 *E. coli*-seeded NGM plate for one hour and were then moved to another such plate for an hour up to 5 hours after removal from fluensulfone. Hatching was then scored for the eggs that were laid on each plate 24 hours and 48 hours after removal from fluensulfone. This assays for the *in utero* effects of fluensulfone that has been taken up by eggs during drug treatment.

2.3.3 Development assays

10 N2 L4+1 day old *C. elegans* were placed on OP50 *E. coli*-seeded NGM plates modified with either vehicle, 100 μM, 300 μM or 1 mM fluensulfone and were allowed to lay eggs for 1 hour, at which point they were removed. The plates were incubated up to 96 hours at 20^{o}C and the development of the eggs was scored at 24, 43, 66 and 96 hours. Developmental stage was scored as egg, <L4 (including L1 and L2/3), L4 or adult. This was performed with 5 plates for each experimental condition and was repeated on 3 separate occasions.

2.3.4 Paralysis assays

Synchronised L2/3 larvae were incubated in M9 buffer with and without fluensulfone up to 24 hours and percentage paralysis was scored. These assays were conducted in 24 well plates with 400 µl of M9 buffer per well, with either vehicle (0.5% acetone) or fluensulfone (100, 300 µM and 1 mM) (5 replicates for each treatment). A 5 µl suspension of L2/3 *C. elegans* was added to each well, containing approximately 50-100 worms. The number of worms moving was scored up to 24 hours. Worms which failed to move in a 10 second observation period were deemed immotile.

In a further experiment, worms of different developmental stages (L1, L2/3, L4 and L4+1 day adult) were incubated in fluensulfone for 3 hours and paralysis was scored. This experiment was conducted as described above.

To investigate the reversibility of paralysis, at 24 hours exposure time the worms were removed from the treatment solution and washed in M9. Subsequently, the larvae were pipetted onto OP50 *E.coli*-seeded NGM plates and paralysis was again scored 6 hours and 24 hours later.

2.3.5 *C. elegans* thrashing assays

C. elegans motility in liquid was scored in M9 buffer with fluensulfone (100 & 300 µM and 1 mM) or with vehicle (0.5% acetone) by counting "thrashing" behaviour. In liquid, *C. elegans* swim via a flexing motion around the midpoint of the body that is known as thrashing. Individual L2/3 larvae were placed in M9 buffer and after 3 hours the number of thrashes in 30 seconds was scored using a dissecting microscope.

In a further experiment to assess the time dependence of this effect, the thrashing of L2/3 larvae was scored immediately prior to placing them in vehicle, 1 mM fluensulfone or 500 µM aldicarb and then up to 1 hour in the indicated treatments.

2.3.6 Pharyngeal pumping assays in the presence of food

C. elegans were transferred to either fluensulfone- or vehicle-modified plates seeded with OP50. Pharyngeal pumping was measured by counting the movements of the grinder of the pharynx with one movement constituting one pump. Feeding was counted only when the specimen was on the food source. If the worm moved off of the food source then counting stopped until it returned to food.

Where stated, locomotion and pharyngeal pumping were concurrently measured, with locomotion measured by counting body bends. *C. elegans* move in a sinusoidal fashion, bending their body back and forth to move forwards. A body bend was classified as each time the head of the worm moved from its position in a S-shape and the point just behind the pharynx reached a maximum bend in the opposite direction to the last bend. Body bends were counted over 30 seconds.

In a separate experiment, the reversibility of the inhibitory effect of fluensulfone on pharyngeal pumping was assessed by exposing L4+1 day adults to fluensulfone (300µM and 1 mM) or vehicle for 1 hour or 24 hours in the presence of food. Pharyngeal pumping was scored and the worms were then transferred to unmodified, OP50 *E.coli*-seeded plates and pumping was again scored 1 hour after transfer to the plate without drug.

2.3.7 Pharyngeal pumping assays in the absence of food

N2 L4+1 *C. elegans* were placed on OP50 *E. coli* and pharyngeal pumping was counted. These worms were then transferred to a cleaning plate of unseeded, unmodified NGM and allowed to move to remove any excess bacteria for 1 minute. Worms were then transferred to either fluensulfone- or vehicle-modified, unseeded plates. Pump frequency was counted immediately for the first minute on the test plate and was subsequently counted at 3, 5, 10, 15 and 20 minutes and every 10 minutes for up to 2 hours. In addition, independent measures of locomotion were made by counting body bends (see above section 2.3.6). After 2 hours worms were placed on drug-free *E. coli*-seeded NGM plate and pumping was counted again on food after 2 minutes to probe for recovery to drug-induced effects.

2.3.8 Comparing fluensulfone with aldicarb – Body length measurements

Aldicarb and other cholinesterase inhibitors induce hyper-contraction of the body wall muscle and a decrease in body length. To assess the effect of fluensulfone on body length and to allow for comparison with aldicarb (Sigma Aldrich), body length assays were conducted on L4 *C. elegans* (Mulcahy et al., 2013). Individual L4 *C. elegans* were imaged on OP50 *E.coli*-seeded plates in the absence of drug and then transferred to plates modified with either 1 mM fluensulfone or 500 µM aldicarb, where further images were captured 30 and 60 minutes later. ImageJ software was used to obtain a skeleton image of the worm to allow quantification of worm length. For each individual worm, the measurements in the presence of drug were normalised to the length prior to drug exposure.

2.3.9 Comparing fluensulfone with aldicarb – paralysis and mutant analysis

The *unc-17* mutant (strain CB113) is resistant to the paralytic effects of aldicarb (Alfonso et al., 1993). 20 L4+1 day adults, either N2 or *unc-17*, were put onto OP50 *E.coli*-seeded NGM plates modified with either vehicle (control), 500 µM aldicarb or 1 mM fluensulfone. Percentage paralysis was then scored at 24 hours.

2.3.10 Comparing fluensulfone with ivermectin – thrashing assays

The *avr-14; avr-15; glc-1* mutant (strain DA1316) is resistant to the inhibitory action of ivermectin on motility as a result of mutations in three glutamate-gated chloride channel subunits that are major targets for ivermectin (Cully et al., 1994, Dent et al., 2000). This

strain was used to compare the activity of ivermectin (Sigma Aldrich) and fluensulfone. To confirm the resistance of this strain to ivermectin, N2 and *avr-14; avr-15; glc-1* L2/3 larvae were exposed to ivermectin (0.1, 1 & 10 µM) or vehicle (0.5% ethanol) for 1 hour, at which point thrashing was scored. In a separate experiment to determine DA1316 sensitivity to fluensulfone, N2 and *avr-14; avr-15; glc-1* L2/3 larvae were exposed to fluensulfone (100 & 300 µM & 1 mM) for 3 hours, at which point thrashing was scored.

2.3.11 Comparing fluensulfone with ivermectin – pharyngeal pumping assays

Pharyngeal pumping assays were conducted to further investigate *avr-14; avr-15; glc-1* sensitivity to fluensulfone. L4+1 day N2 or *avr-14; avr-15; glc-1* were transferred to OP50-seeded NGM plates modified with either 1 mM fluensulfone or vehicle (control) and pharyngeal pumping was scored after 1 hour.

2.3.12 Pharyngeal pumping and locomotion assays on agar in the presence of serotonin and fluensulfone.

Individual N2 L4+1 *C. elegans* were transferred to unseeded NGM plates and allowed to move for 1 minute to remove bacteria. Subsequently, the worms were transferred to unseeded NGM plates modified with either 10 mM 5-HT (serotonin creatinine sulphate monohydrate complex), 1 mM fluensulfone or 10 mM 5-HT and 1 mM fluensulfone. Body bends and pharyngeal pumping were scored up to 20 mins exposure.

2.3.13 *C. elegans* cut head pharyngeal pumping assays

Well-fed L4+1 day *C. elegans* were transferred to a 5 cm Petri dish containing freshly-made modified Dent's saline (140 mM NaCl, 10 mM HEPES, 10 mM D-glucose, 6 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4 with 1 mM NaOH) with 0.01% bovine serum albumin. A razor blade was used to expose the pharynx by cutting just posterior to the terminal bulb.

For the methiothepin assays, cut heads were transferred to control dishes and pharyngeal pumping was counted for 3 minutes. The cut head was then transferred to either 0.5% ethanol or methiothepin and allowed to soak for 5 minutes, at which point pumping was again scored. Heads were then transferred to 5-HT, nicotine or 100 µM

fluensulfone with 0.5% ethanol or 5-HT, nicotine or 100 µM fluensulfone with methiothepin and pumping was again scored.

2.3.14 *C. elegans* cut head pharyngeal pumping assays – 5-HT receptor mutants

For assays with *ser-1; ser-7* (strain DA2109), N2 and *ser-1; ser-7* L4+1 day worms were treated in exactly the same fashion: heads were cut, pumping was scored in the presence of Dent's saline alone and the heads were transferred to 500 nM 5-HT or 100 µM fluensulfone. N2 and *ser-1; ser-7* experiments were conducted in parallel.

2.3.15 Food leaving assays

Plates were either modified with vehicle or with fluensulfone at the final concentrations of 100 µM or 500 µM. This assay is also sensitive to low concentrations of other solvents. Therefore, comparisons were made between unmodified, vehicle-modified and fluensulfone-modified plates. Plates were seeded with 50 µl OP50 *E. coli* 1 day before use. 7 N2 L4+1 *C. elegans* were transferred to the test plates and 5 minutes were allowed for worms to return to the food spot. The test plates were then observed for 2 hours and the number of leaving events was noted. A food leaving event was defined as an incident in which the whole body of the worm left the food source and the worm did not immediately reverse back onto the food source. Leaving rate was calculated as leaving events/worm/minute.

2.3.16 Mutant screen pumping and body bends assays

Mutants, defective in the indicated neurotransmitter pathways, were screened for their response to fluensulfone. This was carried out on well fed L4+1 day *C. elegans* on 1 mM fluensulfone NGM plates seeded with OP50 *E. coli*. For each mutant comparison there was an N2 control both on 1 mM fluensulfone and on the control plates with 0.5% acetone. Each mutant strain was also tested on a control plate with 0.5% acetone and on 1 mM fluensulfone. 5 worms were transferred onto each plate and at time points over 24 hours pumping and body bends were counted over 30 seconds when the worm was on the OP50 layer. At the 24 hour time point overall mortality was counted. If worms showed no movement their anterior end was prodded to test for the withdrawal response. If no withdrawal was seen the worms were deemed as dead.

2.3.17 *C. elegans* prolonged fluensulfone survival assay

The effects of prolonged exposure of *C. elegans* to lower doses of fluensulfone were investigated by a standard, solid media life span assay (Mulcahy et al., 2013). Worms were exposed to different concentrations of fluensulfone for >20 days. L4+1 *C. elegans* were picked onto OP50-seeded NGM plates modified with different concentrations of fluensulfone. 50 worms were used for each concentration tested and for the control. To prevent starvation, surviving worms were picked at intervals onto new seeded NGM plates with the same concentration of drug. Worms were prodded and those that failed to move or show any pharyngeal pumping were deemed dead and were removed from the experiment. Bagged worms were not scored as dead and were censored from the experiment. The number of bagged worms was noted and is independently shown.

2.3.18 *C. elegans* dauer in a prolonged fluensulfone survival assay

To generate *C. elegans* dauers, NGM plates of mixed stage N2 worms that had recently exhausted their OP50 *E.coli* food source were placed at 28^{o}C for 7 days. Subsequently, around 20 dauers were picked into petri dishes containing control and fluensulfone solutions made in M9 buffer. There were 5 dishes for each treatment. Mortality was scored up to 20 days. To assess mortality, dauers were prodded and those that failed to move were scored as dead and were removed from the dish.

2.3.19 DiS-C_3(3) staining of *C. elegans* and *G. pallida* mitochondria

Synchronised L4+1 day *C. elegans* or J2 *G. pallida* were washed in M9 buffer and pelleted via centrifugation before being re-suspended in 1 ml M9. This was repeated three times before the washed worms were then left in the M9 for 30 minutes to allow for digestion of any residual gut bacteria. After another wash with M9, 800 µM 3,3'-dipropylthiocarbocyanine iodide (DiS-C_3(3)) (Sigma Aldrich) dissolved in DMSO was added to the worm suspensions in a 1 in 200 dilution to give a final concentration of 4 µM in 0.5% DMSO. The worms were then soaked in 4 µM DiS-C_3(3) for 1 hour in the dark under constant rotation. The worm solutions were then diluted 100-fold with M9. Worms were immobilised with 1 mM levamisole and transferred to 2% agarose pads. Worms were imaged under a DsRed filter block on a Nikon eclipse E800 fluorescence microscope (Appendix 2).

2.3.20 *C. elegans* electropharyngeogram recordings

An L4+1 day *C. elegans* cut head was transferred to the recording chamber (volume ~ 3 ml) containing Dent's saline with 0.01% BSA. A borosilicate glass suction pipette (pulled from a 1 mm diameter borosilicate capillary) was back filled with Dent's saline. Suction was then applied to the anterior of the cut head to attach the preparation with the mouth aperture inside the suction pipette (Figure 2.2). The reference electrode, a silver chloride-coated silver pellet in 3M KCl, was connected to the recording chamber via an agar bridge. An Axoclamp SB amplifier (Axon Instruments) connected to a Digidata Box (Axon Instruments) was used to make extracellular voltage recordings in "bridge" mode. Immediately prior to recording, the voltage off-set was used to set the extracellular potential at 0 mV. Data were recorded with a sampling rate of 2 kHz and were acquired using Axoscope (Axon Instruments). The typical background noise of the setup was 0.3-0.4 mV.

Once suction was applied onto the preparation 5 minutes were allowed to ensure that the seal between the head and the pipette was stable and ensure the head was pumping normally (1-10 pumps per minute). Cut heads were exposed to a perfusion of saline at a constant rate of 4.5 ml/min. For the first 5 minutes a perfusion of Dent's saline was applied to gain a pre-drug control.

In the experiments with serotonin, after this 5 minute pre-drug period 500 nM 5-HT was applied to the dissected pharynx via perfusion for 3 minutes. This was followed by a 3 minute washout and another 3 minute 5-HT application. After a 3 minute washout fluensulfone was applied for 5 minutes followed by 3 minute co-application 5-HT and fluensulfone in a mixture. This cycle ensures that the pre-drug activity and the recovery are taken from an individual worm.

During the pharyngeal pump cycle, as the pharyngeal muscle contracts and relaxes this produces a stereotypical EPG waveform (see section 1.15.9). EPG recordings were analysed for changes in pump frequency, pump duration and P spike frequency per pump. Pump duration refers to the time duration between the muscle contraction (E spike) and muscle relaxation (R spike).

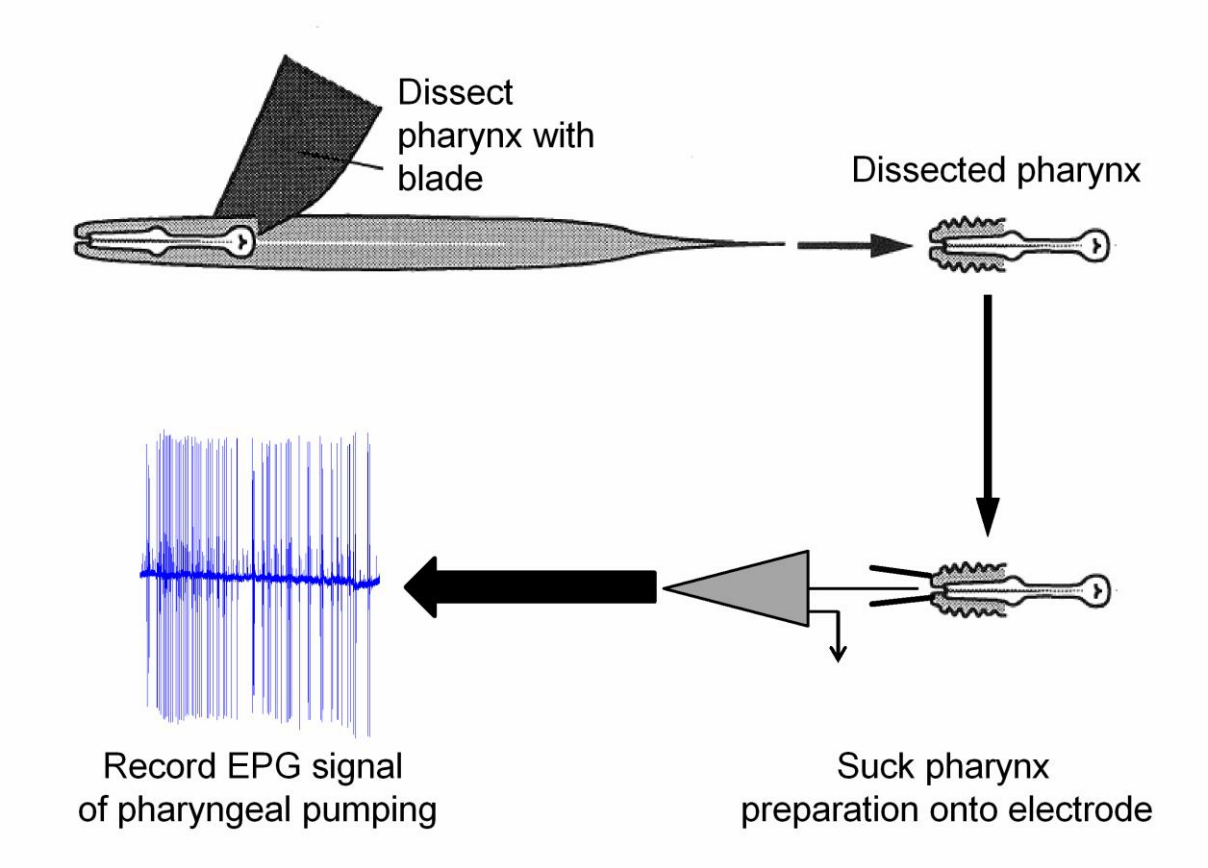

Figure 2.2. A scheme of the key principles of electropharyngeogram (EPG) recordings conducted in this thesis. The pharynx and head of the L4+1 day adult *C. elegans* was dissected away from the body and an extracellular recording electrode was placed over the anterior of the worm, creating a seal. EPG recordings were than conducted in the presence of saline.

2.3.21 Electropharyngeogram analysis

The different components of the EPG waveform have been correlated to neuromuscular events that occur during pharyngeal pumping through the use of mutants, cell ablation and video analysis (see section 1.15.9) (Raizen and Avery, 1994). As such, the EPG waveform can be separated into three phases: the E phase (excitation), the P phase (plateau) and the R phase (relaxation). The E phase contains two positive transients, the small e spike and the larger E spike. This spike is followed by the P phase, which contains a variable number of negative transients called P spikes. The P phase is followed by a large negative transient, the R spike, and the small negative r spike. Annotation of recordings and subsequent analysis was carried out using AutoEPG software developed by Dillon et al. (2009).

2.3.22 Mutagenesis of *C. elegans*

6 plates with large, mixed-stage populations of N2 worms were grown up and washed from the plates with 1 ml M9 buffer into a 20 ml universal tube. The worms were allowed to settle, the supernatant was removed and 20 ml of M9 was added. The worms were again allowed to settle and the supernatant was removed, leaving 2 ml. A further 2 ml of M9, containing 20 µl of liquid ethyl methanesulfonate (EMS) was then added. The universal was then sealed and placed under rotation for 4 hours. Following this, 16 ml of M9 was added and the suspension was allowed to settle. The supernatant was then removed. This was done four times. After removing the supernatant, the worms were mixed and 0.5 ml of the solution was transferred to 4 individual plates seeded with OP50. The L4 worms (The F0 generation) that reached the food source were picked onto individual plates. These L4s were grown to adults and allowed to lay eggs. After 2 days the adults were removed and the eggs (The F1 generation) were grown to adults to self-fertilise. These worms were then bleached and the eggs (The F2 generation) were transferred to 1 mM fluensulfone plates to screen for susceptibility to fluensulfone (see Appendix 1).

To avoid EMS contamination, all of the above procedures were carried out in a fume hood in a dedicated tray with a dedicated Gilson pipette. Double gloves were worn, and all tips, gloves, containers and pipettes that were exposed to the EMS were bathed in 1M sodium hydroxide for 24 hours to hydrolyse the EMS prior to disposal.

2.3.23 Measurement of *C. elegans* oxygen consumption with a phosphorescent oxygen sensitive reagent

The Mito-ID® Extracellular O_2 Sensor kit (High sensitivity) (Enzo Life Sciences) was used to determine the relative oxygen consumption of *C. elegans* under different drug treatments. The sensor kit works via oxygen quenching of phosphorescence (see (Papkovsky and Dmitriev, 2013) for review). As the assay progresses, if oxygen is consumed in the well chamber there is reduced oxygen quenching of probe phosphorescence and the signal increases.

All oxygen consumption measurements were conducted on synchronised L4+1 day *C. elegans*. All oxygen consumption assays were conducted in black, flat and clear-

bottomed 96-well plates (Greiner Bio-one). The solution in each well was made up to a final volume of 150 µl. The stated numbers of *C. elegans* were added to each well in M9 buffer and the solution was made up to 135 µl. To initiate the experiment 15 µl (or 10% of the final solution) of the reconstituted Mito-ID® probe was added to each well. Subsequently, a 50 µl drop of high sensitivity mineral oil was added on top of each well to act as an impermeable seal to prevent back-diffusion of oxygen.

The 96-well plate was then read using a FLUOstar Optima fluorescence plate reader (excitation=380 nm, emission=650 nm) with time-resolved fluorescence (delay time 30 µs, gate time 100 µs) for up to 4 hours, with readings every minute for 30 minutes followed by readings every 5 minutes for up to 210 minutes. Experiments were conducted at room temperature (~20-22^{o}C). Except where stated, the gain settings for the FLUOstar Optima fluorescence plate reader were calculated automatically for each experiment.

Synchronised *C. elegans* were washed from OP50 *E.coli*-seeded NGM plates and washed 4 times to remove bacteria. The *C. elegans* were allowed to sit for 30 minutes to ensure all consumed bacteria were digested. For all experiments, wells with M9 buffer and probe were read as a comparison to wells containing worms. Control wells contained 50 worms with M9 buffer and dye made up to 150 µl. Where fluensulfone was used, diluted ethanol was added to control wells to give a final concentration of 0.5% of total volume. For fluensulfone and fluopyram wells, a stock was made with 100% ethanol which was diluted to 10% ethanol with ddH_2O. 15 µl of the 10% ethanol solution was added to the wells to give the desired final concentration of the drug and 0.5% ethanol. For sodium azide wells, 15 µl of a stock solution made with ddH_2O was added to wells to give the desired final concentration. 0.5% ethanol was also present in sodium azide wells to allow comparison with the control treatment. Drugs were added immediately prior to adding the Mito-ID® probe. Blank wells were conducted for each treatment with 15 µl ddH_2O added rather than Mito-ID® probe. All results shown are blank corrected.

2.3.24 Yeast culture and experimentation

Saccharomyces cerevisiae cultures were grown in YEPD media (1% yeast extract, 2% peptone and 2% D-glucose). Cultures were kept at 5^{o}C on YEPD 2% agar plates for storage. For growth of liquid cultures, individual colonies were picked into YEPD media and grown at 30^{o}C at 180 rpm overnight. Before experimentation, these liquid cultures were diluted to OD_{600} 0.2-0.3 and then grown up to an optical density of 0.6-0.8.

For spot cultures experiments, liquid cultures were grown to OD_{600} 0.6-0.8 as described above. The cultures were then serially diluted to give 1/10, 1/100, 1/1000, 1/10,000, 1/100,000 dilutions. 10 µl of each dilution was pipetted onto YEPD agar plates modified with the stated concentration of fluensulfone. This was done for five separate cultures per plate. The plates were then incubated at 30^{o}C for up to 6 days and were imaged using a G-Box gel imager. Colony number was visually counted.

For liquid culture assessment of fluensulfone and fluopyram sensitivity of yeast, cultures grown overnight were diluted to OD_{600} 0.2-0.3. Fluensulfone or fluopyram, dissolved in 100% acetone or ethanol, respectively, was added to the cultures to give the stated concentration of each drug with 0.5% of the vehicle. Controls were performed with 0.5% vehicle. These cultures were then grown at 30^{o}C at 180 rpm and optical density was measured.

2.4 Plant parasitic nematode behavioural assays

2.4.1 *Globodera pallida* culture

All *G. pallida* cysts and PRD solutions were obtained from Leeds University. *G. pallida* were cultured on potato plants (*Solanum tuberosum* "Desiree"). The potato plants were grown in a mixture of loam soil and sterilised sand (1:1 ratio) that contained *G. pallida* cysts at approximately 25 cysts/g. At 10-12 weeks, a flotation technique with a Fenwick can was used to extract cysts. Once extracted, the cysts were separated from soil and other debris and stored at 4^{o}C.

To induce hatching, cysts were placed in a solution of 1 part potato root diffusate to 3 parts ddH_2O (PRD) at ~20^{o}C in the dark. PRD was generated from potato plants 3-10 weeks post-planting by soaking the roots of the plants in distilled water for 1 hour. This

water was then drained and subjected to gravity filtration through Whatman No.1 filter paper and stored at -20^{o}C.

J2s typically began hatching 3 days after exposure to PRD and continued to hatch for up to 4 weeks. J2s that had hatched within 24 hours were used for experiments. Mixed batches of cysts set up in PRD from 1 week to 4 weeks were used to obtain J2s. 24 hours prior to experimentation all J2 present were pipetted out of the glass dishes containing and were discarded, ensuring that all those experimented upon had hatched within 24 hours. 24 hours prior to experimentation the PRD solution was replaced (Figure 2.3).

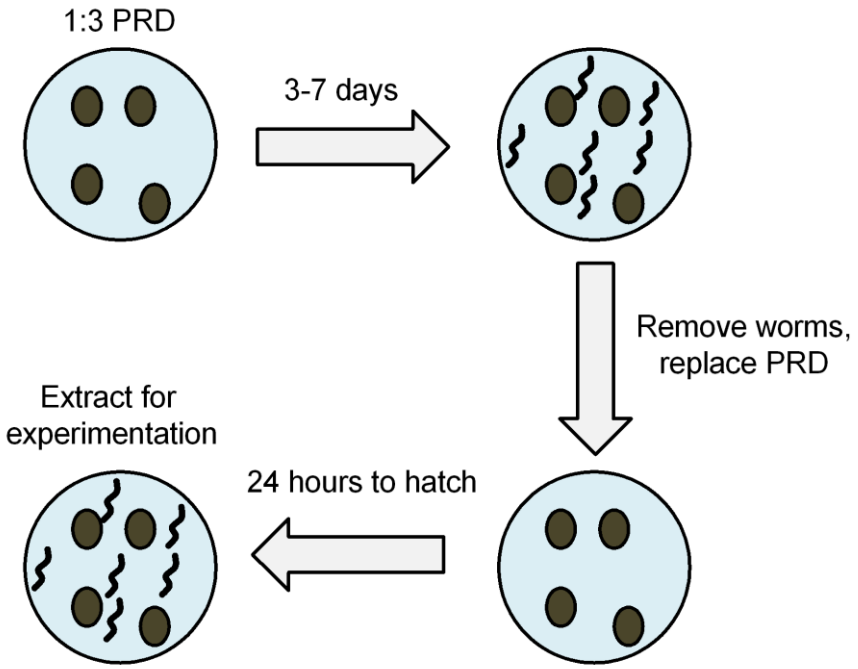

Figure 2.3. The protocol for hatching J2 *G. pallida* from cysts. Stored cysts were transferred to 1 parts PRD to 3 parts ddH_2O and hatching typically began 3-7 days later. To obtain a synchronised population for experiments, all worms were removed 24 hours prior to an experiment and the PRD solution was replaced. 24 hours later, any worms present will have hatched within a 24 hour time period and were removed for experimentation.

2.4.2 Stylet thrusting assays with *G. pallida*

Stylet thrusting assays were conducted in 20 mM HEPES buffer made up with ddH_2O, with pH adjusted to 7.4 with NaOH, except where stated. Cohorts of J2s that had hatched within a 24 hour period were pipetted into test solutions in 30 mm petri dishes or a recording chamber. The number of stylet thrusts per minute was counted at various time points in the presence of the indicated concentrations of 5-HT, fluoxetine

hydrochloride (Sigma Aldrich), fluensulfone, imipramine hydrochloride (Sigma Aldrich), tryptamine hydrochloride (Tocris bioscience), octopamine hydrochloride (Sigma Aldrich), methiothepin mesylate salt (Sigma Aldrich), reserpine phosphate (Tocris bioscience), 4-chloro-DL-phenylalanine methyl ester hydrochloride (Sigma Aldrich) and mixtures applied in sequence and at the concentrations as indicated. A single movement of the stylet knob forwards and then backwards to its original position was counted as one stylet thrust. Controls assays were conducted in the presence of either 20 mM HEPES alone or 20 mM HEPES with vehicle, as indicated in figure captions. All assays were conducted at room temperature (approximately 20-22^{o}C). All drug solutions were made on the day of use.

For experiments with methiothepin, reserpine and 4-chloro-DL-phenylalanine, J2 *G. pallida* were pre-soaked at the indicated concentration for the stated length of time, at which point the J2s were transferred to the stimulant drug solution.

2.4.3 *G. pallida* coiled posture assays

Worms exhibit an induced change in the curvature of their body posture. This was scored by measuring coiling behaviour, in which a coiled worm was defined as any worm that was bent in such a way that either its head or tail was in contact with another part of its body, with this posture consistently maintained for the 10 second observation period (Figure 2.4). ~10 J2 *G. pallida* were soaked in indicated drug and were scored for percentage coiling at the given time. This was repeated 5 times. Coiling and motility were scored contemporaneously (see section 2.4.4, below).

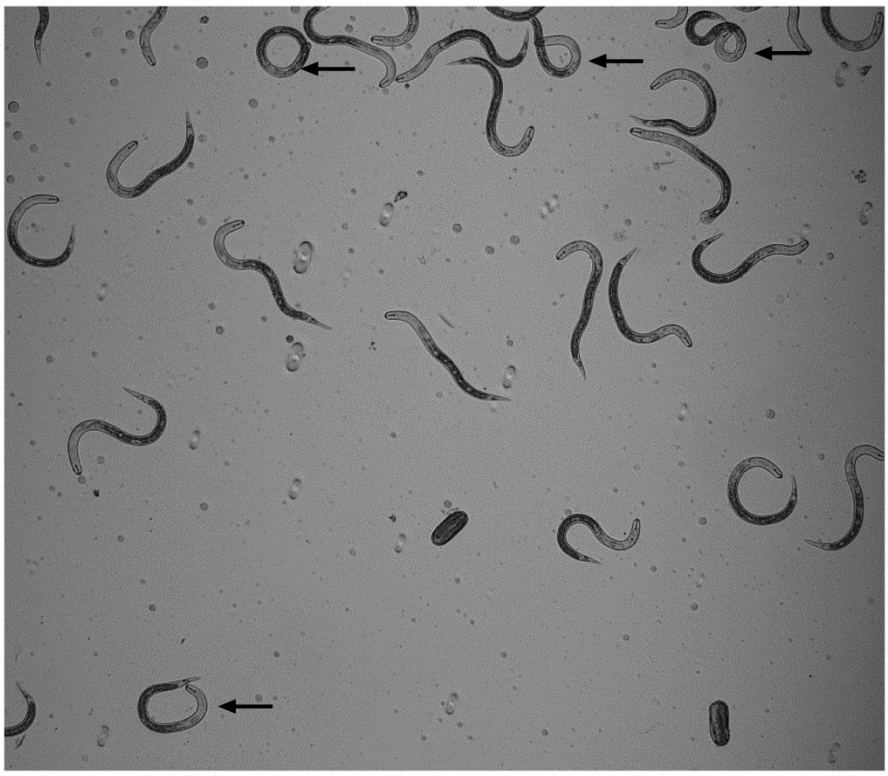

Figure 2.4. *G. pallida* coiling assays. In this group of *G. pallida* J2s, the worms indicated with arrows would be scored as coiled.

2.4.4 Prolonged fluensulfone exposure immobility and granular appearance assays

Assays were performed to examine the effects of prolonged exposure to fluensulfone on the motility and appearance of *G. pallida*. Cohorts of J2s that had hatched within 24 hrs were transferred to ddH_2O (0.01% BSA) to remove PRD and subsequently transferred to 30 mm petri dishes containing the different drug solutions. 7 replicates of each treatment were performed in distinct petri dishes. When scoring motility, J2s that were rod shaped and failed to move during a 10 second observation period were judged to be immotile. In this assay it was noted that during prolonged exposure to fluensulfone the J2s became darker in appearance and looked "granular". This was accompanied with a progressive loss of structure in the internal organs and suggests worm death. Whilst scoring motility the number of J2s that appeared "granular" was also scored. Any worm that was immotile and darkened in appearance was deemed to be granular.

2.4.5 Prolonged fluensulfone exposure MTT assay

MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide) (Sigma Aldrich) is a tetrazolium salt that undergoes a reduction reaction in metabolically active cells and changes from a weak yellow colour to a dark purple colour as it is converted

into an insoluble formazan product (see Berridge et al. (2005) for review). This compound has been used extensively to assay metabolic and proliferative activity in cell cultures (Mattson et al., 1947, Pick et al., 1981, Mosmann, 1983) and has been adapted for use in *C. elegans* to measure worm death (James and Davey, 2007, Smith et al., 2009). We have adapted this method to assay mortality and metabolic activity in *G. pallida*. James and Davey (2007) reported that adult *C. elegans* showed strong staining in the anterior and around the pharynx after soaking for 3 hours in 10 mg.ml MTT, made in M9 buffer. In this work, *C. elegans* stained after 2 hours in 5 mg.ml MTT, yet no staining was seen in *G. pallida* J2s at this time. This may reflect differences in the cuticle or alternatively may reflect reduced metabolic activity in the *G. pallida* J2s. Soaking for 24 hours in 5 mg.ml MTT was found to visibly stain *G. pallida*, predominately in the anterior region (Figure 2.5). No visible change in the distribution or strength of purple MTT staining in *G. pallida* was observed with a staining period of longer than 24 hours, indicating that optimal staining required 24 hour exposure to MTT.

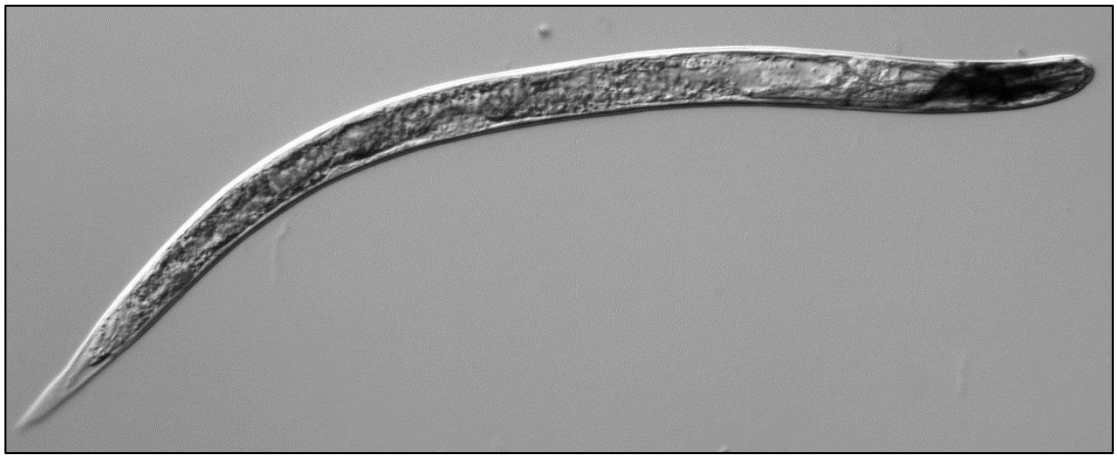

Figure 2.5. A representative image of a stage 2 juvenile *G. pallida* stained with MTT for 24 hours. Note the dark staining in the head region.

For drug assays, freshly hatched (≤24 hrs old) J2 *G. pallida* were washed in ddH_2O (0.01% BSA) and transferred to drug solutions made in ddH_2O, in which they were soaked for up to 14 days. Throughout this time, ~10 J2s were removed from the drug solutions, washed in ddH_2O (0.01% BSA) and then placed in a 24 well plate in 5 mg.ml MTT solution made in ddH_2O. This was performed 7 times for each treatment in seven different wells. The well plates were gently rotated in the dark for 24 hrs. After 24 hours in the MTT solution, each well was observed using a dissecting microscope at X45 magnification and worms were scored for the presence or absence of staining. The J2s

soaked in the control solution typically showed dark purple staining at their anterior end (Figure 2.5). As the intensity and location of the staining varied between J2s, the region of staining was noted as either throughout, predominately anterior, predominately posterior, weakly stained or unstained.

The immotility and MTT staining assays with both methiothepin and fluensulfone were performed as described above, although both compounds were dissolved in 100% ethanol, which was added to the drug solutions at a final concentration of 0.5%.

2.4.6 Cyst hatching assays

G. pallida cysts were washed in ddH_2O and individual cysts were transferred to wells in a 24 well plate, containing ddH_2O, 1:3 PRD or drug solutions made using 1:3 PRD. A vehicle control was performed for each experiment. Fluensulfone and methiothepin were dissolved in 100% ethanol and were added to a solution of 1:3 PRD to give a final ethanol concentration of 0.5%. All other drugs were dissolved in ddH_2O and added to the solution. The cysts were soaked in 1:3 PRD solution in the presence of drug for up to 25 days. During this period hatched J2s were counted and removed from the wells. The solution in which the cysts were soaked was replaced each time a count was taken. The cysts were then removed from the drug solution and washed in ddH_2O and subsequently were transferred to the wells of another 24 well plate containing 1:4 PRD diffusate solution alone to assess hatching recovery. J2 hatching was then counted in the same manner. Throughout each individual experiment the same PRD batch was used. Cumulative hatch of J2s per cyst was plotted over time.

At the conclusion of the hatching experiment, cysts were transferred to ddH_2O and cracked open with a razor to count the number of unhatched eggs per cyst.

2.4.7 Nile Red staining of *G. pallida* with isopropanol fixation

For initial experiments using Nile red (Sigma Aldrich) to quantify differences between starved and freshly hatched worms, one group of juveniles were washed in ddH_2O and left for 14 days. The other group were hatched within 24 hours from the same cysts. The worms were then stained as described below.

Freshly hatched (<24 hrs old) J2s were washed in ddH_2O and transferred to drug solutions made in ddH_2O. For 5-HT experiments, the solutions were replaced with fresh 5-HT daily. J2s were soaked in drug solutions for up to 10 days under constant rotation. At 5 and 10 days, J2s were removed and washed several times in M9 buffer. The worms were then pelleted and 200 µl M9 with 0.01% Triton X-100 was added. The worms were again pelleted and 200 µl of M9 with 40% isopropanol was added. The worms were incubated in 40% isopropanol for 3 minutes with constant agitation and then pelleted and the supernatant was removed. 200 µl Nile red solution (6 µl 0.5 mg/ml Nile red in acetone per 1 ml M9 with 40% isopropanol) was added to the worm pellet and the worms were stained in the dark for 2 hrs with constant rotation. The worms were then washed in M9 with 0.01% Triton X-100 three times and left in 200 µl M9 with 0.01% Triton X-100 in the dark for 30 mins. The worms were subsequently washed in M9 with 0.01% Triton X-100 again and transferred to 2% agarose pads for imaging. Images were taken using a Zeiss Axioplan 2 microscope under a FITC filter block. For each time point, images were taken using the same exposure time (500 ms) and with the same light intensity. This protocol was modified from Pino et al. (2013).

ImageJ software was used to quantify fluorescence intensity in the whole worm. The image (8-bit) was processed by subtracting the background to correct for any unevenness in the background light intensity. The threshold level of each image was then adjusted to distinguish between the worm and the background. The threshold of each image was adjusted to the same level. The worm was then selected using the wand tool and the integrated density value was measured. The integrated density value is sum of the grey scale value of the pixels in the selected area. The data were normalised as a percentage of the mean integrated density value of the control group.

2.5 Statistical Analysis

Data are shown as the mean ± S.E.M, except where stated. Student's t tests, One-way ANOVA's and two-way ANOVA's were used where appropriate and were followed by the stated post-hoc tests (with significance level set at $P<0.05$). All statistics were carried out using GraphPad Prism software. The number of individual worms used to perform the statistical analysis for each experiment is stated in the figure legends, as is the specific statistical test employed. For all statistical testing of the EPG data repeated

measures tests were used. The data shown for the EPG are pooled from multiple experiments.

2.6 Materials

Chemicals and drugs were obtained from standard suppliers.

Fluensulfone was supplied by ADAMA agricultural solutions Ltd.

Borosilicate glass capillaries GC100-10 were purchased from Harvard Apparatus.

Dent's saline (final concentration)

NaCl (140 mM)
HEPES (10 mM)
D-Glucose (10 mM)
KCl (6 mM)
$CaCl_2$ (3 mM)
$MgCl_2$ (1 mM)

pH adjusted to 7.4 using NaOH

Nematode Growth Medium (NGM agar)

Agar 80g
NaCl 12g
Peptone 10g

In 4l ddH_2O

After autoclaving add,
Cholesterol 4 ml
1 M $MgSO_4$ 4 ml
1 M $CaCl_2$ 4 ml
1 M KH_2PO_4 (Phosphate Buffer) 100 ml

Supplemented with drug as indicated

M9 Buffer

KH_2PO_4 3g
Na_2HPO_4 6g
NaCl 5g
1 M $MgSO_4$ 1 ml
In 1 litre distilled water

LB

Bacto-yeast 5g
Bacto-tryptone 10g

NaCl 5g
Distilled water to 1 litre
pH 7

Bleaching Solution (Alkaline Hypochlorite)

Domestos Bleach (20% NaOCl) 1 ml
4 M NaOH 1 ml

Chapter 3: A comparative investigation of the effects of fluensulfone and other nematicides on *C. elegans* indicates a novel mode of action

3.1 Introduction

In this chapter, the work conducted with fluensulfone and *Meloidogyne spp*. will be reviewed in more detail. Published work investigating the effects of fluensulfone on *C. elegans* will also be discussed. Unless stated, all the *C. elegans* experiments included in this introduction were performed by Elizabeth Ludlow and published in (Kearn et al., 2014). Additional data from this thesis from the current author also published in (Kearn et al., 2014) will then be included in the results sections to allow comparison with this published work.

3.1.1 The nematicidal activity of fluensulfone against plant parasitic nematodes

The efficacy of fluensulfone as a nematicide has been tested most rigorously against *Meloidogyne spp*., although other nematodes have been tested, including migratory ectoparasites such as *Xiphinema index*. (Oka et al., 2008, Oka et al., 2009, Oka, 2014). Experiments with fluensulfone have most thoroughly investigated its ability as a crop protection agent, with a focus upon how fluensulfone treatment affects the host plant, including root galling and plant growth (see Chapter 1, section 1.10).

The nematicidal activity of fluensulfone against *M. javanica* has however been scrutinised *in vitro* by Oka et al. (2009) in a series of immobility assays. J2 juvenile *M. javanica* were exposed to fluensulfone in ddH_2O for 48 hours and the number of immotile worms was scored at 24 and 48 hours in a 10 second observation period. The J2s were then washed and immotility was scored again after a 24 hour recovery period. At 24 hours, 1 mg.L (3.4 µM) (For conversion of concentrations see Table 3.1) elicited a slight increase in percentage immobility whilst 4 and 8 mg.L (13.6 and 27.2 µM) induced ≥80% immobility, as compared to <5% in vehicle (control)-treated worms.

At 48 hours ≥80% of J2s exposed to 0.5-8 mg.L were immotile, with near 100% immotility in the presence of 4 and 8 mg.L, relative to <10% immotility in control-treated worms. After washing in ddH_2O, no significant recovery from fluensulfone treatment was observed. It was suggested that this indicates irreversible nematicidal activity, as worms treated with the organophosphates cadusafos and fenamiphos did recover motility to near-control levels after 24 hours. The authors also suggested that

the paralysis that results from fluensulfone exposure is distinct to that resulting from organophosphate exposure. J2s paralysed by both fenamiphos and cadusafos were described as being shrunken and shorter in length relative to control-treated worms with a "wavy" posture, whereas fluensulfone-treated worms were straight and rod-shaped. The authors suggest that this lack of recovery in fluensulfone-treated worms, and the distinctive effects on body shape and posture provide evidence for a novel mode of action for fluensulfone relative to cholinesterase inhibitors. Furthermore, the authors suggest that fluensulfone may have an inhibitory effect on PPN neurotransmission as opposed to the over-excitation resulting from treatment with carbamates or organophosphates.

Molar concentration	Parts per million (ppm)	mg.L
1 mM	292	292
500 µM	146	146
300 µM	87	87
100 µM	29.2	29.2
30 µM	8.76	8.76
10 µM	2.92	2.92
1 µM	0.292	0.292

Table 3.1. Fluensulfone concentration conversion table.

In further experiments, J2s were soaked in fluensulfone for 12 or 24 hours and then rinsed in ddH_2O, after which immotility was scored 2 and 5 days later. 12 hour exposure to fluensulfone had no effect on motility even at 16 mg.L (54.4 µM), the highest concentration tested. Interestingly, even though the worms were then removed from fluensulfone, immotility increased to ≥80% at 1-16 mg.L after a 5 day rinse, relative to 10-20% immotility in control-treated worms. This suggests that when the J2s have absorbed a lethal dose of fluensulfone they will eventually become immotile and die. The authors also remark that after 5 days the body contents of J2s in the control solution became depleted, yet this did not happen in fluensulfone-treated worms. Whilst this was not speculated upon, this depletion of body contents may reflect consumption of lipid

stores by the non-feeding J2s (Storey, 1984). Thus, the inference is that fluensulfone reduces or prevents lipid consumption by *M. javanica*.

The effects of fluensulfone on the hatching of *M. javanica* eggs was also examined. After 3 days soaking, percentage hatching was reduced in the presence of 2, 4 and 8 mg.L fluensulfone relative to the control, although hatching was unaffected by 0.5 and 1 mg.L fluensulfone. The eggs were then rinsed with ddH_2O and hatching was again scored 3 days later to determine recovery. After rinsing, hatching recovered, and was only slightly reduced after treatment with 4 and 8 mg.L fluensulfone (Oka et al., 2009).

Oka et al. (2009) show that fluensulfone has irreversible nematicidal activity against *M. javanica* with 12-48 hours exposure to ≥1 mg.L (3.4 µM), characterised by a rod-shaped posture and potentially reduced consumption of lipid reserves. They further show that fluensulfone has some inhibitory activity against *M. javanica* egg hatching.

The nematicidal activity of fluensulfone *in vitro* has also been tested against the migratory endoparasitic nematodes *Bursaphelenchus xylophilus*, *Aphelenchoides besseyi*, A*phelenchoides fragariae*, *Ditylenchus dipsaci*, *Pratylenchus penetrans*, *Pratylenchus thornei* and *Xiphinema index* (Oka, 2014). Immotility assays in liquid showed that *A. besseyi*, *A. fragariae*, *B. xylophilus* and *D. dipsaci* were relatively unaffected by fluensulfone treatment, with only 16 mg.L (54.4 µM) slightly increasing immotility after a 48 hour soak, although these species were also relatively unaffected by fenamiphos treatment. It has also been previously reported that *D. dipsaci* for example, is highly resistant to nematicides relative to other PPNs (Homeyer and Wagner, 1981). *P. penetrans* and *P. thornei* were more susceptible to the paralytic effect of fluensulfone with a 48 hour exposure to 8 and 16 mg.L increasing percentage immotility to 20-60% relative to <10% in the control solution. After a 48 hour rinse in ddH_2O percentage immotility increased to ≥70% in worms treated with 4, 8 and 16 mg.L, indicating that fluensulfone has irreversible nematicidal activity against *Pratylenchus spp*, albeit at higher concentrations than used against *M. javanica* (Oka et al., 2009). Of the migratory ectoparasitic nematodes tested, *X. index* was the most susceptible to fluensulfone, with 2-16 mg.L significantly increasing immotility at 48 hours, and percentage immotility further increased after a 24 hour rinse in ddH_2O. 48 hour exposure of *X. index* to 1, 2 and 4 mg.L fluensulfone followed by a 24 hour rinse

resulted in 60-80% immotility, whilst 8 and 16 mg.L induced near-100% immotility, relative to 20-30% in the control group. All of these nematodes were less sensitive to fluensulfone than *Meloidogyne spp*., although this may reflect differences in life cycle stages as mixed population with adults and juveniles were tested in experiments on the migratory nematodes whereas only J2 *M. javanica* were tested. The species mentioned above have also been reported to have reduced sensitivity to other nematicides (Voss and Speich, 1976).

These published observations show that fluensulfone has effects on some PPNs at very low concentrations, with ≥0.5 mg.L having nematicidal activity against *M. javanica*. It is clear that other migratory ectoparasitic nematodes are less susceptible, with a threshold of ≥4 mg.L fluensulfone required to show nematicidal activity against *X. index*, *P. penetrans* and *P. thornei*. Several species, including *D. dipsaci* were not susceptible at 16 mg.L. There is clear variation in susceptibility between PPN species. Whilst the immobilising effect of fluensulfone on some PPNs has been investigated, other behaviours such as host localisation, host penetration and feeding have not been investigated and effects on egg hatching have been incompletely examined.

3.1.2 The nematicidal activity of fluensulfone against *C. elegans*

The first aim of this study was to further investigate the effects of fluensulfone on *C. elegans* behaviour. These investigations have since been published and are summarised here (Kearn et al., 2014). The nematicidal activity of fluensulfone against *C. elegans* was determined via mortality assays, in which different developmental stages were soaked in fluensulfone for 24 hours and mortality was visually scored through prodding. 24 hour soaking in 1 mM fluensulfone resulted in high mortality at all life cycle stages, relative to treatment with vehicle control solution, in which >90% of worms at all stages survived (Table 3.2). Mortality was lower at all developmental stages treated with 300 µM fluensulfone, with >75% still alive after 24 hours exposure. More L1, L2/3 and L4 worms died after exposure to 300 µM fluensulfone, with an increase in mortality of 8%, 7% and 18%, respectively, relative to the vehicle control. 100 µM fluensulfone elicited a 19% increase in mortality at the L4 larval stage but did not significantly increase percentage mortality at any other developmental stage. It would appear that there are slight differences in the susceptibility of the different stages of the *C. elegans* life cycle

to fluensulfone, with for example increased mortality of L2/3 larvae in the presence of 300 µM relative to adults.

	% Mortality			
Developmental stage at exposure Concentration of Fluensulfone	L1	L2/L3	L4	Adult
0.5% acetone (vehicle control)	0 ± 0	8 ± 8.0	7.8 ± 1.0	1.4 ± 1.4
100 µM	0 ± 0	0 ± 0	26.6 ± 18.8	4.8 ± 3.3
300 µM	8.3 ± 3.0	14.6 ± 3.0	25.8 ± 18.5	2.3 ± 2.6
1 mM	100 ± 0	97 ± 2.0	81 ± 2.8	97.0 ± 1.2

Table 3.2. Dose-dependent nematicidal effects of fluensulfone at different stages of the *C. elegans* life cycle. 50–100 worms were soaked in M9 buffer for 24 hours at the concentration of fluensulfone indicated. At the end of this incubation period the worms were transferred to an NGM plate and scored for mortality. Immobile worms were prodded and those that failed to respond were deemed dead. Mortality is expressed as a percentage of the initial number of live worms in the sample (n=5 replicate wells for each treatment group, mean ± s.e mean shown). This experiment is representative of two experiments conducted on separate occasions (from (Kearn et al., 2014).

This indicates that *C. elegans* is less susceptible to the nematicidal effects of fluensulfone than some PPNs like *M. javanica*. After 24 hours exposure 1 mM (292 mg.L) fluensulfone brought about near-complete paralysis/ death of *C. elegans* at all life cycle stages whilst exposure to ≥27.2 µM (8 mg.L) for 24 hours induced 40-90% immobility in *M. javanica* J2s (Oka et al., 2009). It is important to note that in the *in vitro* experiments with *M. javanica* only immobility was scored and this may not accurately reflect mortality. The most profound nematicidal activity of fluensulfone against *M. javanica* was seen with a lack recovery of mobility after a 48 hour exposure to ≥3.4 µM (1mg.L) in >90% of worms. No such experiments have been conducted with *C. elegans*. Some migratory ectoparasitic nematodes that have been tested, such as *D. dispaci*, were not susceptible to fluensulfone at 54.4 µM (16 mg.L), the highest

concentration tested and their susceptibility may be nearer to that of *C. elegans* than *Meloidogyne spp*.

Whilst *C. elegans* was found to be less susceptible to fluensulfone than some PPNs, it has qualitatively similar effects on PPNs and *C. elegans*. Fluensulfone exposure irreversibly inhibits motility in both *C. elegans* and PPNs, characterised by a non-spastic paralysis (Oka et al., 2009) (see section 3.1.7, Figure 3.6). Furthermore, fluensulfone inhibits egg hatching in both *C. elegans* and PPNs (see section 3.1.4, Figure 3.2) (Oka et al., 2009). Given these similarities between both species, *C. elegans* was used to study and quantify the effects of fluensulfone on a range of behaviours that are not easily assayed in sedentary endoparasitic PPNs, including reproduction, feeding and development. *C. elegans* has been used successfully as a model organism in mode of action studies for several anthelmintics, and the genetic tools that are available in *C. elegans* can be used to investigate the pathways that mediate the behavioural effects of fluensulfone (Schaeffer et al., 1992, Dent et al., 2000, Guest et al., 2007, Hu et al., 2009, Crisford et al., 2011).

Therefore, to gain a greater understanding of the nematicidal activity of fluensulfone, behavioural, electrophysiological and genetic investigations were conducted in *C. elegans* to systematically characterise the effects of fluensulfone (Kearn et al., 2014). The behaviour and biology of *C. elegans* is better understood than PPNs and can be studied using techniques that are not tractable for PPNs. As mentioned, previous work compared the activity of fluensulfone against the organophosphate pesticides, fenamiphos and cadusafos, and this comparison suggested a novel mechanism of action for fluensulfone (Oka et al., 2009). Comparisons with previously and currently used nematicides with known mechanisms of action can provide insight into the activity of compounds like fluensulfone. Therefore, in work conducted with *C. elegans*, fluensulfone was also compared to another anticholinesterase pesticide, aldicarb. Conducting this comparison using *C. elegans* enabled an investigation of the genetic factors underlying susceptibility.

3.1.3 A forward genetic approach did not identify any mediators of fluensulfone susceptibility

Prior to the investigations described above (Kearn et al., 2014), the nematicidal effect of 1 mM fluensulfone against *C. elegans* was used as a selection protocol in a forward genetic screen, utilising mutagenesis via EMS to identify targets that conferred resistance to the nematicidal effects of fluensulfone (Johnsen and Baillie, 1997) (Appendix A). The mutagenesis experiment did produce strains that had low-level resistance to 1 mM fluensulfone that were able to develop past the L1 larval stage. These strains still propagated poorly in the presence of 1 mM fluensulfone however, and the majority of worms still failed to develop beyond the L1 stage. There are several reasons that could explain why the mutagenesis failed to generate high-level resistance. It is possible that at a concentration of 1 mM, fluensulfone interacts with multiple targets to inhibit development and to kill worms. If so, this could mean that any single mutation may be insufficient to produce significant resistance. It is also possible that the target(s) of fluensulfone are integral to the survival and development of *C. elegans* and therefore any mutations in the genes encoding these targets are lethal or profoundly affect worm viability, and thus no resistant strains were generated.

Whilst it was possible that repeating the mutagenesis could still generate more strongly resistant strains, the effects of fluensulfone on *C. elegans* behaviour were firstly fully characterised, with a view to gaining a greater understanding of fluensulfone and clues as to its target(s).

3.1.4 Fluensulfone adversely affects *C. elegans* development and reproduction

The effects of fluensulfone on the dynamics of *C. elegans* development were investigated by cultivating worms from eggs in the constant presence of either fluensulfone or vehicle (control) (Kearn et al., 2014). The developmental stage reached was scored up to 96 hours to determine the rate of development relative to the control. During this time, the N2 worm undergoes full development. On vehicle control plates, >90% of *C. elegans* had reached the adult stage at 66 hours (Figure 3.1).

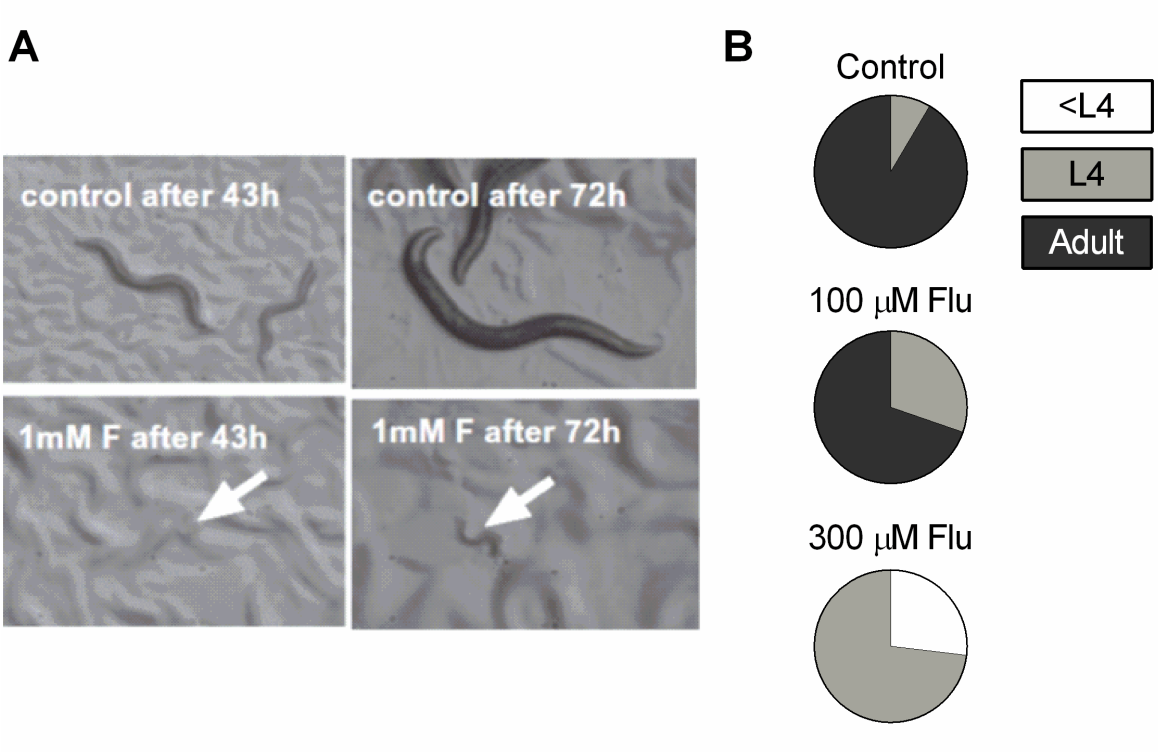

Figure 3.1. Dose-dependent effects of fluensulfone on *C. elegans* development. Eggs were grown to adults in the presence of either vehicle (control) or fluensulfone at different concentrations for 96 hours and developmental stage was scored. **A**) Representative images of eggs cultivated in the presence of either vehicle (control) or 1 mM fluensulfone. After 43 hours in the presence of vehicle worms have developed to the L2/3 and reach the young adult stage by 72 hours. In the presence of 1mM fluensulfone larvae failed to develop beyond the L1 stage even after 72 hours. **B**) At 66 hours (data shown) development was delayed by fluensulfone at 100 µM and 300 µM (The assay was repeated on 3 separate occasions with 5 plates tested for each condition each time, mean shown) (from (Kearn et al., 2014)).

1 mM fluensulfone prevented any development past the L1 stage, as the L1 worms arrested (Figure 3.1A). Lower concentrations did not prevent development, rather development was delayed relative to the vehicle control (Figure 3.1B). 100 µM fluensulfone slightly delayed development at 66 hours, with fewer worms reaching the adult stage relative to those grown in the presence of vehicle (control). 300 µM fluensulfone caused a more pronounced delay in development with no worms reaching the adult stage and some having failed to reach the L4 stage. It is clear that fluensulfone delays *C. elegans* development in a concentration-dependent manner.

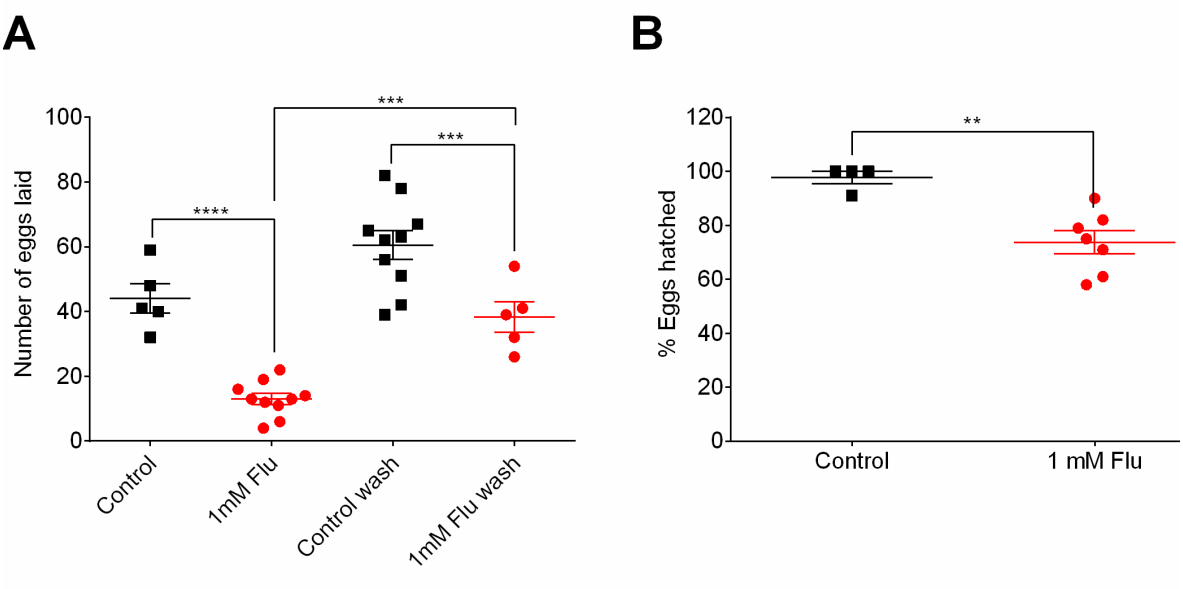

Figure 3.2. The effect of fluensulfone on egg-laying and egg hatching in *C. elegans*. A) After 1 hour fewer eggs were laid by 10 gravid hermaphrodites in the presence of 1 mM fluensulfone (flu) than in the presence of vehicle (control). Worms were removed after 1 hour and egg laying was again scored after 1 hour in the absence of drug. On removal to unmodified, OP50-seeded plates egg laying recovered slightly (n=5-10 plates with 10 worms per plate, one-way ANOVA with Tukey post-hoc tests). **B**) 24 hours later, the eggs that were laid on the vehicle- and 1 mM fluensulfone-modified plates were scored for hatching. Egg hatching was significantly reduced in the presence of 1 mM fluensulfone (n=4-7 plates with 10 worms per plate, unpaired T-test. Data are mean ± s.e mean for all figures except where stated. In all figures * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, n.s = not significant) (from (Kearn et al., 2014).

As mentioned, experiments have shown that fluensulfone elicits a slight decrease in egg hatching in the PPN *M. incognita* (Oka et al., 2012). Fluensulfone was therefore also tested for inhibitory effects on the hatching of *C. elegans* eggs to validate these observations and to allow for comparison with a PPN. Whilst assaying egg hatching behaviour, effects on the egg laying of adult hermaphrodites was also scored. Adult L4+1 day *C. elegans* were placed on food in the presence of fluensulfone or vehicle (control) and the number of eggs laid was scored. Fewer eggs were laid in the presence of 1 mM fluensulfone relative to the control, with a 71% reduction in the number of eggs laid (Figure 3.2A). Indeed, visual observation showed that eggs were retained inside the hermaphrodite, which subsequently displayed a "bagging behaviour", where the eggs hatched within the adult worm. This acute inhibition of egg laying was partially reversible, as after removal to food plates without fluensulfone, egg laying increased. 24 hours later the eggs laid in the presence of fluensulfone were scored for hatching and there was a 24.6% reduction in hatching relative to the vehicle control (Figure 3.2B). Of the eggs that did hatch, none progressed beyond the L1 larval stage

(data not shown). These results indicate that fluensulfone elicits a decrease in egg hatching in both *C. elegans* and the PPN *M. javanica* (Oka et al., 2009).

Fluensulfone reduced the viability of eggs *in utero* (Figure 3.3). In these experiments, gravid hermaphrodites were exposed to 1 mM fluensulfone for 1 hour in the presence of food. The worms were then removed to a new agar plate in the presence of food with no drug and allowed to lay eggs. The eggs that were laid were then scored for hatching 24 hours (Figure 3.3A) and 48 hours later (Figure 3.3B). The viability of eggs laid within 1 hour of removal from fluensulfone was unaffected, with nearly 100% hatching. Interestingly, there was a reduction in the viability of eggs laid 2-5 hours subsequent to fluensulfone exposure, with percentage hatch significantly lower than the vehicle control. Even 48 hours after egg laying the eggs did not hatch, indicating a complete inhibition of the developmental cycle, as opposed to merely delayed hatching. It was hypothesised that the exposure of the adult worm to fluensulfone could impair events such as fertilisation. Therefore, eggs hatched in the presence of fluensulfone were scrutinised using DIC microscopy and it was found that the unhatched eggs contained embryos, indicating that fluensulfone does not impair the self-fertilisation process (Figure 3.3C). For both eggs laid on control plates and those laid in the presence of fluensulfone, >90% of eggs were fertilised and contained embryos. This suggests that fluensulfone prevents hatching by direct effects on the unhatched embryo, most likely fluensulfone completely inhibits embryo development or kills the developing worm.

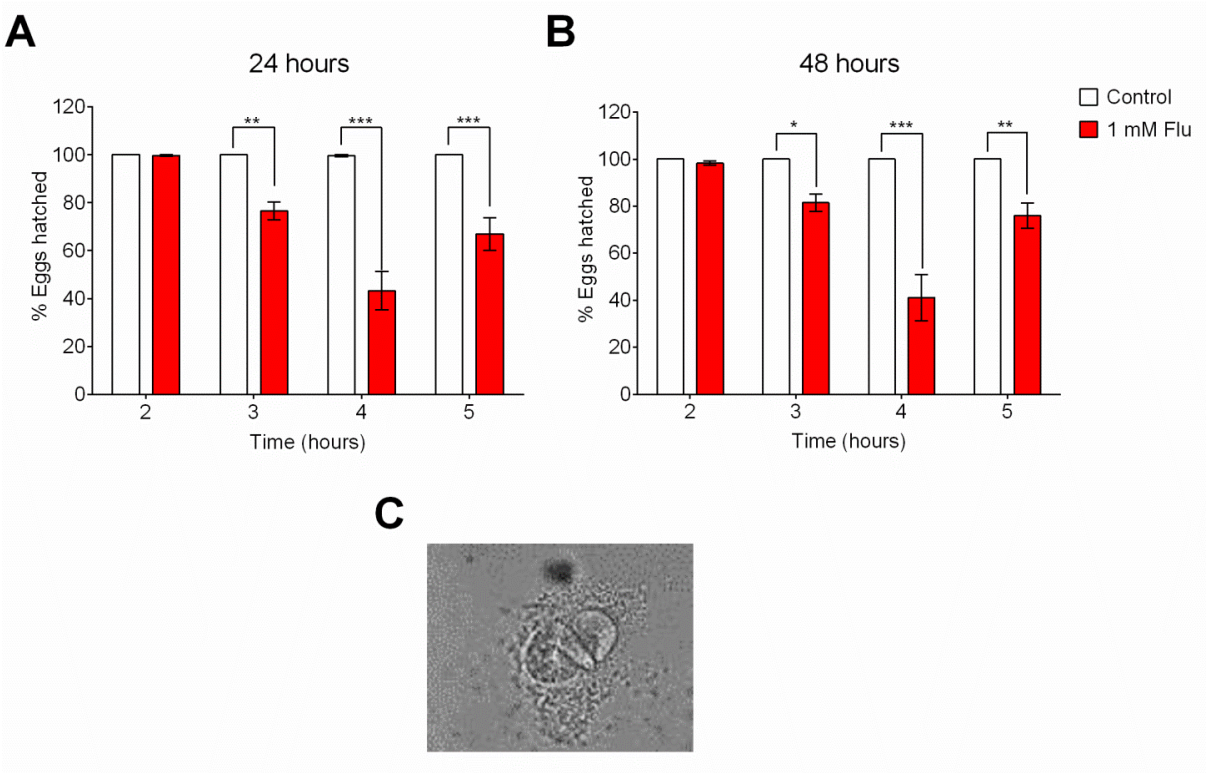

Figure 3.3. Fluensulfone exposure reduces subsequent embryo viability. 10 gravid hermaphrodites were transferred to OP50-seeded plates modified with either vehicle (control) or 1 mM fluensulfone (flu) for 1 hour. The worms were then removed to unmodified OP50-seeded plates every hour up to 5 hours and the eggs laid on the plates were scored for hatching 24 hours (**A**) and 48 hours (**B**) post-exposure. Hatching was reduced in eggs laid 3-5 hours after removal of the hermaphrodites from fluensulfone and these eggs also did not hatch 48 hours later (n=10 plates each treatment. Two-way ANOVA with Bonferroni post-hoc tests). **C**) Eggs from this experiment were observed via DIC microscopy. The image is representative of a typical unhatched egg on 1 mM fluensulfone (from (Kearn et al., 2014)).

3.1.5 Fluensulfone inhibits *C. elegans* motility in liquid

Fluensulfone has been shown to have inhibitory effects on the motility of *Meloidogyne spp*. with chronic exposure, with increased immobility becoming apparent at 24 hours (Oka et al., 2008, Oka et al., 2009). Experiments were therefore conducted to characterise the effects of fluensulfone on *C. elegans* motility in M9 buffer. L2/3 larvae were soaked in fluensulfone for up to 24 hours and paralysis was scored at intervals by counting the number moving in a 10 second period (Figure 3.4A). Exposure to 1 mM fluensulfone resulted in significant paralysis relative to the vehicle control, with increased paralysis observed from 1-6 hours relative to the vehicle control.

300 µM fluensulfone increased percentage paralysis at 6 hours, although 100 µM fluensulfone had no effect on percentage paralysis, relative to the vehicle control. At 24 hours nearly 100% of all larvae, including those in the control treatment group, were paralysed. The larvae were then washed and subsequently transferred to OP50-seeded NGM plates for 24 hours to assess recovery. Nearly all larvae recovered from paralysis in the control, 100 µM and 300 µM fluensulfone treatment groups yet there was no significant recovery in larvae treated with 1 mM fluensulfone, indicating irreversible nematicidal effects following 24 hour exposure.

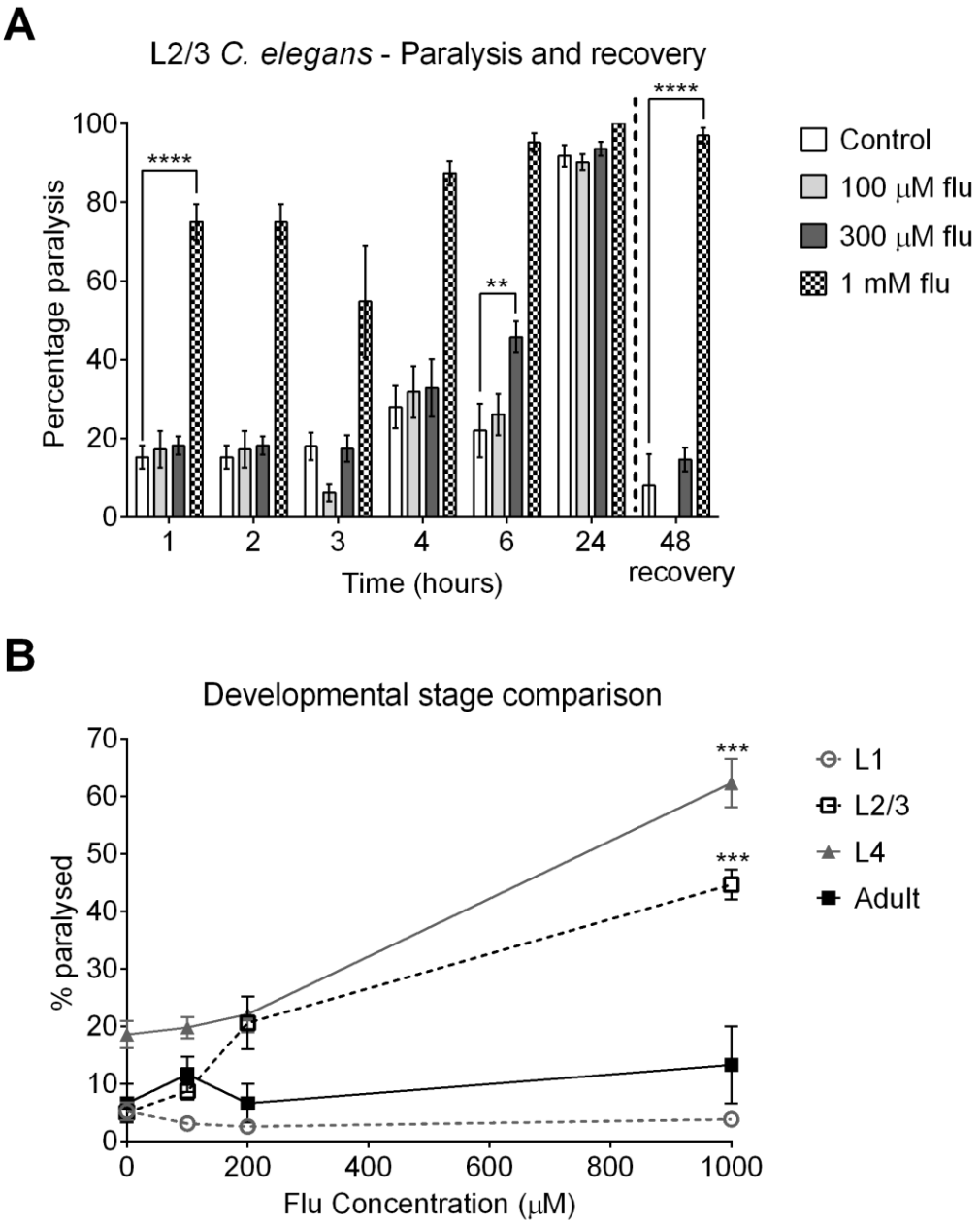

Figure 3.4. Fluensulfone paralyses *C. elegans* in liquid. A) L2/3 larvae were exposed to fluensulfone or vehicle (control) in M9 buffer and percentage paralysis was scored over 24 hours. To assess recovery, the larvae were washed in buffer 3 times and transferred to NGM plates seeded with OP50 *E.coli* (n=5 dishes with 50 worms per dish, mean ± s.e mean shown, two-way ANOVA with Dunnett post-hoc tests). **B**) 50-100 synchronised worms at different stages were transferred to 96 well plates and soaked in either vehicle (0 μM) or fluensulfone for 3 hours. Percentage paralysis was then scored (n=5 replicate wells per treatment, mean ± s.e mean shown, one way ANOVA with Bonferroni post-hoc tests) (from (Kearn et al., 2014)).

L2/3 larvae were used for this experiment to allow comparison with previous observations on mobility made using second-stage juvenile *M. javanica* (Oka et al., 2009, Oka et al., 2012). In order to determine any differences in susceptibility to fluensulfone between the different developmental stages, synchronised worms from L1 to adult were exposed to fluensulfone for 3 hours and percentage paralysis was scored (Figure 3.4B). Percentage paralysis of L2/3 and L4 larvae was increased by treatment with 1 mM fluensulfone for 3 hours yet the same treatment did not induce paralysis in both adults and L1 larvae. This indicates that the L2/3 and L4 larval stages are more susceptible to the inhibitory action of fluensulfone on motility during acute exposure than adults and L1 larvae, although fluensulfone has nematicidal effects against all stages.

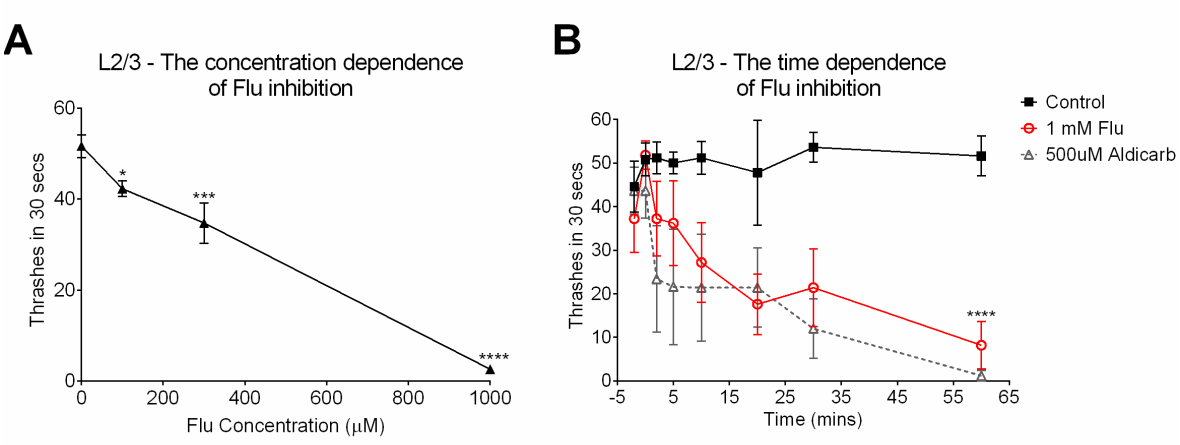

Figure 3.5. Acute exposure to fluensulfone inhibits the rate of movement of *C. elegans* L2/3 larvae in liquid over a similar time course to aldicarb. A) L2/3 larvae were placed in M9 buffer containing either vehicle (control) or fluensulfone (Flu). After 3 hours the number of thrashes in 30 secs was counted for each worm (n=9-10 worms, mean ± s.e mean, one way ANOVA with Bonferroni post-hoc tests). **B**) The thrashing rate of L2/L3 larvae in M9 buffer was measured 2 minutes prior to placing them either in M9 containing either vehicle (control), 1 mM fluensulfone (Flu) or 500 µM aldicarb. Time zero is the point when the worms were transferred. The number of thrashes made by each worm in 30 secs was visually scored throughout the next hour. (n=5 worms, mean ± s.e mean, two-way ANOVA with Dunnett post-hoc tests) (from (Kearn et al., 2014)).

The paralysis experiments indicated that fluensulfone acutely inhibits motility in liquid at 1 mM. To characterise this further, the thrashing behaviour of *C. elegans* in liquid was quantified in the presence of fluensulfone. L2/3 larvae were used to allow for comparison with the J2 juvenile infective stage of PPNs. A 3 hour incubation of L2/3 larvae in fluensulfone showed a concentration-dependent inhibition of thrashing behaviour (Figure 3.4A). After 3 hours in the presence of vehicle alone, L2/3 thrashed 52 times per minute. In comparison, the rate of thrashing was 42 thrashes per minute in 100 µM and 35 thrashes per minute in 300 µM fluensulfone. Thrashing was nearly completely inhibited after 3 hours in 1 mM fluensulfone, with a mean rate of 2.6 thrashes per minute. A time course for the inhibitory effect of 1 mM fluensulfone on thrashing was also conducted, showing that the rate of thrashing gradually decreased in fluensulfone until near-maximal inhibition was reached after 1 hour (Figure 3.4B). The time course for 1 mM fluensulfone inhibition of thrashing was similar to that for 500 µM aldicarb. Fluensulfone and aldicarb were then more rigorously compared.

3.1.6 Comparing the effects of fluensulfone with the anticholinesterase aldicarb

Since the withdrawal of the majority of fumigant nematicides, such as methyl bromide, PPN infestation has been controlled predominately through the use of organophosphate and carbamate soluble nematicides (Chitwood, 2003a). Both of these groups of nematicides work via inhibition of cholinesterases, which results in excessive acetylcholine signalling and thus spastic paralysis (see general introduction). As acetylcholine is an integral neurotransmitter throughout the animal kingdom, these nematicides have undesirable toxicity towards non-target organisms (Husain et al., 2010, Risher et al., 1987). Given the importance of this mode of action in PPN control, experiments were performed to compare the effects of fluensulfone to the carbamate aldicarb. Aldicarb was chosen as a comparison to fluensulfone as its paralytic effects have been studied extensively in *C. elegans* (Nguyen et al., 1995). These experiments also provided evidence that the mode of action of fluensulfone is distinct from anticholinesterases and that it is unlikely to interact with cholinergic targets.

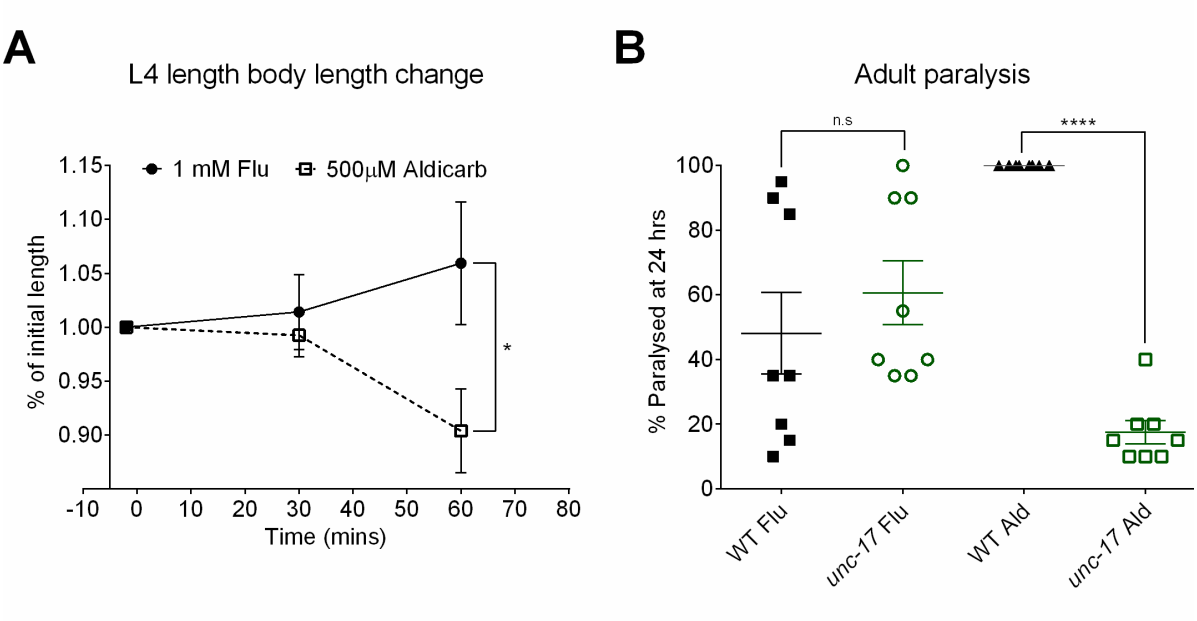

Figure 3.6. A comparison of the effects of fluensulfone and aldicarb on *C. elegans*. A) Aldicarb elicits a shortening of body length whereas fluensulfone does not. Individual L4 larvae were imaged on food plates in the absence of compound (shown as -2 mins) and then placed on agar plates containing either 1 mM fluensulfone (flu) or 500 µM aldicarb (ald). Further images were captured at time-points after placing them on the compound. ImageJ software, which provides a skeleton image of the worm, was used to measure its length. Measurements were normalised to the initial length of each individual worm (n=6 worms, mean ± s.e mean; two-way ANOVA with Bonferroni post-hoc tests). **B**) 20 L4+1 day worms, wild-type (WT) or the *unc-17* mutant, were put onto individual OP50/NGM plates containing either vehicle (control), 1 mM fluensulfone (Flu) or 500 µM aldicarb (Ald). The number of paralysed worms after 24 hrs was scored. Fewer *unc-17* were paralysed than wild-type in the presence of aldicarb but % paralysis was not significantly different from wild-type when in the presence of fluensulfone (n=8 plates per treatment, mean ± s.e mean, one-way ANOVA with Sidak post-hoc tests) (from (Kearn et al., 2014)).

As mentioned, fluensulfone was found to inhibit motility acutely and to induce full paralysis with chronic exposure. Aldicarb, like fluensulfone, inhibits motility followed by paralysis (Figure 3.6B). Paralysis of *M. javanica* by fluensulfone appears to be distinct from that induced by anticholinesterases, with fluensulfone causing a straight, rod-shaped posture and organophosphates inducing a "wavy" posture with a shortening of body length (Oka et al., 2009). Therefore, the paralysis induced by fluensulfone and aldicarb was examined in *C. elegans*, with a view to comparing their respective modes of action. This was done by measuring posture and body length in the presence of fluensulfone and aldicarb. As mentioned, aldicarb induces a spastic paralysis via hyper-contraction of the body wall muscle. This results in a quantifiable decrease in body length. L4 larvae were exposed to either 1 mM fluensulfone or 500 µM aldicarb and images taken before and after treatment with the drugs were used to quantify changes in body length (Figure 3.6A). As expected, aldicarb treatment induced a reduction in body

length. Fluensulfone on the other hand had no significant effect on the body length of the treated worms over the 1 hour course of the experiment. This indicates that whilst both fluensulfone and aldicarb induce paralysis, the fluensulfone-induced paralysis is not due to hyper-contraction, and that the mode of action of fluensulfone is distinct from anticholinesterases such as aldicarb. In addition, these experiments suggest that mode of action of fluensulfone is also distinct from cholinergic agonists used as anthelmintics such as levamisole, which also induce a shortening of body length resulting from hypercontraction (Mulcahy et al., 2013). Furthermore, this indicates a distinct mode of action from other pesticides such as the neonicotinoids, which also act as cholinergic agonists and have been shown to have activity against *C. elegans* (Ruan et al., 2009).

3.1.7 Mutant analysis confirms a distinct mode of action for fluensulfone relative to anticholinesterases

As a means of further comparing the effects of these two nematicides, an *unc-17* (strain CB113) mutant was utilised. *unc-17* encodes the vesicular acetylcholine transporter required for the loading of acetylcholine into vesicles for neurotransmission (Alfonso et al., 1993). The hypomorphic mutation in the strain used results in greatly diminished acetylcholine release at the neuromuscular junction and thus confers resistance to cholinesterase inhibitors such as aldicarb. Wild type and *unc-17* L4+1 day *C. elegans* were exposed to either 1 mM fluensulfone or 500 µM aldicarb and after 24 hours percentage paralysis was scored (Figure 3.6B). Whilst 100% of wild type worms were paralysed after 24 hours exposure to 500 µM aldicarb, the *unc-17* strain showed a marked resistance to aldicarb with fewer than 20% paralysed. There was however no difference in percentage paralysis between wild type and *unc-17* worms exposed to 1 mM fluensulfone. This provides further evidence that fluensulfone does not act like a cholinesterase inhibitor to bring about paralysis in *C. elegans*.

3.1.8 Comparing the effects of fluensulfone with the macrocyclic lactone anthelmintic ivermectin

The effects of the macrocyclic lactone ivermectin on *C. elegans* and other nematodes are qualitatively similar to the effects of fluensulfone in that it also induces paralysis without hyper-contraction, inhibits feeding, slows development and inhibits egg hatching (Dent et al., 1997, Brownlee et al., 1997, Kass et al., 1980). Ivermectin exerts its effects via interaction with a family of glutamate-gated chloride channels that are only present in invertebrate phyla such as nematoda (Wolstenholme, 2012).

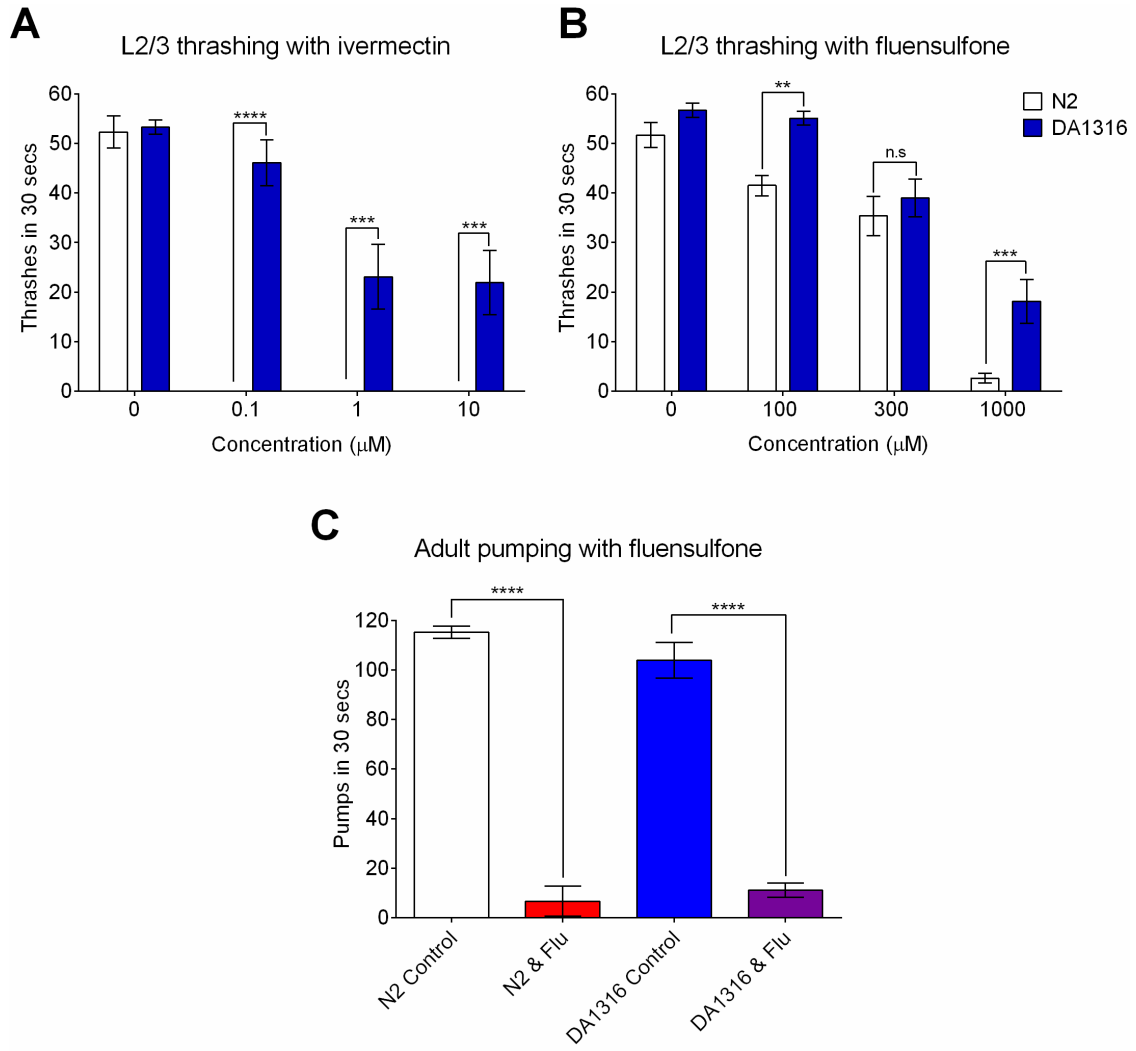

Figure 3.7. A comparison of the effects of ivermectin and fluensulfone on *C. elegans* thrashing and feeding behaviour. The rate of thrashing of wild type N2 L2/3 larvae was compared to the ivermectin-resistant DA1316 (*avr-14; avr-15; glc-1*) in the presence of vehicle (0 μM), ivermectin (**A**) or fluensulfone (**B**). The rate of thrashing was scored at either 1 hour (A) or 3 hours (B) (n=10 worms, mean ± s.e mean shown, two-way ANOVA with Sidak post-hoc tests). **C**) N2 or *avr-14; avr-15; glc-1* (DA1316) worms were exposed to either vehicle (control) or 1 mM fluensulfone for 1 hour and pharyngeal pumping was scored (n= 10, mean ± s.e mean, one-way ANOVA with Tukey post-hoc tests) (from (Kearn et al., 2014)).

To determine whether glutamate-gated chloride channels play any role in mediating the effects of fluensulfone, an ivermectin resistant strain, DA1316, with mutations in three glutamate-gated chloride channel subunits (*avr-14; avr-15; glc-1*) was utilised (Dent et al., 2000). The ivermectin resistant strain was tested for susceptibility to ivermectin and fluensulfone by scoring thrashing, which is inhibited by both fluensulfone and ivermectin (Dent et al., 2000). Wild type L2/3 larvae exposed to ≥100 nM ivermectin for 1 hour were completely paralysed and did not thrash (Figure 3.7A). On the contrary, *avr-14; avr-15; glc-1* mutants displayed a high level of resistance to ivermectin, with 100 nM ivermectin having no significant effect on thrashing after 1 hour and larvae still thrashing in the presence of 10 µM ivermectin. The thrashing rate of wild type L2/3 larvae was slightly inhibited after 3 hours exposure to 100 and 300 µM fluensulfone and strongly inhibited by 1 mM fluensulfone (Figure 3.7B).

Whilst *avr-14; avr-15; glc-1* mutants were highly resistant to ivermectin, thrashing was still inhibited in the presence of fluensulfone relative to the vehicle control. This inhibition was however to a lesser extent than occurred in wild type, potentially indicating partial resistance. To investigate this further, the effects of fluensulfone on pharyngeal pumping in wild type and *avr-14; avr-15; glc-1* mutants were compared. *avr-14; avr-15; glc-1* mutants are highly resistant to the inhibitory effect of ivermectin on pharyngeal pumping (Dent et al., 2000). Exposure of L4+1 day adult worms to 1 mM fluensulfone for 1 hour elicited an almost complete inhibition of pharyngeal pumping in both wild type and *avr-14; avr-15; glc-1* mutants (Figure 3.7C). The level of inhibition was not significantly different between wild type and *avr-14; avr-15; glc-1* mutants, indicating that glutamate-gated chloride channels are not the major target of fluensulfone.

In summary, before initiating the experiments described below, it was known that ≥3.4 µM fluensulfone induces the paralysis of *Meloidogyne spp*. with 24-48 hours exposure (Oka et al., 2009). It was also known that ≥13.6 µM fluensulfone reduces the hatching of *M. javanica* eggs. Earlier experiments showed that whilst fluensulfone does have nematicidal activity against *C. elegans*, this is only evident at 1 mM, over 100-fold higher (Kearn et al., 2014). 1 mM fluensulfone also inhibits *C. elegans* egg laying and the hatching of eggs. Further investigation found that fluensulfone inhibits *C. elegans* development in a concentration-dependent manner, with some effect at 100 µM. ≥100

µM fluensulfone was also found to have an inhibitory effect of *C. elegans* motility. A comparison with aldicarb showed that fluensulfone does not act as an anticholinesterase and is unlikely to have a cholinergic mode of action. Further comparison with ivermectin showed that glutamate-gated chloride channels are not likely to be a major target of fluensulfone.

In this chapter, the effects of fluensulfone on *C. elegans* behaviour are further characterised and investigated. The effects of fluensulfone on locomotion and pharyngeal pumping are examined under different conditions and mutants are screened for susceptibility to fluensulfone. The effects of fluensulfone on *C. elegans* were established to gain an insight into its mechanism of action and to provide a platform for further studies in PPN species. The effects of fluensulfone on pharyngeal pumping were investigated via electrophysiology as this has been used as a route for mechanism of action studies on the anthelmintics ivermectin and emodepside (Dent et al., 1997, Pemberton et al., 2001, Crisford et al., 2011).

Species	Concentration (µM)	Assay	Time (hours)	Effect
M. javanica	3.4-27.2	Immobility	48	80-100% immobility, no recovery
X. index	6.8-54.4	Immobility	48	60-100% immobility, no recovery
P. penetrans, *P. thornei*	13.6-54.4	Immobility	48	>70% immobility, no recovery
A. besseyi, *A. fragariae*, *B. xylophilus*, *D. dispaci*	54.4	Immobility	48	Unaffected
C. elegans	1000	Mortality	24	90-100% mortality

Table 3.3. A summary of the susceptibility of several nematode species to fluensulfone *in vitro*. These data are from (Oka et al., 2009, Oka, 2014, Kearn et al., 2014).

Behaviour	Concentration (µM)	Time (hours)	Effect
Development	100	66	Delayed
	300	66	Delayed
	1000	66	Arrested development
Egg laying	1000	1	71% reduction
Egg hatching	1000	24	25% reduction
Egg viability *in utero*	1000	3-5	60% reduction
L2/3 thrashing	100	3	20% reduction
	300	3	30% reduction
	1000	3	100% reduction

Table 3.4. A summary of the effects of fluensulfone on *C. elegans* behaviour described in this introduction (Kearn et al., 2014).

3.1.9 Electropharyngeogram (EPG) investigation of pharyngeal effects

In earlier work and the experiments described in this chapter, fluensulfone was found to inhibit *C. elegans* pharyngeal pumping and the concentration-dependence of this inhibition was altered in the presence and absence of food (section 3.2.3) (Kearn et al., 2014). The regulatory mechanisms underpinning the pharyngeal system and the neuropharmacology of its behaviours are well understood (see Chapter 1, section 1.15). As a result, the pharynx has been used as a model system for investigations into the effects and mechanisms of action of several anthelmintics and for assaying neuroactive drugs (Avery and Horvitz, 1990, Dent et al., 1997, Pemberton et al., 2001, Crisford et al., 2011). The effects of fluensulfone on pharyngeal pumping were therefore further investigated using the EPG technique, which generates an electrophysiological readout of pharyngeal activity (see chapter 1, section 1.15.9) (Raizen and Avery, 1994). The EPG technique provides a means of investigating the effects of fluensulfone on pharyngeal activity with a greater level of detail, relative to visually counting pumping. EPG recordings allow analysis of drug effects on several parameters of pharyngeal

pumping that cannot be determined through visual observation, such as the duration of each pump. Analysis of the spikes of the EPG waveform can also inform on alterations in neural activity and the strength of the pharyngeal muscle contraction (Dillon et al., 2009). The rapid, acute effects of ≥300 μM fluensulfone on pharyngeal pumping and other behaviours suggest that it may interact with neural signalling and thus electrophysiological investigation may provide information regarding the pathways with which it interacts.

EPG recordings were performed on cut head preparations, in which the head is severed from the rest of the body by cutting just behind the terminal bulb. The pharynx, and the associated pharyngeal nervous system, remains intact and undamaged in cut heads (Avery et al., 1995). Pharyngeal pumping continues to occur spontaneously in cut heads for several hours, if the cut head is left in physiological Dent's saline, typically at a rate of 1-6 pumps per minute (*unpublished observations*). The pharyngeal muscle and nervous system in cut heads is exposed to the surrounding saline solution and thus drugs have direct access without the cuticle presenting a barrier. Drugs such as 5-HT, for example can be applied to stimulate the rate of pharyngeal pumping.

Having summarised and compared the published work on the effects of fluensulfone on *Meloidogyne spp*. and *C. elegans*, the aim of this chapter is to further characterise and investigate the effects of fluensulfone on *C. elegans* behaviour, with a view to gaining an insight into its mechanism of action.

3.2 Results

3.2.1 Fluensulfone has context-dependent effects on *C. elegans* locomotion

As shown, ≥100 μM fluensulfone inhibits *C. elegans* motility and thrashing in liquid, with inhibition seen within 3 hours (Figure 3.4, Figure 3.5) (Kearn et al., 2014). When on a solid medium *C. elegans* move differently, via sinusoidal body bends, and this movement is affected by environmental cues such as food (Zheng et al., 1999). These distinct forms of movement are regulated by differential neurotransmitter and neuromodulator signalling (Vidal-Gadea et al., 2012). Thus, examining the effects of fluensulfone on worm behaviour under different contexts may enable further understanding of the pathways with which it interacts.

When in liquid, *C. elegans* do not exhibit pharyngeal pumping, but pharyngeal pumping does occur on a solid media, both in the presence and absence of a food source. For many drugs, the primary route of access to the worm is thought to be through ingestion and subsequent diffusion from the gut into other tissues (Meyer and Williams, 2014). Diffusion through the cuticle is another route via which drugs can enter the worm. Examining the effects of fluensulfone and the concentration-dependence of these effects, on a solid media in both the presence and absence of food provides an insight into how fluensulfone accesses *C. elegans* and whether susceptibility is influenced by the route of drug uptake. This approach also allowed concomitant investigation of the effects of fluensulfone on *C. elegans* pharyngeal pumping. Pharyngeal pumping is a well-characterised behaviour, with the neural and pharmacological mechanisms underpinning it well understood. As such, pharyngeal pumping is a useful system for examining how neural signalling is affected by drugs such as fluensulfone and has been used previously in this respect for other chemicals and drugs (Dent et al., 1997, Crisford et al., 2011, Meyer and Williams, 2014).

Body bend assays were conducted in the presence and absence of food on agar plates. These body bend assays were conducted concomitant with pharyngeal pumping measurements, where indicated. For this reason, L4+1 day adults were used to allow for visualisation of pharyngeal pumping. Firstly, adult worms were exposed to fluensulfone on agar plates modified with the drug in the absence of food (Figure 3.8A). Body bends

per minute in the presence of vehicle remained steady at 33-41 body bends per minute over the course of 2 hours and 100 µM fluensulfone had no effect on body bends per minute relative to the control. In contrast, body bends declined in the presence of 500 µM fluensulfone, falling to 27 bends per minute after 30 minutes and to 16 bends per minute after 80 minutes. In a separate experiment, body bends were quantified in the absence of food in the presence of 1 mM fluensulfone (Figure 3.14B). Body bends per minute were not different from the vehicle control up to 4 minutes but then fell from 33 bends per minute to just 2 bends per minute after 21 minutes exposure. This indicates that fluensulfone inhibits body bends in the absence of food more rapidly than thrashing.

In contrast, the same investigation of fluensulfone-induced changes in body bends in the presence of food found the opposite effect, with an increase in body bends per minute. When food is present, worms typically exhibit dwelling behaviour, which is characterised by low movement and high reversals, allowing maximal feeding (Fujiwara et al., 2002). Thus, the rate of body bends in the control treated worms was much lower in the presence of food than in the absence of food. When exposed to fluensulfone in the presence of food *C. elegans* adults exhibited roaming behaviour, characterised by high levels of movement and few reversals. The effects of fluensulfone on locomotion are therefore context-dependent. Body bends were higher relative to control in the presence of both 500 µM and 1 mM fluensulfone when measured on food, with 6 bends per minute in the presence of vehicle, relative to 10 and 18 bends per minute in the presence of 500 µM and 1 mM fluensulfone, respectively (Figure 3.8B). On transfer to the test plates, the worms moved rapidly in the presence of both vehicle and fluensulfone, due to mechanical stimulation. The worms on the control plates slowed their locomotion within 5-10 minutes but those on fluensulfone plates continued moving more quickly. This stimulatory effect on locomotion was transient and at 5 hours locomotion was not significantly different from the control (Figure 3.8C). Based upon the thrashing assays and immobility, it would be expected that most, if not all, *C. elegans* would be immotile after 5 hours in the presence of 1 mM fluensulfone (Figure 3.4, Figure 3.5). This indicates that the effects of fluensulfone on locomotion are context-dependent, in that in the presence of food locomotion is transiently stimulated whereas after the same exposure time in the absence of food in both liquid and on solid media only an

inhibition occurs. This observation suggests that the effects of fluensulfone on behaviour are dependent upon the behavioural state of the worm.

The lack of dwelling behaviour seen in worms that are exposed to ≥500 µM fluensulfone and the increased rate of locomotion suggests that fluensulfone may be perceived as a noxious stimulus by the worm or that fluensulfone prevents detection of the presence of a food source.

Food leaving assays were therefore conducted to ascertain if fluensulfone promotes food-leaving behaviour. On a food source of sufficient quality, N2 *C. elegans* typically exhibit very low levels of food leaving if the food source is not overpopulated and free from potential harmful stimuli such as pathogenic organisms (Shtonda and Avery, 2006, Pradel et al., 2007, Harvey, 2009, Milward et al., 2011). When *C. elegans* do leave a food source this indicates that the food is perceived as being of poor quality and as a result, food leaving increases to enhance the probability of finding a superior food source. 100 µM had no effect on food leaving behaviour. The rate of food leaving in the presence of 500 µM fluensulfone increased to 0.018 leaving events per worm per minute, relative to 0.007 in the presence of vehicle alone (Figure 3.8D). This indicates that the transient increase in locomotion induced by fluensulfone is also associated with an increase in food leaving.

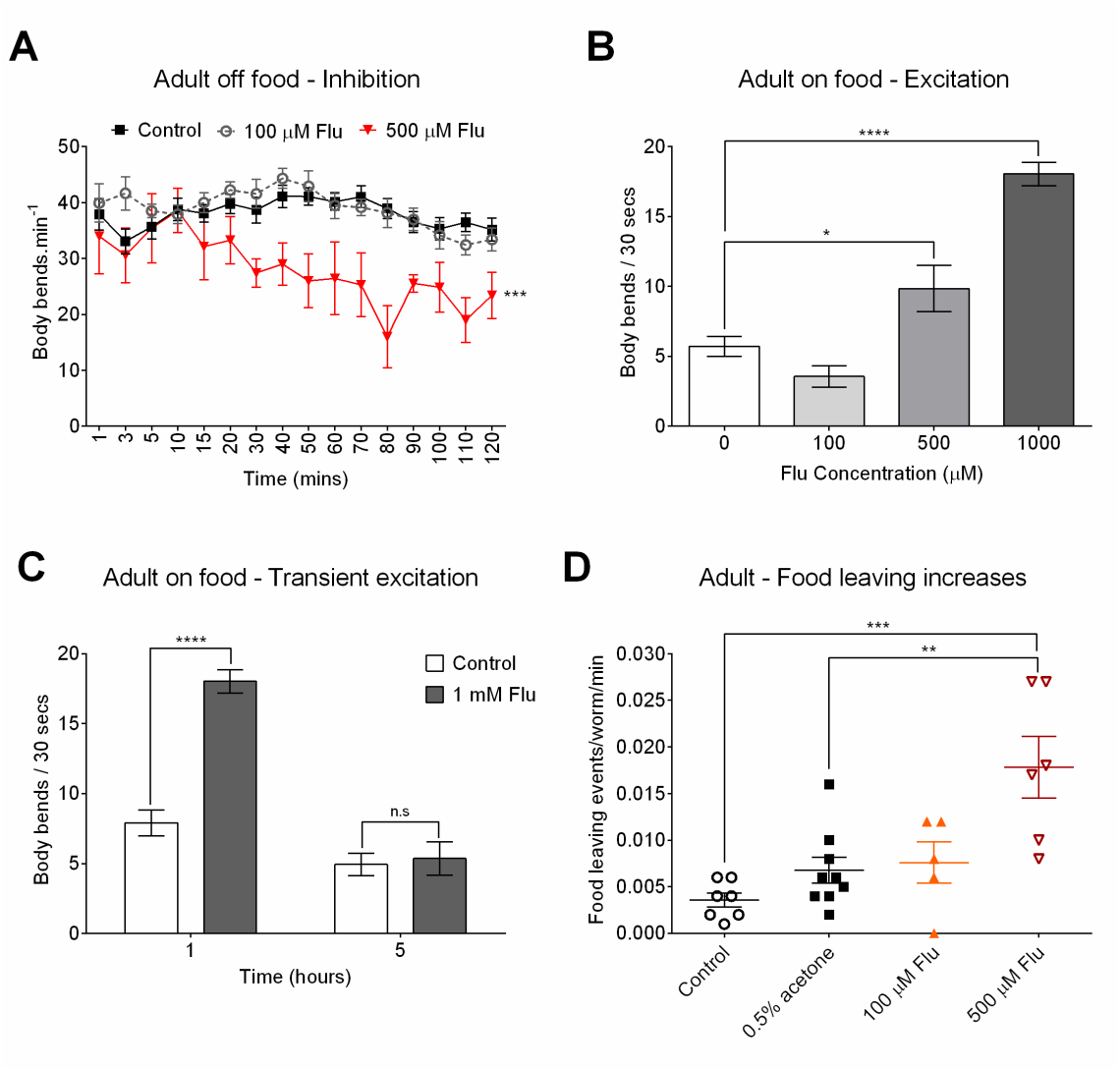

Figure 3.8. The effects of fluensulfone on locomotion in the presence and absence of food on agar and its effects on food leaving. A) In the absence of food, fluensulfone reduces the rate of body bends of *C. elegans* moving on an agar plate. L4+1 day worms were picked onto an unseeded 9 cm plate modified with either vehicle (control) or fluensulfone (flu). The frequency of body bends for each worm was counted for at indicated intervals for 2 hours (control n=10 worms, 100 µM flu n=12, 500 µM flu n=7, mean ± s.e mean shown, two-way ANOVA with Bonferroni post-hoc tests, P<0.001). The x-axis has been plotted on an expanded scale for the first 30 mins to reveal the response for the early time points. **B**) Dose-dependent effects of fluensulfone on the locomotion of L4+1 *C. elegans* on a bacterial lawn. The plates contained fluensulfone at the indicated concentration and after 1 hr the rate of body bends was counted for individual worms for 1 min. (Control n=51 worms, 100 µM n=20, 500 µM n =19, 1000 µM n=28; mean ± s.e mean; one way ANOVA with Bonferroni post-hoc tests, P<0.0001). **C**) 1 mM fluensulfone caused an elevated level of locomotion at the 1 hr time point but this returned to near-control levels at 5 hrs (Control n=30 worms, flu n=28 worms, two-way ANOVA with Bonferroni post-hoc tests, P<0.0001). **D**) Exposure to 500 µM fluensulfone causes an increase in the frequency of food leaving events relative to agar and vehicle controls over a 2 hr period. 100 µM fluensulfone did not increase food leaving (control n=7 plates, 0.5% acetone n=9, 100 µM n=5 plates, 500 µM n=6 plates, with 7 worms per plate; one-way ANOVA with Bonferroni post-hoc tests, P<0.05).

3.2.2 Fluensulfone is not aversive to *C. elegans*

The increased food leaving and locomotion when fluensulfone is present suggested that fluensulfone may induce aversive behaviour. To test this hypothesis, a standard assay for avoidance was utilised in which a drop of drug is placed directly in the path of a freely moving worm and the response of the worm is recorded (Hart et al., 1995). Of 8 worms tested, 7 continued on their path and swam through the drop of 1 mM fluensulfone whereas only 1 worm reversed and moved away from the droplet (data not shown). This indicates that fluensulfone is not aversive to *C. elegans* and that the stimulation of locomotion and food leaving are due to other factors.

3.2.3 Fluensulfone has context-dependent effects on *C. elegans* pharyngeal pumping

Pharyngeal pumping was scored concurrently with locomotion to ascertain the effects of fluensulfone on feeding behaviour. L4+1 day adult *C. elegans* were exposed to fluensulfone in the presence of food and pharyngeal pumping was scored over 24 hours (Figure 3.9A). 100 µM fluensulfone had no effect on pharyngeal pumping in the presence of food. The rate of pharyngeal pumping was profoundly reduced in the presence of both 500 µM and 1 mM fluensulfone relative to the vehicle control. 1 mM fluensulfone completely inhibited pumping after 1 hour's exposure. Pharyngeal pumping still occurred in the presence of 500 µM fluensulfone at all time points, albeit at a much reduced rate. The greatest level of inhibition of pumping by 500 µM fluensulfone occurred at 3 hours whilst a slight recovery was observed at 5 hours and 7 hours, increasing from 32 pumps per minute to 207 pumps per minute at 7 hours. In a separate experiment, 1 mM fluensulfone was found to rapidly inhibit pumping, with a 40% reduction in pump rate in 2 minutes and a 90% reduction within 5 minutes, relative to the vehicle control (data not shown).

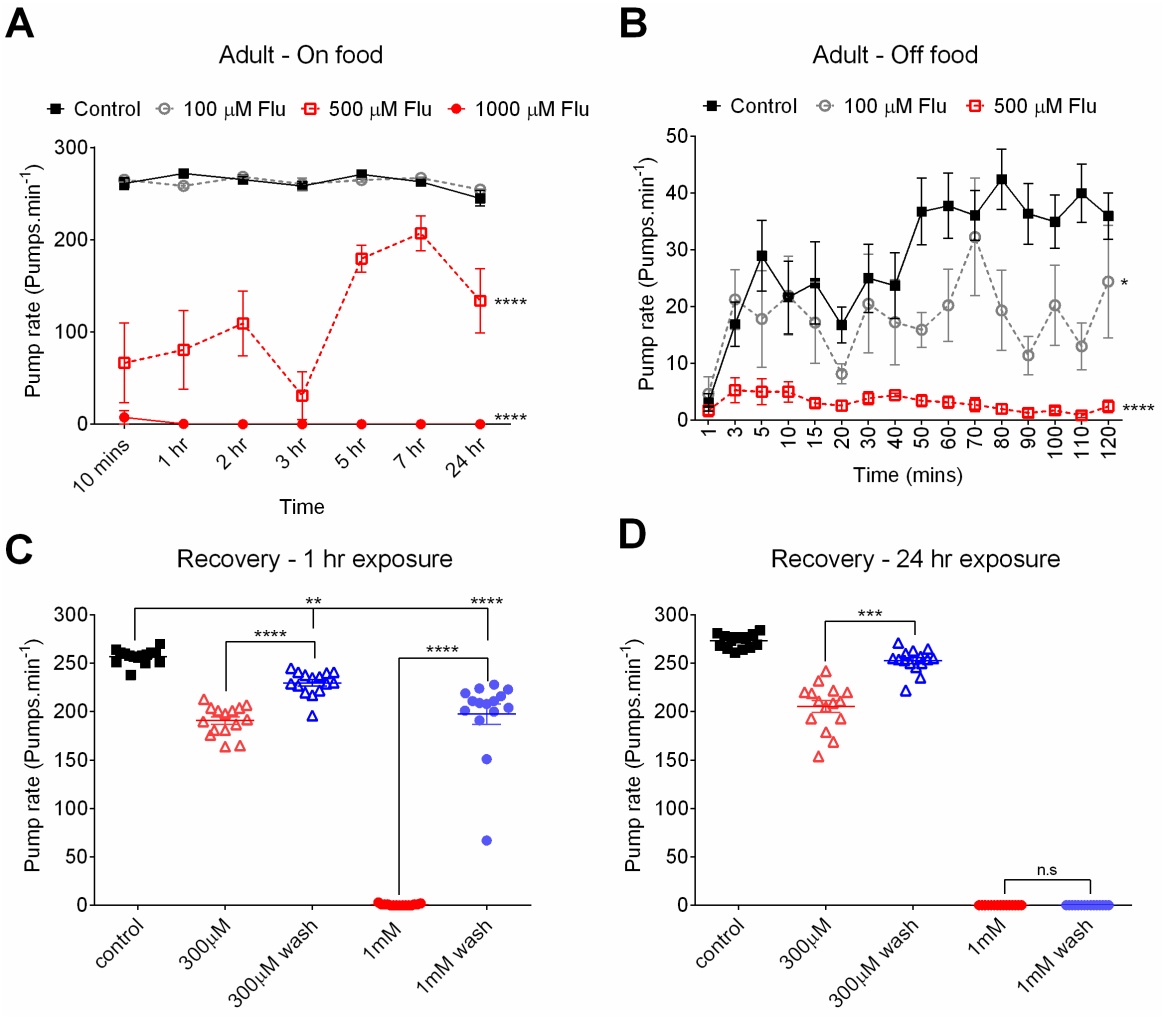

Figure 3.9. Acute exposure to fluensulfone both in the presence and in the absence of food inhibits *C. elegans* pharyngeal pumping. A) L4+1 day worms were placed onto plates seeded with a bacterial lawn that were modified with either vehicle (control) or fluensulfone (flu) and pharyngeal pumping was visually scored over 24 hrs. Pumping was unaffected by 100 µM fluensulfone but was inhibited by 500 µM and completely abolished by 1 mM fluensulfone (control n=13 worms, 100 µM n=7, 500 µM n=6, 1 mM n=6; mean ± s.e mean; two-way ANOVA with Bonferroni post-hoc tests, P<0.0001. The data are pooled from 2 separate experiments). **B**) Worms were placed, via a cleaning plate, onto unseeded 9 cm plates modified with either vehicle (control) or fluensulfone (flu) and pharyngeal pumping was visually scored over 2 hrs. Pumping was inhibited by both 100 µM and 500 µM fluensulfone (control n=19 worms, 100 µM n=12, 500 µM n=7; mean ± s.e mean shown; two-way ANOVA with Bonferroni post-hoc tests, P<0.05. The data are pooled from 2 separate experiments). The x-axis has been plotted on an expanded scale for the first 30 mins to reveal the response for the early time points. **C**) L4+1 day worms were placed on agar plates in the presence of food with vehicle (control), 300 µM or 1 mM fluensulfone and pumping was scored after 1 hour. Worms were then removed to plates without drug in the presence of food (wash) and pumping was scored 1 hour later (n=15 worms, individual data points and mean ± s.e mean shown, one way ANOVA with Tukey post-hoc tests, P<0.0001). **D**) L4+1 day worms were place on agar plates in the presence of food with vehicle (control), 300 µM or 1 mM fluensulfone and pumping was scored after 24 hours. Worms were then removed to plates without drug in the presence of food and pumping was scored 1 hour later (n=15 worms, individual data points and mean ± s.e mean shown, one way ANOVA with Tukey post-hoc tests, P<0.0001). Elizabeth Ludlow conducted the experiments shown in C and D.

To assess the potential for recovery of the rate of pharyngeal pumping, adult worms were exposed to vehicle (control), 300 µM or 1 mM fluensulfone for either 1 hour (Figure 3.9C) or 24 hours (Figure 3.9D) in the presence of food and pumping was scored. The worms were subsequently transferred to agar plates on food in the absence of drug and pumping was again scored. Exposure to 300 µM and 1 mM fluensulfone for 1 hour inhibited pumping and this inhibition was reversible on removal from drug. The rate of pumping did not however fully recover to vehicle control levels. A similar inhibition was seen with 24 hours exposure to 300 µM and 1 mM fluensulfone, however pharyngeal pumping did not recover in worms exposed to 1 mM fluensulfone. This indicates that the reversibility of the inhibition is dependent upon exposure time. Visual observation found that many of the worms exposed to 1 mM fluensulfone for 24 hours appeared dead or bagged. In all experiments, bagging only occurred in the presence of 1 mM fluensulfone, indicating a concentration threshold for this effect (data not shown).

The effects of fluensulfone on the pharyngeal pumping of L4+1 day adult *C. elegans* in the absence of food was also quantified (Figure 3.9B). When food is present the rate of feeding of adult *C. elegans* typically exceeds 250 pumps per minute. In the absence of food, the rate of pumping is far lower and is much more variable. The rate of pumping in worms placed on unseeded NGM plates modified with vehicle in the absence of food increased with time after the initial transfer to the agar plate. This is consistent with observations made using unmodified NGM plates (*personal communication,* Nicolas Dallière). This increase in pump rate did not occur in the presence of 500 µM fluensulfone and pumping was almost completely inhibited after 2 hours. 100 µM fluensulfone elicited a slight but significant reduction in the rate of pharyngeal pumping in the absence of food over the course of 2 hours relative to control-treated worms. This appears to be a selective effect in the absence of food, as 100 µM fluensulfone had no discernible effect on feeding in the presence of food. This again highlights the context-dependent effects of fluensulfone on *C. elegans* behaviour. The concentration-dependence of the inhibitory effect of fluensulfone seems to shift to lower concentrations when food is absent.

3.2.4 Susceptibility to fluensulfone is unaffected by mutations in major neurotransmitter pathways

A reverse genetic screen was conducted using mutants deficient in most of the major neurotransmitter and neuromodulator pathways. The mutants were exposed to 1 mM fluensulfone in the presence of food for 24 hours and pharyngeal pumping and body bends were scored and compared to an N2 control. Mortality was also scored through responsiveness to prodding. Mutants defective in glutamate, serotonin, octopamine and neuropeptide signalling were tested. Broadly, no significant differences in the susceptibility of these strains to the effects of 1 mM fluensulfone on feeding, locomotion or mortality were observed (Table 3.5).

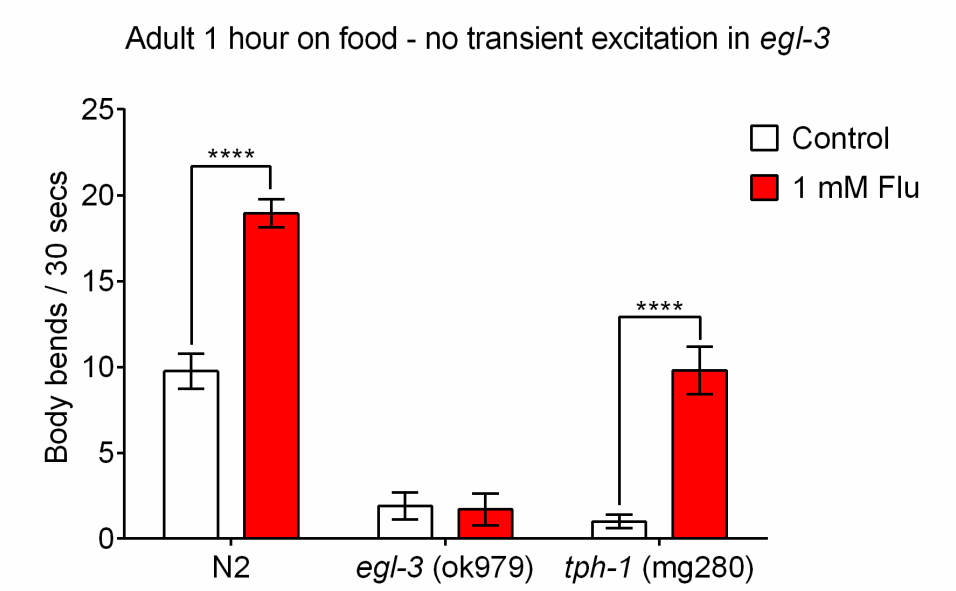

Figure 3.10. Fluensulfone does not elicit a transient excitation of locomotion in an *egl-3* mutant. 10 L4+1 day N2, *egl-3* and *tph-1* were placed onto food-seeded plates in the presence of either vehicle (control) or 1 mM fluensulfone (flu) and body bends were scored after 1 hour. Both mutants were conducted concurrently with an N2 comparison. Fluensulfone did not increase body bends in *egl-3* but did in *tph-1* (control n=19 worms, *egl-3* & *tph-1* n=10, mean ± s.e mean shown, two-way ANOVA with Sidak post-hoc tests, P<0.0001).

Interestingly, whilst 1 mM fluensulfone elicits a transient increase in locomotion in N2 *C. elegans*, no such increase occurred in *egl-3* mutants (Figure 3.10). *egl-3* encodes a proprotein convertase that is crucial for the generation of neuropeptides by cleaving proprotein peptide precursors into their biologically active form (Husson et al., 2006). The tested strain VC671 can only generate 1 of the known 75 neuropeptides found in *C. elegans* (Husson et al., 2006). After exposure to 1 mM fluensulfone for 1 hour, N2

locomotion was increased relative to the vehicle control-treated worms whereas no such increase occurred in the *egl-3* mutant (Figure 3.10).

Gene	Protein role	Pharyngeal pumping	Locomotion	Mortality at 24 hours
N2	N/A	Inhibited	Initial stimulation followed by inhibition	40-75%
eat-4 (n2472)	Vesicular glutamate transporter	Inhibited	Initial stimulation followed by inhibition	13%
avr-14; avr-15; glc-1 (ad1302, ad1051, pk54)	Glutamate-gated chloride channel subunits	Inhibited	Initial stimulation followed by inhibition	33%
tph-1 (mg280)	Tryptophan hydroxylase (5-HT synthetic enzyme)	Inhibited	Initial stimulation followed by inhibition	100%
tbh-1 (ok1196)	Tyrosine decarboxylase (octopamine synthetic enzyme)	Inhibited	Initial stimulation followed by inhibition	63%
egl-3 (ok979)	Proprotein convertase 2 (neuropeptide activating enzyme)	Inhibited	No transient increase in movement at 1 hour	100%
slo-1 (js379)	Voltage- and calcium-activated potassium channel, emodepside resistant	Inhibited	Initial stimulation followed by inhibition	30%

Table 3.5. The effects of fluensulfone on *C. elegans* mutants deficient in neurotransmitter pathways. The columns describe the susceptibility of the mutants to the effects of 1 mM fluensulfone on feeding, locomotion and mortality relative to a paired N2 control. L4+1 day *C. elegans* were placed on food plates in the presence of either vehicle (control) or 1 mM fluensulfone and pharyngeal pumping, locomotion and mortality were scored at 1 hour and 24 hours. An N2 comparison was performed with each mutant (n=10 worms per treatment).

The rate of locomotion of the *egl-3* mutant was significantly lower than N2 in the absence of drug. It is therefore possible that the transient increase in locomotion does

not occur in the *egl-3* mutant due to its highly compromised locomotory capability. However, the *tph-1* null mutant that was tested is also defective in locomotion (Ben Arous et al., 2009) in the absence of drug but fluensulfone did elicit a transient increase in locomotion in this strain. This suggests that neuropeptide signalling may be necessary for the increase in locomotion induced by fluensulfone. Both *egl-3* and *tph-1* were more susceptible to the nematicidal effects of 1 mM fluensulfone than N2, with 100% mortality after 24 hours compared to 40% and 75%, respectively, for N2 on 1 mM fluensulfone. This may however reflect the compromised physiology of the *tph-1* and *egl-3* mutants.

3.2.5 Electrophysiological analysis of the effects of fluensulfone on *C. elegans* cut head preparations

Fluensulfone was shown to inhibit *C. elegans* pharyngeal pumping at ≥300 µM in the presence of food and ≥100 µM in the absence of food in intact worms (see section 3.2.3). Here, EPG recordings were conducted on cut head preparations of L4+1 day worms in the presence of comparable doses of fluensulfone. All recordings were conducted under constant perfusion of saline into the recording chamber, with a 5 minute saline application before and after fluensulfone application to assess any changes induced by fluensulfone and to evaluate recovery. All experiments were paired, with control recordings with vehicle (0.5% acetone) performed on the same day as fluensulfone tests. 0.5% acetone alone had no effect on the rate of pharyngeal pumping, although the rate of pumping did gradually increase with time in control recordings, independent of treatment with vehicle. This phenomenon has been observed and noted previously (Dillon et al., 2013).

A concentration-response curve of the effect of fluensulfone on pump rate in cut heads demonstrated that 10 minute application of 50 and 100 µM fluensulfone elicited an increased pump rate relative to the intrinsic, spontaneous activity of the pharynx (Figure 3.11B). This is contrary to observations made in intact worms, in which 100 µM fluensulfone had no effect on pharyngeal pumping in the presence of food and slightly inhibited pumping in the absence of food (Figure 3.9). The rate of pumping was not however significantly affected by 10 µM or 250 µM fluensulfone. When 500 µM fluensulfone was applied, a rapid and transient excitatory effect was observed that was swiftly followed by a gross inhibition (Figure 3.11A, D). When these effects were

plotted, they indicated a bell-shaped concentration-response curve, with an excitatory effect on pumping at 50-100 µM and an inhibition at 500 µM, with 250 µM lying at the threshold between the concentrations required to excite and inhibit (Figure 3.11B). In a separate series of experiments, 30 µM fluensulfone was also found to increase the rate of pharyngeal pumping (Kearn et al., 2014). The threshold for the excitatory effect therefore appears to lie somewhere between 10 µM and 30 µM fluensulfone, >10 fold lower than the concentrations that affect motility in intact *C. elegans*. Both the stimulatory low concentration effect and the inhibitory high concentration effect were rapidly reversible and pump rate returned to control levels within the 5 minutes saline wash (Figure 3.11). Overall, this indicates that fluensulfone has complex effects on the pharyngeal system of *C. elegans*, with a low concentration excitatory effect seen in cut heads that is not seen in intact worms under any context. Furthermore, higher concentrations transiently elicit an increase in pumping that is rapidly followed by a complete inhibition.

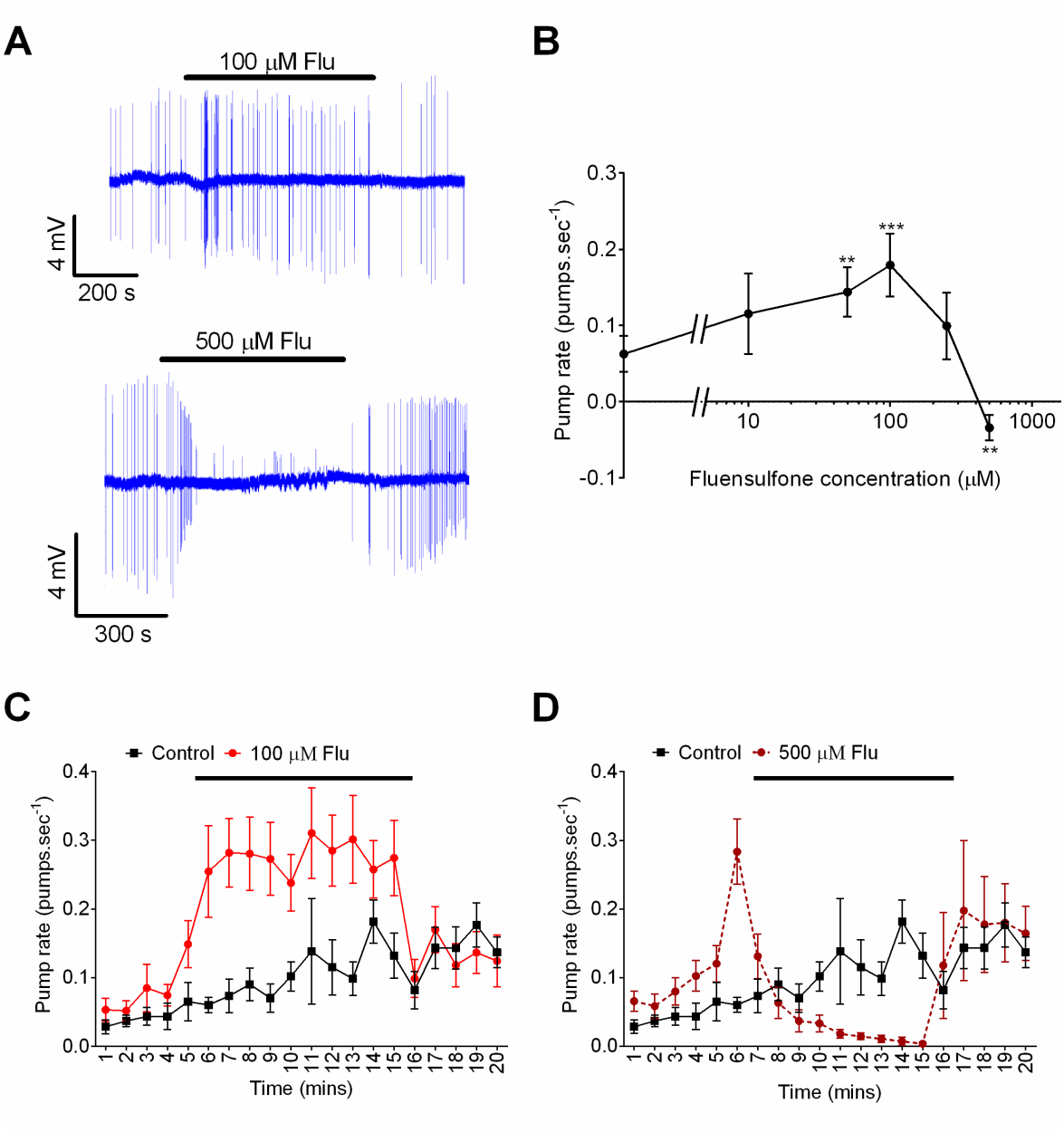

Figure 3.11. Electrophysiological analysis of the effects of fluensulfone on *C. elegans* pharyngeal activity. Electropharyngeogram (EPG) recordings were conducted on dissected pharynx preparations of wild type L4+1 day worms. Saline was constantly perfused onto the preparation and fluensulfone (flu) was applied and washed out by perfusion. **A**) Representative traces showing brief (10 mins) application of fluensulfone onto the preparation, followed by a recovery period in saline. Each vertical spike corresponds to a single pharyngeal pump. The horizontal line above the trace indicates the period of fluensulfone application. **B**) The concentration-response of fluensulfone on pump rate. The response is the difference between the average pump rate for 5 mins before fluensulfone application and the pump rate for the during the last 5 mins of fluensulfone application (n=8-16, mean ± s.e mean, one-way ANOVA with Bonferroni post-hoc tests, P<0.0001). **C**+**D**) The time course for the effects of 100 µM fluensulfone (C) and 500 µM fluensulfone (D) on pump rate. The horizontal bar indicates the duration of fluensulfone perfusion (Control n=10, 100 µM n=11, 500 µM n=14, mean ± s.e mean, P<0.001).

Application of 50 and 100 µM fluensulfone increased pump frequency but no other features of the EPG were affected, with pump duration, pump amplitude and P-spike frequency unaffected relative to the vehicle control (data not shown). 500 µM fluensulfone however, induced a rapid decline in waveform amplitude over a 5 minute interval (Figure 3.12). This decline was also associated with the distortion of the stereotypical EPG waveform relative to the pre-drug control saline application, which was characterised by the loss of the key features of the waveform such as the E and R spikes (Figure 3.12). These effects on pump amplitude and waveform shape were rapidly reversible with a saline wash.

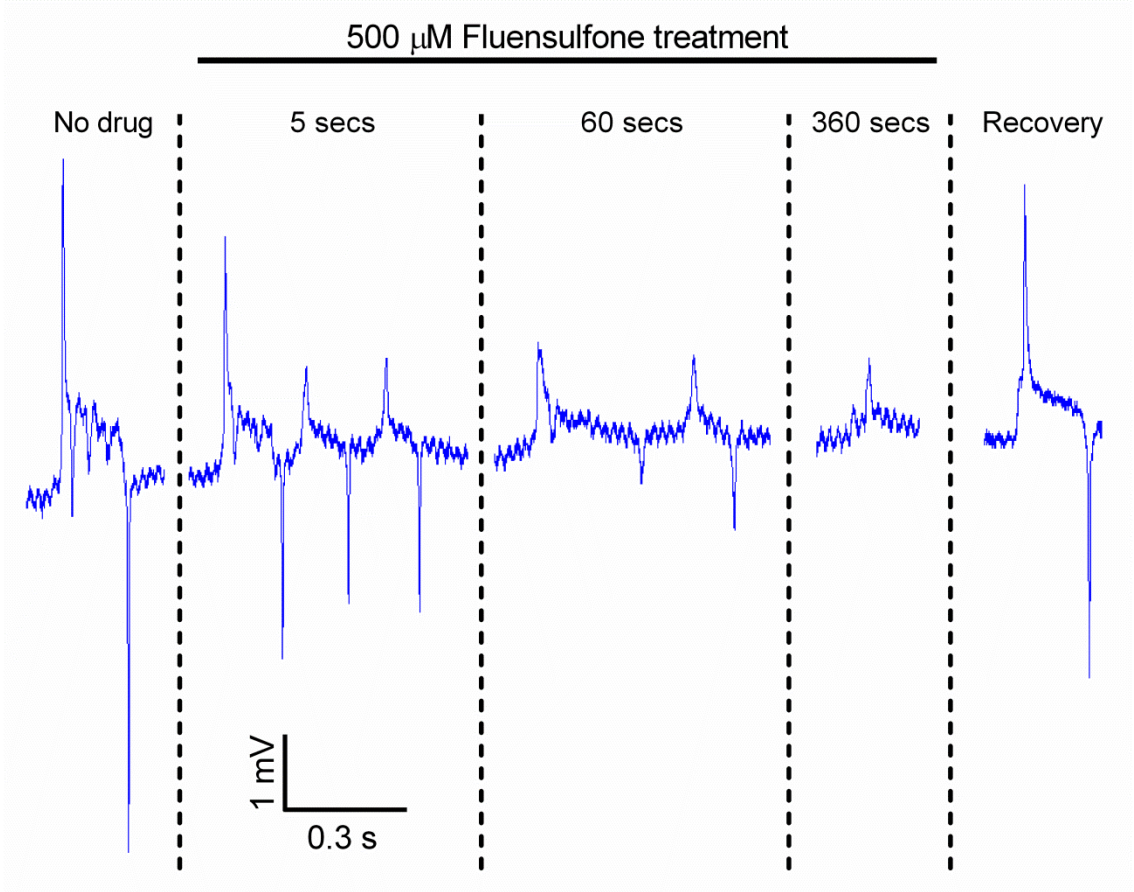

Figure 3.12. The effect of 500 µM fluensulfone on the EPG waveform. Treatment with 500 µM fluensulfone induced a rapid reduction in EPG amplitude and distortion of the EPG waveform. Amplitude is diminished within 5 seconds of fluensulfone application and distortion of the waveform can be seen by 60 seconds. After 5 minutes application few recognisable pumps occur although occasional spikes are seen. After 3 minutes wash with saline recognisable pumps resume, displaying the stereotypical E and R spikes.

3.2.6 The biomechanics of pharyngeal pumping in the presence of fluensulfone

The observed decline in EPG waveform amplitude and distortion of the waveform in the presence of 500 µM fluensulfone implies impairment of the contraction-relaxation cycle that occurs during pharyngeal pumping and implicates a direct effect on the pharyngeal muscle. Therefore, videos were taken to examine the effects of fluensulfone on pharyngeal muscle contraction and relaxation during pumping. Pumps appeared similar between the pre-drug control and when exposed to 100 µM fluensulfone, with no observable change in the force of the pharyngeal contraction or the physical appearance of each pump, although pump rate increased. This is consistent with the stimulatory effect of 100 µM fluensulfone observed in EPG recordings from dissected pharynx preparations. This may suggest that low concentrations of fluensulfone increase pumping by activity at the level of the nerve or by increasing the excitability of the pharyngeal muscle itself. When exposed to 500 µM fluensulfone however, the movements of the grinder of the terminal bulb and the opening and closing of the pharyngeal lumen appeared weaker and were of a shorter duration than before exposure to fluensulfone. The movements of the terminal bulb and the lumen were also uncoordinated, which is consistent with the reduction in EPG amplitude caused by 500 µM fluensulfone in cut head preparations (data not shown).

3.2.7 Electrophysiological analysis of the effects of fluensulfone on 5-HT-stimulated pharyngeal pumping

Experiments with intact worms showed that fluensulfone blocks pharyngeal pumping stimulated by the presence of food (Figure 3.9). Therefore, to determine the effects of fluensulfone on stimulated pumping in cut heads, EPG recordings were conducted where 5-HT was applied in the presence and absence of fluensulfone (Figure 3.13). 500 nM 5-HT was applied onto dissected pharynxes by perfusion in 3-minute bursts. These bursts were followed by a 5 minute wash with saline. Each pharynx was exposed to 5-HT twice to determine the average response. Subsequently, fluensulfone, at either 100 or 500 µM, was perfused onto the pharynx for 5 minutes, following which the pharynx was again exposed to 5-HT but in the continued presence of fluensulfone. The same protocol was also conducted with vehicle (0.5% acetone) as a control. 100 and 500 µM fluensulfone had no effect on the rate of pumping in the presence of 5-HT, relative to the both the pre-fluensulfone 5-HT responses and relative to the vehicle control

(Figure 3.13A). Whilst the rate of 5-HT-induced pumping was unaffected by 500 µM fluensulfone, the amplitude of the EPG waveform was reduced by 50% when fluensulfone was present relative to the pre-fluensulfone amplitude (Figure 3.13B, C). On visual inspection of cut heads treated with both 5-HT and 500 µM fluensulfone the contraction of the terminal bulb and corpus of the pharynx appeared weaker and less complete when compared to cut heads treated with 5-HT alone. The movements of the pharynx when exposed to 5-HT and fluensulfone were however identifiable as pharyngeal pumps, with coordinated movement of the muscle, unlike the uncoordinated twitches observed in the presence of 500 µM fluensulfone alone (data not shown). Pump amplitude returned to pre-fluensulfone levels after a wash with saline (data not shown).

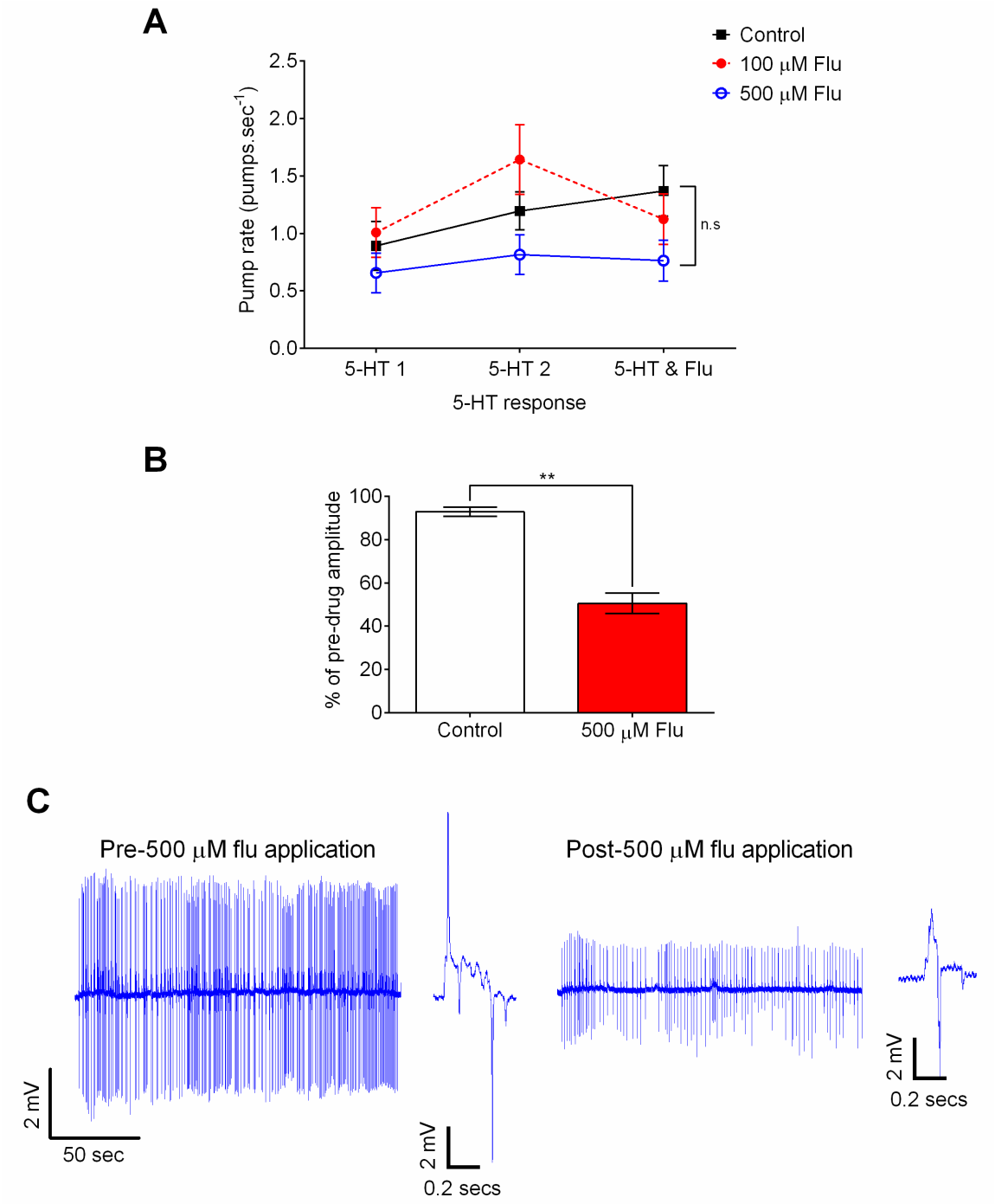

Figure 3.13. The effects of fluensulfone on 5-HT-stimulated pharyngeal pumping in *C. elegans* dissected pharynxes. Dissected pharynxes were exposed to 3 perfusions of 500 nM 5-HT for 3 mins, separated by 5 minute washes with saline. For the 1st and 2nd responses 5-HT alone was applied whereas for the 3rd response 5-HT was applied with vehicle (control), 100 µM or 500 µM fluensulfone (flu). Fluensulfone treatment had no effect on pump rate in the presence of 5-HT. **A**) The presence of fluensulfone did not significantly alter pump frequency (n=5-6 worms, mean ± s.e mean, two-way ANOVA with Dunnett post-hoc tests, P=0.15). **B**) The amplitude of 5-HT-stimulated pumping was reduced in the presence of 500 µM fluensulfone relative to pre-drug amplitude. Vehicle had np significant effect on pump amplitude (P<0.01). **C**) Representative traces of 5-HT application before fluensulfone treatment (left image) and in the presence of 500 µM fluensulfone (right image). When 500 µM fluensulfone was applied concurrently with 5-HT the EPG amplitude was consistently lower than prior to fluensulfone application. Representative individual waveforms are also shown for pre-(left) and post-fluensulfone application.

3.2.8 The interaction between fluensulfone and 5-HT-stimulated pharyngeal pumping in intact *C. elegans*

It is interesting that ≥500 μM fluensulfone inhibits basal pharyngeal pumping in dissected pharynxes and inhibits pumping in intact worms yet does not affect pump rate in the presence of 5-HT in cut head preparations. The effects of fluensulfone on 5-HT-stimulated pharyngeal pumping in intact *C. elegans* were therefore investigated to compare sensitivity with cut heads, as described above. N2 L4+1 day worms were transferred onto agar plates without food, containing vehicle, 10 mM 5-HT, 1 mM fluensulfone or both fluensulfone and 5-HT. After this transfer, pharyngeal pumping and body bends were visually scored from 1-20 minutes (Figure 3.14). The rate of pharyngeal pumping on the vehicle control plates was 12-24 pumps per minute throughout the course of the experiment (Figure 3.14A), which is consistent with previous results (Figure 3.9B). In comparison, 5-HT stimulated pharyngeal pumping within 1 minute, and the rate of pumping remained consistent at a steady-state level of 204-246 pumps per minute throughout the experiment. In the presence of both 5-HT and fluensulfone, the 5-HT effect was immediately reduced with 103 pumps per minute and complete inhibition occurring at 15 minutes. 1 mM fluensulfone alone also completely inhibited pumping, relative to the vehicle control. Fluensulfone treatment had no effect on 5-HT-induced paralysis. Treatment with fluensulfone alone resulted in a progressive decrease in body bends, resulting in near-complete paralysis at 21 minutes. This is in contrast to the increased movement seen in the presence of 1 mM fluensulfone when food is present over a similar time course (Figure 3.8).

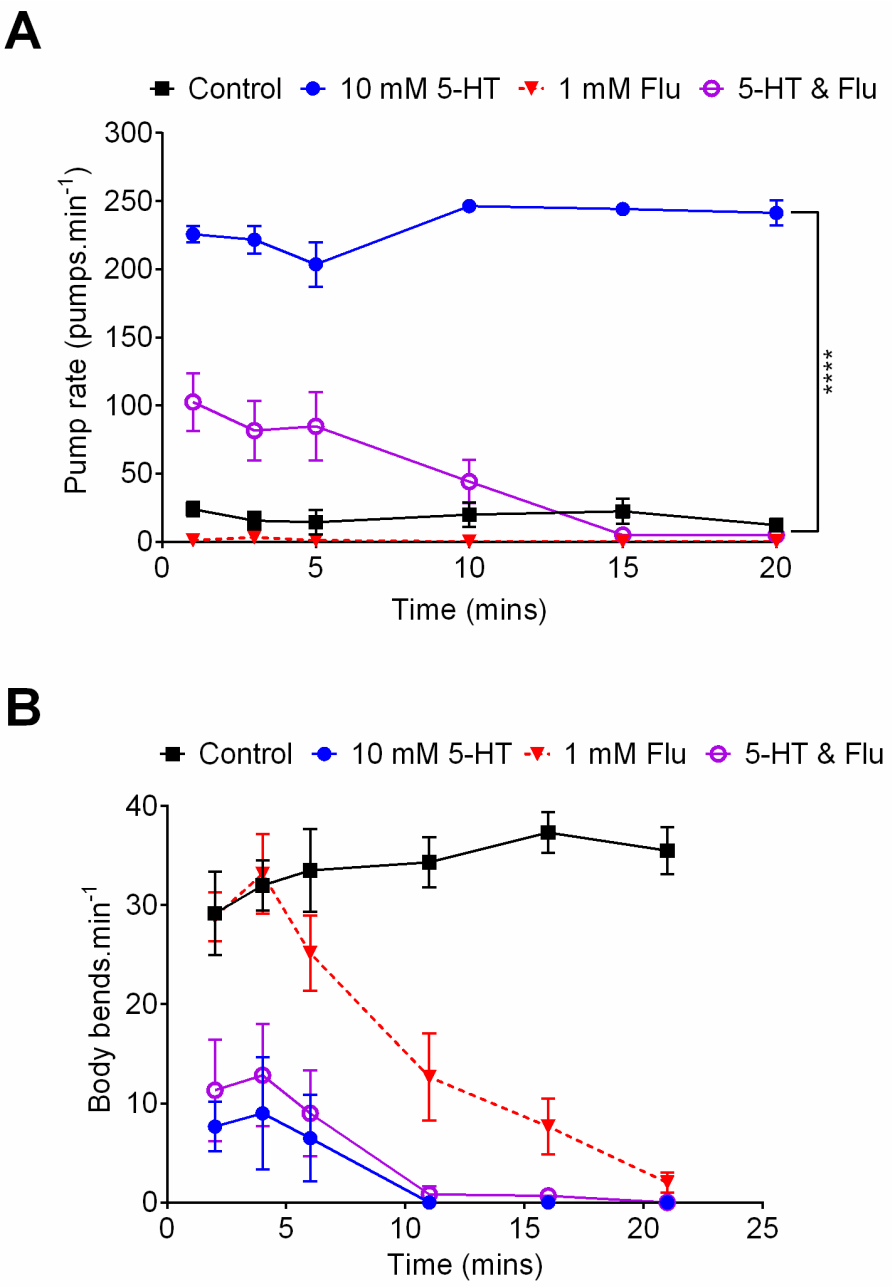

Figure 3.14. The effects of fluensulfone on 5-HT-induced behaviour in intact *C. elegans* off food. L4+1 day worms were transferred to unseeded plates modified with either vehicle (control), 10 mM 5-HT, 1 mM fluensulfone (flu) or 5-HT and fluensulfone. The worms were scored for pharyngeal pumping (**A**) and body bends (**B**). Fluensulfone inhibited 5-HT-induced feeding behaviour but had no effect on 5-HT-induced inhibition of locomotion (n=6 worms per treatment, mean ± s.e mean, two-way ANOVA with Tukey post-hoc tests, P<0.0001 for A+B).

3.2.9 EPG analysis of fluensulfone in mutant backgrounds

Fluensulfone had distinct effects on the *C. elegans* pharyngeal system in cut heads relative to the intact animal, with stimulatory effects seen at lower concentrations (Figure 3.11). Cut heads were also more sensitive to fluensulfone, with activity at 30 µM (see section 3.2.5). Mutants were therefore analysed using the EPG technique, to investigate the mechanisms underpinning the distinct effects fluensulfone on cut heads.

Firstly, *avr-14; avr-15; glc-1* (DA1316) was tested, due to the altered susceptibility of this strain to fluensulfone-mediated inhibition of thrashing (Figure **3.7**). Glutamate-gated chloride channels are also known to be important mediators of inhibitory signalling in the pharyngeal system (Dent et al., 1997, Pemberton et al., 2001). N2 and *avr-14; avr-15; glc-1* L4+1 day adult cut heads were exposed to 500 µM fluensulfone in the EPG setup, following a 5 minute period in the presence of saline alone (Figure 3.15). As previously described (Figure 13), the rate of pumping of N2 cut heads was transiently stimulated in the presence of 500 µM fluensulfone, yet was followed by a near-complete inhibition of pumping within 5 minutes. In contrast, the rate of pumping of *avr-14; avr-15; glc-1* cut heads remained elevated in 500 µM fluensulfone for 10 minutes and was not inhibited relative to the pre-drug control throughout the recording (Figure 3.15A). It was observed in previous experiments with N2 *C. elegans* that 500 µM fluensulfone reduced the amplitude of the EPG waveform, due to impairment of the contraction-relaxation cycle of the pharyngeal muscle (Figure 3.11, sections 3.2.5 & 3.2.6). This was repeated here, with an 80% reduction in waveform amplitude after 10 minutes exposure to 500 µM (Figure 3.15B). Waveform amplitude was also inhibited in *avr-14; avr-15; glc-1* cut heads, with the level of inhibition not significantly different from the control. This indicates that despite the differential effects of fluensulfone on pump rate in N2 and *avr-14; avr-15; glc-1* cut heads, *avr-14; avr-15; glc-1* was susceptible to the inhibitory action of fluensulfone.

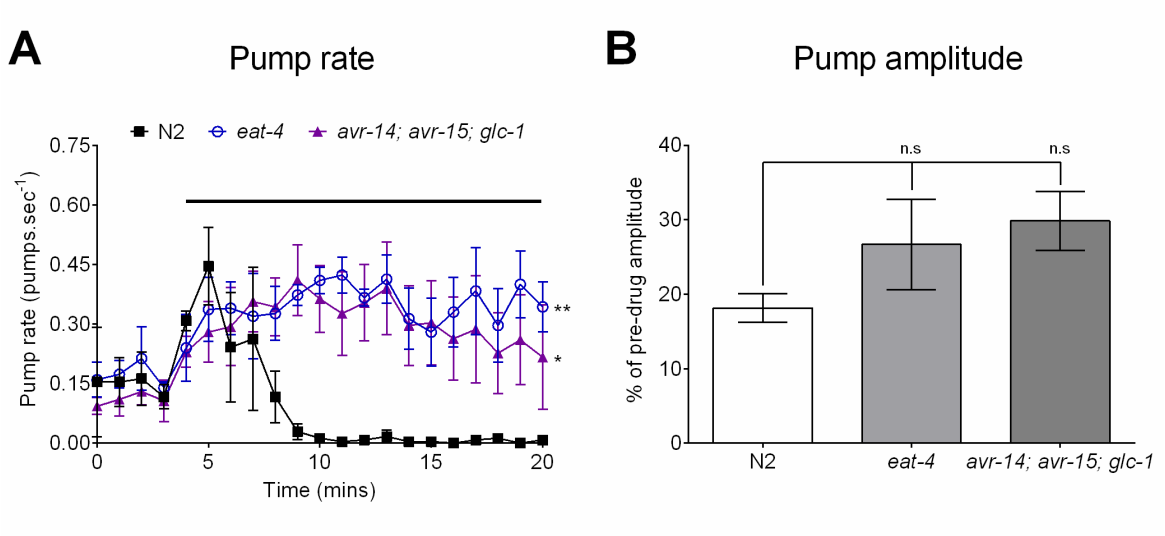

Figure 3.15. The response of *eat-4* and *avr-14; avr-15; glc-1* mutant cut heads to fluensulfone. L4+1 day N2, *eat-4* and *avr-14; avr-15; glc-1* cut heads were exposed to 500 µM fluensulfone in the EPG setup after a 4 minute pre-drug control with saline alone. The black line on the graph indicates the duration of fluensulfone perfusion. **A**) Pump rate is differentially affected by fluensulfone in *eat-4* and *avr-14; avr-15; glc-1* cut heads relative to N2. Pump rate is elevated initially on exposure 500 µM fluensulfone but is then rapidly inhibited within 5 minutes, whereas pump rate remained elevated in *eat-4* and *avr-14; avr-15; glc-1* (n=5 worms per strain, mean ± s.e mean, two-way ANOVA with Dunnett post-hoc tests, $P<0.05$). **B**) 10 minutes exposure to 500 µM fluensulfone decreased pump amplitude relative to the pre-drug control in N2, *eat-4* and *avr-14; avr-15; glc-1*, with no difference in % inhibition between strains (n=4, mean ± s.e mean, one-way ANOVA with Dunnett post-hoc tests, $P=0.31$). An N2 control was conducted for each strain on the same day.

The different effects of fluensulfone on pump rate in N2 and *avr-14; avr-15; glc-1* could be due to the altered inhibitory tone of the motornervous system of *avr-14; avr-15; glc-1* animals. Potentially, due to a reduction in inhibitory glutamatergic signalling, *avr-14; avr-15; glc-1* animals have heightened excitability in the motor circuitry and thus the inhibitory action of fluensulfone is less effective. Thus, mutations in signalling pathways that are not targeted by fluensulfone could alter susceptibility, as has been seen with other nematicidal compounds (Holden-Dye et al., 2012). To investigate this possibility, *eat-4* cut heads were also tested in the presence of 500 µM fluensulfone and were found to show a similar response to fluensulfone to *avr-14; avr-15; glc-1*, with an elevated rate of pumping in the presence of 500 µM fluensulfone relative to N2 but the same inhibition of waveform amplitude (Figure 3.15). The observation that *avr-14; avr-15; glc-1* and *eat-4* have a similar response to fluensulfone suggests that the altered susceptibility of these strains to the effects of 500 µM fluensulfone on cut heads is probably due to reduced inhibitory glutamatergic signalling.

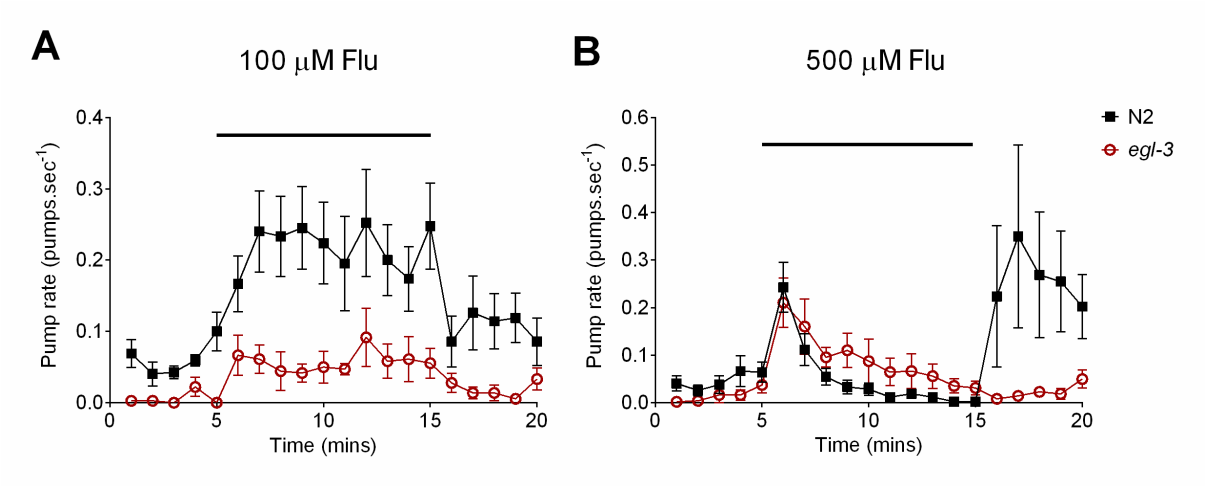

Figure 3.16. The response of *egl-3* mutant cut heads to fluensulfone. A) L4+1 day N2 and *egl-3* cut heads were exposed to 100 µM fluensulfone in the EPG setup after a 5 minute pre-drug control with saline alone. Pump rate was reduced in *egl-3* relative to N2 (n=6-7 worms, mean ± s.e mean, two-way ANOVA with Sidak post-hoc tests, P<0.01). **B**) L4+1 day N2 and *egl-3* cut heads were exposed to 500 µM fluensulfone in the EPG setup after a 5 minute pre-drug control with saline alone. There was no difference in the pump rate of N2 and *egl-3* (n=7-8, , mean ± s.e mean, two-way ANOVA with Sidak post-hoc tests, P=0.19). The black line on the graphs indicates the duration of fluensulfone perfusion. An N2 control was conducted for each strain on the same day.

egl-3 was also tested in the EPG setup for responsiveness to fluensulfone, due to the altered responsiveness of this strain to the fluensulfone-meditated stimulation of locomotion in the presence of food (Figure 3.10). N2 and *egl-3* L4+1 day cut heads were exposed to 100 µM (Figure 3.16A) and 500 µM fluensulfone (Figure 3.16B). As before (Figure 3.11), 100 µM stimulated pharyngeal pumping relative to the pre-drug control, with a 350% increase in pump rate relative to the pre-drug control rate. In *egl-3* cut heads pharyngeal pumping was stimulated by 100 µM fluensulfone yet this stimulation was lower than that seen in N2 cut heads. In the absence of fluensulfone, N2 cut heads exhibited 0.04-0.1 pumps per second, whereas *egl-3* displayed a very low basal pump rate of 0-0.02 pumps per second. This suggests that the reduced stimulation of pumping by 100 µM fluensulfone in an *egl-3* background most likely reflects reduced intrinsic pumping. *egl-3* cut heads also did not respond to 500 µM fluensulfone differently relative to N2.

Behaviour	Concentration (μM)	Time (hours)	Effect
Locomotion - Thrashing	300	3	30% inhibition
	1000	3	100% inhibition
Locomotion – Off food body bends	500	2	50% inhibition
	1000	0.3	100% inhibition
Locomotion – On food body bends	500 & 1000	1-2	50-100% increase

Table 3.6. A summary of results presented in this chapter.

Intact animals			
	Concentration (µM)	Time (hours)	Effect
Pharyngeal pumping on food	500	0.2	70% inhibition
	1000	0.2	100% inhibition
Pharyngeal pumping off food	100	2	25% inhibition
	500	2	80% inhibition
	1000	0.4	100% inhibition
Pharyngeal pumping – 5-HT	1000	0.4	100% inhibition
Cut heads			
EPG	30-100	Immediate	100-150% increase
	500	Immediate	Stimulation followed by inhibition
EPG– 5-HT	500	Immediate	50% amplitude reduction

Table 3.7. A summary of the effects of fluensulfone on the *C. elegans* pharyngeal system.

3.3 Discussion

3.3.1 *C. elegans* are less susceptible to fluensulfone than *Meloidogyne spp*.

The aim of this chapter was to characterise the effects of fluensulfone on *C. elegans* to gain an insight into its mode of action. Fluensulfone has qualitatively similar effects on PPNs such as *M. javanica* and on *C. elegans*, causing an irreversible, non-spastic paralysis and a reduction in egg hatching (Oka et al., 2009, Kearn et al., 2014). There is however, a considerable discrepancy in the concentrations of fluensulfone that kill *C. elegans* and *M. javanica*, with nematicidal activity seen at 3.4 µM in *M. javanica* (Oka et al., 2009), as compared to ≥300 µM in *C. elegans* (Kearn et al., 2014). The experimental data for *M. javanica* was obtained via immotility assays, in which 2nd stage juveniles (J2s) were exposed to fluensulfone in ddH_2O and scored over a 10 second period for movement (Oka et al., 2009). ≥80% immotility was observed after 48 hours in the presence of ≥3.4 µM fluensulfone and there was no recovery from this paralysis 24 hours later. On the other hand, *C. elegans* of all developmental stages were exposed to fluensulfone in an M9 phosphate buffer for 24 hours, at which point the worms were transferred to an agar plate and prodded to assess responsiveness and mortality. 15% mortality of L2/3 *C. elegans* occurred after 24 hours in the presence of 300 µM fluensulfone, with near-100% mortality at 1 mM (Kearn et al., 2014).

There are several possible reasons for this difference. As ≥80% paralysis of *M. javanica* was only observed after 48 hours in the presence of 3.4 µM fluensulfone, it is possible that exposing *C. elegans* to fluensulfone for more time may see nematicidal activity at lower concentrations. Differences in experimental conditions may also account for some of the difference between the species. For example, *C. elegans* mortality was scored through prodding on agar and may be a more rigorous measure of death than the immotility that was scored in experiments with *M. javanica* (Oka et al., 2009, Kearn et al., 2014). The difference could also result from altered drug pharmacokinetics between *C. elegans* and *M. javanica*, resulting from differential cuticle permeability between species. This explanation seems unlikely however, as ≥500 µM fluensulfone almost immediately affects *C. elegans* locomotion and pharyngeal pumping, indicating rapid diffusion across the cuticle. Another reason is differential expression and/or sensitivity of the molecular target(s) of fluensulfone between *C. elegans* and *M. javanica*. The target(s) of fluensulfone may also play different roles or have altered importance

between *C. elegans* and *M. javanica*. For example, there may be considerable metabolic distinctions between the non-feeding *M. javanica* J2 and feeding *C. elegans*.

Susceptibility to fluensulfone has also been found to vary considerably between PPN species (Oka et al., 2009, Oka, 2014). The migratory nematodes *A. fragariae*, *B. xylophilus*, *D. dispaci*, have reduced susceptibility to fluensulfone relative to *M. javanica*, with no increase in immotility after 48 hours exposure to 54.4 µM (Oka, 2014). The susceptibility of these species to fluensulfone may therefore be nearer to that of *C. elegans* than *M. javanica*. This discrepancy between *C. elegans*, *M. javanica* and other PPN species must be investigated more rigorously via direct comparison of fluensulfone nematicidal activity. This would provide information that could aid in the use fluensulfone in the field, for example the quantities of fluensulfone applied. This would also provide a greater insight into the potential ecological effects of fluensulfone application. The relative sensitivity of *C. elegans* and *G. pallida* is investigated in chapter 5.

3.3.2 Fluensulfone affects *C. elegans* development and reproductive behaviours

The behavioural effects of fluensulfone on *C. elegans* were characterised due to the qualitative similarities between the effects of fluensulfone on *C. elegans* and PPNs and the relative experimental amenability of *C. elegans*. Fluensulfone was found to delay and inhibit *C. elegans* development. Fluensulfone also inhibited *C. elegans* egg laying and the subsequent hatching of these eggs.

100-300 µM fluensulfone delayed development and 1 mM completely inhibited development, causing L1 larval arrest. It is possible that this delayed development results from the inhibition of pharyngeal pumping in the presence of fluensulfone. L1 larvae for example, are known to enter a state of diapause in the absence of food, where development is halted and the next larval moult will not occur until food becomes available (Padilla and Ladage, 2012). As 1 mM fluensulfone completely inhibits pharyngeal pumping in the presence of food, L1 larvae may arrest even in the presence of abundant food. Whilst 100 µM fluensulfone was not found to affect the pharyngeal pumping of adults in the presence of food, it is possible that the larval stages are more sensitive to the inhibitory action of fluensulfone on pumping. If this is so, the delayed development in the presence of 100 µM and 300 µM fluensulfone could be caused by a

slight reduction of food intake. Fluensulfone could also delay and inhibit development through inhibition of mitochondrial function and inhibition of metabolism. For example, both antimycin A and siccanin, which inhibit cytochrome C reductase (complex III) and succinate dehydrogenase (complex II) of the mitochondrial electron transport chain (Slater, 1973, Mogi et al., 2009), respectively, arrest larval development (Yamamuro et al., 2011, Padilla and Ladage, 2012). Fluensulfone could also delay development through interfering with the larval moulting process or through direct inhibition of the developmental cycle. Alternatively, the physiological stress that results from fluensulfone exposure may result in delayed development.

Exposure to 1 mM fluensulfone was found to reduce the number of egg laid by adults and promoted the retention of eggs and bagging. Egg laying occurs through the contraction of the vulval muscles (Schafer, 2005) and is regulated, at least in part, by 5-HT signalling. It has been suggested that 5-HT signalling through the SER-1 and SER-7 G protein-coupled receptors (GPCR) promotes egg laying whereas signalling through another GPCR SER-4 and the 5-HT-gated chloride channel MOD-1 inhibits egg laying (Hapiak et al., 2009). There is also some evidence that neuropeptides may regulate egg laying as mutations in *egl-3*, which encodes a proprotein convertase required for neuropeptide processing, and *egl-21*, which encodes a carboxypeptidase required for neuropeptide synthesis, both impair egg laying (Trent et al., 1983, Jacob and Kaplan, 2003). Acetylcholine signalling through the metabotropic G protein-coupled acetylcholine receptor, GAR-2 also inhibits egg laying (Bany et al., 2003). Fluensulfone could interfere with these neurotransmitter and neuromodulator signalling pathways to reduce egg laying. Alternatively, fluensulfone may reduce egg laying through impairment of the contraction of the vulval muscles. The inhibition of feeding by fluensulfone could also bring about a reduction in egg laying, as it has been observed that adult hermaphrodites can enter a reproductive diapause under starvation conditions during which egg laying is substantially reduced (Angelo and Van Gilst, 2009). Ethanol has been found to inhibit *C. elegans* egg laying (Morgan and Sedensky, 1995, Davies et al., 2003). Mutagenesis experiments have revealed that the inhibition of egg laying by ethanol is mediated, at least in part, by activation of SLO-1 (Davies et al., 2003), a voltage- and calcium-activated potassium channel (Wang et al., 2001). Activation of SLO-1 is therefore another means by which fluensulfone could impair egg laying.

1 mM fluensulfone also reduced egg hatching, which is consistent with the reduction in hatching of *M. javanica* in the presence of fluensulfone (Oka et al., 2009). This inhibitory activity does however occur at 1 mM in *C. elegans* as compared to 15.2 µM in *M. javanica*. In addition, fluensulfone also reduced the viability of eggs *in utero*. Fluensulfone may also impair the viability of PPN eggs *in utero*, which could contribute to its crop protective capabilities. It is probable that the inhibition of hatching and reduction of egg viability are due to the same mechanisms.

Exposure to sodium azide has been found to cause cell cycle arrest during embryo development (Hajeri et al., 2010). Sodium azide inhibits the electron transport chain and acts via cytochrome C (complex IV) (Duncan and Mackler, 1966). It has been postulated that this inhibition of mitochondrial respiration halts cell cycle progression due to the high energy requirements of this process (Padilla and Ladage, 2012). Fluensulfone could prevent hatching and reduce embryo viability through inhibition of mitochondrial respiration or other metabolic processes. The microtubule inhibitor benomyl has also been found to cause embryonic arrest (Encalada et al., 2005) and interference with components of the cytoskeleton is another means through which fluensulfone could impair embryo viability.

3.3.3 Fluensulfone has complex, context- and concentration-dependent effects on *C. elegans* locomotion

Fluensulfone had several effects on locomotion that varied with concentration, and the concentration- and time-dependence of these effects differed in the presence and absence of a food source and differed between a solid substrate and liquid media. In M9 buffer, 1 mM fluensulfone caused near-complete inhibition of thrashing within 1 hour, whilst exposure to 100 and 300 µM for 3 hours slightly reduced the rate of thrashing. On agar in the absence of food, 1 mM fluensulfone completely inhibited body bends within 20 minutes, whilst 500 µM caused a slight reduction in body bends over the course of 2 hours. On agar in the presence of food, 500 µM and 1 mM fluensulfone transiently excited locomotion, with an increase in body bends at 1-2 hours and locomotion returning to near control-levels at 5 hours.

Whilst fluensulfone generally acts to inhibit *C. elegans* locomotion, it is interesting that movement is elevated on initial exposure in the presence of food. This elevated

locomotion was concurrent with an inhibition of pharyngeal pumping and an increase in food leaving behaviour. There are several possible reasons for this increase in locomotion. Fluensulfone could for example, be perceived as a noxious stimulus by *C. elegans* and so trigger an aversive response, increasing movement velocity and avoidance behaviour. Acute avoidance assays demonstrated however, that fluensulfone is not aversive to *C. elegans*. It is however possible that fluensulfone could be aversive to *C. elegans* with a longer period of exposure. As with the inhibition of development and egg laying that occurs in the presence of fluensulfone, this increase in locomotion and food leaving could result from the concomitant inhibition of pharyngeal pumping. As feeding is inhibited, the worm adopts a behavioural state associated with low food abundance. When on a food source that is deemed to be of poor quality, or when food is entirely absent a worm will exhibit more roaming behaviour, which is characterised by fast, linear movement (Shtonda and Avery, 2006). Roaming enhances the probability of locating a food source of higher quality and enhances the probability of leaving a food patch. In contrast, when on a food source that is deemed to be of high quality; that is a food source that is not overpopulated and lacks noxious compounds or pathogenic organisms, a worm will typically show high levels of dwelling behaviour (Shtonda and Avery, 2006). Dwelling behaviour is characterised by low levels of locomotion and frequent reversals to permit efficient feeding and to ensure proximity to a recognised food source is maintained (Shtonda and Avery, 2006). Indeed, it has been reported that worms possessing mutations that make feeding more difficult, such as *eat-2*, which encodes an acetylcholine-gated ion channel subunit that is crucial in the upregulation of pharyngeal pumping (Raizen et al., 1995), exhibit increased roaming behaviour on mediocre quality food sources (Avery and Shtonda, 2003, Rankin, 2006). These effects could also be seen if fluensulfone interferes with the ability of *C. elegans* to sense a food source.

This increase in locomotion would not occur in the absence of food as locomotion is already elevated. In the absence of food however, 1 mM fluensulfone rapidly inhibits both locomotion and pharyngeal pumping. As pharyngeal pumping is inhibited by fluensulfone both on and off food yet locomotion is only stimulated in the presence of food, it is less likely that the increased locomotion on food results from inhibition of pumping. It is possible that worms respond to fluensulfone differently in the presence and absence of food due differences in the physiological state of the worm. For example, it has been reported that the presence of food results in an enhanced avoidance

response to repellents, resulting from increased dopamine signalling causing elevated responsiveness of the ASH nociceptive neurons (Ezcurra et al., 2011). It is possible that the presence of food heightens the sensitivity of *C. elegans* to the presence of fluensulfone, resulting in an avoidance response characterised by heightened locomotion and food leaving. This avoidance response could temporarily overcome the direct reduction of locomotion in the presence of fluensulfone.

It is interesting that *egl-3* mutants do not exhibit the elevated locomotion on food in the presence of fluensulfone that is seen in N2. *egl-3* encodes proprotein convertase 2, and mutants cannot generate 74 of the 75 functional neuropeptides that are found in N2 (Berridge et al., 2005). It is possible that *egl-3* mutants do not exhibit increased locomotion in the presence of fluensulfone due to their already compromised physiology. This may mean they are less able to increase locomotion or that being compromised, they are more sensitive to the inhibitory effects of fluensulfone and thus an inhibition becomes manifest earlier than in N2. Alternatively, neuropeptide signalling may play a role in the upregulation of locomotion that occurs in the presence of fluensulfone. Research into *C. elegans* foraging and food leaving behaviour has shown that neuropeptide signalling can both enhance and reduce roaming and food leaving behaviour. For example, mutants defective in *flp-18*, which encodes an FMRFamide-related peptide, fail to exhibit dispersal behaviour following removal from food and continue to perform local area search behaviours (Cohen et al., 2009). In N2 *C. elegans*, FLP-18 peptides are released from the amphid interneurons AIY and this promotes dispersal and forward movement associated with the absence of food (Cohen et al., 2009). As *egl-3* mutants do not exhibit roaming in the presence of fluensulfone, this suggests that fluensulfone may increase food leaving and dispersal through inhibition of feeding. *npr-1* KO mutants however exhibit increased food leaving and roaming (Melo and Ruvkun, 2012). Despite this, the absence of neuropeptide signalling in *egl-3* mutants may prevent the neural signalling that promotes food leaving and roaming.

Despite the transient excitation that was observed in the presence of food, locomotion was ultimately inhibited in the presence and absence of food and in liquid. Comparison with aldicarb showed that the paralysis that results from treatment with 1 mM fluensulfone is non-spastic and therefore is not due to hyper-excitation of the body wall muscle (Kearn et al., 2014). This indicates that fluensulfone has an inhibitory effect on

the *C. elegans* neuromuscular system. Further evidence for an inhibitory effect on the neuromuscular system is the heightened and diminished susceptibility of the *unc-17* and *avr-14; avr-15; glc-1* mutants, respectively, to the action of fluensulfone (Kearn et al., 2014). Fluensulfone could inhibit *C. elegans* locomotion through direct interaction with the body wall muscle, through interference with neurotransmitter signalling pathways or through metabolic impairment.

3.3.4 Fluensulfone has both excitatory and inhibitory effects on *C. elegans* pharyngeal pumping

In the intact animal, 1 mM fluensulfone completely inhibits pharyngeal pumping in both the presence and absence of food, whilst both 300 and 500 µM cause a reduction in the rate of pharyngeal pumping. 100 µM fluensulfone causes a slight reduction in the rate of pharyngeal pumping off food but not on food, which indicates increased efficacy in the absence of food. A parsimonious explanation for this observation is that the rate of pharyngeal pumping is far lower off food than on food and thus inhibitory effects are more overt when food is absent.

In cut head preparations, 100 µM fluensulfone actually stimulates pharyngeal pumping, in contrast to the inhibition that occurs in the intact worm in the absence of food. 500 µM fluensulfone has a transient excitatory effect on pharyngeal pumping in cut head preparations, followed by a gross inhibition. This dual excitatory and inhibitory effect on pharyngeal pumping has not been seen with other anthelmintics or nematicides (Dent et al., 1997, Crisford et al., 2011). The inhibitory effect of 500 µM fluensulfone was characterised by a reduction in EPG waveform amplitude and weak, uncoordinated movement of the pharyngeal muscle. This implies impairment of the contraction-relaxation cycle of pharyngeal pumping. On the other hand, in the presence of 50 and 100 µM fluensulfone, pharyngeal pumping appeared normal. The excitatory effect is rapid and is rapidly reversible, which may indicate a neuromuscular target.

It is interesting that at 500 µM fluensulfone, a transient excitation precedes the complete inhibition of pharyngeal pumping. A simple explanation for this is that an insufficient concentration of fluensulfone reaches the cut head on initial application via perfusion and thus pumping is stimulated until a higher concentration has built up and an inhibition is seen. Alternatively, fluensulfone could have a biphasic effect, initially

increasing muscle excitability and then causing an inhibition. It is possible that fluensulfone increases the excitability of the pharyngeal muscle at lower concentrations, resulting in an increased rate of activity of the myogenic pharyngeal muscle. The excitatory and inhibitory action may be due to a depolarising block (Raghavendra, 2002). Lower concentrations of fluensulfone may weakly depolarise the pharyngeal muscle and increase excitability, whilst higher concentrations cause greater depolarisation, rendering the muscle incapable of contraction. This explanation is consistent with the uncoordinated twitching that the pharyngeal muscle undergoes in the presence of 500 µM fluensulfone, as a depolarising block can cause muscular fasciculation (Raghavendra, 2002). A depolarising block could also explain the inhibition of egg laying in *C. elegans* that occurs in the presence of fluensulfone. Fluensulfone is unlikely to inhibit locomotion via this mechanism however, as a depolarising block would result in hypercontraction and a spastic paralysis of the body wall muscle, whereas fluensulfone causes a non-spastic paralysis.

The uncoordinated nature of the muscle motions in the presence of 500 µM fluensulfone could also be due to interaction with gap junctions, which are known to be crucial to the synchronisation of pharyngeal muscle contraction (Starich et al., 1996, Li et al., 1997, Li et al., 2003). This could be tested via fluorescent dyes such as carboxyfluorescein, which has been used to demonstrate the uncoupling effects of ethanol on the pharyngeal pumping contraction-relaxation cycle (Dillon et al., 2013). The stimulation of pumping by lower concentrations of fluensulfone may also be due to weak agonistic activity at 5-HT, acetylcholine or neuropeptide receptors, as 5-HT, cholinergic agonists and some neuropeptides have all been shown to stimulate pharyngeal pumping (Horvitz et al., 1982, Avery and Horvitz, 1990, Papaioannou et al., 2008). Intracellular recordings from the pharyngeal muscle are needed to further investigate this phenomenon.

The effects of fluensulfone on cut heads as measured by the EPG technique are similar to the effects of ethanol, which stimulates pumping at 50 mM, whilst 300 mM elicited an initial increase in pump rate that shifts to a gross inhibition (Dillon et al., 2013). 300 mM ethanol also resulted in the reduction and distortion of the EPG waveform and the apparent incoordination of the pharyngeal muscle contraction cycle. A *slo-1* mutant did not exhibit increased pumping in the presence of 50 mM ethanol yet pumping was still inhibited at higher concentrations. This suggests that the stimulation of pumping by low concentrations of ethanol may be due to interaction with SLO-1. 50 mM ethanol was

also found to stimulate pharyngeal pumping in intact worms, yet no such stimulatory effect was observed with fluensulfone in intact animals. In spite of this difference, fluensulfone could also interact with SLO-1 to bring increase pump rate in cut heads and this is an avenue that would be interesting to explore.

It is unclear why cut heads would respond to fluensulfone differently to intact animals. It has been reported that cut heads can have different responses to drugs (Dent et al., 2000). For example, ivermectin completely inhibits pharyngeal pumping and locomotion in *avr-15* null mutants. If the head is cut away from an *avr-15* mutant in the presence of ivermectin however, pharyngeal pumping resumes (Dent et al., 2000). This is because ivermectin also targets AVR-14 and GLC-1, which are expressed outside the pharynx. This observation suggests an extra-pharyngeal humoral or neuronal signal that somehow prevents an excitation of pharyngeal pumping occurring in an intact animal. This could be the case for fluensulfone as well.

The effects of fluensulfone on 5-HT-stimulated pharyngeal pumping were also investigated. In the intact worm, 1 mM fluensulfone completely inhibited pumping stimulated by 10 mM 5-HT. In cut heads, 500 µM fluensulfone did not reduce the rate of pharyngeal pumping in the presence of 500 nM 5-HT yet the amplitude of the EPG waveform was reduced and the movements of the pharyngeal muscle were weaker. It is likely that a longer incubation in fluensulfone would have elicited an inhibition of 5-HT-stimulated pumping in cut heads, as experiments in which pumping was visually scored have shown that 1 hour exposure to 300 µM and 1 mM fluensulfone reduces the rate of pharyngeal pumping in the presence of 300 nM 5-HT (Kearn et al., 2014).

The inhibition of 5-HT stimulated pumping in the intact worm was rapid, occurring over a similar time course to that seen in the presence of food, with a >50% reduction in pump rate within 1 minute and complete inhibition at 20 minutes. This indicates that inhibition of 5-HT-stimulated pumping occurs more slowly in cut heads. It would be interesting to incubate cut heads in fluensulfone for longer and to see how this affects pharyngeal pumping via the EPG technique.

3.3.5 Indications as to the mode of action of fluensulfone

Analysis of *unc-17* and *avr-14; avr-15; glc-1* mutants indicates that fluensulfone has a mode of action that is distinct from cholinesterase inhibitors such as aldicarb and glutamate-gated chloride activators, such as ivermectin (Dent et al., 2000, Rich et al., 2004, Kearn et al., 2014). The *unc-17* mutant, which has diminished acetylcholine release and thus reduced excitatory neurotransmission, was found to be more susceptible to fluensulfone. In contrast, worms with mutations in the glutamate-gated chloride channel subunit encoding genes *avr-14*, *avr-15* and *glc-1* were slightly less susceptible to fluensulfone.

The reduction in susceptibility of *avr-14; avr-15; glc-1* worms to the effects of fluensulfone on thrashing in intact worms and pharyngeal pumping in cut heads may indicate that fluensulfone does interact with glutamate-gated chloride channels. *avr-14; avr-15; glc-1* is tolerant of ivermectin at concentrations >1000-fold greater than N2 (Dent et al., 2000). It is therefore unlikely that the slight reduction of susceptibility of *avr-14; avr-15; glc-1* to fluensulfone suggests that glutamate-gated chloride channels are a major target of fluensulfone. Furthermore, the mutant screen that was conducted showed no change in susceptibility relative to N2, and intact *avr-14; avr-15; glc-1* worms were as susceptible as N2 to the inhibition of pharyngeal pumping by fluensulfone. The *eat-4* mutant, which has diminished glutamate release, showed a similar reduction in susceptibility to the inhibition of pumping in cut heads. This indicates that the slight reduction of susceptibility of *avr-14; avr-15; glc-1* to fluensulfone in some assays may be due to reduced glutamate release resulting in reduced inhibitory tone of the motor nervous system. In *avr-14; avr-15; glc-1*, the motor nervous system has heightened excitability and thus is less susceptible to the inhibitory activity of fluensulfone. Indeed, it has been reported that mutations in pathways not containing the molecular target can alter susceptibility to anthelmintics (Holden-Dye et al., 2012). Reduced excitatory neurotransmission in *unc-17* mutants could also account for the greater susceptibility of this strain to fluensulfone relative to N2.

The effects of fluensulfone on *C. elegans* behaviour are qualitatively similar to those of ethanol, as both have stimulatory and inhibitory effects on pharyngeal pumping and both inhibit locomotion and egg laying (Davies et al., 2003). Ethanol is known to

activate the voltage- and calcium- activated potassium channel SLO-1 and this is responsible for some of these effects (Davies et al., 2003, Dillon et al., 2013). *slo-1* mutants are resistant to the effects of ethanol on locomotion but were not found to respond to fluensulfone treatment differently to wild-type worms. This suggests that SLO-1 does not mediate the nematicidal activity of fluensulfone. Fluensulfone may however still interact with SLO-1 to stimulate pumping in cut heads.

Fluensulfone has a broad range of effects on *C. elegans* behaviour, including effects on pharyngeal pumping, locomotion and egg laying. Some of the effects of fluensulfone, in particular the effects on cut head preparations, are rapidly reversible. These observations may indicate a neuromuscular target at these concentrations in *C. elegans*. The reverse genetic screen did not however reveal any profound alterations in susceptibility in mutants deficient in several neurotransmitter pathways. Likewise, EMS mutagenesis did not generate any strains with profound resistance to 1 mM fluensulfone. This may indicate that at these concentrations, fluensulfone interacts with multiple targets. If fluensulfone does interact with multiple targets to achieve its effects, then multiple mutations must occur for a mutagenesis to generate a strain with high-level resistance. Larger forward and reverse genetic screens are necessary to gain an insight into the target(s) of fluensulfone. There are also several neurotransmitter pathways that were unexplored in the reverse genetic screen that was conducted, for example the GABAergic system. Subsequent reverse genetic screens would yield more information through looking at multiple concentrations of fluensulfone and through analysing effects on several different behaviours.

Whilst many of the observations made suggest a neurobiological target for fluensulfone, the inhibition of egg hatching and development by fluensulfone may indicate effects on metabolism and other physiological processes. Other compounds that have been found to inhibit egg hatching and development are known to act as metabolic toxins or to impair cytoskeletal processes (Yamamuro et al., 2011, Hajeri et al., 2010).

3.3.6 Summary

In this chapter, fluensulfone was found to be nematicidal towards *C. elegans*, but only at concentrations substantially higher than those effective against PPNs. At these concentrations, fluensulfone acutely affected several *C. elegans* behaviours, including

pharyngeal pumping, locomotion and egg laying. These effects were dependent upon the context in which the measurements were conducted and are indicative of interaction with neural targets. Fluensulfone also affected *C. elegans* development and egg viability, which may result from interaction with non-neural targets. Forward and reverse genetics failed to identify a target and this suggests, along with the complex action of fluensulfone, that it may be interacting with multiple targets at these concentrations.

The next chapter will focus upon stylet behaviour in a PPN, to allow validation of the effects of fluensulfone on *C. elegans* and how this compares to its effects on a true target organism. Fluensulfone has multiple actions on *C. elegans* pharyngeal pumping and this indicates that it may also influence the behaviour of the analogous stylet.

Chapter 4: A neuropharmacological investigation of stylet behaviour in *G. pallida* and the effects of fluensulfone on this system

4.1 Introduction

The complex action of fluensulfone on *C. elegans*, as exemplified by its effects on the pharyngeal system (see chapter 3) indicated that it may have effects on a range of comparable behaviours in PPNs. Whilst *C. elegans* has been proposed as a model for PPNs and used to further our understanding of other nematodes (Burglin et al., 1998, Costa et al., 2007), the effects of fluensulfone on PPN behaviour needed to be understood, as a means of validating the observations made in *C. elegans* and their relevance to its activity in the field. The relevance of the observations in chapter 3 also need to be addressed, given the high concentration required to induce these effects. Therefore, using the effects of fluensulfone on *C. elegans* as a guide, experiments were performed with a PPN to determine and measure the effects of fluensulfone on a tractable parasitic nematode behaviour. The infective J2 is the stage against which fluensulfone is believed to act predominately to reduce crop damage (see section 1.10). Here, the potato cyst nematode *Globodera pallida* (see sections 1.4 and 1.6), a major pest that is prevalent throughout Europe and has more recently been found in the U.S.A (O'Dell and Hoffman, 2006), was used as a comparison with *C. elegans*. All experiments were conducted on J2 juveniles, as this is the only free-living stage of the *G. pallida* life cycle, with the other stages only found *in planta*.

G. pallida and the majority of PPNs, possess a stylet, a feeding structure that is common to the vast majority of PPNs, which is crucial to the parasitic life cycle (see section 1.5) (Baldwin et al., 2004b). Stylet behaviour is characterised by the movement of the needle-like structure out of the mouth of the worm and its subsequent retraction, known as stylet thrusting. Stylet thrusting is required for the hatching of juvenile PPNs from the egg, for penetration and invasion of the host's tissues and for feeding from the host. Stylet behaviour can be studied as a direct comparison with *C. elegans* pharyngeal pumping. As a behaviour, it presents a potential target for chemical control of PPNs (see section 1.5 & 1.8.6).

The regulatory mechanisms and the neuropharmacology underpinning control of stylet activity are poorly understood (see section 1.8.7). Several pharmacological studies have indicated the involvement of serotonergic signalling in upregulating stylet thrusting behaviour (McClure and von Mende, 1987, Robertson et al., 1999, Masler, 2008) (see section 1.8.7). The mechanisms underpinning PPN movement are also poorly

understood, although it is thought that these mechanisms are likely to be conserved throughout the nematode phylum, as studies with free-living and animal parasitic nematodes have indicated a role of acetylcholine and GABA signalling in the control of nematode movement (see section 1.8) (Martin et al., 1991, Gjorgjieva et al., 2014). Pharmacological studies have also indicated that glutamate, biogenic amines and neuropeptides are also likely to modulate PPN movement (Masler, 2007, Masler, 2008, Masler et al., 2012) (see section 1.8).

Given the importance of 5-HT in increasing pharyngeal pumping and isthmus peristalsis in *C. elegans*, this suggests evolutionary similarities that extend to the neurochemical control of stylet and pharyngeal behaviour in PPNs and *C. elegans*, respectively. Given the importance of the stylet in the parasitic life cycle, achieving a greater understanding of the regulatory mechanisms behind this behaviour could provide potential targets for the control of PPNs and further its use as a platform for drug studies in PPNs. Furthermore, investigation of the regulatory mechanisms controlling stylet activity are required to provide insight into the effects of fluensulfone on this behaviour. Pharmacological studies on the stylet also provide ideas and tools for scrutinising drug effects on this system.

Here, the well understood neuropharmacology of *C. elegans* pharyngeal pumping was used to inform investigation of stylet activity, In the *C. elegans* pharyngeal system, 5-HT elicits an increase in pharyngeal pumping via increased acetylcholine and glutamate release from the MC and M3 pharyngeal neurons, respectively (McKay et al., 2004, Song and Avery, 2012) (see section 1.15). Pharmacological studies have also found that the biogenic amines octopamine and tyramine inhibit 5-HT-stimulated pharyngeal pumping and it has been suggested that these neuromodulators could act as physiological antagonists of 5-HT and negative regulators of pharyngeal pumping (Horvitz et al., 1982, Alkema et al., 2005).

In the experiments described in this chapter, stylet thrusting assays were conducted in HEPES-buffered (pH 7.4) ddH_2O and the effects of drugs on stylet activity were visually scored. In addition, changes in body posture or movement were recorded. In the absence of any drugs, J2 *G. pallida* rarely exhibited any stylet thrusting. In liquid, *G. pallida* J2s move in an uncoordinated fashion via "swaying" motions that lack directionality or consistency. J2 *G. pallida* do not adopt a set posture in the absence of

drugs. Therefore, it is difficult to accurately quantify *G. pallida* J2 movement in liquid by visual inspection alone.

The aim of this chapter was to pharmacologically investigate *G. pallida* stylet thrusting behaviour, via exogenously applied neuroactive compounds, using the *C. elegans* pharynx as a guide to build up a putative model of the regulatory pathways controlling stylet activity. This pharmacological approach provided a template, which was then used to interrogate the effects of fluensulfone on stylet behaviour to gain insight into the potential pathways affected by fluensulfone.

4.2 Results

4.2.1 An investigation into the neuropharmacology of stylet behaviour – A potential platform for drug studies in PPNs

It has been shown in several species of PPN, including *M. incognita*, *H. glycines* and *H. schachtii*, that 5-HT stimulates stylet activity (McClure and von Mende, 1987, Masler, 2007). A concentration-response was therefore conducted for 5-HT on stylet thrusting. For all stylet thrusting assays, J2 *G. pallida* were soaked in petri dishes in drug solutions. In a series of experiments, J2 *G. pallida* were incubated in 5-HT, tryptamine, fluoxetine and imipramine and stylet thrusting was scored at intervals throughout 1 hour. When investigating with these compounds the number of worms that showed a stylet response as a percentage of the total number was scored, as was the number of stylet thrusts made per worm.

5-HT was found to stimulate stylet activity in a concentration-dependent manner (Figure 4.1A). In the absence of any drug or vehicle, no stylet thrusting was observed. 5 mM, 10 mM and 20 mM 5-HT all stimulated stylet thrusting over the course of 1 hour, with 20 mM achieving the highest rate of thrusting at 74.1 ± 12.6 stylet thrusts per minute. Some stylet thrusting was observed in the presence of 500 µM, 1 mM and 2 mM 5-HT, although the rate of activity was not significantly different from the control as a large percentage of the worms tested did not exhibit any stylet activity (Figure 4.2A). When stylet thrusting was initiated in response to 5-HT, it does so within 10 minutes and this response was maintained over 1 hour. The rate of stylet thrusting increased with time for 5 mM and 10 mM 5-HT, although this had plateaued out at 60 minutes, with a mean maximal rate of around 70 stylet thrusts per minute. Some worms were however seen to stylet thrust up to 115 times per minute. At all concentrations of 5-HT, there were some J2s that showed no stylet activity and appeared to be unaffected by 5-HT treatment, suggesting a distinct subset of unresponsive worms (see section 4.2.3).

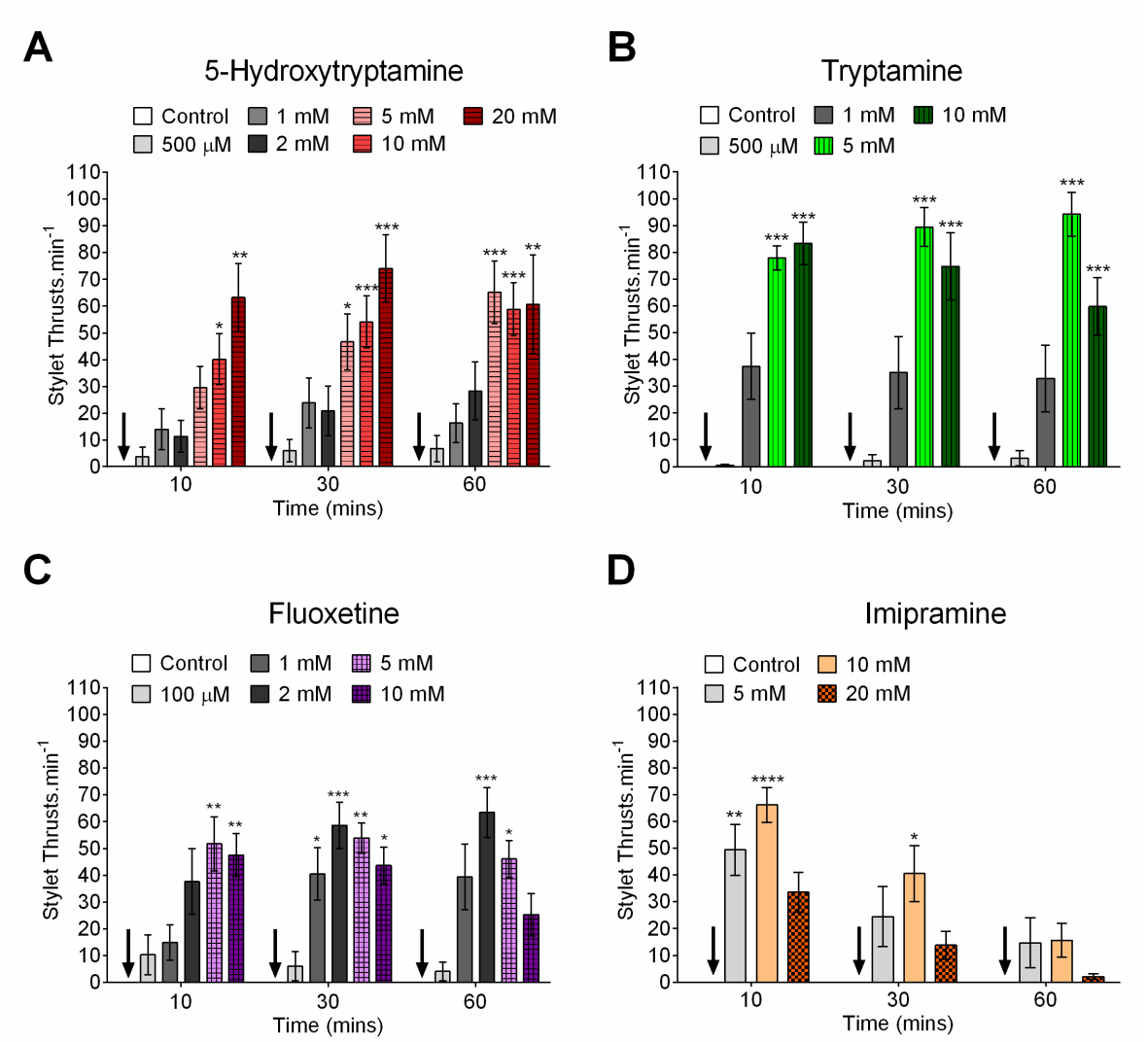

Figure 4.1. 5-HT, tryptamine and the serotonin re-uptake inhibitors fluoxetine and imipramine stimulate *G. pallida* stylet activity. 5-HT (**A**), tryptamine (**B**), fluoxetine (**C**) and imipramine (**D**) were tested for stimulatory effects on *G. pallida* stylet behaviour. For each concentration of each drug ≥9 worms were transferred to the drug solution and stylet thrusting was visually scored up to 1hr. For 5-HT the data shown are collated from 2 separate experiments (n≥9 worms, mean ± s.e mean, two-way ANOVA with Bonferroni post-hoc tests, P<0.0001 for A, B, C, D). Arrows on the graphs indicate where no stylet activity was observed.

A concentration-response was also conducted for the effects of the monoamine tryptamine, as it has been shown to act as a potent agonist at *C. elegans* 5-HT receptors and for other animal species (Van Oekelen et al., 2002, Hobson et al., 2003). Tryptamine was also found to induce stylet thrusting in a concentration-dependent manner (Figure 4.1B). Indeed, tryptamine was a more efficacious stimulator of stylet activity, with a mean rate of 94.2 ± 8.2 stylet thrusts per minute observed in the presence of 5 mM tryptamine. It is interesting to note that at 10 mM, the highest dose tested, the rate of stylet thrusting began to decrease at 60 minutes. Stylet thrusting did

occur in the presence of 1 mM tryptamine however, this was not significantly different from the control due to the percentage of J2s that did not exhibit any stylet activity (Figure 4.2B).

These data confirm that exogenous application of 5-HT and other 5-HT receptor agonists stimulates stylet thrusting in *G. pallida*. To assess the potential role for endogenous release of 5-HT in the control of stylet thrusting, J2 *G. pallida* were also exposed to fluoxetine. Fluoxetine acts as a selective serotonin re-uptake inhibitor, blocking the activity of the serotonin transporter (SERT), and preventing the re-uptake of 5-HT. This results in increased extra-synaptic 5-HT levels (Tatsumi et al., 1997). Fluoxetine therefore provides evidence for a role for endogenous 5-HT in the control of stylet activity. Fluoxetine induced a dose-dependent increase in stylet thrusting behaviour, with 2 mM eliciting the highest rate of stylet activity at 63.4 ± 9.4 stylet thrusts per minute (Figure 4.1C). At 10 mM, the highest dose of fluoxetine tested, there was a decrease in the rate of stylet thrusting at 1 hour relative to 2 mM. Imipramine, a monoamine re-uptake inhibitor has the same net effect of increasing extrasynaptic 5-HT, also stimulated stylet thrusting. This stimulation was weaker than the other compounds tested and after the initial stimulation at 10 minutes the rate of stylet activity decreased to levels not significantly different from the control at 60 minutes (Figure 4.1D).

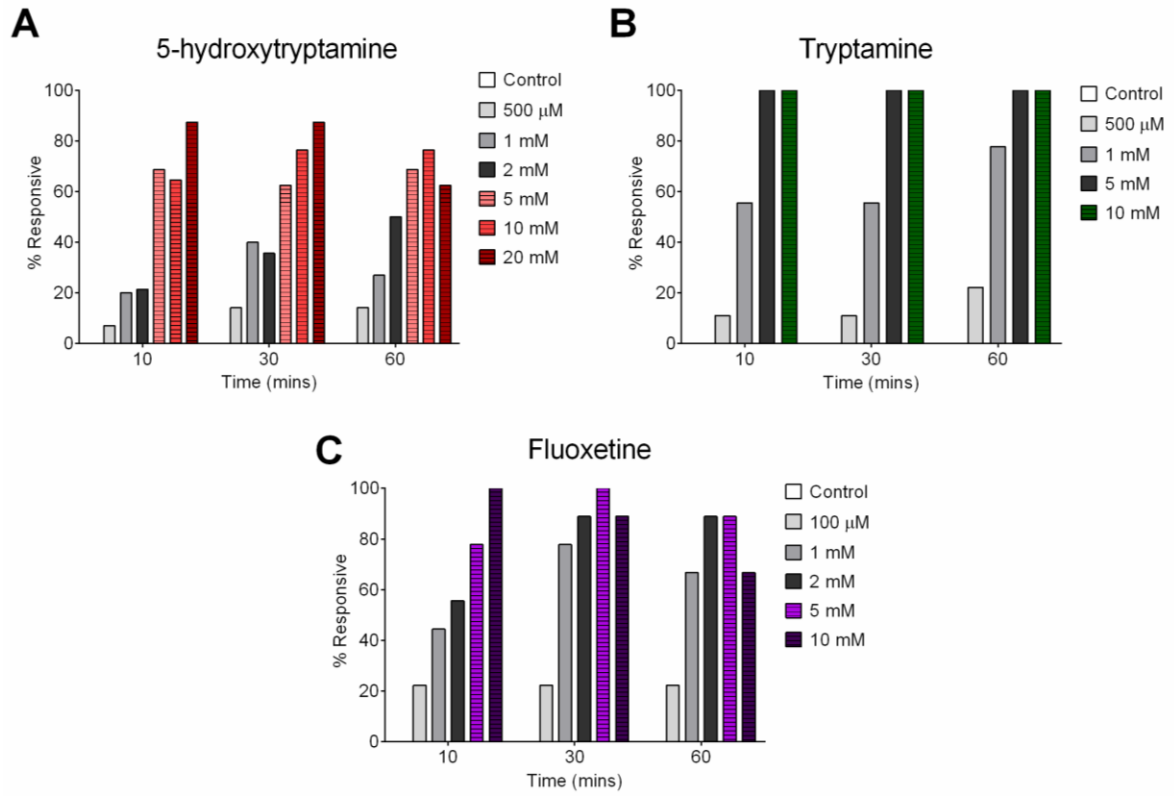

Figure 4.2. An analysis of the responsiveness of *G. pallida* J2s to serotonergic drug treatment. The percentage of J2s in the experiments shown in Figure **4.1** that showed ≥1 stylet thrust per minute in the presence of 5-HT (**A**), tryptamine (**B**) and fluoxetine (**C**) (n ≥9 worms per concentration shown, % responsive from one experiment).

4.2.2 5-HT and other compounds affecting the serotonergic system induce changes in movement and posture

It was also observed that when stylet thrusting was initiated in the presence of 5-HT, fluoxetine and tryptamine, the juveniles typically assumed a distinct posture, characterised by reduced or no movement, an S-shaped body posture and the kinking of the head to one side (Figure 4.3B). When in the absence of any drugs, J2s normally move freely and rarely adopt a set posture for extended periods (Figure 4.3A). Vigorous stylet thrusting was usually associated with this discrete posture (see section 4.2.3).

Figure 4.3. 5-HT induces an S-shaped body posture in J2 *G. pallida*. A) J2s in ddH_2O move freely and rarely adopt a posture for a sustained period. **B**) J2s in 10 mM 5-HT typically adopt a stereotypical S-shaped posture characterised by little/no movement and a kinking of the head (as indicated by arrow) that is associated with stylet thrusting.

4.2.3 A subset of *G. pallida* are unresponsive to pharmacological stimulation of stylet activity

When conducting these stylet thrusting assays, it became clear that a subset of the juveniles failed to display any stylet activity in the presence of the stimulatory drugs, even at the highest doses tested. For example, after 60 minutes in 20 mM 5-HT the mean rate of stylet activity was 60.6 ± 18.5 thrusts per minute. Of the 8 worms tested at this point 3 worms showed no stylet activity at all whereas the mean rate of stylet activity in the juveniles that responded was 97 ± 8.4 thrusts per minute. This occurred for all of the compounds tested, although it is noteworthy that 100% of the worms exposed to 5 mM and 10 mM tryptamine showed at least some stylet activity. The worms that failed to display stylet behaviour in the presence of the stimulatory compounds also failed to assume the S-shaped posture that is associated with stylet thrusting and continued moving freely, much like worms that were not treated with any drug. All of the results described here include unresponsive J2s.

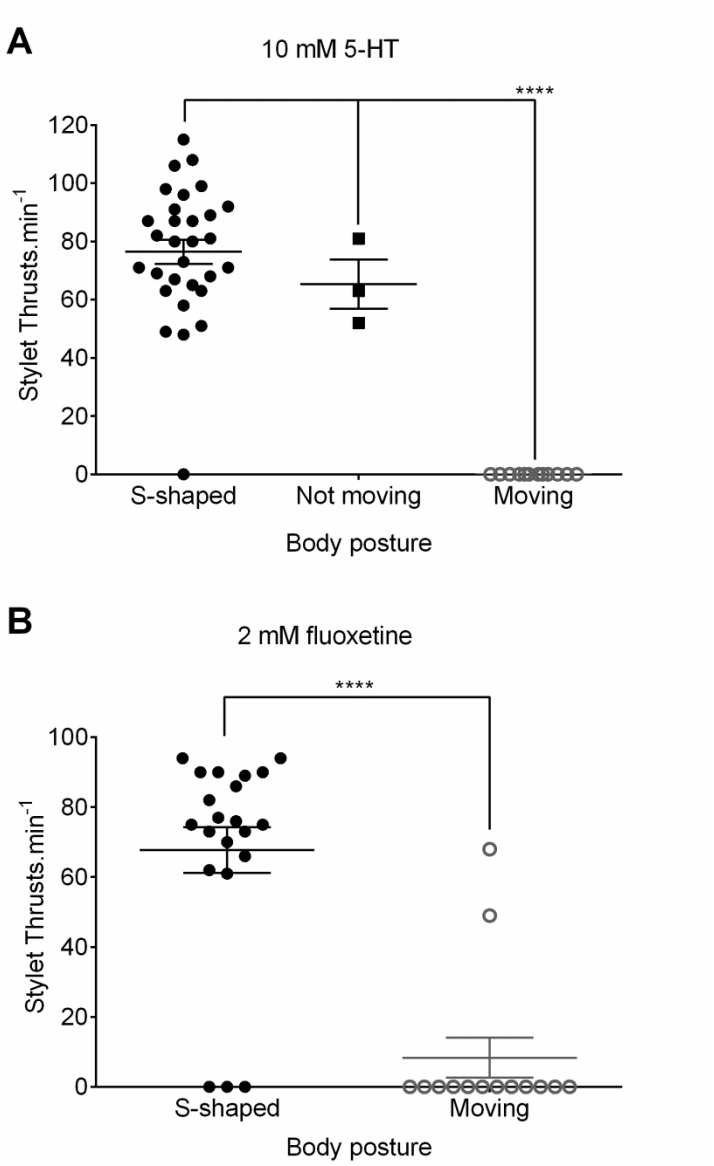

Figure 4.4. The rate of 5-HT- and fluoxetine-induced stylet thrusting is linked to associated changes in body posture. J2 *G. pallida* were exposed to 10 mM 5-HT (**A**) and 2 mM fluoxetine (**B**) for 30 minutes and stylet thrusting, body posture and movement was visually scored or noted. Posture and movement were scored as either moving, not moving or S-shaped. Of the worms tested, a subset failed to show stylet activity. Scatter plot analysis indicates that the rate of stylet thrusting was higher in worms that adopted an S-shaped posture or stopped moving, relative to worms that continued to move. The majority of worms that did not exhibit stylet thrusting continued moving (individual data points shown with mean ± s.e mean, A) n=45, one way-ANOVA with Tukey post-hoc tests, $P<0.0001$), B) n=35, student's t-test, $P<0.0001$). The data are pooled from multiple experiments.

Scatter plot analysis reveals distinct populations of responsive and unresponsive worms within experiments (Figure 4.4). In a series of experiments, J2 *G. pallida* were exposed to 10 mM 5-HT (Figure 4.4A) or 2 mM fluoxetine (Figure 4.4B) for 30 minutes and stylet thrusting was scored and body posture was noted. After 30 minutes in the presence of 10 mM 5-HT, the average rate of stylet activity in worms that exhibited an

S-shaped posture and reduced movement was 77 thrusts per minute, whereas stylet activity was 0 thrusts per minute in the worms that continued moving and did not adopt an S-shaped posture. Similar results were obtained after treatment with 2 mM fluoxetine. This observation was consistent throughout the experiments shown in this chapter, with a subset of worms in each experiment that showed little or no stylet activity and failed to exhibit any changes in movement or posture. This suggests that within each experimental population there is a sub-population that is insensitive to pharmacological manipulation of stylet thrusting.

4.2.4 Investigation of the regulation of stylet behaviour using the 5-HT antagonist methiothepin

To further investigate the role of 5-HT in the regulation of stylet behaviour, the 5-HT receptor antagonist methiothepin was utilised. Methiothepin has been demonstrated to have affinity towards several mammalian 5-HT receptors (Monachon et al., 1972) and more notably has been shown to have affinity for several *C. elegans* 5-HT receptors in the Nano molar (nM) range, including SER-7 and the 5-HT-gated chloride channel MOD-1. Methiothepin was also found to block 5-HT-mediated behaviours associated with these receptors (Hobson et al., 2003, Ranganathan et al., 2000).

J2 *G. pallida* were incubated in methiothepin for 1 hour and then transferred to 5-HT, tryptamine or fluoxetine, in the continued presence of methiothepin (Figure 4.5). The stimulation of stylet thrusting by 7 mM 5-HT was then compared after pre-treatment in a solution with vehicle (0.5% ethanol) alone or with increasing concentrations of methiothepin. In the presence of 5-HT alone, 55-85 stylet thrusts per minute were observed throughout the experiment. 100 µM methiothepin partially blocked the stimulation of stylet activity by 7 mM 5-HT, reducing stylet activity to 11-47 thrusts per minute (Figure 4.5A), whereas a more complete block was effected by 300 µM methiothepin, reducing stylet activity to 3-9 thrusts per minute (Figure 4.5B). The stimulatory effects of both 2 mM fluoxetine and 2 mM tryptamine were also strongly inhibited by pre-treatment with 300 µM methiothepin (Figure 4.5C, D). Methiothepin not only blocked the stimulation of stylet thrusting but also prevented the decrease in spontaneous movement and the induction of the S-shaped body posture that results from treatment with these drugs in the absence of methiothepin. For example, the average rate of stylet activity of vehicle-treated worms after 60 minutes in 7 mM 5-HT was 84

thrusts per minute, with 8 of 9 worms tested exhibiting an S-shaped posture and reduced movement. On the other hand, the average rate of stylet activity of methiothepin-treated worms was 9 thrusts per minute, and 8 of the 9 worms tested did not adopt an S-shaped posture and continued moving.

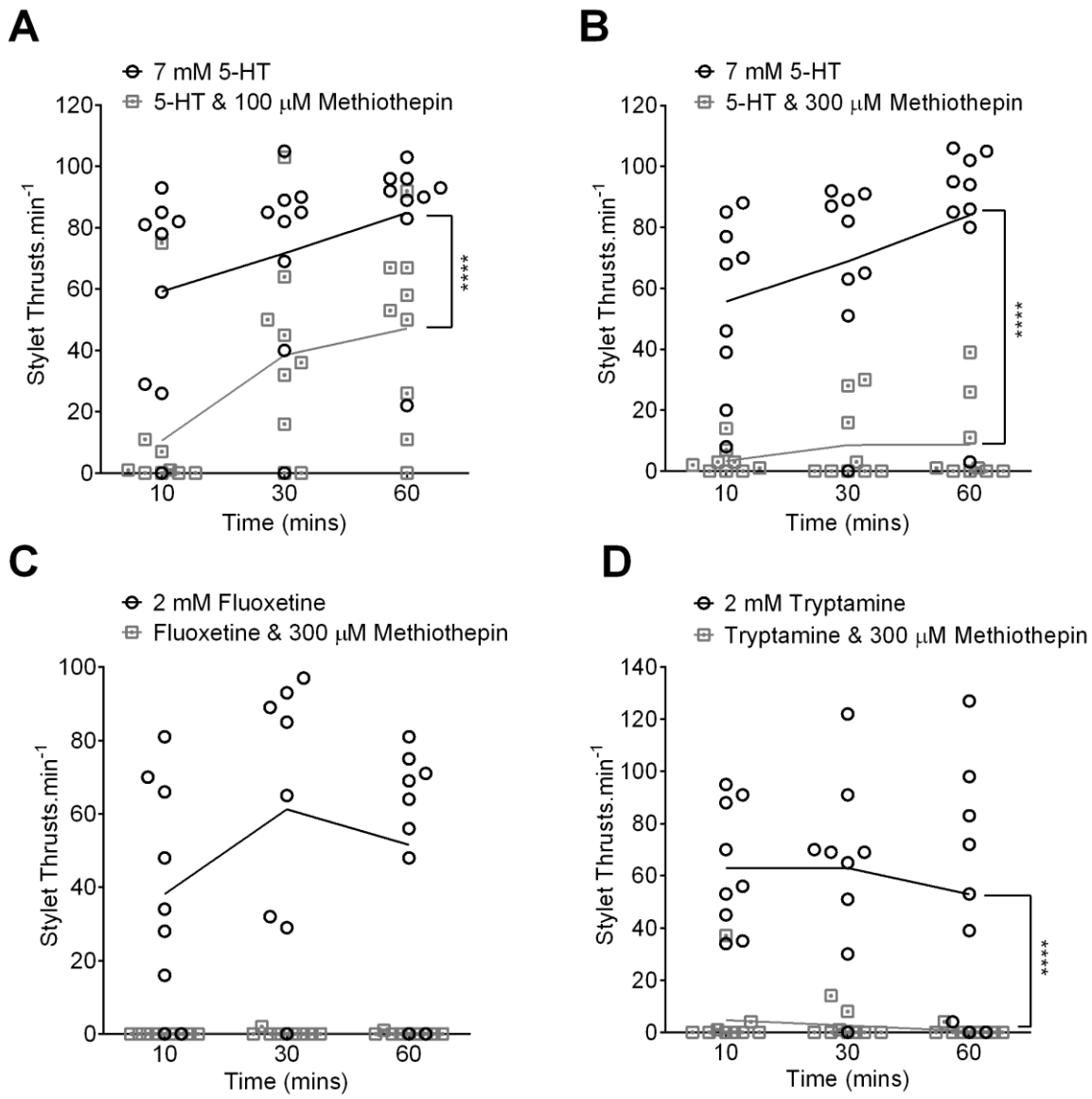

Figure 4.5. The serotonin receptor antagonist methiothepin blocks 5-HT-, tryptamine- and fluoxetine-stimulated stylet activity in *G. pallida*. J2s were incubated in either a control solution of 0.5% ethanol or methiothepin for 1 hour. The J2s incubated in the control solution were then transferred to 7 mM 5-HT (**A**, **B**), 2 mM fluoxetine (**C**) or 2 mM tryptamine (**D**). The J2s soaked in methiothepin were transferred to 7 mM 5-HT & 100 µM methiothepin (A), 7 mM 5-HT & 300 µM methiothepin (B), 2 mM fluoxetine and 300 µM methiothepin (C) or 2 mM tryptamine & 300 µM methiothepin (D). Stylet thrusting was counted at 10, 30 and 60 minutes (n=9 worms, individual data points shown with mean, two-way ANOVA with Bonferroni post-hoc tests, P<0.0001 for A, B, C and D).

This inhibition by methiothepin suggests the presence of 5-HT receptors in *G. pallida* and a potential target for chemical control of PPNs. The concentration required to induce this block is much higher than the 7.8 nM IC_{50} value that has been found for methiothepin binding to the *C. elegans* SER-7 receptor (Hobson et al., 2003). Whilst such binding assays do not necessarily reflect the concentrations that are effective in the intact organism, this does suggest that lower concentrations may also block 5-HT-stimulated activity. It is possible that methiothepin could have non-selective effects at such high doses via interactions with non-serotonergic targets that bring about this block. It is also possible that with shorter exposure times, due to the pharmacokinetic barrier of the cuticle, only very low concentrations of methiothepin are present at the receptor sites. To address this, in a separate experiment J2 *G. pallida* were soaked in several doses of methiothepin for 24 hours and then exposed to 5-HT or fluoxetine in the continued presence of methiothepin. With a longer incubation period, methiothepin was found to significantly reduce the rate of 5-HT-induced stylet thrusting by 58% at 1 µM and to reduce the rate of fluoxetine-induced stylet thrusting by 86% at 250 nM (Figure 4.6). 5-HT- and fluoxetine-stimulated stylet activity was completely blocked by 24 hour pre-treatment with 30 µM and 1 µM methiothepin, respectively. Pre-treatment with 0.25-300 µM methiothepin had no obvious effects on worm movement, posture or viability but blocked the 5-HT- and fluoxetine-induced reduction in movement and S-shaped posture that occurs in the absence of methiothepin (data not shown).

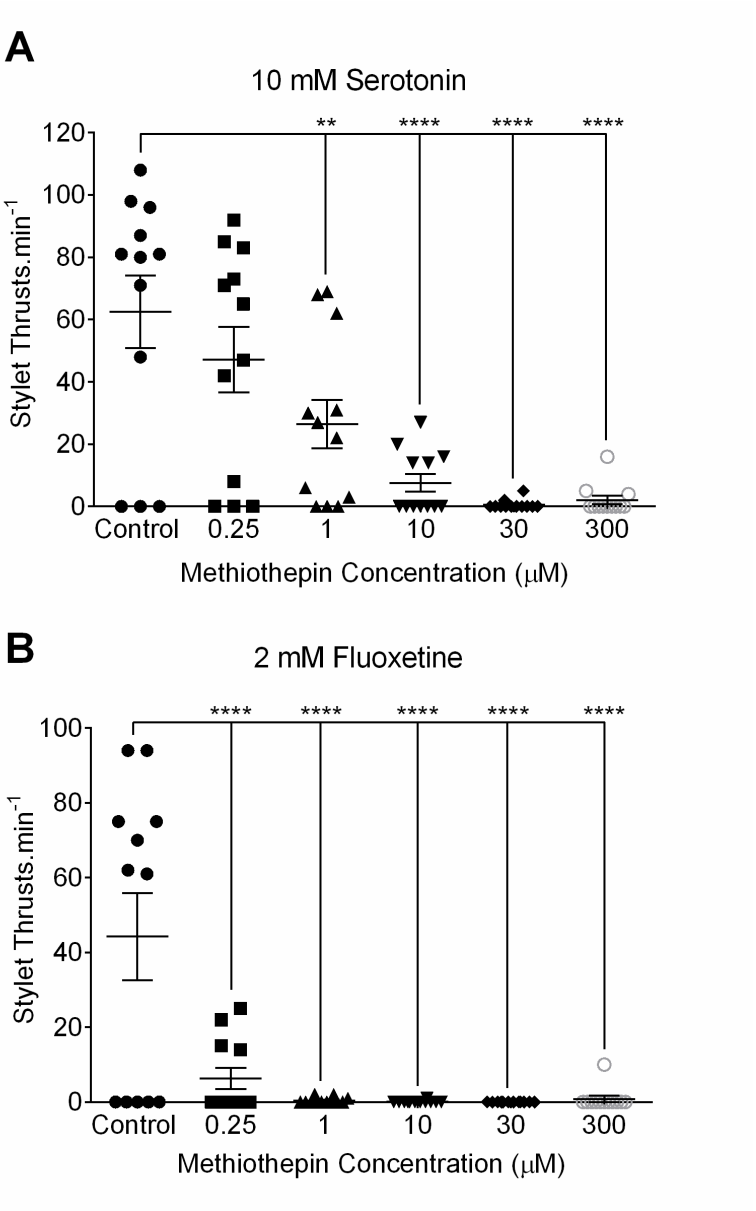

Figure 4.6. 24 hour pre-treatment with Nanomolar concentrations of methiothepin block 5-HT- and fluoxetine-stimulated stylet behaviour in *G. pallida*. J2s were soaked in either a control solution of 0.5% ethanol or methiothepin at different concentrations for 24 hrs. From these solutions the J2s were then transferred to either 10 mM 5-HT (**A**) or 2 mM fluoxetine (**B**) and the rate of stylet thrusting was counted after 30 minutes had elapsed (n=12, individual data points and mean ± s.e mean shown, one-way ANOVA with Bonferroni post-hoc tests, P<0.0001 for A and B).

4.2.5 Investigation of the role of 5-HT in stylet regulation with reserpine

The data presented here suggest that *G. pallida* possess 5-HT receptors and suggest a role for endogenous 5-HT in the regulation of stylet behaviour, as evidenced by stimulatory effects of the SSRI fluoxetine and the monoamine reuptake inhibitor imipramine (Figure 4.1). Fluoxetine inhibits the serotonin transporter and imipramine inhibits the monoamine transporter (Tatsumi et al., 1997). Therefore, fluoxetine and imipramine prevent the reuptake of 5-HT from the synaptic cleft and increase extrasynaptic 5-HT levels. If they are acting in this capacity, this suggests that *G. pallida* J2s possess 5-HT and that its signalling increases stylet activity. There is a wealth of evidence however, that shows the direct interaction of both fluoxetine and imipramine with several 5-HT receptor subtypes, in the capacity of agonists and antagonists, for both mammalian and *C. elegans* receptors (Ni and Miledi, 1997, Eisensamer et al., 2003, Kullyev et al., 2010, Dempsey et al., 2005). There is also further evidence implying the interaction of SSRIs with non-serotonergic targets (Kullyev et al., 2010). It is therefore possible that the stimulatory effects of both fluoxetine and imipramine on stylet behaviour are due to interactions with 5-HT receptors rather than inhibition of 5-HT re-uptake.

To investigate a physiological role for 5-HT in regulating stylet activity, the pharmacological agent reserpine was utilised. Reserpine acts by selectively blocking the activity of the vesicular monoamine transporter(s), thus preventing the loading of 5-HT and other monoamines into vesicles and thereby preventing serotonergic and monoaminergic neurotransmission (Erickson et al., 1992, Reckziegel et al., 2015). If fluoxetine is indeed acting as an SSRI to induce stylet thrusting, then reserpine treatment will block the stimulatory effect of fluoxetine but not exogenous 5-HT. The release of other monoamines, such as dopamine, octopamine and tyramine, should also be blocked by reserpine treatment. Dopamine, octopamine and tyramine however were tested for effects on stylet behaviour at concentrations up to 10 mM and had no effect on stylet behaviour (data not shown).

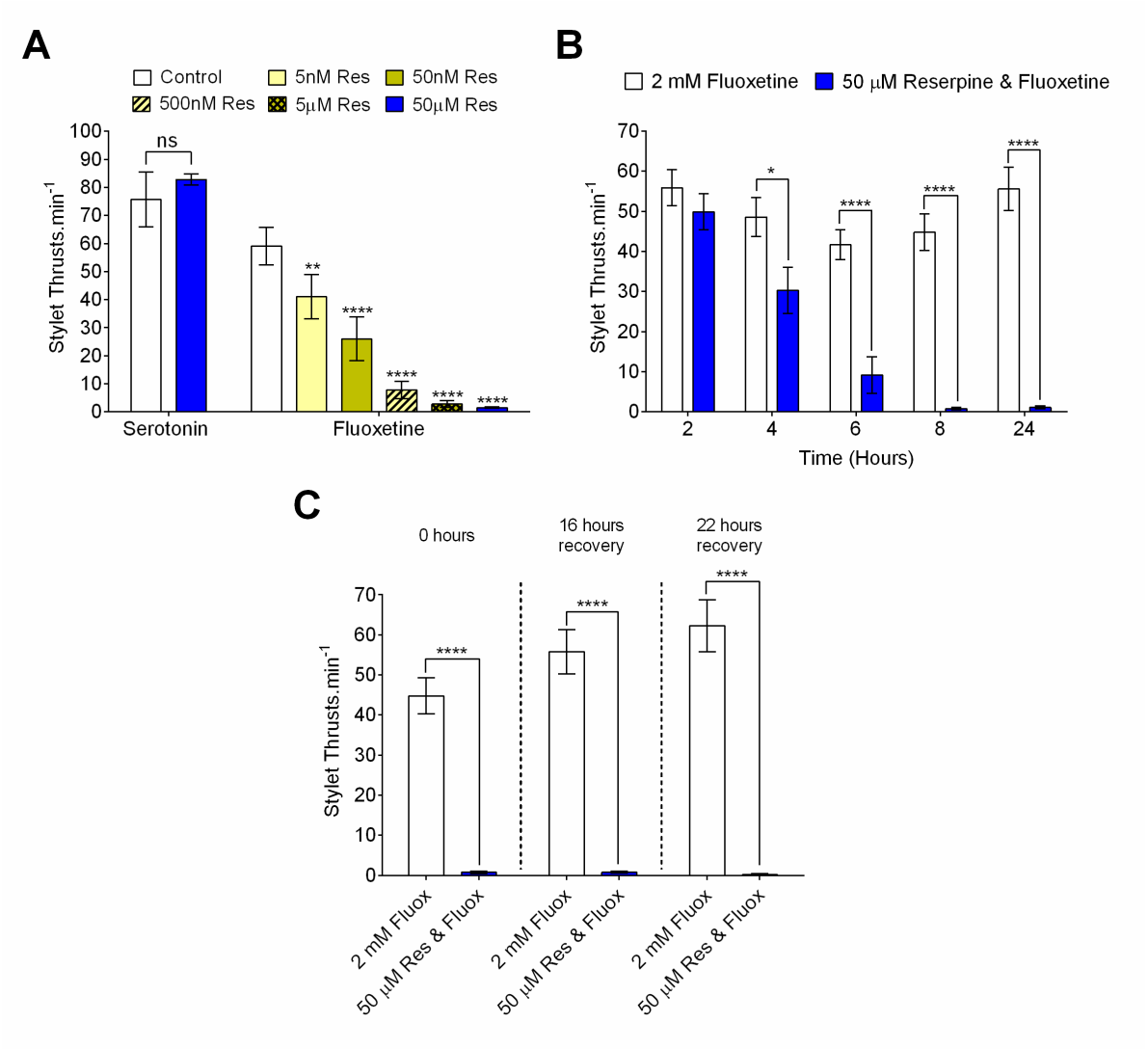

Figure 4.7. The vesicular monoamine transporter reserpine prevents the stimulatory effect of the serotonin reuptake inhibitor fluoxetine on stylet activity but not exogenous serotonin. A) To test for effects of reserpine (Res) on stylet thrusting stimulated by exogenous or endogenous 5-HT, J2 *G. pallida* were soaked in either reserpine or ddH_2O for 24 hours. Reserpine-treated J2s were then transferred to a solution of 10 mM 5-HT and reserpine or 2 mM fluoxetine (fluox) and reserpine and ddH_2O-treated J2s were transferred to either 5-HT or fluoxetine alone, where stylet thrusting was counted after 30 mins (n=10 worms, mean ± s.e mean, two-way ANOVA with Bonferroni post-hoc tests). **B**) To assess the time dependence of the inhibitory effect of reserpine, J2s were exposed to 50 µM reserpine or ddH_2O for 24 hours, with some being removed at 2, 4, 6, 8 and 24 hours and transferred into 2 mM fluoxetine where stylet activity was scored after 30 mins. Full inhibition occurred at 8 hrs (n=10, mean ± s.e mean, two-way ANOVA with Bonferroni post-hoc tests). **C**) The reversibility of the reserpine effect following protracted washing was assessed by taking J2s treated with 50 µM for 24 hrs and washing in ddH_2O. No recovery of the stylet response was seen after 22 hrs wash (n=10, mean ± s.e mean, two-way ANOVA with Bonferroni post-hoc tests, $P<0.0001$ for A, B and C). These experiments were conducted in collaboration with Francesca Keefe.

J2 *G. pallida* were soaked for 24 hours in different concentrations of reserpine and subsequently exposed to either 10 mM 5-HT or 2 mM fluoxetine for 30 mins, at which point stylet thrusting was counted (Figure 4.7). Whilst reserpine had no effect on the stimulation of stylet activity by 5-HT at any concentration, a concentration-dependent inhibition of fluoxetine-induced activity was seen (Figure 4.7A), with a significant reduction seen at concentrations as low as 5 nM reserpine. 5 and 50 µM reserpine induced a near-complete block of the stimulatory effect of fluoxetine. As with methiothepin, reserpine also blocked the S-shaped posture and reduced movement that is typically induced by fluoxetine exposure. Reserpine had no independent stimulatory effect on stylet activity (data not shown). In a separate experiment, *G. pallida* were soaked in 50 µM reserpine for differing lengths of time to assess the time course of reserpine activity. The reduction of fluoxetine-stimulated stylet activity was evident after 4 hours in reserpine and the full block was reached at 8 hours exposure (Figure 4.7B). Subsequent to 24 hours soak in 50 µM reserpine a sample of juveniles were removed and soaked in water to assess the potential for recovery of the stylet response (Figure 4.7C). Up to 22 hours post-reserpine treatment the juveniles were tested for fluoxetine-induced stylet thrusting yet no recovery of the response occurred, indicating a long-lasting block of serotonergic transmission by reserpine.

This confirms the activity of fluoxetine as an SSRI in *G. pallida* and that its stimulation of stylet behaviour is due to inhibition of 5-HT re-uptake, thus validating a role for 5-HT signalling in the control of stylet activity. This also provides evidence for the presence of the vesicular monoamine transporter in *G. pallida*. Indeed, an ortholog of *cat-1*, the *C. elegans* vesicular monoamine transporter, has been identified in the *G. pallida* genome (Cotton et al., 2014).

To further investigate the role of 5-HT signalling in stylet behaviour, the effects of 4-Chloro-DL-phenylalanine (4Cl) were studied. 4-Chloro-DL-phenylalanine inhibits tryptophan hydroxylase, the rate-limiting enzyme in the synthesis of 5-HT (Sze et al., 2000, Wang et al., 2002, Bao et al., 2010). If 4-Chloro-DL-phenylalanine inhibited fluoxetine-induced stylet activity then this would indicate the presence of the tryptophan hydroxylase enzyme in *G. pallida* and highlight another potential target for control of PPNs. A 24 hour soak with 4-Chloro-DL-phenylalanine reduced the rate of fluoxetine-induced stylet thrusting by 70% but had no effect on the rate of 5-HT-induced stylet thrusting (Figure 4.8).

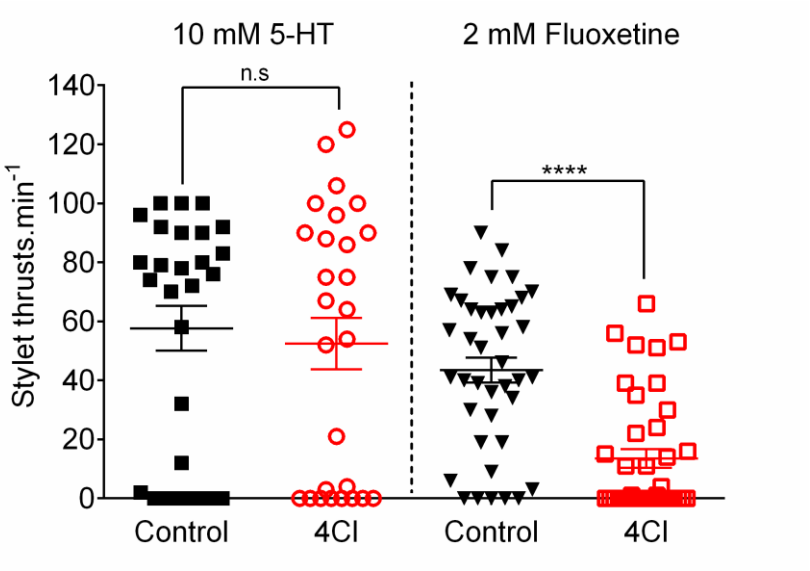

Figure 4.8. Pharmacological inhibition of tryptophan hydroxylase blocks the stimulatory effect of fluoxetine on stylet activity but not 5-HT. J2 *G. pallida* were soaked in 10 µM 4-Chloro-DL-phenylalanine methyl ester hydrochloride (4Cl) or ddH_2O for 24 hrs. 4Cl-treated J2s were transferred to either 10 mM 5-HT and 4Cl or 2 mM fluoxetine and 4Cl. Control J2s were transferred to 5-HT or fluoxetine alone. 4Cl treatment had no effect on 5-HT-mediated stimulation of stylet activity but reduced fluoxetine-mediated stimulation (5-HT n=27 worms, fluoxetine n=40 with data pooled from 2 separate experiments. Mean ± s.e mean shown, two-way ANOVA with Bonferroni post-hoc tests, $P<0.0001$). This experiment was conducted in collaboration with Anna Crisford.

4.2.6 Octopamine inhibits the effects of 5-HT on *G. pallida* behaviour

In addition to the investigation of the role of 5-HT in stylet behaviour, other compounds were also tested for effects on stylet thrusting. Other monoamines, such as octopamine, have been shown to have effects on pharyngeal pumping in *C. elegans* (Niacaris and Avery, 2003, Horvitz et al., 1982, Rex et al., 2004). As mentioned, 10 mM dopamine, tyramine and octopamine had no effect on stylet thrusting (data not shown). It has been suggested that octopamine functions as a physiological antagonist to 5-HT in *C. elegans* (see section 1.15) (Horvitz et al., 1982, Segalat et al., 1995, Niacaris and Avery, 2003). To determine whether octopamine acts antagonistically to 5-HT in *G. pallida*, juveniles were treated with 5 mM 5-HT and a mixture of 5-HT and 5 mM octopamine and stylet thrusting was scored (Figure 4.9). Octopamine completely and rapidly blocked 5-HT-induced stimulation of stylet thrusting and blocked the S-shaped body posture and reduction of locomotion elicited in the presence of 5-HT alone. This suggests that octopamine also functions as a physiological antagonist to 5-HT in *G. pallida*.

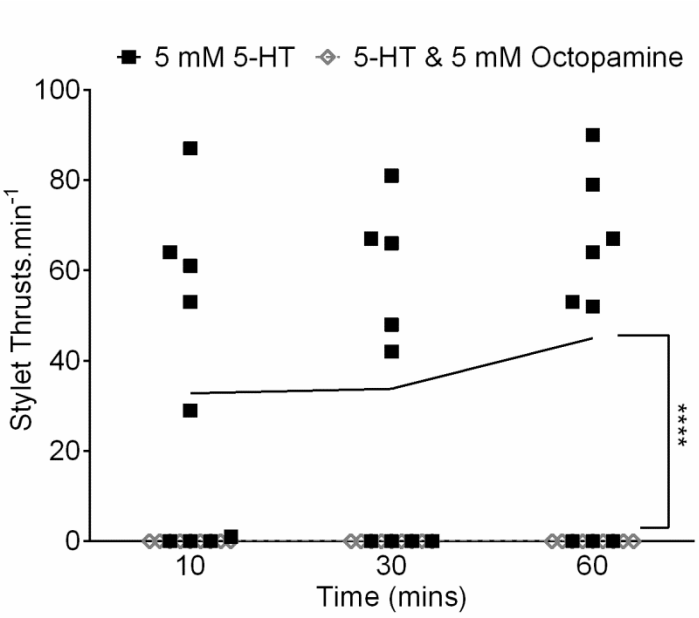

Figure 4.9. Octopamine completely blocks the stimulatory effects of 5-HT on stylet activity. J2 *G. pallida* were exposed to either 5 mM 5-HT alone or 5-HT and 5 mM octopamine and stylet thrusting was scored at 10, 30 and 60 minutes. Octopamine prevented stimulation of stylet thrusting by 5-HT (n=9 worms, individual data points and mean shown, two-way ANOVA with Tukey post-hoc tests, P<0.0001). J2s were also exposed to a control solution and octopamine alone although octopamine had no stimulatory effect on stylet thrusting (data not shown).

4.2.7 Investigation of the role of cholinergic signalling in stylet behaviour

Whilst research with *C. elegans* suggests that 5-HT is a key regulator of pharyngeal activity there is further evidence that cholinergic signalling via the MC motor neuron is important in the up-regulation of pumping, with acetylcholine released downstream of 5-HT (see section 1.15) (Raizen et al., 1995, McKay et al., 2004). It has also been shown that cholinergic agonists such as nicotine stimulate *C. elegans* pharyngeal pumping and that nicotinic receptor antagonists such as D-tubocurarine and mecamylamine inhibit stimulated pumping (Avery and Horvitz, 1990, Chiang et al., 2006, Ruiz-Lancheros et al., 2011). To investigate whether cholinergic signalling may be involved in stylet activation aldicarb was applied to J2 *G. pallida* and stylet thrusting was counted. Aldicarb is a cholinesterase inhibitor that prevents the degradation of endogenously-released extra-synaptic acetylcholine, thus leading to elevated acetylcholine at receptor sites (Opperman and Chang, 1991).

Aldicarb induced stylet activity (Figure 4.10), although the rates of stylet thrusting reached were lower than those achieved in the presence of 5-HT, tryptamine or

fluoxetine. In the presence of aldicarb the highest mean rate of stylet activity was 22.9 ± 3.8 thrusts per minute at 5 mM. After an initial stimulation at 10 minutes, the rate of stylet thrusting decreased with time in the presence of 10 and 20 mM aldicarb until no stylet thrusting occurred at 60 minutes (Figure 4.10). 10 and 20 mM aldicarb led to an eventual paralysis of the worms. Aldicarb treatment also resulted in the chronic protrusion of the stylet in some worms, during which the stylet did not retract, perhaps due to spastic paralysis of the stylet protractor muscles.

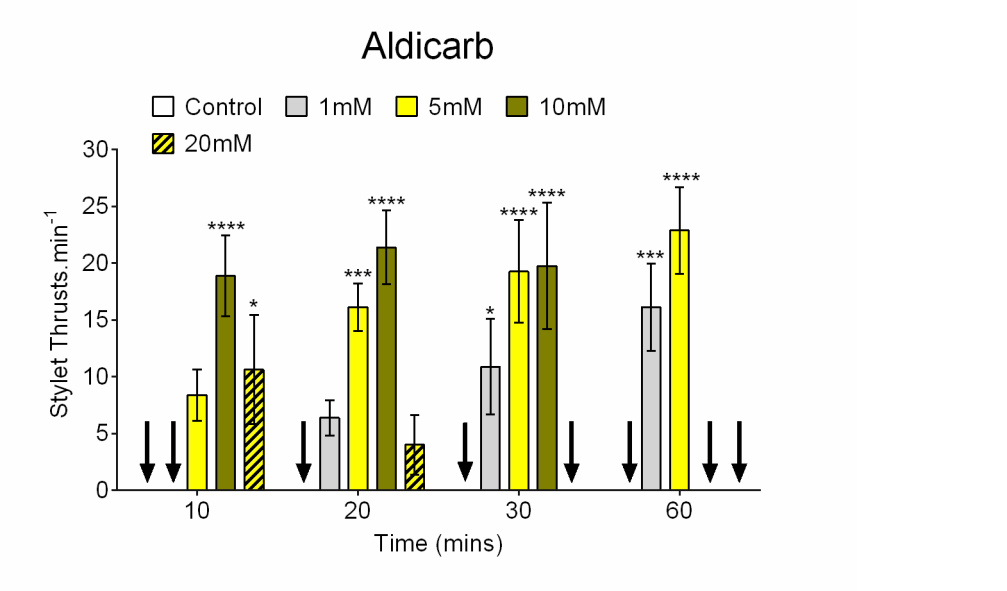

Figure 4.10. The cholinesterase inhibitor aldicarb weakly stimulates stylet thrusting. Aldicarb stimulated *G. pallida* stylet thrusting. For each concentration, eight worms were transferred to the drug solution and stylet thrusting was counted at 10, 30 and 60 minutes (n=8 worms, mean ± s.e mean shown, two-way ANOVA with Bonferroni post-hoc tests, P<0.0001). This experiment was conducted in collaboration with Adam Dorey.

4.2.8 Investigation into interactions between serotonergic and nicotinic compounds

The stimulation of stylet thrusting by aldicarb suggests that cholinergic signalling can regulate stylet activity. Acetylcholine release occurs downstream of 5-HT signalling in *C. elegans* pharyngeal pumping (McKay et al., 2004). Conversely, in the regulation of locomotion 5-HT acts to inhibit the pre-synaptic release of acetylcholine that drives locomotion (Sawin et al., 2000). To investigate whether 5-HT may interact with cholinergic signalling in the control of stylet thrusting and body posture, 5-HT was applied to J2s after treatment with the nicotinic antagonist mecamylamine (Figure 4.11). J2s were scored for stylet thrusting, posture and movement in the presence of the mecamylamine and this was compared to co-application with 5-HT. It was predicted that if acetylcholine signalling is induced by 5-HT signalling, mecamylamine would

alter the behavioural effects of 5-HT on *G. pallida* J2s. Mecamylamine was also co-applied with nicotine to assess its ability to directly affect or block behaviours manipulated with nicotine.

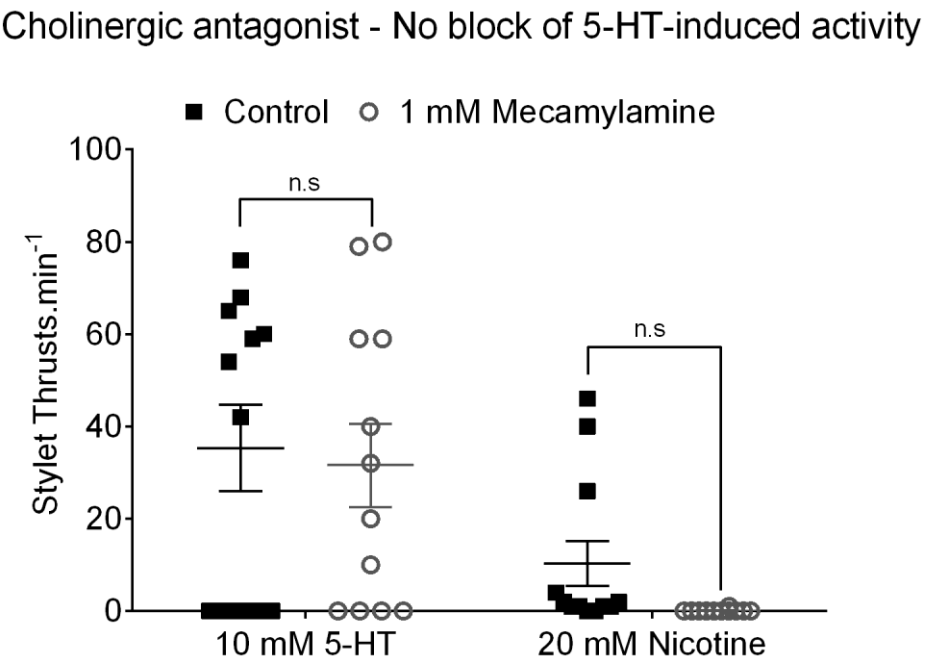

Figure 4.11. 5-HT-induced stylet thrusting is not affected by the nicotinic receptor antagonist mecamylamine. J2s were exposed to either 1 mM mecamylamine or a ddH_2O control for 24 hours and subsequently transferred to either 10 mM 5-HT or 20 mM nicotine and stylet thrusting was counted at 30 minutes. Mecamylamine did not affect 5-HT-induced thrusting. Mecamylamine did not affect nicotine stimulated stylet thrusting (n=12, individual data points shown with mean ± s.e mean, two-way ANOVA with Sidak post-hoc tests, P=0.78).

The juveniles were soaked for 24 hours in 1 mM mecamylamine and subsequently exposed to 10 mM 5-HT, or 20 mM nicotine in the continued presence of mecamylamine (Figure 4.11). This group was compared to another soaked in ddH_2O alone. No difference was found in the rate of 5-HT-induced stylet thrusting between worms soaked in mecamylamine overnight or those soaked in water. Mecamylamine treatment also had no significant effect on nicotine-stimulated stylet activity, although the rate of stylet thrusting in nicotine was lower after pre-treatment with mecamylamine. It is clear that further experiments with other nicotinic antagonists need to be conducted to resolve whether cholinergic signalling is involved in the 5-HT-mediated upregulation of stylet thrusting.

4.2.9 Investigation of the acute effects of fluensulfone on *G. pallida* stylet behaviour and body posture

Having delineated some of the key features of the pharmacology of *G. pallida* stylet behaviour, the effects of fluensulfone on this system were characterised.

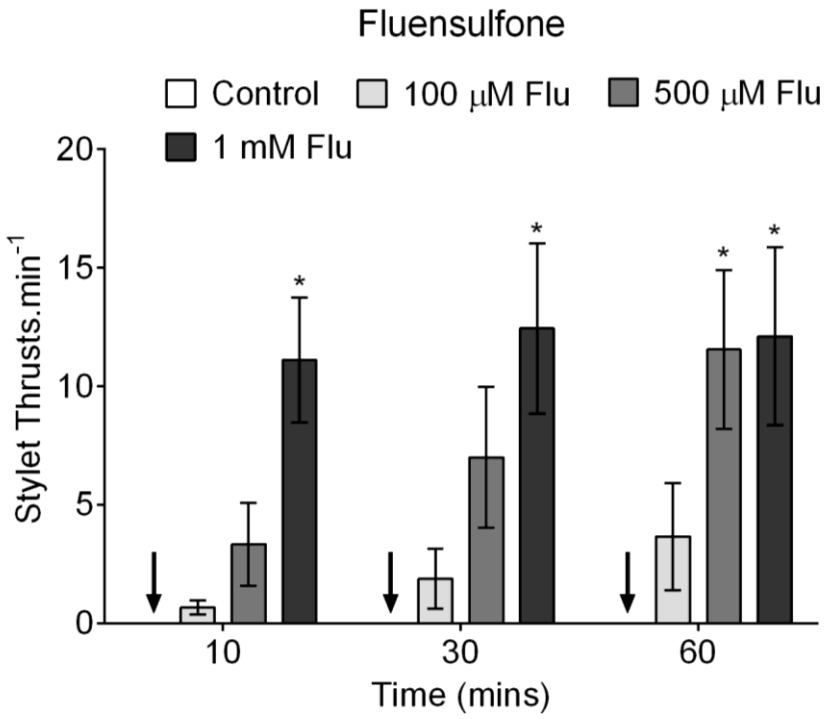

Figure 4.12. Fluensulfone induces stylet thrusting in *G. pallida* juveniles. J2 *G. pallida* were exposed to fluensulfone and stylet thrusting was visually scored at 10, 30 and 60 minutes. Both 500 µM and 1 mM caused a significant induction of stylet thrusting (n=9 worms per concentration, mean ± s.e mean, two-way ANOVA with Dunnett post-hoc tests, P<0.0001).

To determine if fluensulfone affects stylet behaviour, a concentration-response was conducted in which the stylet thrusting of J2 *G. pallida* was visually scored in the presence and absence of fluensulfone over time (Figure 4.12). No stylet thrusting behaviour was seen in vehicle-treated worms. Stylet thrusting was however induced in the presence of fluensulfone and was significantly stimulated by both 500 µM and 1 mM fluensulfone. Whilst stylet thrusting was observed in the presence of 100 µM fluensulfone, this was not significantly different from the control. The highest mean rate of stylet thrusting was 12.4 ± 3.6 thrusts per minute after 30 minutes in 1 mM fluensulfone, with 31 thrusts per minute the maximal rate seen. This is substantially lower than the 74.1 ± 12.6 thrusts per minute seen in the presence of 5-HT (Figure 4.1). The stimulatory effect occurred rapidly, within 10 minutes and was maintained at a steady state over the course of 1 hour. This stimulatory effect is redolent of that seen in cut heads, although the concentration required to elicit stimulation is around 10-fold

higher in *G. pallida* to cut heads. This discrepancy could potentially be explained by the presence of the cuticle as a barrier to drug penetration in the stylet thrusting assay. Stimulation of the pharyngeal pumping of intact *C. elegans* by fluensulfone has not been observed in any context, however (see section 3.2.3).

4.2.10 Fluensulfone has distinct effects on *G. pallida* body posture and movement

When fluensulfone stimulated stylet activity there was a concomitant alteration in the body posture and movement of the juveniles, with a coiled body posture that was rarely observed in untreated *G. pallida*. This coiled posture appeared distinct from the S-shaped, kinked posture seen in the presence of 5-HT (Figure 4.3). To quantify this, J2s were soaked in fluensulfone in Petri dishes for up to 5 hours and percentage coiling was visually scored at intervals. A worm was defined as coiled if the anterior and/or posterior of the body was looped over or back onto or over another part of the body throughout the 10 second observation period. In the absence of drug, J2s moved freely in liquid via seemingly random motions of both the head and tail and rarely adopted a coiled posture. In the presence of ≥500 µM fluensulfone however, movement was reduced and 60-70% of the juveniles adopted a coiled posture for extensive periods (Figure 4.13). Some coiling was also observed in the presence of 10 and 100 µM fluensulfone yet this was not consistently maintained. This coiled posture was adopted in both liquid and on a solid agar medium (data not shown). 500 µM fluensulfone induced coiling for at least 5 hours, however this effect was not seen at 24 hours (data not shown).

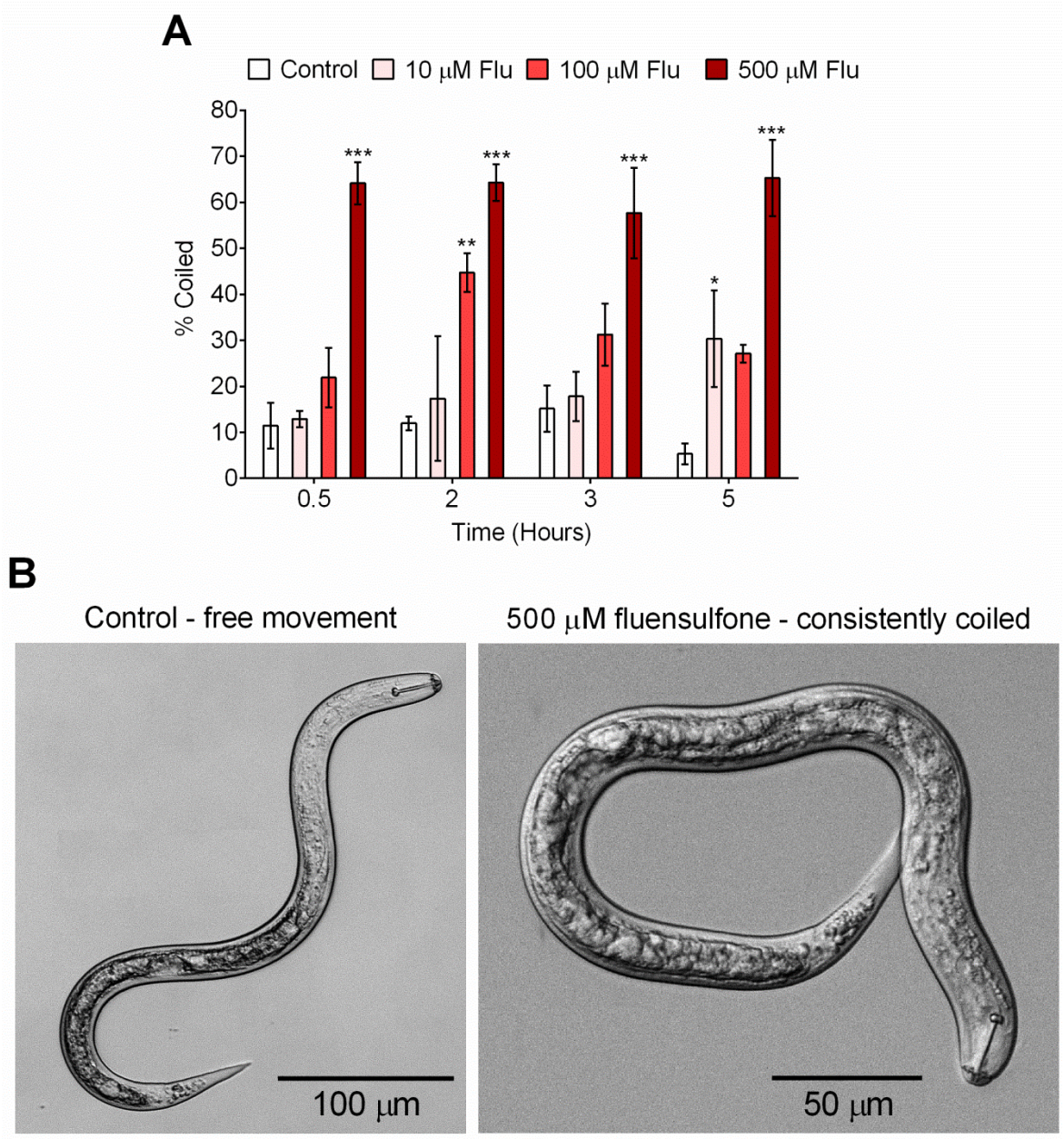

Figure 4.13. Acute exposure to fluensulfone induces a coiled posture in *G. pallida*. A) ~10 juveniles were placed in ddH_2O containing fluensulfone and vehicle and visually scored over 5 hours for percentage coiling. A worm's posture was defined as coiled if either the anterior or posterior of the worm was looped back over its body and this posture was maintained for the 10 second observation period. 500 µM fluensulfone consistently elicited protracted coiling in 60-70% of worms observed (n=5 plates per treatment, mean ± s.e mean, two-way ANOVA with Bonferroni post-hoc tests, P<0.0001). **B**) Representative images of juveniles after 15 minutes in the presence of vehicle (left) and 500 µM fluensulfone (right).

4.2.11 Investigating the effects of fluensulfone on serotonergic pharmacological stimulation of stylet behaviour

Due to the suggested importance of 5-HT signalling in stylet activation, and the observed inhibition of 5-HT-stimulated pumping in *C. elegans*, fluensulfone was applied concomitantly with 5-HT, to test for any interaction between serotonergic signalling and the stimulatory effect of fluensulfone (Figure 4.14A). Juveniles were exposed to either 5 mM 5-HT, 1 mM fluensulfone or a mixture of 5-HT and fluensulfone and stylet thrusting was scored. In the presence of 5-HT alone, the rate of stylet activity reached 70 thrusts per minute at 60 minutes whilst stylet activity was consistently 7-10 thrusts per minute in the presence of fluensulfone alone. Stylet thrusting was consistently lower in the presence of 5-HT and fluensulfone than when in 5-HT alone, with a maximal rate of just 20 thrusts per minute. The rate of stylet thrusting of worms treated with 5-HT and fluensulfone, whilst different from 5-HT-treated worms was not significantly different from fluensulfone-treated worms. It is important to note that the reduced rate of stylet thrusting when fluensulfone and 5-HT were present was not due an increased number of unresponsive worms. For example, at 60 minutes all worms treated with 5-HT and fluensulfone showed at least some stylet activity. The highest rate of stylet thrusting with 5-HT and fluensulfone was 26 thrusts per minute, compared to 105 thrusts per minute in the presence of 5-HT alone.

Worms treated with 5-HT alone typically adopted an S-shaped posture, whilst worms treated with fluensulfone alone exhibited a coiled body posture. Worms treated with a mixture of 5-HT and fluensulfone displayed a coiled posture and were not seen to adopt an S-shaped posture. This suggests that fluensulfone blocks both the increased stylet thrusting and the S-shaped posture that occurs in the presence of 5-HT.

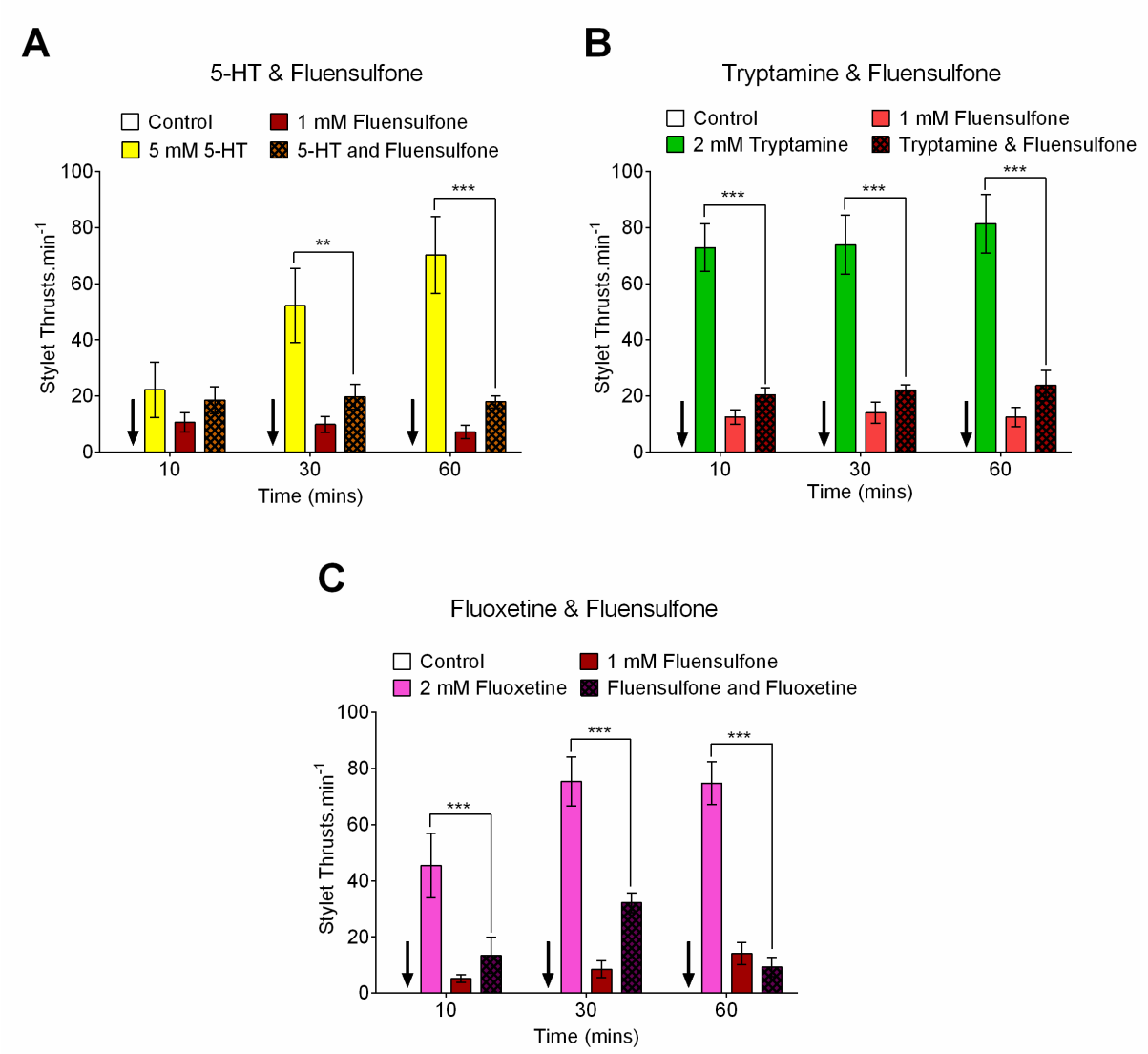

Figure 4.14. Fluensulfone inhibits 5-HT-, tryptamine- and fluoxetine-stimulated stylet thrusting. A, **B**, **C**) Inhibitory actions of fluensulfone on stylet activity were tested by exposing J2 *G. pallida* to the stimulants 5-HT, tryptamine and fluoxetine alone and concurrently exposing a separate group of worms to these stimulants mixed with 1 mM fluensulfone. The stimulatory effects of 5-HT (A), tryptamine (B) and fluoxetine (C) were all blocked by fluensulfone, with the rate of stylet thrusting similar to that of worms treated with fluensulfone alone. (For A, B, C and D control *n*=5 worms, all other treatments n=9, mean ± s.e mean, two-way ANOVA with Bonferroni post-hoc tests, P<0.0001 for all experiments).

To determine whether fluensulfone has the same blocking effect on stylet activity stimulated by endogenous 5-HT, the same experiment was conducted with 2 mM fluoxetine and 1 mM fluensulfone co-applied (Figure 4.14C). As with 5-HT, stylet thrusting was lower when fluoxetine and fluensulfone were co-applied as compared to fluoxetine alone. The rate of stylet activity in the presence of fluoxetine and fluensulfone was again not significantly different from that seen in the presence of fluensulfone alone. Similar results were also obtained with tryptamine and fluensulfone

(Figure 4.14B). Fluensulfone treatment also prevented the induction of an S-shaped posture by fluoxetine and tryptamine (data not shown).

4.2.12 Investigating fluensulfone-stimulated stylet thrusting via pharmacological manipulation of the serotonergic system

The 5-HT receptor antagonist methiothepin was used to investigate whether there is any interaction between fluensulfone and the serotonergic system. Juveniles were soaked in 300 µM methiothepin or a vehicle control solution for 1 hour and subsequently transferred to 1 mM fluensulfone. Stylet thrusting was then scored over 1 hour. Stylet thrusting was reduced in worms soaked in methiothepin relative to the vehicle control (Figure 4.15). Methiothepin treatment did not however block the effects of fluensulfone on body posture and movement, with treated worms still exhibiting the distinctive coiled posture induced by fluensulfone (data not shown).

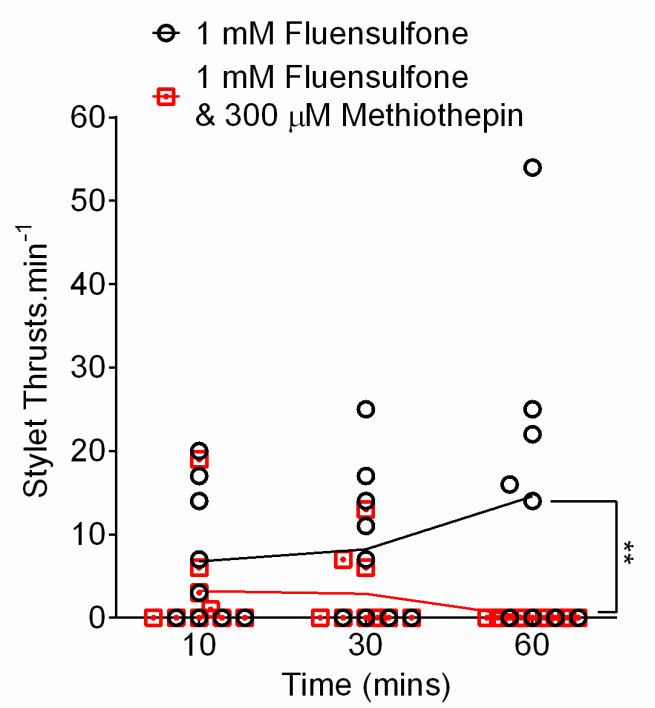

Figure 4.15. Methiothepin inhibits fluensulfone-induced stylet activity. J2s were soaked in either a control solution of 0.5% ethanol or 300 µM methiothepin for 1 hour. The J2s soaked in the control solution were then transferred to 1 mM fluensulfone and the J2s soaked in methiothepin were transferred to methiothepin & fluensulfone. Stylet thrusting was scored over 1 hour (n=9 worms, individual data point with mean ± s.e mean, two-way ANOVA with Sidak post-hoc tests, $P<0.01$).

In experiments with methiothepin, 5-HT and fluoxetine, a 24 hour pre-incubation in methiothepin revealed antagonistic activity at concentrations as low as 250 nM

(Figure 4.6). To ascertain whether fluensulfone-stimulated stylet activity could be blocked by lower concentrations of methiothepin, J2 *G. pallida* were soaked 1 and 30 µM methiothepin for 24 hours and then exposed to either 1 mM fluensulfone or 10 mM 5-HT and stylet thrusting was scored. As reported earlier in this chapter, 1 µM methiothepin partially blocked 5-HT-stimulated stylet activity relative to the vehicle control, whilst treatment with 30 µM methiothepin elicited a near-complete block of 5-HT-induced stylet thrusting (Figure 4.16A). In the control treatment group, an average rate of 11.8 ± 2.3 stylet thrusts per minute was observed in the presence of 1 mM fluensulfone. Stylet thrusting in J2s treated with 1 µM methiothepin was reduced relative to control-treated J2s, with a mean rate of 3.2 ± 1.3 thrusts per minute (Figure 4.16B). Treatment with 30 µM methiothepin elicited a near-complete inhibition of stylet thrusting relative to treatment with a control solution. Methiothepin treatment however did not inhibit the effects of fluensulfone on body posture and movement, with both control- and methiothepin-treated worms found to coil and exhibit reduced movement in the presence of 1 mM fluensulfone.

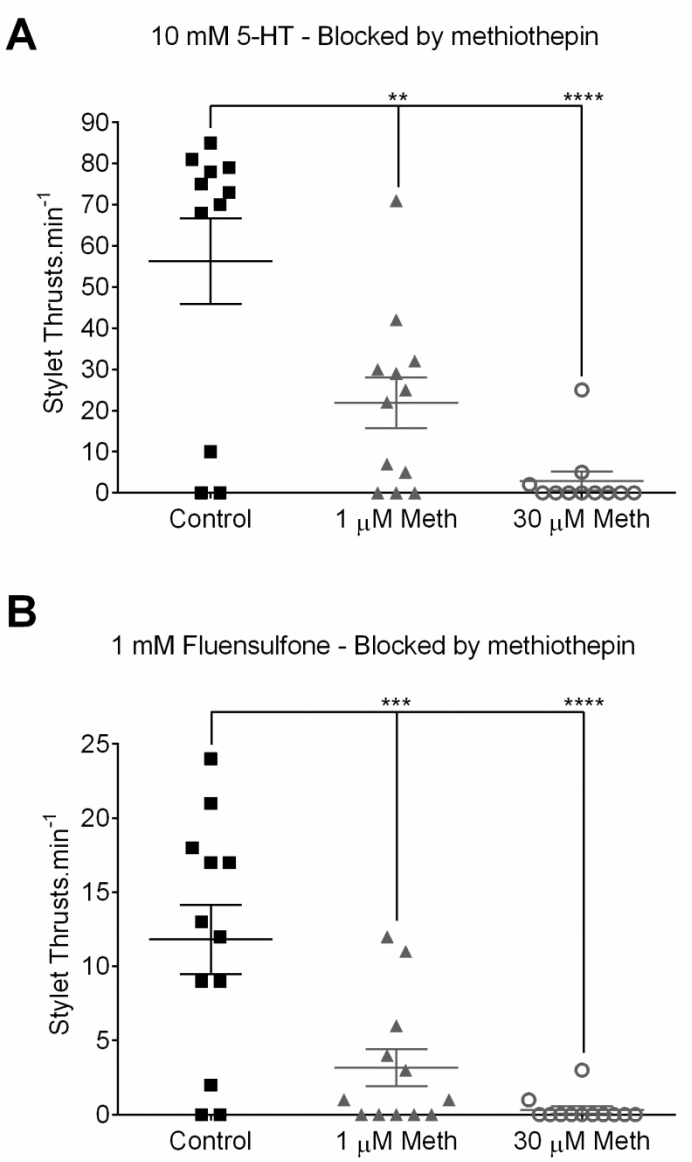

Figure 4.16. Protracted incubation in low concentrations of methiothepin blocks fluensulfone-stimulated stylet activity. J2s were soaked in either a control solution of 0.5% ethanol, 1 µM methiothepin or 30 µM methiothepin for 24 hours. **A**) J2s soaked in the control solution were transferred to 10 mM 5-HT with 0.5% ethanol, whilst J2s soaked in methiothepin were transferred to 10 mM 5-HT and the corresponding methiothepin concentration. Stylet thrusting was scored at 20 minutes. Both 1 and 30 µM methiothepin blocked 5-HT-stimulated stylet thrusting (n=12 worms, individual data point with mean ± s.e mean, one-way ANOVA with Dunnett post-hoc tests, P<0.0001). **B**) J2s soaked in the control solution were transferred to 1 mM fluensulfone, whilst J2s soaked in methiothepin were transferred to 1 mM fluensulfone and methiothepin. Stylet thrusting was scored at 20 minutes. Both 1 and 30 µM methiothepin blocked fluensulfone-stimulated stylet thrusting (n=12 worms, individual data point with mean ± s.e mean, one-way ANOVA with Dunnett post-hoc tests, P<0.0001).

The block of fluensulfone-stimulated stylet thrusting by methiothepin implies an interaction between fluensulfone and the serotonergic system. Reserpine was therefore used as a means of testing whether fluensulfone increases stylet activity by increasing extra-synaptic 5-HT. Juveniles were soaked in either 50 µM reserpine or a control solution for 24 hours and subsequently transferred to either 10 mM 5-HT, 2 mM fluoxetine or 1 mM fluensulfone and stylet thrusting was scored after 30 minutes (Figure 4.17).

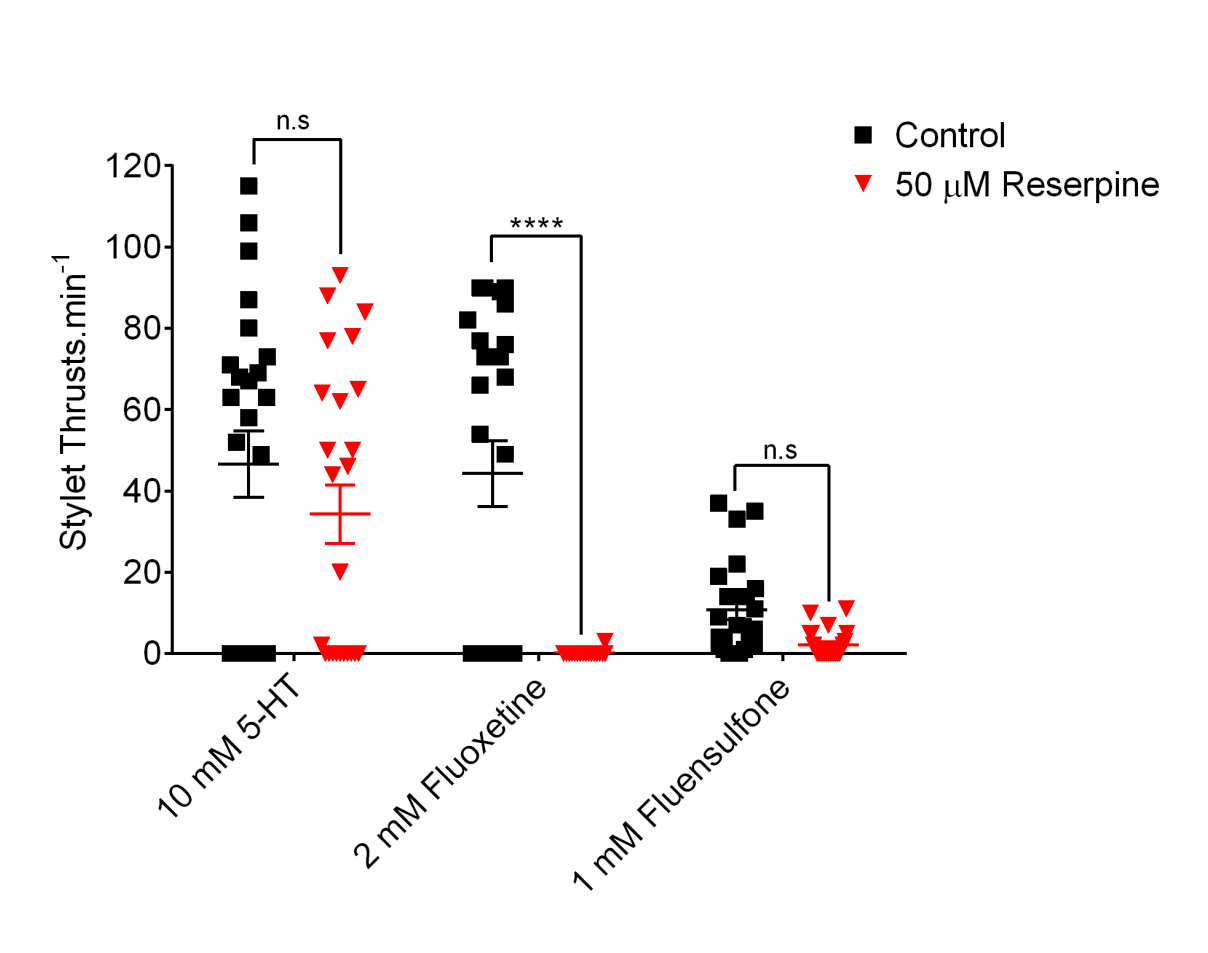

Figure 4.17. The effects of reserpine treatment on fluensulfone-stimulated stylet thrusting. J2 *G. pallida* were soaked in 50 µM reserpine or ddH_2O as a control for 24 hrs. Reserpine-treated worms were transferred to solutions of either 10 mM 5-HT and reserpine, 2 mM fluoxetine and reserpine or 1 mM fluensulfone and reserpine and ddH_2O-treated worms were transferred to either 5-HT, fluoxetine or fluensulfone alone. Reserpine blocked the stimulatory effect of fluoxetine but did not block the stimulatory effects of 5-HT or fluensulfone. (n=24 worms, data pooled from two separate experiments, individual data points and mean ± s.e mean shown, $P>0.05$, two-way ANOVA with Sidak post-hoc tests).

The rate of stylet thrusting in the presence of fluensulfone was not significantly different between reserpine-treated and control-treated worms when compared to the inhibition of fluoxetine-induced stylet thrusting by reserpine (Figure 4.17). When analysed independently however, reserpine-treated worms exhibited reduced stylet thrusting in the presence of fluensuflone relative to untreated worms (unpaired t-test, P<0.001). This indicates that increased extrasynaptic 5-HT may account for the induction of stylet thrusting by fluensulfone. Body posture in the presence of fluensulfone was unaffected by reserpine treatment, with coiling still osberved in both reserpine- and control-treated worms. For both experiments controls were conducted with 5-HT and fluoxetine and 5-HT-stimulated stylet thrusting was unaffected by reserpine treatment but fluoxetine-stimulated stylet activity was completely blocked (see also Figure 4.7).

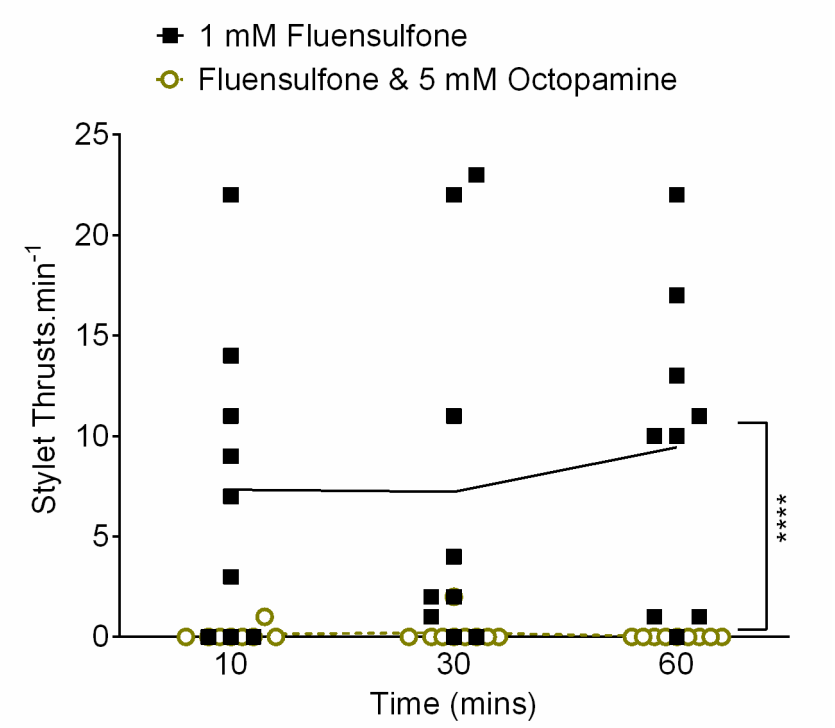

Figure 4.18. Octopamine blocks the stimulatory effect of fluensulfone on stylet activity. J2 *G. pallida* were exposed to either 1 mM fluensulfone alone or fluensulfone and 5 mM octopamine and stylet thrusting was scored (individual data points and mean shown, two-way ANOVA with Tukey post-hoc tests, P<0.0001).

Octopamine was shown to block 5-HT-stimulated stylet activity. Therefore, in another experiment juveniles were exposed to 1 mM fluensulfone in the presence and absence of 5 mM octopamine (Figure 4.18). Stylet activity was significantly lower in the presence of fluensulfone and octopamine when compared to fluensulfone alone. The presence of octopamine did not however prevent fluensulfone-induced coiling.

4.2.13 Using *C. elegans* cut heads to investigate potential serotonergic effects of fluensulfone on feeding behaviour

Having established the effects of fluensulfone on *G. pallida* stylet behaviour and how these effects were altered by pharmacological manipulation, these observations were cross-referenced to the *C. elegans* pharynx. These experiments were conducted through visual scoring of pharyngeal pumping in cut heads. Cut heads were exposed to methiothepin or vehicle and subsequently transferred to 5-HT, nicotine or fluensulfone to determine whether methiothepin also blocks both 5-HT- and fluensulfone-stimulated activity in *C. elegans*. Pharyngeal pumping was scored in the presence of saline alone to determine basal pumping. The pharynxes were then transferred to 10 µM methiothepin or a vehicle control solution and soaked for 5 minutes, after which pharyngeal pumping was scored. The cut heads were then transferred to either 500 nM 5-HT, 1 µM nicotine or 100 µM fluensulfone in the continued presence of methiothepin and pharyngeal pumping was scored (Figure 4.19). As a control, dissected pharynxes were transferred to 5-HT, nicotine or fluensulfone alone.

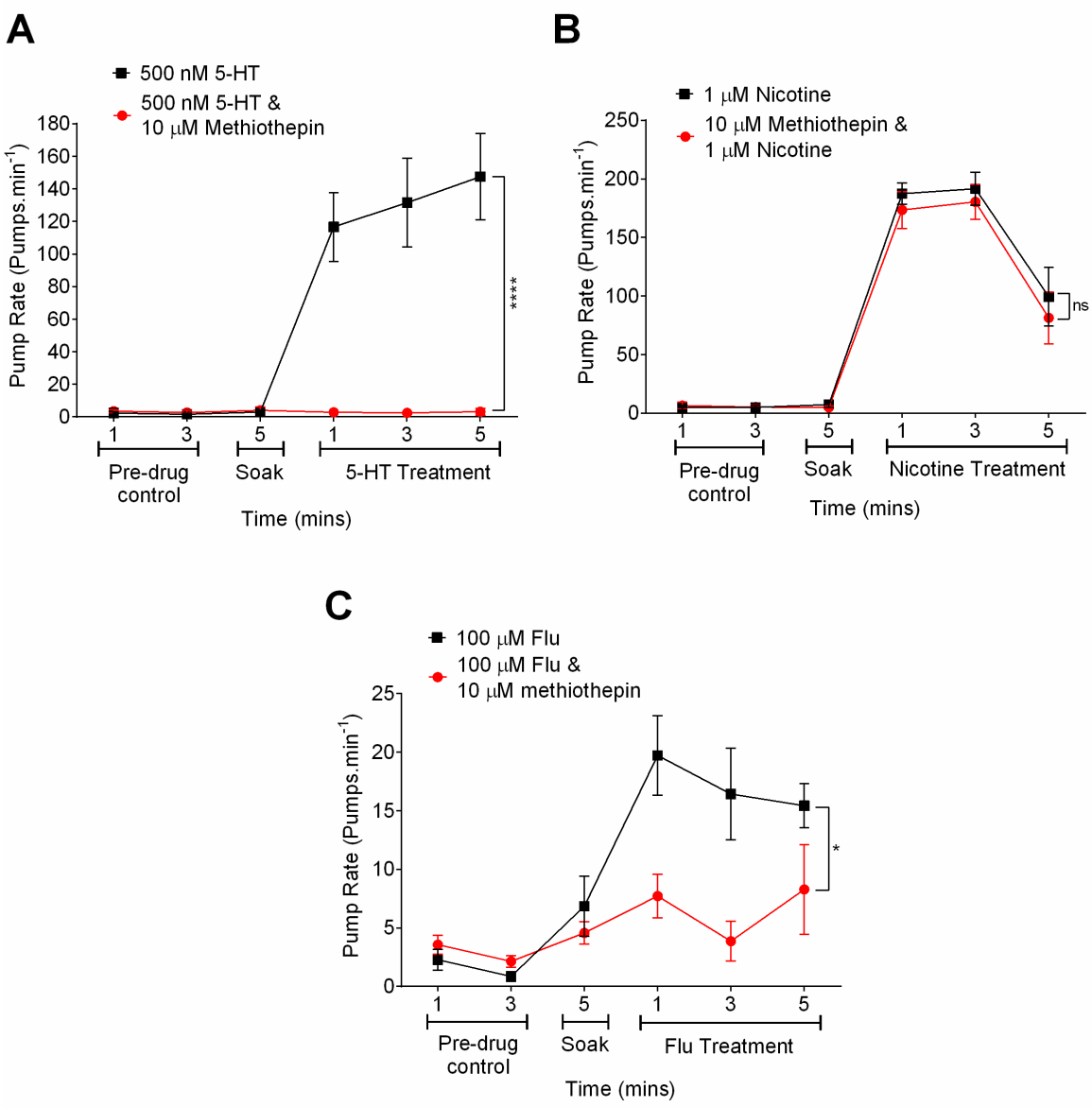

Figure 4.19. The stimulatory effect of fluensulfone on *C. elegans* dissected pharynxes is blocked by methiothepin. L4+1 day *C. elegans* were dissected to isolate the pharynx and visually scored for pharyngeal pumping. The pharynxes were subsequently soaked in either ddH_2O (control) or 10 µM methiothepin for 5 mins and then transferred to 500 nM 5-HT (**A**), 1 µM nicotine (**B**) or 100 µM fluensulfone (**C**) and pumping was again scored. Methiothepin blocked and reduced stimulation of pumping by 5-HT and fluensulfone, respectively, but had no effect on nicotine-stimulated pumping. Methiothepin treatments were always paired with controls (5-HT n=6 worms, nicotine n=5, fluensulfone n=7, mean ± s.e mean, two-way ANOVA with Bonferroni post-hoc tests, A $P<0.0001$, C $P<0.01$).

Exposure to methiothepin completely blocked stimulation of pumping by 5-HT (Figure 4.19A). Methiothepin treatment had no effect on nicotine-stimulated pumping, consistent with a selective block of 5-HT receptors as opposed to a general inhibition of pharyngeal pumping (Figure 4.19B). After pre-treatment with a control solution, 15-20 pumps per minute occurred in the presence of 100 µM fluensulfone. The stimulation of pumping by 100 µM fluensulfone was reduced by pre-treatment with methiothepin,

with 4-8 pumps per minute observed (Figure 4.19C). This suggests that fluensulfone acts via the same target to induce an increase in feeding behaviour in *C. elegans* and *G. pallida*, and that this target could be serotonergic. Methiothepin alone had no effect on the rate of pumping.

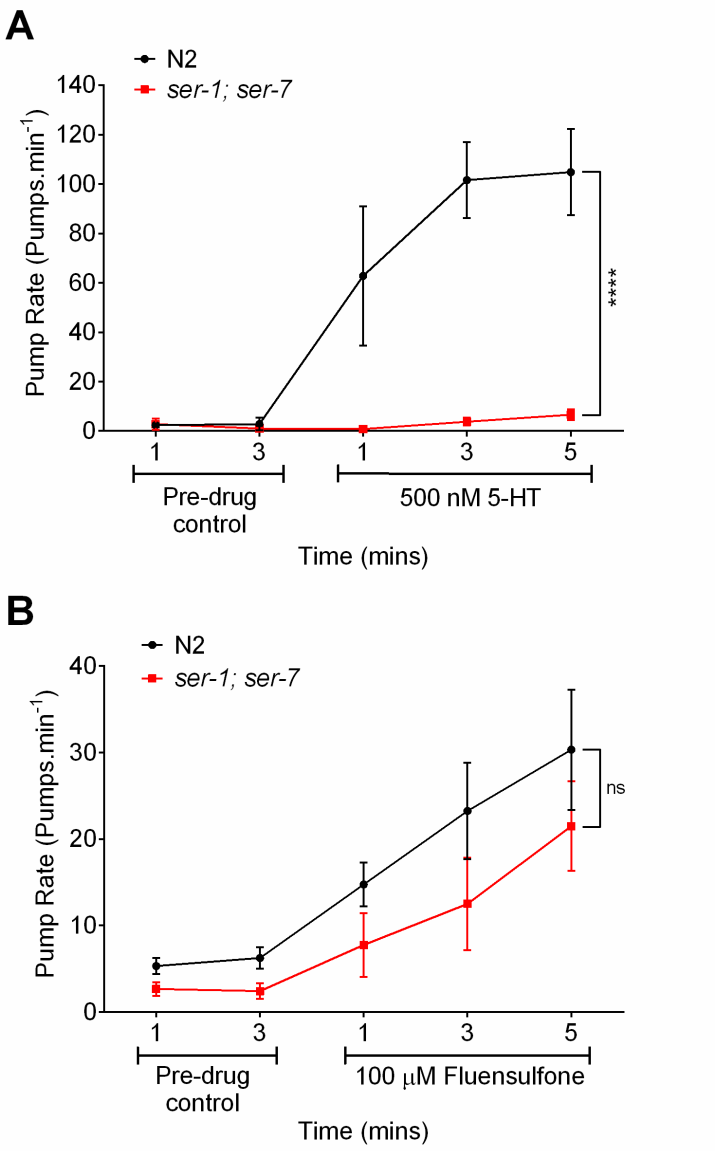

Figure 4.20. 100 µM fluensulfone stimulates pharyngeal pumping in both N2 and *ser-1; ser-7* mutant dissected pharynxes. N2 and *ser-1; ser-7* L4+1 day *C. elegans* were dissected to isolate the pharynx and visually scored for pharyngeal pumping. The pharynxes were then transferred to either 500 nM 5-HT (**A**) or 100 µM fluensulfone (**B**) and again scored for pharyngeal activity. Responsiveness to 5-HT was greatly reduced in *ser-1; ser-7* but the response to fluensulfone was not significantly different from N2. An N2 control was always paired with *ser-1; ser-7* (5-HT n=5 worms, flu n=12, mean ± s.e mean, two-way ANOVA with Bonferroni post-hoc tests, A $P<0.0001$, B $P=0.18$).

In the *C. elegans* pharyngeal system, the principle 5-HT receptors involved in the up-regulation of pump rate are SER-1 and SER-7 (Hobson et al., 2006). To determine whether fluensulfone acts via either of these receptors, *ser-1; ser-7* dissected pharynxes were exposed to 100 µM fluensulfone and pump rate was scored (Figure 4.20). Relative to the N2 control, *ser-1; ser-7* exhibited no difference in the rate of pumping in the presence of 100 µM fluensulfone (Figure 4.20B). On the other hand, *ser-1; ser-7* pharynxes were almost completely unresponsive to 500 nM 5-HT (Figure 4.20A). This suggests that if fluensulfone does indeed interact with a 5-HT receptor to induce pharyngeal pumping, then the receptor is unlikely to be either SER-1 or SER-7.

4.3 Discussion

4.3.1 5-HT is a key regulator of stylet thrusting in *G. pallida*

The aim of this chapter was to investigate the pharmacology of stylet behaviour in the PPN *G. pallida*, with a view to understanding a behaviour that is crucial to plant parasitism and to develop what is an attractive platform for drug studies in PPNs. Subsequently, the knowledge gained was used to investigate the effects of fluensulfone on the stylet system and the potential mechanisms underlying these effects.

As has been reported in other species (see section 1.8.7) (McClure and von Mende, 1987, Masler, 2007, Robertson et al., 1999), 5-HT stimulated stylet thrusting in *G. pallida*. Stylet thrusting was also stimulated by the SSRI fluoxetine, the monoamine re-uptake inhibitor imipramine and by tryptamine (Tatsumi et al., 1997). This suggests that endogenous 5-HT release can upregulate stylet thrusting in *G. pallida*. There is however evidence that both fluoxetine and imipramine can also directly interact with 5-HT receptors in both *C. elegans* and in mammals (Ni and Miledi, 1997, Eisensamer et al., 2003, Kullyev et al., 2010). The stimulation of stylet activity in the presence of fluoxetine may therefore be due to mechanisms other than 5-HT re-uptake inhibition. Treatment with reserpine and 4-chloro-DL phenylalanine selectively blocked fluoxetine-stimulated stylet thrusting without diminishing 5-HT-stimulated stylet thrusting. This suggests that the stimulation of stylet thrusting by fluoxetine is due to inhibition of 5-HT re-uptake and is not due to direct receptor interaction. Furthermore, this suggests that endogenous 5-HT is a key regulator of stylet activity in *G. pallida* and potentially other PPNs.

The stimulation of stylet thrusting by 5-HT, tryptamine and fluoxetine was concentration-dependent, with 20 mM 5-HT, 5 mM tryptamine and 2 mM fluoxetine most strongly stimulating stylet activity for each drug (section 4.2.1). At these high concentrations, the mean rate of stylet thrusting was found to drop at 60 minutes relative to 30 minutes. The rate of stylet thrusting in the presence of imipramine also decreased with time. This may be due to receptor desensitisation, resulting from the high concentrations used. Imipramine-treated worms began moving and lost the S-shaped posture, which does suggest desensitisation. In contrast, in the presence of 5-HT, tryptamine and fluoxetine, as the rate of stylet thrusting decreased, the treated J2s

remained in the distinct S-shaped posture and moved little, which indicates that these compounds were still affecting body posture. It may be that different receptors mediate the effects of 5-HT, tryptamine and fluoxetine on movement and stylet activity and these different receptors desensitise at different rates. It has been reported that different receptor subtypes exhibit different rates of desensitisation in the presence of 5-HT (Briddon et al., 1998). Some of the worms that showed reduced stylet thrusting exhibited prolonged protrusion of the stylet. This may be due to hyper-excitation of the stylet protractor muscles resulting in their spastic paralysis, preventing withdrawal of the stylet. This could also be due to non-specific drug effects due to interaction with other targets.

Whilst the concentrations tested for 5-HT and tryptamine were relatively high, similar concentrations of 5-HT and tryptamine are required to stimulate pharyngeal pumping in *C. elegans* (Horvitz et al., 1982) (*unpublished observations, James Kearn*), which suggests that 5-HT and tryptamine diffuse poorly across the cuticle of both *G. pallida* and *C. elegans*. It would be interesting to incubate J2s in these drugs for longer to see if similar rates of stylet activity can be achieved at lower concentrations. A slight reduction in the rate of stylet thrusting was seen at the highest concentrations of 5-HT, tryptamine and fluoxetine tested after 60 minutes. It is possible that this reflects non-specific effects because of the high concentrations used.

Tryptamine was a more potent stimulator of stylet activity than 5-HT, with a greater rate of stylet thrusting seen in the presence of tryptamine and a higher percentage responsiveness to tryptamine. Tryptamine has been found to have high binding affinity for the *C. elegans* SER-7 5-HT receptor (Hobson et al., 2003). It is possible that the more potent stimulation of stylet thrusting by tryptamine reflects its higher binding affinity for the 5-HT receptor(s) present in *G. pallida* relative to 5-HT. Tryptamine has been found to enhance the release of other neurotransmitters (Friedman et al., 1986) and could stimulate stylet thrusting through this mechanism of action. Alternatively, the tryptamine hydrochloride used could pass more easily across the cuticle of *G. pallida* than serotonin creatinine sulphate monohydrate. Tryptamine could also be interacting with tryptamine receptors rather than 5-HT receptors to stimulate stylet thrusting. Tryptamine may act as a neurotransmitter or neuromodulator in some invertebrates (see (Gardner and Walker, 1982) for review) though there is currently no evidence for a role for endogenous tryptamine in nematodes. The tryptamine response was blocked by

methiothepin, which has been shown to act as a competitive antagonist at several 5-HT receptor subtypes but not at tryptamine receptors. This indicates that tryptamine is likely to be acting through 5-HT receptors to stimulate stylet activity.

4.3.2 The effects of 5-HT on *G. pallida* posture

When in the presence of 5-HT, tryptamine and fluoxetine worms that exhibited stylet thrusting adopted a distinct S-shaped posture, typically characterised by the kinking of the head to one side and decreased movement (section 4.2.2). This "kinked" posture has been observed after incubation of *Heterodera glycines* in 20 mM 5-HT (Komuniecki et al., 2012). This posture may be linked to the invasion of the host plant by some PPNs, during which vigorous stylet thrusting occurs. It has, for example, been observed that the ectoparasite *Belonolaimus longicaudatus* "arches" its anterior body when probing the host root for an appropriate feeding site so as to orientate the stylet so that it is perpendicular to the root surface (Huang and Becker, 1997). This "kinked" posture seen in *H. glycines* and *G. pallida* in 5-HT may mimic the posture that occurs during host invasion. Given the body posture that is assumed by PPNs in the presence of 5-HT, it is possible that serotonergic signalling is intricately involved in PPN feeding behaviour, controlling not only stylet thrusting and pharyngeal pumping but also the body posture and movement that allows efficient root penetration. The adoption of this S-shaped posture in the presence of 5-HT was blocked by methiothepin and octopamine, whilst reserpine blocked the adoption of this posture in the presence of fluoxetine. There is a parallel for this in *C. elegans*, in which 5-HT upregulates pharyngeal pumping and reduces locomotion, so as to increase feeding efficiency and maintain contact with the food source in starved animals (Sawin et al., 2000). It would therefore be interesting to see how J2s treated with methiothepin, reserpine or 4-chloro-DL-phenylalanine interact with roots and whether these compounds alter their movement, posture or invasion behaviour.

4.3.3 A subset of *G. pallida* J2s are unresponsive to pharmacological stimulation of stylet thrusting

During the course of these experiments, it was observed that a subset of J2s failed to exhibit stylet thrusting in the presence of 5-HT, fluoxetine and tryptamine (section 4.2.3). This was observed in all experiments, although the number of unresponsive

worms varied between experiments. The percentage of unresponsive J2s in the presence of concentrations of 5-HT, tryptamine and imipramine that optimally stimulated stylet thrusting varied from 0-50%. These unresponsive worms also failed to adopt the S-shaped posture and reduced movement that was seen in the majority of responsive worms. This means that either these worms are completely unresponsive to the effects of 5-HT on all behaviours, or that the posture and stylet effects are inextricably linked. This observation may also indicate that stylet thrusting, and the associated posture changes are all-or-none, in that worms either adopt an S-shaped posture and stylet thrust vigorously or do not adopt an S-shaped posture and do not stylet thrust.

There are many possible explanations for why a subset of worms does not respond to 5-HT. *G. pallida* reproduce sexually and thus are likely to be more genetically diverse than other species, such as *M. incognita*, which reproduces via mitotic parthenogenesis (Triantaphyllou, 1985). The J2s that are used in experiments are taken from a large number of different cysts and differing responsiveness may reflect genetic variation. It would be interesting to see how other species respond to 5-HT and if other species have an unresponsive subset of worms. It seems unlikely however, that genetic variation could account for such a profound difference between worms that have had similar treatment prior to 5-HT exposure.

The J2s used in the experiments described in this chapter will have been taken from groups of cysts that had been soaked in PRD for different lengths of time. Worms were taken from groups of cysts that were soaked in PRD from 3-50 days and there may be physiological or genetic differences between worms that hatch out of cysts shortly after exposure to root diffusate and those that hatch out later. For example, it has been reported that after stimulation with PRD, *G. rostochiensis* J2s that fail to hatch from cysts over 20 day period still deplete lipid reserves and have reduced infectivity relative to J2s that hatch shortly after stimulation with PRD (Robinson et al., 1985). *G. pallida* are however known to hatch over a more protracted period after exposure to root diffusates relative to *G. rostochiensis* (Forrest and Perry, 1980, Salazar and Ritter, 1993). Furthermore, there were no apparent differences in the movement or appearance of J2s prior to addition of 5-HT. If J2s that hatch from older cysts are unresponsive due to reduced lipid reserves, it might be expected that the movement of these J2s would be reduced and that the internal contents of the worms would be reduced. It would be interesting to measure lipid levels in worms that are responsive to 5-HT and those that

fail to respond to see if differences in lipid reserves could account for differences in responsiveness.

In the stylet thrusting experiments described here, stylet thrusting was scored in groups of worms. It therefore cannot be known if unresponsive worms always remain unresponsive to 5-HT treatment. It would be interesting to score stylet thrusting in individual worms over a longer time course. If individual worms were exposed to the drug for longer, unresponsive worms may become responsive. It would also be useful to isolate unresponsive J2s to see if they are also unresponsive to other drugs that stimulate stylet thrusting, for example nicotine. This would indicate whether these worms are specifically unresponsive to 5-HT treatment or if they are resistant/ unresponsive to all drug treatments. Unresponsive J2s should also be screened for the ability to infect host plants, as these observations may be relevant to the ability of nematodes to damage crops in the field. Furthermore, thorough examination of morphology, potentially by electron microscopy, could reveal differences that might account for differing responsiveness to drug treatment, for example differences in the cuticle that might impair drug diffusion into the worm. The *C. elegans* dauer cuticle for example has more tyrosine cross-links between cuticlins, an insoluble component of the cuticle (Page and Johnstone, 2007).

4.3.4 Serotonergic signalling – A potential target for chemical control of PPNs?

The experiments described in this chapter suggest that endogenous 5-HT signalling is a key regulator of stylet activity in *G. pallida*. Fluoxetine stimulates stylet thrusting, and this is blocked by reserpine, which inhibits VMAT (section 4.2.5). Fluoxetine-stimulated stylet thrusting is also blocked by 4-chloro-DL-phenylalanine, which inhibits tryptophan hydroxylase (Erickson et al., 1992, Jequier et al., 1967). Both reserpine and 4-chloro-DL-phenylalanine also blocked the adoption of the S-shaped posture that typically occurs in the presence of fluoxetine. This further suggests that the stimulation of stylet thrusting by fluoxetine is due to endogenous 5-HT signalling and that 5-HT signalling is a crucial regulator of stylet thrusting in *G. pallida*, and potentially other PPNs. As stylet behaviour is essential to plant parasitism in PPNs, serotonergic signalling is a pathway that could be targeted to control PPNs in the field.

Reserpine treatment elicited some reduction in fluoxetine-induced stylet thrusting at concentrations as low as 5 nM. Furthermore, after treatment with reserpine, the stylet response to fluoxetine had not recovered after washing for 22 hours. This is consistent with the mammalian literature, as it is known that the effects of reserpine treatment are long lasting (Stitzel, 1976). Whilst the potential for recovery was not assessed beyond 22 hours, it is possible that reserpine could block stylet thrusting over a more protracted period. If so, the VMAT of *G. pallida* is a possible target for chemical control agents. Indeed, genome sequencing has predicted the presence of an ortholog of *cat-1*, the *C. elegans* VMAT (Cotton et al., 2014). Blocking stylet activity is also a non-lethal means of preventing nematode damage to crops, and therefore may provide an avenue to less environmentally hazardous crop protection.

Treatment with 4-chloro-DL-phenylalanine also blocked fluoxetine-induced stylet activity, though not as profoundly as reserpine. It is possible that 4-chloro-DL-phenylalanine does not completely block tryptophan hydroxylase activity in *G. pallida* and therefore does not completely prevent 5-HT release. Indeed, it has been found that 4-chloro-DL-phenylalanine does not completely deplete brain 5-HT in studies with rats (Koe and Weissman, 1966, Stokes et al., 2000). This inhibitory activity of 4-chloro-DL-phenylalanine does however indicate that 5-HT synthesis is required for the upregulation of stylet activity and that tryptophan hydroxylase is the rate-limiting enzyme in 5-HT synthesis in *G. pallida*. Genome sequencing by Cotton et al. (2014) has also indicated the presence of an ortholog of the *C. elegans tph-1* gene in *G. pallida*. Tryptophan hydroxylase is therefore another potential target for chemical control of PPNs.

Methiothepin was found to block 5-HT-, fluoxetine- and tryptamine-induced stylet activity, with a significant reduction in the rate of thrusting observed after 24 hours at concentrations as low as 250 nM (section 4.2.4). Methiothepin is a 5-HT receptor antagonist and binds at a number of distinct 5-HT receptor families and subtypes. In mammalian studies, methiothepin has been shown to significant affinity towards 5-HT_1, 5-HT_2, 5-HT_5, 5-HT_6 and 5-HT_7 receptors (Boess et al., 1997, Newman-Tancredi et al., 1998, Thomas et al., 1998, Grailhe et al., 2001, Knight et al., 2004). In studies with *C. elegans*, methiothepin has been shown to bind SER-7, SER-4 and the 5-HT-gated chloride channel MOD-1 (Ranganathan et al., 2000, Hobson et al., 2003, Petrascheck et al., 2007). There is no direct evidence that methiothepin has affinity for SER-1. As

described here however, methiothepin completely blocks 5-HT-stimulated pharyngeal pumping in *C. elegans* cut heads, which suggests that methiothepin interacts with SER-1 as well as SER-7, given role of both of these receptors in the upregulation of pharyngeal pumping (Hobson et al., 2006, Srinivasan et al., 2008). It is not known if methiothepin interacts with the putative 5-HT receptor SER-5 (Hapiak et al., 2009). *G. pallida* is predicted to have two GPCR 5-HT receptors and one 5-HT-gated ion channel (Cotton et al., 2014). Whilst the results with methiothepin suggest the presence of 5-HT receptors, it would be interesting to see if stylet thrusting can be blocked using antagonists that bind to specific 5-HT receptors to gain a greater insight into the receptors involved in regulating stylet activity. Lurasidone, for example, has high and selective affinity for mammalian 5-HT_7 receptors (Ishibashi et al., 2010) and could be used to investigate whether the role of the 5-HT_7-like receptor SER-7 is conserved between *C. elegans* and *G. pallida*.

It would be interesting to see how treatment with reserpine, 4-chloro-DL-phenylalanine and methiothepin affects root invasion by *G. pallida* and other PPNs. The data in this chapter suggests that 5-HT signalling is likely to play a key role in the upregulation of stylet thrusting that occurs during host invasion. If these compounds can block *G. pallida* root invasion, 5-HT receptors, the VMAT or tryptophan hydroxylase are potential targets for chemical control of PPNs.

4.3.5 Cholinergic signalling may also have a role in regulating stylet behaviour

The stimulation of stylet thrusting by aldicarb and nicotine suggests that cholinergic signalling may also play a role in the upregulation of stylet activity. The rate of stylet activity in the presence of nicotine and aldicarb is however far lower than in the presence of 5-HT, tryptamine or fluoxetine. In intact *C. elegans*, exogenous nicotine also stimulates pharyngeal pumping, though at a lower rate than that seen in the presence of exogenous 5-HT (Horvitz et al., 1982, Avery and Horvitz, 1990). Here, 5-HT and nicotine were found to stimulate pumping in cut heads and similar rates were observed in the presence of both drugs, although pump rate fell in the presence of nicotine. This may be due to receptor desensitisation or non-specific drug effects. In the *C. elegans* pharyngeal system, 5-HT signalling results in acetylcholine release, which interacts with the EAT-2/EAT-18 receptor on the pharyngeal muscle to increase the rate of pumping (McKay et al., 2004). Given the other apparent similarities between the

pharmacology of the *G. pallida* stylet and the *C. elegans* pharynx, it was postulated that 5-HT may also act upstream of acetylcholine to upregulate stylet thrusting. Experiments with the nicotinic receptor antagonist mecamylamine were inconclusive, as it did not block either 5-HT- or nicotine-stimulated stylet activity. Based on the data presented in this chapter, few conclusions can be made about the role of acetylcholine signalling in stylet thrusting and potential interactions with the serotonergic system. More experiments must be conducted, particularly with other cholinergic antagonists to investigate this further.

4.3.6 Other biogenic amines may have a modulatory role in the regulation of the stylet

The biogenic amines octopamine, tyramine and dopamine failed to stimulate stylet activity (data not shown). Interestingly, octopamine completely blocked the effects of 5-HT on *G. pallida* stylet thrusting and body posture (section 4.2.6). Octopamine signalling is therefore another route that could be targeted in PPN control. In *C. elegans* it has been postulated that octopamine acts as a physiological antagonist to 5-HT, blocking the stimulation of pharyngeal pumping and egg laying by 5-HT (Horvitz et al., 1982). It is possible that octopamine plays a similar role in *G. pallida* and other PPNs. It has however been suggested that octopamine can stimulate pharyngeal pumping in *G. pallida*, as it has been used to induce the uptake of FITC into the pharyngeal lumen (Urwin et al., 2002). This is in contrast to its inhibitory effect on pharyngeal pumping in *C. elegans*. Octopamine was found to induce FITC uptake into the pharyngeal lumen at 50 mM and so it is possible that at such a high concentration non-specific effects will occur. It would be interesting to test for interactions between tyramine, dopamine and 5-HT. For example, tyramine has been found to inhibit *C. elegans* pharyngeal pumping (Tsalik et al., 2003).

4.3.7 A putative model of the neuropharmacological regulation of stylet thrusting

Based on the results presented in this chapter, a putative model of the neuropharmacological regulation of stylet thrusting in *G. pallida* was developed (Figure 4.21). There appear to be significant similarities between the neurochemical regulation of stylet thrusting in *G. pallida* and *C. elegans* pharyngeal pumping. There are however many avenues that need to be investigated further. There are numerous

other signalling pathways, neuromodulators and neurotransmitters that may be involved in stylet activity, including glutamatergic, GABAergic and muscarinic signalling. Glutamate signalling is known to be important in determining the duration of each pharyngeal pump in *C. elegans* and cholinergic signalling through muscarinic acetylcholine receptors is believed to regulate pharyngeal muscle contraction (Avery, 1993b, Dent et al., 1997, Niacaris and Avery, 2003, Steger and Avery, 2004). The regulation of pharyngeal activity in PPNs should also be explored, given the crucial role of this behaviour in parasitism (see general introduction) (Wyss et al., 1992, Wyss, 1992). Whilst stylet thrusting assays with neuroactive drugs are useful, more informative techniques are needed to truly elucidate the neuropharmacological regulation of stylet behaviour and pharyngeal pumping in PPNs. For example, immunolocalisation would reveal where 5-HT was present in *G. pallida*, which could indicate whether 5-HT signalling is neuronal or humoral (Sze et al., 2002). In *C. elegans* there is evidence that 5-HT acts as neurohormone (Jafari et al., 2011) and this could be the case with *G. pallida* and other PPNs. Techniques such as formaldehyde-induced fluorescence could be used to this aim (Horvitz et al., 1982). With the genome of *G. pallida* now sequenced, heterologous expression of receptors and other components of neural signalling pathways can allow for the characterisation and investigation of these components and their roles in stylet and pharyngeal behaviour (Cotton et al., 2014). RNA interference is another avenue that is now open to explore the neuromolecular basis of stylet thrusting behaviour (Kimber et al., 2007, Fleming et al., 2007, Lilley et al., 2012).

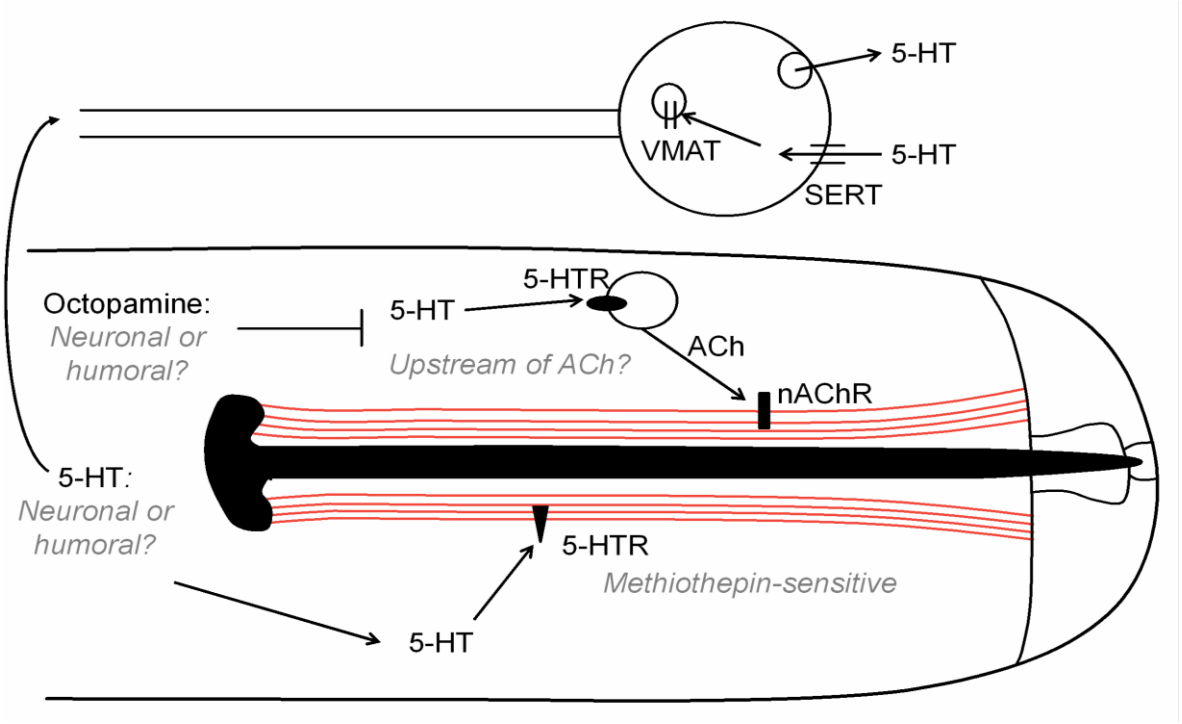

Figure 4.21. A putative model of the neuropharmacological regulation of stylet thrusting in *G. pallida*. In the experiments described here, 5-HT was found to be a key regulator of stylet thrusting. Methiothepin block of 5-HT-stimulated thrusting indicates the presence of 5-HT receptor in *G. pallida*. The stimulatory action of fluoxetine suggests that *G. pallida* J2s possess the serotonin transporter (SERT) and the block of fluoxetine-stimulated thrusting by reserpine indicates the presence of the vesicular monoamine transporter (VMAT). Stimulation of stylet thrusting by aldicarb and nicotine suggests that cholinergic signalling may also function to regulate stylet behaviour. In C. elegans, 5-HT signalling increases acetylcholine release onto the pharyngeal muscle (Raizen et al., 1995, McKay et al., 2004) and this may also occur in the regulation of stylet thrusting. Octopamine functions as a physiological antagonist to 5-HT in *C. elegans* (Horvitz et al., 1982, Niacaris and Avery, 2003) and may play a similar role in *G. pallida*.

4.3.8 Fluensulfone may interact with components of the serotonergic signalling pathway to stimulate stylet thrusting

≥500 µM fluensulfone was also found to stimulate stylet thrusting, with effects seen within 10 minutes (section 4.2.9). Furthermore, fluensulfone induced a coiled posture and a distinctive, slower pattern of movement. This coiled posture was distinct from the S-shaped posture observed in the presence of 5-HT or fluoxetine. Interestingly, fluensulfone also blocked the induction of stylet thrusting by 5-HT, fluoxetine and tryptamine and their effects on body posture (4.2.11). These observations suggested that fluensulfone might interact with the serotonergic system. To investigate this, J2s pre-treated with methiothepin were exposed to fluensulfone and were found to have a reduced rate of stylet thrusting relative to untreated J2s. This further suggests that

fluensulfone might interact with serotonergic signalling to both stimulate and block stylet thrusting.

The stimulation of stylet thrusting by fluensulfone can be compared to the stimulation of pharyngeal pumping in *C. elegans* cut heads and both these effects may result from interaction with the same target in both species. The rate of stylet thrusting in the presence of fluensulfone was lower than that achieved in the presence of 5-HT and this is similar for the stimulation of pumping in *C. elegans* cut heads. There is a discrepancy in the concentrations that stimulate activity between the species however, with ≥500 µM required to stimulate stylet thrusting, as compared to 50 µM in *C. elegans* cut heads. ≥500 µM fluensulfone strongly inhibits pharyngeal pumping in both *C. elegans* cut heads and intact animals. It is difficult to compare the concentrations that stimulate stylet thrusting and *C. elegans* pharyngeal pumping however, as stimulation in *C. elegans* only occurs in cut heads. These discrepancies do not however mean that the stimulatory effects of fluensulfone in *C. elegans* and *G. pallida* are due to interaction with different targets. Indeed, methiothepin also blocked fluensulfone-stimulated pumping in *C. elegans* cut heads.

Whilst the fluensulfone-mediated stimulation of stylet thrusting was blocked by methiothepin (section 4.2.12), it is possible that at 300 µM, methiothepin has non-specific effects and could block the stimulatory effect of fluensulfone through interaction with non-serotonergic targets. In a subsequent experiment, a 24 hour pre-incubation in 1 µM methiothepin partially blocked fluensulfone-stimulated stylet thrusting, whilst a 24 hour soak in 30 µM methiothepin caused near-complete inhibition of fluensulfone-stimulated stylet thrusting. This blocking activity at lower concentrations of methiothepin suggests that fluensulfone-induced stylet thrusting may occur through interaction with the serotonergic signalling pathway. Octopamine was found to block both 5-HT- and fluensulfone-stimulated stylet activity (section 4.2.12), which also points towards interaction with serotonergic signalling, given the role of octopamine as a physiological antagonist of 5-HT in *C. elegans* (Horvitz et al., 1982). Reserpine treatment did not significantly reduce fluensulfone-stimulated stylet activity when compared with the inhibition of fluoxetine-induced thrusting by reserpine (section 4.2.12). When analysed alone however, reserpine did reduce fluensulfone-induced stylet activity (unpaired student's T-test). This indicates that increased extrasynaptic 5-HT is responsible, at least in part, for the stimulation of stylet thrusting by fluensulfone. It is

possible that fluensulfone exposure evokes a stress response in both *C. elegans* and *G. pallida* at these concentrations, which could increase 5-HT signalling, as 5-HT is known to regulate stress behaviour in *C. elegans* (Liang et al., 2006).

As the non-serotonergic compounds tested were not found to strongly stimulate stylet thrusting, it was difficult to assess the specificity of the methiothepin block of stylet thrusting. Therefore, methiothepin was used in *C. elegans* cut heads and was found to partially block both 5-HT- and fluensulfone-stimulated pharyngeal pumping. Methiothepin had no effect on nicotine-stimulated pumping in cut heads, which suggests that it selectively blocks pharyngeal pumping stimulated through either agonistic activity at 5-HT receptors or increased endogenous 5-HT signalling. Despite the reduction in fluensulfone-induced pharyngeal pumping in cut heads treated with methiothepin, fluensulfone still stimulated pumping in *ser-1; ser-7* mutant cut heads. In contrast, *ser-1; ser-7* mutant cut heads did not exhibit any increase in pharyngeal pumping in the presence of 5-HT. This indicates that if fluensulfone does indeed interact with 5-HT receptors, SER-1 and SER-7 may not be the primary molecular targets involved in mediating the increased pharyngeal pumping in the presence of fluensulfone. Other 5-HT receptors that fluensulfone may interact with to increase pharyngeal pumping include SER-4, SER-5, SER-6 and MOD-1, all of which are expressed in either pharyngeal neurons or other head neurons (Tsalik et al., 2003, Carre-Pierrat et al., 2006, Mills et al., 2011, Li et al., 2012). None of these receptors has however been found to have any role in the regulation of the rate of pharyngeal pumping. Methiothepin has been shown to have affinity for both SER-4 and MOD-1 (Ranganathan et al., 2000, Petrascheck et al., 2007), and it would be interesting to see if fluensulfone stimulates pumping in *mod-1* and *ser-4* mutant cut heads. There is also evidence for other putative 5-HT GPCRs in *C. elegans*, some of which were found to be expressed in pharyngeal or head neurons and have been found to have a minor role in mediating the rate of pharyngeal pumping via RNA interference (Carre-Pierrat et al., 2006). Fluensulfone may increase pump rate in *C. elegans* cut heads via interaction with one of these putative receptors.

As fluensulfone stimulates pharyngeal pumping in *C. elegans* cut heads and stylet thrusting in *G. pallida* yet also blocks the stimulatory effect of 5-HT it is possible that fluensulfone acts as a partial agonist at a 5-HT receptor. Interestingly, the rate of stylet thrusting in the presence of 5-HT and fluensulfone was similar to that of fluensulfone

alone, which lends support to this hypothesis. It seems unlikely that the block of 5-HT-stimulated activity by fluensulfone could be caused by increased extrasynaptic 5-HT, which indicates that fluensulfone may also act directly at the receptor level. A partial agonist can reduce the response to a full agonist, like 5-HT by reducing the number of receptors that are available for the full agonist to occupy (Rang et al., 2011). It would be interesting to see if fluensulfone is capable of blocking cut head pharyngeal pumping or stylet thrusting induced by non-serotonergic compounds, for example nicotine in cut heads. This would indicate whether fluensulfone selectively blocks 5-HT-stimulated activity or is a more general inhibitor of pharyngeal and stylet activity. It would also be interesting to try co-application of different concentrations of 5-HT and fluensulfone in both cut heads and in *G. pallida*. This might indicate whether the block of 5-HT-induced activity can be overcome, and would reveal more about the nature of the inhibition, for example whether it is competitive or non-competitive.

Fluensulfone also prevented the induction of an S-shaped posture in the presence of 5-HT, with a coiled posture typical of fluensulfone treatment observed instead. The coiled posture that occurs in the presence of fluensulfone was not however blocked by methiothepin treatment. This suggests that the fluensulfone interacts with a non-serotonergic target to induce this coiled posture in *G. pallida* J2s and that fluensulfone is likely to interact with multiple targets at ≥500 µM. It has been reported that the PPN *Aphelenchus avenae* exhibit a coiled posture in the presence of the carbamate aldicarb and the organophosphate phorate (Keetch, 1974). Experiments with *C. elegans* however, indicate that fluensulfone is unlikely to induce coiling through anticholinesterase activity (Kearn et al., 2014). A coiled posture has also been reported in *G. pallida* J2s following gene silencing of the neuropeptide *flp-1* by RNA interference (Kimber et al., 2001). Interaction with cholinergic or neuropeptidergic pathways could perhaps result in the coiled posture that occurs in the presence of fluensulfone. There are no other reports of drug-induced coiling in PPNs. In *C. elegans* coiling results from an imbalance of excitatory and inhibitory signalling at the body wall muscle. Fluensulfone may therefore cause coiling by affecting pathways that regulate body wall muscle contraction or relaxation in *G. pallida*. Testing other nematicides and neuroactive compounds for effects on *G. pallida* body posture would reveal more regarding the neuropharmacological basis of this behaviour and may provide an insight into the target(s) fluensulfone is interacting with to affect posture.

4.3.9 Summary

In this chapter, the pharmacology of the stylet of *G. pallida* was characterised and was found to share similarities with the pharmacological regulation of the *C. elegans* pharynx. 5-HT was found to be the key regulator of stylet activity and evidence was provided for a role for cholinergic signalling. Other biogenic amines, in particular octopamine, are likely to play a modulatory role in stylet behaviour. Serotonergic signalling pathways were sensitive to low concentrations of methiothepin and reserpine, indicating that this could be a potential target for crop protection.

High concentrations of fluensulfone were found to acutely affect *G. pallida* behaviour, with fluensulfone weakly stimulating stylet thrusting and inducing a distinct coiled posture. Fluensulfone was found to block 5-HT- and fluoxetine-stimulated stylet thrusting. Furthermore, the stimulatory effect of fluensulfone was partially inhibited by octopamine, methiothepin and reserpine, indicating interaction with 5-HT signalling pathways. Experiments with *C. elegans* cut heads indicated that similar pathways are affected to stimulate pharyngeal pumping. The effects of fluensulfone on *G. pallida* posture and movement were not blocked by pharmacological inhibition of serotonergic signalling, indicating interaction with multiple targets at these high concentrations.

The next chapter will focus upon characterising the nematicidal activity and behavioural effects of lower concentrations of fluensulfone on *G. pallida* that are more relevant to its action in the field.

Assay	Fluensulfone Concentration (µM)	Effect
Stylet thrusting	500-1000	Increase from 0 to 10 thrusts per minute
Stylet thrusting – 5-HT co-application	1000	Decrease from 60-70 to 15-20 thrusts per minute
Body posture	500-1000	Coiling

Table 4.1. Summary of fluensulfone effects presented in Chapter 4.

Behaviour (organism)	Drug/ strain	Effect on fluensulfone-stimulation
Stylet thrusting (*G. pallida*)	Methiothepin (300 µM)	From 7-15 thrusts per minute to 0-3 thrusts per minute
	Octopamine (5 mM)	From 7-10 thrusts per minute to 0 thrusts per minute
	Reserpine (50 µM)	From 11 thrusts per minute to 2 thrusts per minute
	Nicotine (20 mM)	No effect (data not shown)
Cut head pumping (*C. elegans*)	Methiothepin (10 µM)	From 15-19 pumps per minute to 4- 8 pumps per minute
	ser-1; ser-7	No effect

Table 4.2. Summary of data pertaining to the mode of action of fluensulfone in chapter 4.

Chapter 5: Selective low concentration nematicidal activity of fluensulfone on *G. pallida* with chronic exposure

5.1 Introduction

It was shown in chapter 4 that fluensulfone has acute effects *G. pallida* behaviour at ≥500 µM, including stimulation of stylet activity and induction of a coiled posture. Fluensulfone also has a number of acute effects on *C. elegans* behaviour at similar concentrations and has nematicidal activity against *C. elegans* at 1 mM after 24 hours (see chapter 3). The effects on intact *C. elegans* and the acute effects on *G. pallida* are only seen at >100 µM. This is nearly 100-fold higher than the concentrations that have reported nematicidal activity against some PPN species such as *Meloidogyne spp* (see chapter 3- introduction for in-depth discussion) (Oka et al., 2008, Oka et al., 2009, Kearn et al., 2014). For example, with *C. elegans*, 1 mM fluensulfone, or 292 ppm, is nematicidal after 24 hours exposure whilst ≥1.7 ppm has nematicidal activity against the PPN *M. javanica* after 48 hours exposure (Oka et al., 2009). Furthermore, there is evidence that the highest concentration that PPNs are likely to be exposed to immediately after fluensulfone application in the field is <30 µM (Norshie, 2014). Therefore, the estimated concentrations that will be distributed in soil after fluensulfone application are likely to be lower than the ≥100 µM that produces overt acute effects in *C. elegans* and *G. pallida in vitro*.

The considerable difference in the *in vitro* concentrations that induce mortality between *C. elegans* and some PPNs may reflect the differences in their respective biology. The experiments in chapter 4 however suggest that *G. pallida* is susceptible at similar concentrations to *C. elegans*. The work with *G. pallida* has thus far focussed on acute exposure. It is therefore possible that with longer exposure times, effects will be seen at lower concentrations.

Throughout this chapter, assays were performed in which *G. pallida* were soaked in fluensulfone for increasing periods of time up to 14 days. The worms were exposed to a range of fluensulfone concentrations, including higher concentrations that were found to have overt acute effects on both intact *C. elegans* and *G. pallida* (200-500 µM) and lower concentrations that had no obvious effects on either species with acute exposure (1-30 µM) (see chapter 4).

Given the acute effects of fluensulfone on stylet activity, worms were exposed to lower concentrations for protracted periods and stylet behaviour was scored. It was observed

that with time, there was a progressive increase in immotility of J2s, with a rod-shaped posture and that this lead to the apparent eventual death of the worms (see section 5.2.1). This was seen at 30 µM, with nearly 100% immotility ensuing at 7-10 days. Subsequently, experiments were performed to investigate the concentration- and time-dependence of this immotility effect and to scrutinise the potential mechanisms underlying the nematicidal activity of fluensulfone against *G. pallida*.

As prolonged incubation of *G. pallida* in fluensulfone revealed nematicidal activity at lower concentrations, experiments were conducted to ascertain if prolonged fluensulfone exposure has similar low concentration nematicidal activity against *C. elegans*. Due to the differences in the biology of *G. pallida* and *C. elegans*, long-term mortality assays cannot be conducted in the same manner on the two species. J2 juvenile *G. pallida* are non-feeding and can survive in water alone for up to 21 days after hatching (data not shown). Due to issues with osmolarity, the survival of *C. elegans* is negatively affected by protracted incubation in ddH_2O (*unpublished observations*). This can be overcome using media such as phosphate-buffered M9, but *C. elegans* adults undergo bagging if food is not present. If food is present then other chemicals such as fluorodeoxyuridine (FUDR) must be included to prevent the growth of progeny that would otherwise disrupt the assay (Sutphin and Kaeberlein, 2009). FUDR has been shown however to have protective effects against proteotoxic stress (Angeli et al., 2013). FUDR has also been shown to modify lifespan (Aitlhadj and Sturzenbaum, 2010) and to have effects on motility (Glenn et al., 2004). It therefore cannot be ruled out that the presence of other chemicals, such as FUDR, may alter the effects of fluensulfone on *C. elegans*.

The effects of fluensulfone on *C. elegans* lifespan and mortality were therefore investigated using the established solid-media lifespan assay (Mulcahy et al., 2013), where *C. elegans* L4+1 day adults are grown on agar plates seeded with OP50 *E. coli* in the presence of the drug and time of death is scored. Any worm that is immotile and does not exhibit any other signs of life, such as pharyngeal pumping, and fails to respond to prodding is deemed dead.

The scoring of immotility and other visual scoring of death with PPNs exposed to fluensulfone and other nematicides is relatively subjective. The length of the observation period in which immotility is scored is likely to affect the results obtained.

In studies with *C. elegans*, prodding is often used to ascertain responsiveness (Mulcahy et al., 2013) yet this is problematic in PPNs as the internal pressure of the worm can cause it to rupture (Oka et al., 2009). PPNs can recover from the immotility induced by some compounds, such as the organophosphates and carbamates (Oka et al., 2009) and thus immotility, whilst useful, does not necessarily indicate death. Death in nematodes can be measured via several methods, including stains and dyes for viability such as MTT, acridine orange, sytox and propidium iodide and through assessment of metabolic activity by measurement of oxygen consumption or ATP production (James and Davey, 2007, Schouest et al., 2009, Van Raamsdonk et al., 2010a, Yang and Hekimi, 2010, Mouchiroud et al., 2013, Lant and Derry, 2014, Ferreira et al., 2015).

Here, the time-dependence of the onset of death resulting from fluensulfone exposure was further investigated using the tetrazolium dye MTT (2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Berridge et al., 2005). Tetrazolium dyes, MTT in particular, have been used extensively in the field of cell biology as a means of quantifying cell growth and cell metabolic activity (Mosmann, 1983). When dissolved in water, MTT is a salt with yellow colouration. When MTT undergoes a reduction reaction, its tetrazole ring is disrupted and this results in the formation of an insoluble formazan product with a bright purple colour (Mattson et al., 1947). In cellular-based assays, this reduction reaction will occur in metabolically active cells, dependent upon a number of NAD(P)H-dependent oxidoreductase and dehydrogenase enzymes. This reduction reaction requires NAD(P)H (Nicotinamide adenine dinucleotide phosphate) or succinate as a reducing agent (Berridge et al., 2005). Thus, if these reducing agents are not present, or are only sparingly present, MTT reduction will be limited and less of its formazan product will be generated (Figure 5.1A). These reducing agents are generated through several metabolic pathways, including glycolysis, the tricarboxylic acid cycle and β-oxidation (Berg et al., 2007). Therefore, whilst metabolically active cells will stain purple in the presence of MTT, cells with reduced metabolic activity will have reduced purple staining and dead cells will not stain purple.

As mentioned, it is not known which specific enzymes catalyse this reduction reaction. There is also some uncertainty regarding the major site of MTT reduction within metabolically active cells and the major reducing agent in the reaction (see (Berridge et al., 2005) for review). When converted to its formazan product, MTT forms insoluble crystals, which can rupture out of and between cells and so the site of MTT reduction is

difficult to determine. It is accepted however that MTT conversion can be used as a general measure of metabolic activity. MTT has also been used as a measure of death in *C. elegans* (James and Davey, 2007, Smith et al., 2009) (Figure **5.1**B). In this chapter, MTT was therefore used to more precisely define the nematicidal activity of fluensulfone on *G. pallida* and to gain an insight into whether fluensulfone treatment has any effect on *G. pallida* metabolic capability.

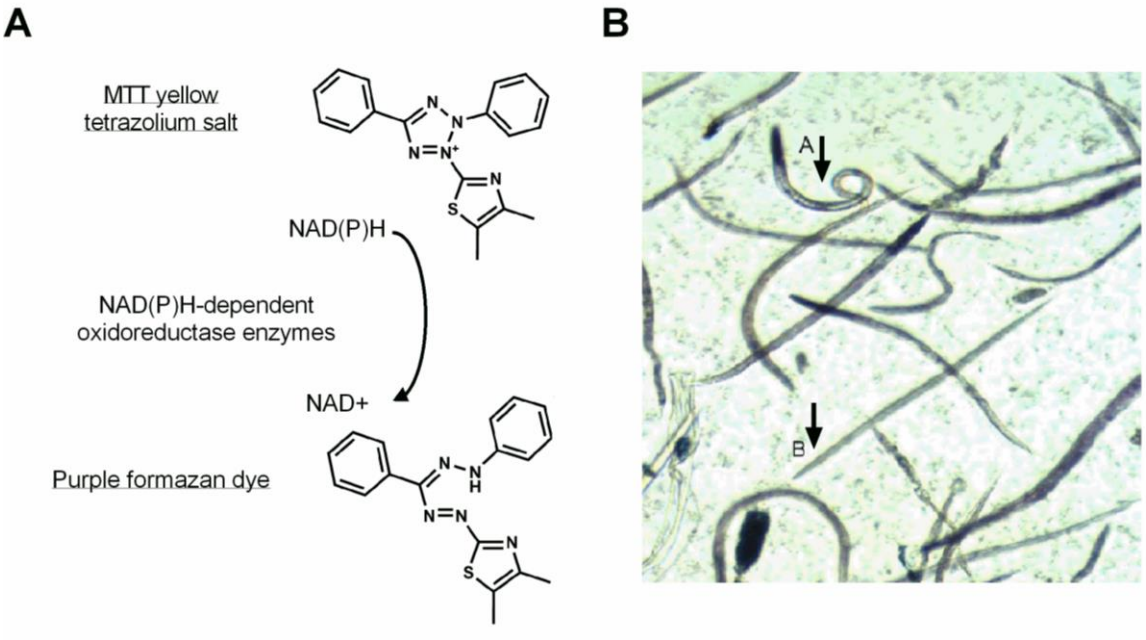

Figure 5.1. The tetrazolium dye MTT can be used to indicate metabolic integrity and nematode death. A) MTT is a yellow tetrazolium salt that can be used to indicate the metabolic activity of an organism or cell. When taken up by a metabolically active cell MTT undergoes a reduction reaction that is dependent upon oxidoreductase enzymes and is converted to a purple formazan product. This reaction requires NAD(P)H as an electron donor. Purple staining indicates that a cell or organism is alive and metabolically active; a lack of staining indicates death or metabolic impairment. **B**) MTT has been developed as an assay for viability of *C. elegans* by James and Davey (2007) and Smith et al. (2009). In the image, the arrow and A indicate a live worm that shows purple staining in the head region after staining with MTT. The arrow and B in the image indicate a dead worm that has no purple staining (from (Smith et al., 2009)).

As the chronic nature of the nematicidal activity of lower concentrations of fluensulfone emerged, it was postulated that these concentrations might affect cyst hatching of *G. pallida* with protracted exposure in the field. Fluensulfone has also been shown elicit a reduction in the hatching of *M. javanica* at 27.2 µM (8 mg.L) (Oka et al., 2009) and therefore may also affect *G. pallida* hatching. Therefore, another aim of this chapter was to examine the effects of lower concentrations of fluensulfone on *G. pallida* hatching from cysts. Few *G. pallida* hatch from cysts unless potato root diffusate (PRD) is present (see section 1.7 for the background to hatching in PPNs). Therefore, in the cyst

hatching assays, cysts were soaked in 1 part (PRD) to 3 parts ddH_2O in the presence of either vehicle (0.5% acetone) or fluensulfone and hatching was scored over time. Studies have found that the half-life of fluensulfone in soil is around 24 days (Norshie, 2014). Therefore, after 24-25 days, the cysts were removed from both fluensulfone and vehicle, washed and then placed in a solution of 1 part PRD to 3 parts ddH_2O to assess the potential for recovery from any inhibition of hatching.

It has been shown that PPNs exhibit stylet thrusting immediately prior to hatching and it has been speculated that this stylet thrusting is important in the emergence of the juvenile from the protective eggshell (Perry and Moens, 2013). As shown in chapter 4, serotonergic transmission appears to be a key regulator of *G. pallida* stylet activity and can be blocked by pharmacological agents such as methiothepin, reserpine and 4-chloro-DL-phenylalanine. Therefore, in a further series of experiments these drugs were used to assess the role of serotonergic transmission in the regulation of *G. pallida* hatching, using the same method as with fluensulfone.

The aim of this chapter was to investigate low concentration chronic exposure of *G. pallida* to fluensulfone and to compare this with the same concentrations in *C. elegans*. Another aim was to investigate metabolic impairment as a potential route to the nematicidal activity of fluensulfone, particularly with chronic exposure to lower concentrations. Finally, extended bio-assays were conducted to investigate potential effects of fluensulfone on cyst hatching of *G. pallida*.

5.2 Results

5.2.1 The effects of chronic fluensulfone exposure on *G. pallida* stylet activity

As reported in chapter 4, fluensulfone acutely stimulates stylet thrusting behaviour but also inhibits 5-HT- and fluoxetine-stimulated stylet activity (see chapter 4). These effects are seen at ≥500 µM fluensulfone, which is equivalent to 146 ppm, far higher than the 1 ppm that has been shown to have irreversible nematicidal activity against PPNs (Oka et al., 2009). 1 ppm (3.4 µM) has been shown to paralyse *M. javanica* irreversibly after 48 hours exposure. It was therefore postulated that fluensulfone may have effects on stylet activity at lower concentrations when the juveniles have been exposed for a longer period. Freshly hatched J2 *G. pallida* were exposed to fluensulfone for up to 14 days, during which time stylet activity was counted (Figure 5.2A). After measuring stylet activity in the presence of fluensulfone alone, the worms were transferred to 10 mM 5-HT in the continued presence of fluensulfone and stylet thrusting was again counted after 10 minutes to assess any block of stimulated stylet activity (Figure 5.2B). 30 µM fluensulfone (8.76 ppm) was used as the lower concentration as it has been determined that this is the peak soil concentration immediately-post application and is therefore the maximal concentration that PPNs are likely to be exposed to in the field (Norshie, 2014). 30 µM is also the lowest concentration that was found to have any effect on *C. elegans* behaviour, with an increase in pharyngeal pumping in cut heads observed at this concentration (see section 3.2.7).

Little or no stylet activity was observed in worms treated with vehicle alone throughout the course of the experiment. In contrast, stylet activity was modestly stimulated by ≥200 µM fluensulfone to 7 stylet thrusts per minute at 5 hours exposure, which is consistent with earlier observations (see chapter 4). 30 µM fluensulfone did not stimulate stylet activity (Figure 5.2A). The stimulatory effect of ≥200 µM fluensulfone was transient and no significant stimulation of stylet thrusting occurred beyond 5 hours exposure, with no stylet activity seen at all after 24 hours (data not shown).

Worms in the control group showed some response to a 10 minute treatment with 10 mM 5-HT throughout the experiment, with stylet activity still induced by 5-HT after 14 days soaking in the presence of vehicle. Over the course of the experiment there was

considerable variability in the response of worms soaked in vehicle before being transferred to 5-HT, with a maximal mean rate of 62 thrusts per minute and a minimum rate of 23 thrusts per minute. This highlights the considerable day-to-day variability that became clear in the experiments shown in chapter 4.

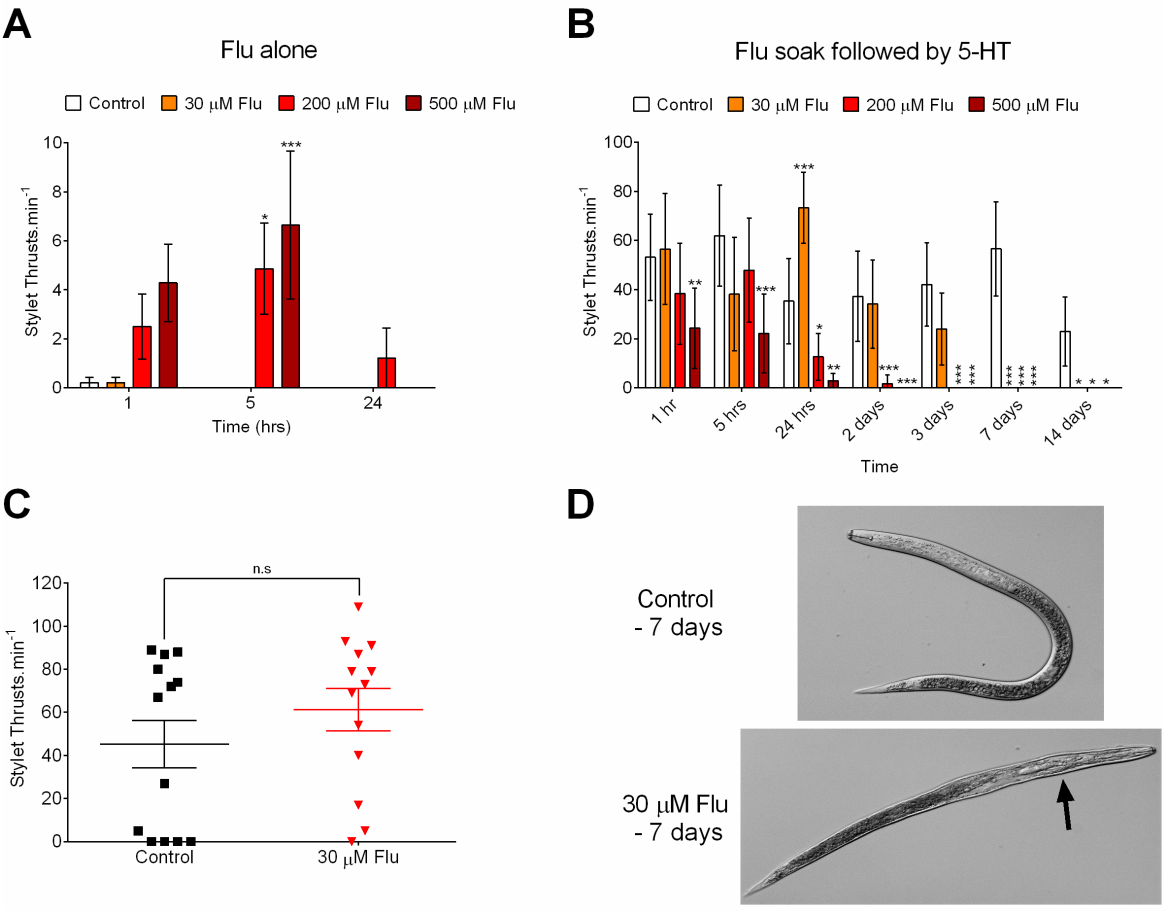

Figure 5.2. The effects of fluensulfone on *G. pallida* stylet activity with prolonged exposure. **A**) J2 *G. pallida* were soaked for up to 14 days in fluensulfone (flu) or ddH_2O (control) and stylet activity was scored during this period. Stylet activity was stimulated at ≥200 µM at 1-5 hours but not beyond 24 hours (n=14 worms, mean ± s.e mean, two-way ANOVA with Bonferroni post-hoc tests, P<0.001). **B**) At each time point, J2s were transferred to 10 mM 5-HT and stylet activity was scored after 10 mins. 5-HT-induced stylet thrusting was reduced at all concentrations, including 30 µM after ≥3 days, most likely due to nematicidal effects. 30 µM fluensulfone appeared to potentiate the effect of 5-HT on stylet thrusting at 24 hours (n=16, mean ± s.e mean shown, two-way ANOVA with Sidak post-hoc tests, P<0.0001). **C**) This effect was not however repeatable in a subsequent experiment (n= 13, student's unpaired T-test, P=0.29). **D**) Representative images of worms soaked for 7 days in vehicle and 30 µM fluensulfone. Fluensulfone-treated worms were "granular" with apparent disintegration of internal organs and distortion of the cuticle. Note the "rough" appearance at the anterior of the fluensulfone treated J2, as indicated by the arrow.

500 µM fluensulfone significantly reduced 5-HT-induced stylet activity relative to the vehicle control after 1 and 5 hours exposure, with a 64% reduction in the rate of stylet

activity when compared to control-treated worms. Both 200 and 500 µM fluensulfone reduced stylet activity in the presence of 5-HT relative to the control at 24 hours exposure and at all other time points up to 14 days. No 5-HT-induced stylet activity was observed after 2 days in 500 µM fluensulfone and 3 days in 200 µM. This seems consistent with observations made in chapter 4, where fluensulfone and 5-HT were co-applied (see section 4.2.11). Surprisingly, 5-HT-induced stylet thrusting was increased by 108% relative to the vehicle control after 24 hour soaking in 30 µM fluensulfone (Figure 5.2B). This effect was not however repeatable in a separate experiment, with no significant difference in the response to 10 mM 5-HT between worms soaked in vehicle or 30 µM fluensulfone for 24 hours, with a mean rate of stylet activity of 45.3 ± 11 and 61.2 ± 9.8 thrusts per minute, respectively (Figure 5.2C). 5-HT did not induce stylet activity after 7 and 14 days exposure to 30 µM fluensulfone, whereas stylet activity was still observed after treatment with vehicle (Figure 5.2B). This inhibitory action of fluensulfone on 5-HT-induced stylet thrusting was however accompanied by a progressive increase in the number of immotile juveniles at all concentrations, characterised by a rod-shaped posture (Figure 5.3). Previous work has described that *M. javanica* exposed to fluensulfone for 24-48 hours adopt a rod-shaped posture when paralysed (Oka et al., 2009).

After ≥2 days exposure to 200 and 500 µM fluensulfone some immotile worms began to look "granular" with indistinct internal organs and a darkened appearance. Worms that appeared granular did not move and did not respond to mechanical stimulation by prodding. Worms exhibiting this deteriorated appearance increased with time, and exhibited progression to a more complete disintegration of internal organs. It was assumed that this "granular" appearance indicates worm death (Figure 5.2D). These morphological effects that appeared and further progressed after 2 days exposure to 200 and 500 µM fluensulfone also became apparent in a delayed fashion at lower concentrations. After ≥7 days exposure to 30 µM fluensulfone this deteriorated appearance became apparent and progressively more worms became granular, with the majority of worms showing these morphological changes after 14 days. In contrast, this appearance was not observed in worms treated with vehicle alone, even at 14 days. Control-treated worms did not exhibit any deterioration of internal structures.

After 3 days in the presence of 200 and 500 µM fluensulfone and 7 days in 30 µM fluensulfone, 5-HT-induced stylet activity does not occur though is still seen in control

treated worms. At all concentrations of fluensulfone there was an increase in the number of rod-shaped, immotile worms with time and this increase occurs over 2-3 days at ≥200 µM and 3-14 days at 30 µM. This is followed by deterioration of internal structures, suggesting worm death. These changes seem to correlate temporally with the reduction in stylet thrusting. This suggests that the reduction in the 5-HT response with protracted incubation in fluensulfone is non-selective and may be due to nematicidal effects rather than a direct block of stylet activity.

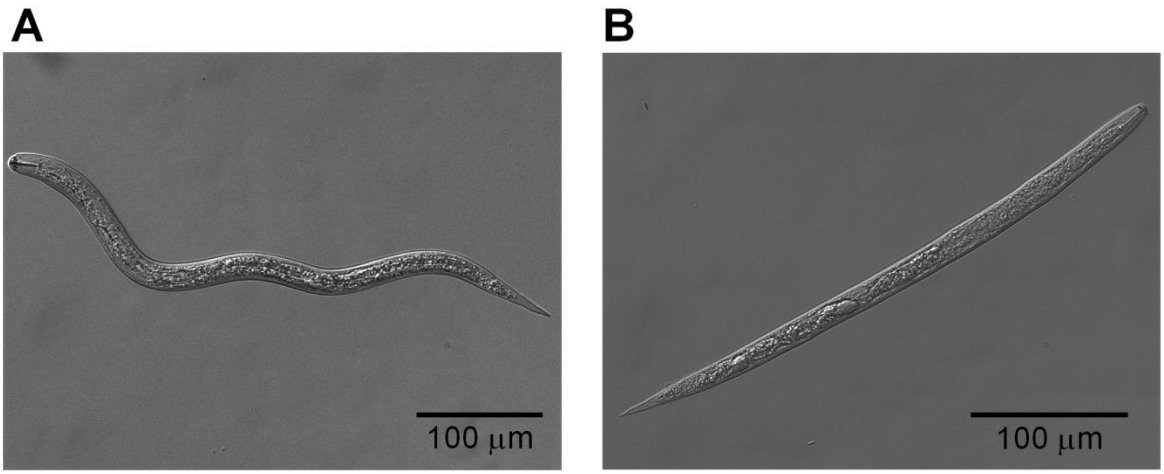

Figure 5.3. Prolonged exposure to fluensulfone induces a rod-shaped posture in *G. pallida*. Representative images of *G. pallida* juveniles soaked for 7 days in the presence of either vehicle (**A**) or 30 µM fluensulfone (**B**). The control-treated worm is still moving whereas the fluensulfone-treated worm is paralysed, as characterised by a rod-shaped posture from which the worms did not recover.

5.2.2 Chronic exposure of *G. pallida* to lower concentrations of fluensulfone elicits a progressive increase in immotility and death

The observations made during the experiments shown in section 5.2.1 suggest that chronic exposure to 30 µM fluensulfone has progressive paralytic and nematicidal effects against *G. pallida* J2s. This is >10-fold lower than the concentrations that acutely affect stylet thrusting in *G. pallida* and the concentrations that have acute effects on *C. elegans* behaviour in the intact worm (see chapter 3 and 4). This is also >20-fold lower than the concentrations seen to have nematicidal activity against *C. elegans* after 24 hours exposure (Kearn et al., 2014).

The concentration- and time-dependence of the apparent paralytic and nematicidal effects of chronic fluensulfone exposure on *G. pallida* juveniles was further investigated via motility assays and by noting morphological observations that suggest worm death

(Figure 5.4). Freshly hatched J2s were soaked in fluensulfone or a vehicle (0.5% acetone, control) solution made with ddH_2O and percentage immotility was scored in two separate experiments (Figure 5.4A+B). Immotility was scored under the criteria that if, during a 10 second observation period, a worm failed to move and was rod-shaped it was immotile.

In both experiments, percentage immotility gradually increased in worms exposed to vehicle alone, with 7-17% of worms immotile after 1 day, increasing to 40-60% immotility at 14 days. This gradual reduction of movement in the absence of drug is probably due to depletion of lipid reserves by the non-feeding J2s (*personal communication, Justyna Pertek*) (Reversat, 1981, Storey, 1984). Exposure to 500 µM fluensulfone for 24 hours elicited a 29% increase in percentage immotility relative to the control, whilst 24 hour exposure to 200 µM induced a 9-30% increase in immotility in the two experiments. All worms exposed to 500 µM fluensulfone were immotile after 2 days, whilst in both experiments 200 µM fluensulfone induced complete immotility at 2-7 days. Exposure to 30 µM fluensulfone also resulted in a slight increase in the number of immotile worms at 24 hours in both experiments, with a 20-33% increase in the number of immotile J2s relative to the vehicle control. Whilst the onset of complete paralysis was relatively rapid in the presence of 200 and 500 µM fluensulfone, 30 µM elicited a slower progressive increase in percentage immotility, with immotility of all worms achieved at 10-14 days exposure in both experiments. In the second experiment (Figure 5.4A), a significant increase in immotility occurred in the presence of 1 and 10 µM at 10 days exposure, with a 40% and 50% increase in percentage immotility at 10 days when compared to the control, respectively. 1 and 10 µM did not however cause immotility in all the worms even after 14 days.

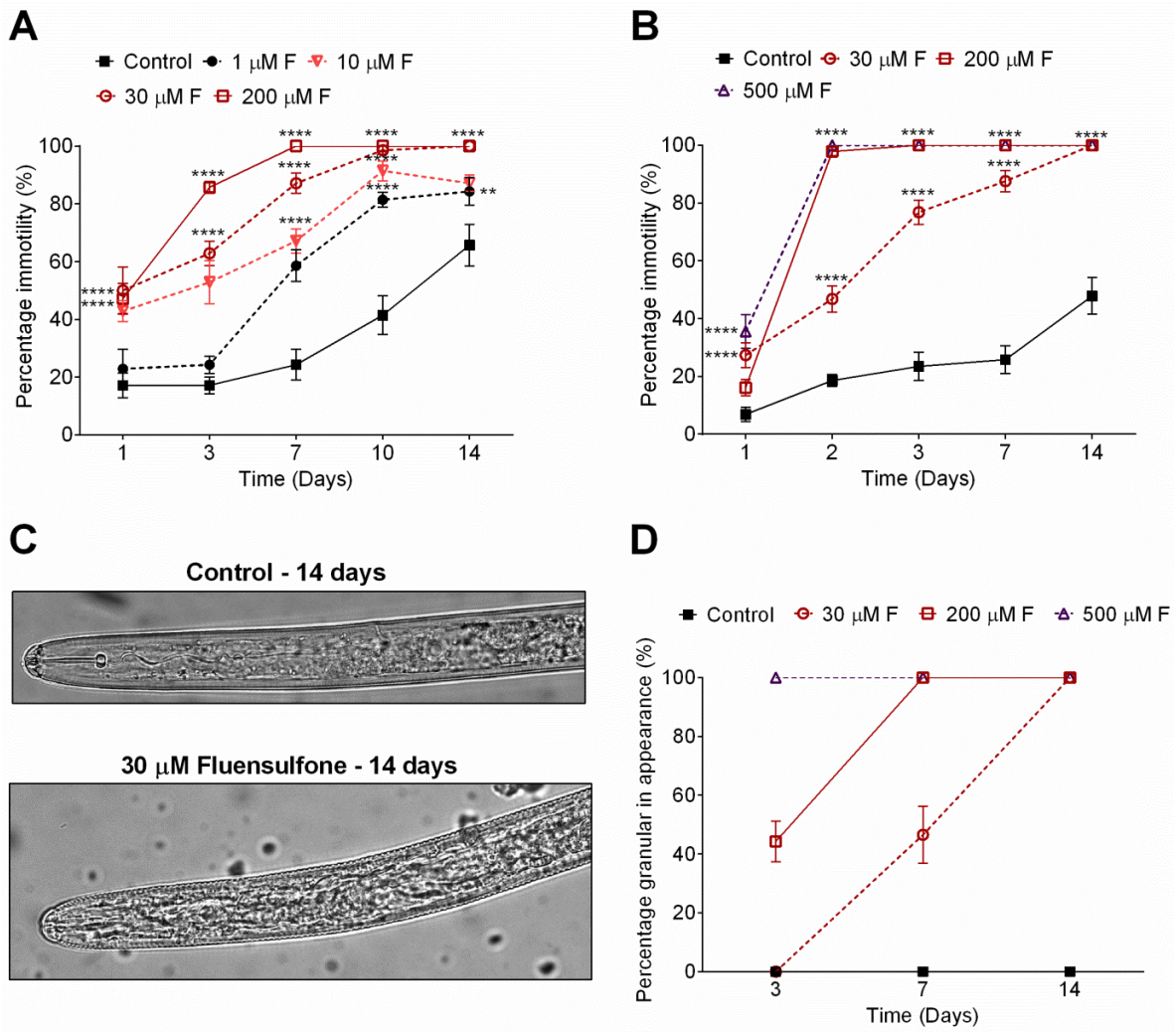

Figure 5.4. Prolonged exposure of J2 *G. pallida* to fluensulfone induces a progressive increase in paralysis, which is followed by death. J2 juveniles were exposed to fluensulfone in ddH_2O in 2 separate experiments (**A**+**B**) and scored for immotility up to 14 days. J2s that were rod-shaped and failed to move were deemed immotile. For both the first (**A**) and second (**B**) repeats worms from different groups of cysts were soaked in 7 dishes with around 10 worms per dish (mean % immotility ± s.e mean, two-way ANOVA with Bonferroni post-hoc tests, P<0.0001). **C**+**D**) Immotility in the presence of ≥30μM fluensulfone was followed by a darkening of appearance and the apparent loss of integrity and structure of internal organs, with worms appearing "granular" (**C**). No J2s in the control treatment group appeared granular after 14 days whereas there was a progressive increase in the number of J2s that were granular in appearance in the presence of ≥30μM fluensulfone (**D**). Granular appearance was quantified by visual scoring in the first (**A**) experiment (mean ± s.e mean from 7 dishes with ~10 worms, two-way ANOVA with Bonferroni post-hoc tests P<0.0001).

In the experiments described in section 5.2.1, it became clear that with protracted exposure to fluensulfone there was an increase in the number of "granular" worms and this appearance indicates worm death. For the experiment shown in Figure 5.4B, the number of worms "granular" in appearance was also scored, as a means of identifying the stage at which the worms progressed from paralysis to death. Worms that failed to move, were rod-shaped and were darkened in appearance with indistinguishable internal

organs were deemed granular and therefore dead (Figure 5.4C). No granular J2s were observed in any of the treatment groups at 1 and 2 days exposure (Figure 5.4D). All J2s were granular at 3 days in the 500 µM treatment group. At 3 days around 40% of worms treated with 200 µM fluensulfone were granular in appearance and 100% were granular at 7 days exposure. No worms appeared granular at 3 days exposure to 30 µM though this increased with time, with around 40% of worms granular at 7 days and 100% at 14 days. No worms that were granular in appearance were observed throughout the experiment in the control treatment group.

The results from these experiments suggest that the effects of fluensulfone motility and morphology are concentration-dependent and that the time course of these effects varies with concentration. ≥200 µM fluensulfone has acute behavioural effects on J2 *G. pallida*, including the induction of a coiled body posture, altered movement and stylet thrusting (see chapter 4), culminating in paralysis and death at 2-3 days. On the other hand, concentrations of 30 µM and less have no obvious effects on behaviour at <24 hours but induce a slower progressive increase in paralysis, reaching full paralysis and death at 10-14 days exposure. These results further suggest that *G. pallida* are susceptible to fluensulfone at similar concentrations to *M. javanica* yet a longer exposure period is necessary to achieve *G. pallida* mortality.

5.2.3 Lower concentrations of fluensulfone are not nematicidal against *C. elegans* adults with protracted exposure

These results indicate there may be a considerable difference between the concentrations required to elicit immotility and death in different species of nematode (see chapter 3 – introduction). ≥1.7 µM fluensulfone elicits near-100% immotility in *M. javanica* with 48 hours exposure and 10-30 µM fluensulfone has nematicidal activity against *G. pallida* with 7-10 days exposure (Figure 5.4). In contrast, 1 mM fluensulfone is nematicidal against *C. elegans* with 24 hours exposure (Kearn et al., 2014) (see chapter 3 – introduction). ≤100 µM fluensulfone is not nematicidal towards *C. elegans* with 24 hours exposure. ≤100 µM may have nematicidal activity on *C. elegans* with more protracted exposure. To test this directly, *C. elegans* were subjected to a protocol that allowed them to be exposed to fluensulfone for a longer period, to determine if this species is susceptible to the nematicidal effects of lower concentrations.

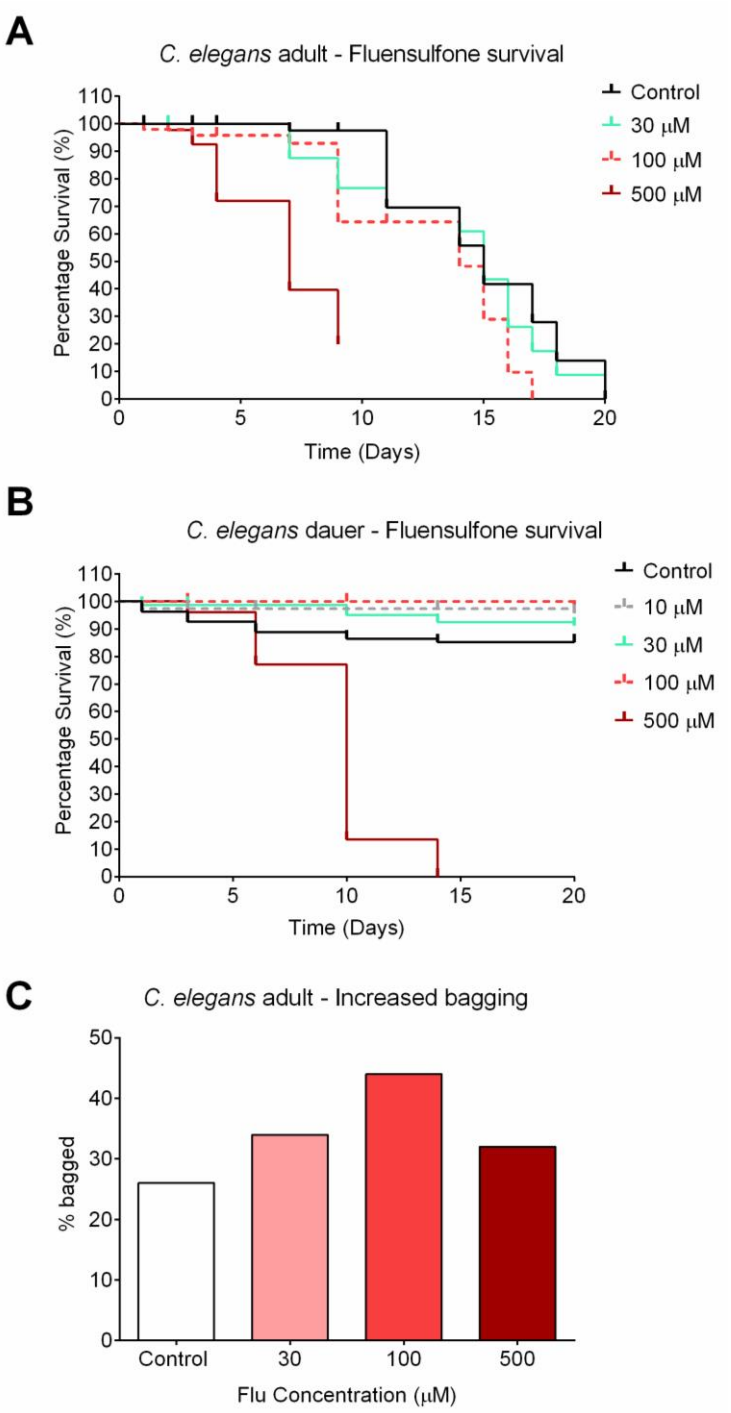

Figure 5.5. Fluensulfone does not significantly affect adult or dauer *C. elegans* survival or lifespan at concentrations comparable to those effective against *G. pallida*. **A**) L4+1 day *C. elegans* were picked onto OP50-seeded plates modified with either vehicle (control) or fluensulfone (flu) and scored for death by prodding. Constant exposure of adult *C. elegans* to 30 µM or 100 µM fluensulfone when on food did not have any effect on survival. 500 µM fluensulfone did reduce lifespan and did affect survival ($P<0.0001$) (data represent 1 experiment with 50 worms per treatment, log-rank Mantel-Cox test, $P=0.21$). **B**) Dauer *C. elegans* were transferred to petri dishes containing M9 buffer in the presence of either vehicle (control) or fluensulfone (flu) and scored for death. Only 500 µM fluensulfone had any effect on mortality (data represent 1 experiment with 67-82 worms, log rank Mantel-Cox test). **C**) The number of worms that bagged for each treatment in experiment A. A greater percentage of worms bagged in the presence of 30 and 100 µM fluensulfone than vehicle (% from one experiment shown).

The effects of fluensulfone on *C. elegans* lifespan and mortality were investigated using the established solid-media lifespan assay (Mulcahy et al., 2013), where *C. elegans* L4+1 day adults were grown on agar plates seeded with OP50 *E. coli*, with fluensulfone or vehicle (control) dissolved into the agar and time of death of individual worms was recorded (Figure 5.5A). Any worms that were immotile and did not exhibit any pharyngeal pumping and failed to respond to prodding were deemed dead.

In the presence of vehicle alone, significant death only began after 11 days and all worms were dead after 20 days (Figure 5.5A). In the worms treated with 500 µM fluensulfone worms began dying after 3 days, and no worms survived beyond 9 days. Concurrent with the mortality seen at 3 days, >30% of worms bagged and were therefore censored from the experiment. Bagged worms were not included in the death count and were censored (Figure 5.5C). In contrast, the survival curves obtained for worms exposed to 30 and 100 µM fluensulfone were not significantly different from the vehicle control. A slight increase in bagging behaviour occurred in worms exposed to 30 and 100 µM fluensulfone relative to those exposed to vehicle alone, however. This suggests that fluensulfone may be having some effect on *C. elegans* at concentrations that are nematicidal to *G. pallida* and other PPNs (Oka et al., 2009). This increase in bagging is not however comparable to the profound immobilising and nematicidal effects of ≤30 µM fluensulfone on *G. pallida*. Exposure to 30 µM for ≥7 days results in complete immotility of *G. pallida*, as compared to 20-30% immotility in the control group (Figure 5.4).

5.2.4 Lower concentrations of fluensulfone are not nematicidal against *C. elegans* dauers with protracted exposure

There are a number of differences in the biology of *G. pallida* J2s and *C. elegans* (see General Introduction). The infective J2 stage of sedentary endoparasitic PPNs is non-feeding and relatively metabolically inactive and has therefore been compared to the *C. elegans* dauer stage (Bird and Opperman, 1998). As such, the *C. elegans* dauer stage is arguably a better comparison for the sedentary endoparasitic J2 stage of PPNs than either *C. elegans* adults or L2 larvae. *C. elegans* dauers are unaffected by extended incubation in liquid media in the absence of food and do not produce progeny and as such mortality can be assayed via similar means to those used for *G. pallida*.

Dauers were soaked in fluensulfone in M9 buffer and lifespan and mortality were scored (Figure 5.5B). Dauers exhibit little spontaneous movement and so were prodded to determine whether they were dead or alive. Live dauers rapidly swam away on being vigorously prodded whereas dauers that did not move and were in a rod-shaped posture were defined as dead. In the presence of vehicle alone, very few dauers died throughout the course of the experiment, with 85% still alive after 20 days. The survival curves of dauers in the presence of 10, 30 and 100 µM fluensulfone did not differ from the vehicle control, with few worms dying over 20 days. Only 500 µM fluensulfone caused a reduction in survival, with a large number of deaths occurring at 10 days and 100% mortality at 14 days. It appears that *C. elegans* dauers have a similar susceptibility to the nematicidal effect of fluensulfone as *C. elegans* adults, and that *C. elegans* of all stages are less sensitive to fluensulfone than *G. pallida*.

5.2.5 Fluensulfone affects *G. pallida* metabolism prior to its nematicidal effects

As shown above, protracted exposure to 30 µM fluensulfone elicits a gradual increase in immotility, followed by death, as evidenced by the granular appearance that becomes prevalent (see section 5.2.2, Figure 5.4D). It was unclear however when death occurred, as granular appearance, and the associated disintegration of internal organs, presumably occurs some time after death.

To further understand the process leading to death, the tetrazolium dye MTT was utilised (James and Davey, 2007). MTT can be used to determine mortality and can indicate potential metabolic effects (see 5.2 introduction) (Figure 5.6A). *G. pallida* J2s were incubated in fluensulfone in ddH_2O or a control solution in vehicle for up to 14 days and worms were removed throughout this period and stained with MTT for 24 hours. The percentage of worms stained was scored, as was the distribution of the staining. This was scored using a dissecting microscope at X45 magnification. Typically, the majority of untreated J2s soaked in MTT for 24 hours are strongly stained purple in the head region, with fewer staining at the posterior (Figure 5.6B). Some worms however were seen to stain predominately in the tail whilst some showed strong purple staining throughout the worm. Some worms showed faint spots of purple staining not strongly localised to any region and these were defined as weakly stained.

In the first experiment, worms were soaked in a concentration range from 30-500 µM fluensulfone, with the carbamate aldicarb as a comparison (Figure 5.6B). 30-500 µM fluensulfone was tested to permit comparison between the higher concentrations (200 & 500 µM) that have overt acute effects on *G. pallida* and are nematicidal at 2-3 days, and a lower concentration (30 µM) that is nematicidal with chronic exposure for 7-14 days (Figure 5.4). Aldicarb was used to allow comparison with a previously used chemical control for PPNs that acts as a nematostatic and is not nematicidal (Haydock et al., 2014). In the second experiment, the concentration range was expanded to include lower concentrations of fluensulfone that do not appear to achieve complete mortality, even at 14 days exposure (1 & 10 µM). Fluopyram, a recently unveiled nematicide, was also used as a comparison with fluensulfone (Figure 5.6C). Fluopyram is a mitochondrial inhibitor, and acts through interaction with succinate dehydrogenase, which is the enzyme complex constituting complex II of the electron transport chain in mitochondria (Veloukas and Karaoglanidis, 2012). Inhibition of succinate dehydrogenase limits mitochondrial potential for generating ATP. If fluopyram inhibits mitochondrial function in *G. pallida* it might be expected that fluopyram treatment would rapidly reduce MTT staining.

Throughout the experiment, nearly 100% of control treated worms exhibited purple MTT staining, with >95% of worms still stained after 14 days. In contrast, exposure to the highest concentration of fluensulfone tested, 500 µM, resulted in a substantial reduction in the percentage of worms showing clear MTT staining with 24 hours exposure, with only 35% of worms showing any staining as compared to the 99% that stained in the control treatment group (Figure 5.6D). This suggests that metabolic impairment occurs in tandem with the increase in immotility. After 3 days exposure to 500 µM 0% of worms stained, indicating full metabolic impairment and death. 500 µM results in 100% paralysis of treated J2s after 2 days (Figure 5.6B) yet some of these worms are still staining and thus showing metabolic activity, and are not dead. 200 µM fluensulfone had a similar effect on MTT staining to 500 µM, with some reduction in the number of worms staining after 1 day exposure in one experiment and almost no observable staining seen after 3 days.

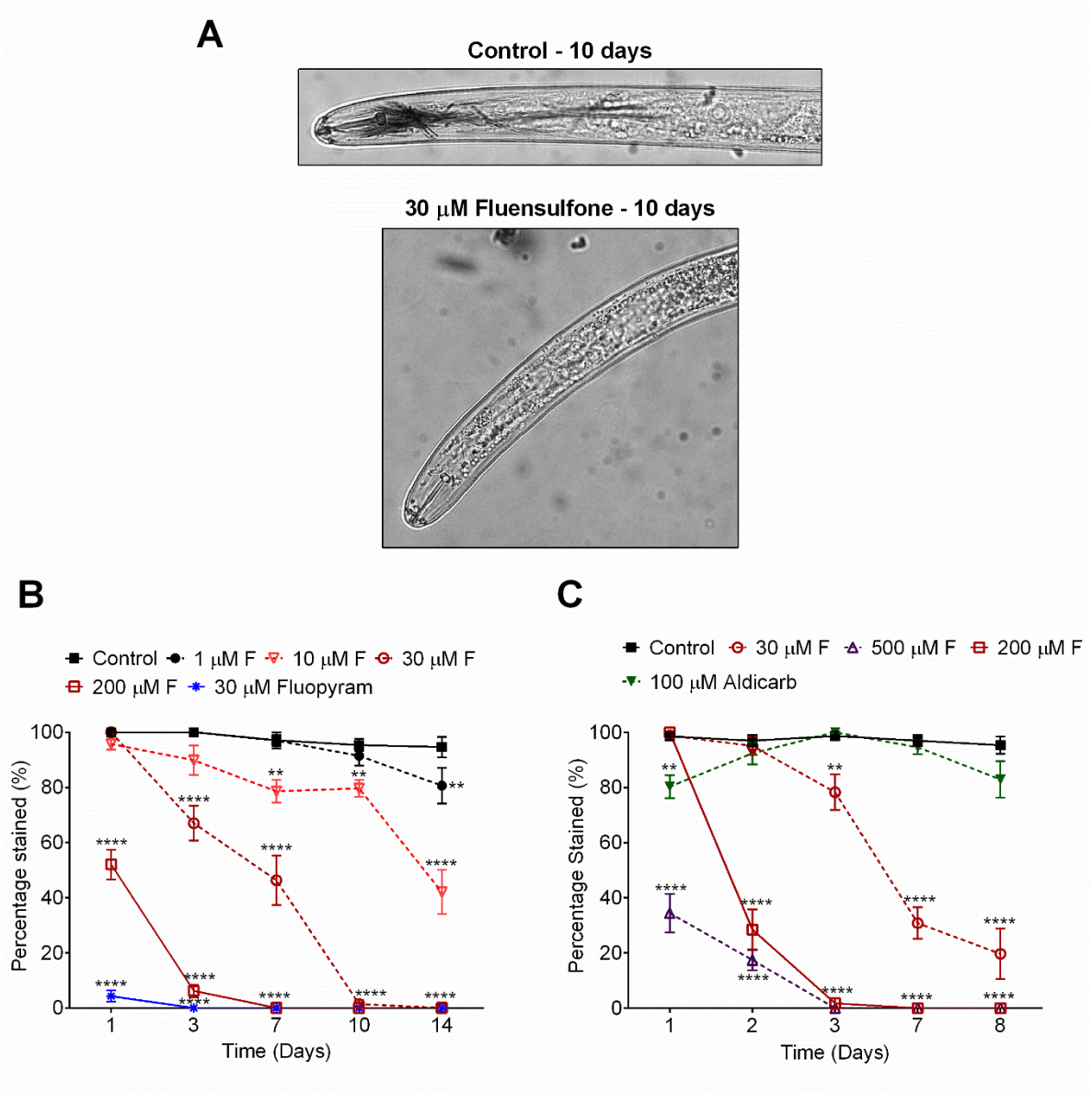

Figure 5.6. Prolonged exposure to fluensulfone causes a progressive reduction in the number of *G. pallida* that stain in the presence of MTT. A+ B+C) In 2 separate experiments J2 *G. pallida* were soaked in fluensulfone, aldicarb or fluopyram in ddH_2O for up to 14 days and were subsequently moved, washed and transferred to wells containing MTT solution. After staining, worms were visually scored for staining. The control-treated worms persistently stained, predominately around the anterior region up to 14 days (B), whereas there was a progressive decrease in the number of worms that stained in the presence of fluensulfone. Fluopyram induced a rapid loss of staining and aldicarb had no effect on the proportion of stained worms relative to the control. (For both experiments worms from different batches of cysts were soaked in 7 dishes and around 10 worms were removed from each dish for staining at each time point, mean ± s.e mean, two-way ANOVA with Bonferroni post-hoc tests, $P<0.0001$).

Exposure to 30 µM fluensulfone had no effect on the percentage of worms with observable MTT staining after 1 or 2 days exposure in both experiments (Figure 5.6C+D). In the two experiments, at 3 days 20-30% of juveniles treated with 30 µM had no visible staining and with longer exposure there was a progressive decrease

in the percentage of worms with MTT staining. After 10 days exposure to 30 µM staining was almost completely absent and no staining was observed at 14 days (Figure 5.6B+C). This reduction in the percentage of total worms staining appears closely tied to the increase in percentage immotility that occurs in the presence of fluensulfone (Figure 5.4).

A smaller reduction in the percentage of worms showing clear MTT staining was also observed in worms treated with 10 µM fluensulfone, at 7 and 10 days, with a 15% and 18% reduction in staining relative to the control, respectively. There was a sharp drop in percentage staining between 10 and 14 days, with only 42% of worms treated with 10 µM staining at 14 days compared to 95% of control-treated worms. This suggests that longer exposure to 10 µM fluensulfone could result in a similar endpoint to 30 µM, with complete immotility and death. A significant, 14% reduction in percentage staining also occurred after 14 days exposure to 1 µM fluensulfone, relative to the vehicle control.

It is interesting that nearly 100% of juveniles treated with vehicle alone still stained up to 14 days, despite the increasing percentage immotility that occurred (Figure 5.4A). Indeed, in a separate experiment 75% of juveniles still stained after 21 days in a vehicle control solution, despite 75% of the worms being immotile (data not shown). This implies that even at 1 µM, fluensulfone treatment reduces *G. pallida* metabolism. This also shows that despite the increasing immotility with time that occurs in the non-feeding J2s subsequent to hatching, the majority are still alive and metabolically active. The reduction in staining at 21 days does however suggest that J2s begin to lose metabolic activity and may also begin dying, probably as a result of near-complete depletion of lipid reserves (Storey, 1984).

J2s were also exposed to 100 µM aldicarb as a comparison (Figure 5.6C). Overall, no reduction in MTT staining occurred as a result of exposure to aldicarb and percentage staining did not differ from the control even at 8 days. This confirms that the carbamate aldicarb is nematostatic rather than nematicidal and highlights the distinct nematicidal effects of fluensulfone on PPNs relative to currently used chemical controls. The lack of death or compromised metabolic capability in aldicarb-exposed worms also implies a potential for worm recovery on removal of the drug that is not seen in worms exposed to fluensulfone.

Treatment with 30 µM fluopyram induced near-100% paralysis within 1 day (data not shown) and almost completely prevented MTT staining at 24 hours. No staining was observed in fluopyram-treated *G. pallida* after 2 days exposure (Figure 5.6B). This indicates that metabolic inhibition does indeed elicit a reduction in MTT staining. Fluopyram prevented MTT staining and induced paralysis over a shorter time course than all doses of fluensulfone tested, which may indicate that these compounds act through distinct targets.

5.2.6 Fluensulfone treatment induces a spatial shift in the pattern of MTT staining

It was noted that fluensulfone treatment appeared to cause a shift in the distribution of staining from predominately in the head region in control- and aldicarb-treated worms to the tail region after fluensulfone treatment. To examine this further, after staining in MTT, the pattern of staining in each individual worm was visually scored as either throughout, anterior, posterior, weakly or unstained. The term "throughout" described strong staining both in the head and tail regions, whilst worms classed as "anterior" or "posterior" were stained strongly and exclusively in the head and tail regions, respectively. Worms described as weakly stained showed some faint purple spots or colouring that was not strongly localised to any region, whilst unstained worms had no apparent staining. These categorisations were given after observation through a dissecting microscope at X45 magnification.

Worms in the vehicle control solution stained mainly in the head region up to 10 days, with 91% of worms showing strong anterior staining after 1 day in the control solution and 80% staining in the head after 10 days (Figure 5.7). The proportion of worms staining in the anterior region dropped however at 14 days, with only 44% staining in the anterior region whereas 48% were deemed weakly stained (data not shown). It is possible that this reflects weakened metabolic activity in the worms resulting from the increased depletion of lipid stores with time after hatching.

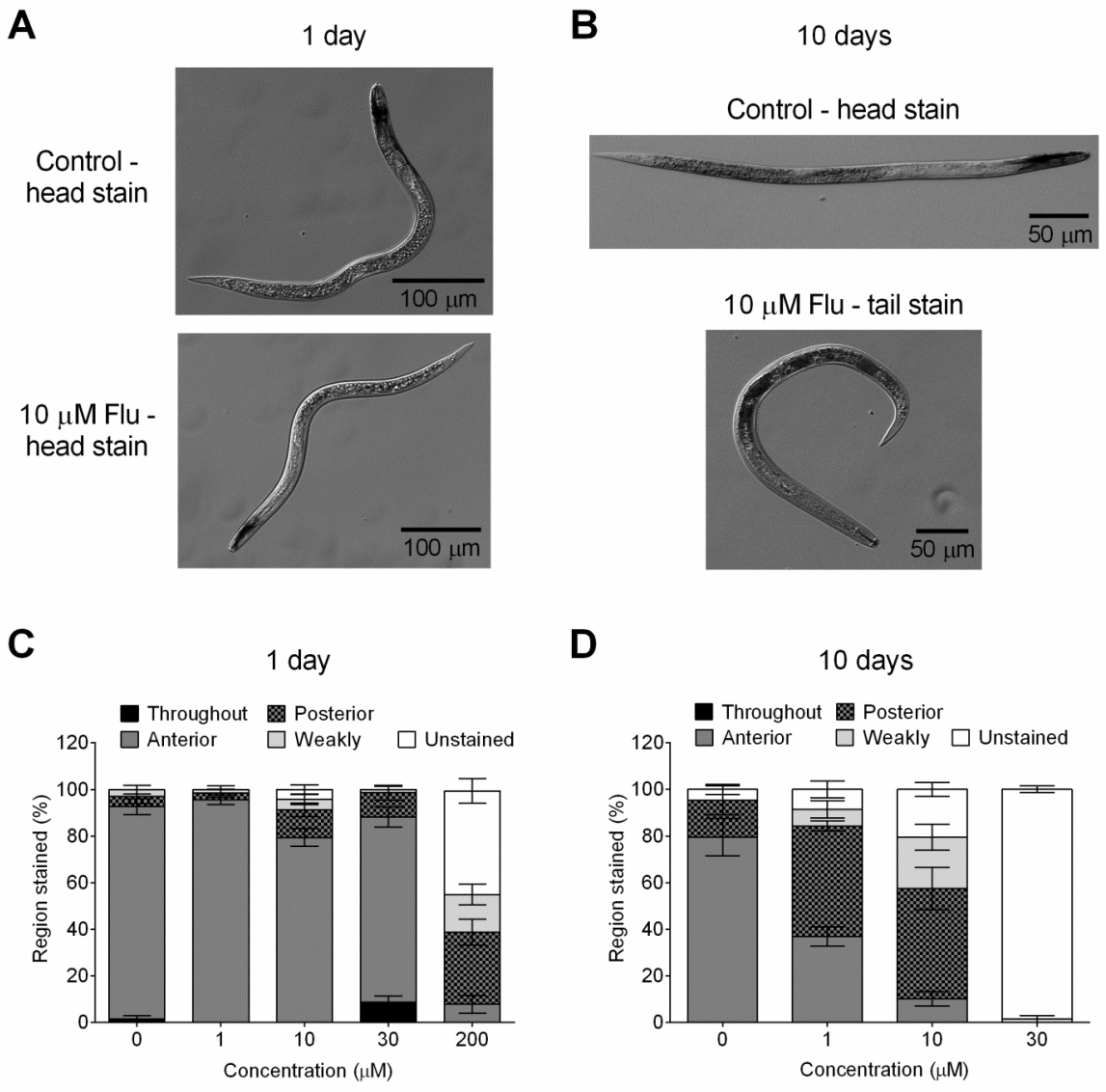

Figure 5.7. Prolonged exposure of *G. pallida* to fluensulfone induces a shift in the distribution of MTT staining from the head region to the tail as a prelude to complete loss of staining. A+**C**) 24 hrs exposure to 1-30 µM fluensulfone does not alter the pattern of MTT staining. J2 *G. pallida* exposed to both 10 and 30 µM fluensulfone still stain predominately in the head region like those in the control group (0 µM). A greater percentage of worms treated with 200 µM fluensulfone stain in the tail relative to the control (n=7 groups of worms from 1 experiment, mean ± s.e mean, one-way ANOVA with Dunnett post-hoc tests, P<0.0001). **B**+**D**) At 10 days exposure, whilst control worms still stain in the head region, those treated with 10 µM fluensulfone exhibit a shift in staining to the tail region whilst those treated with 30 µM fluensulfone show a lack of staining (n=7 groups of worms from 1 experiment, mean ± s.e mean, one-way ANOVA with Dunnett post-hoc tests, P<0.0001).

Treatment with 1, 10 and 30 µM fluensulfone for 1 day had no effect on the distribution of staining, with the majority of worms staining in the head region as with worms treated with vehicle alone (Figure 5.7A,C). Whilst 1 day exposure to 200 µM fluensulfone reduced the overall percentage of worms that stained to 55%, of the worms that did show staining, 56% were exclusively stained in the tail. Treatment with 1 and

10 µM fluensulfone for 10 days also resulted in a shift in staining from the head to the tail (Figure **5.7**B, D). Of the worms that did stain after treatment with 1 and 10 µM fluensulfone, 52% and 60% stained in the tail, respectively, as compared to 17% in the control treatment group. This shift in staining to the tail was also seen at 7 days exposure (data not shown). Interestingly, staining did not shift from the head to the tail at any stage of treatment with the control solution. These results indicate a general trend of MTT staining shifting from the anterior of the worm to the posterior after treatment with fluensulfone. The results also suggest that the shift in staining to the tail precedes the overall loss of staining that is a consequence of prolonged fluensulfone treatment.

Treatment with 100 µM aldicarb did not induce any significant shift in the pattern of staining when compared to the control and did not result in weakened staining (Figure 5.8).

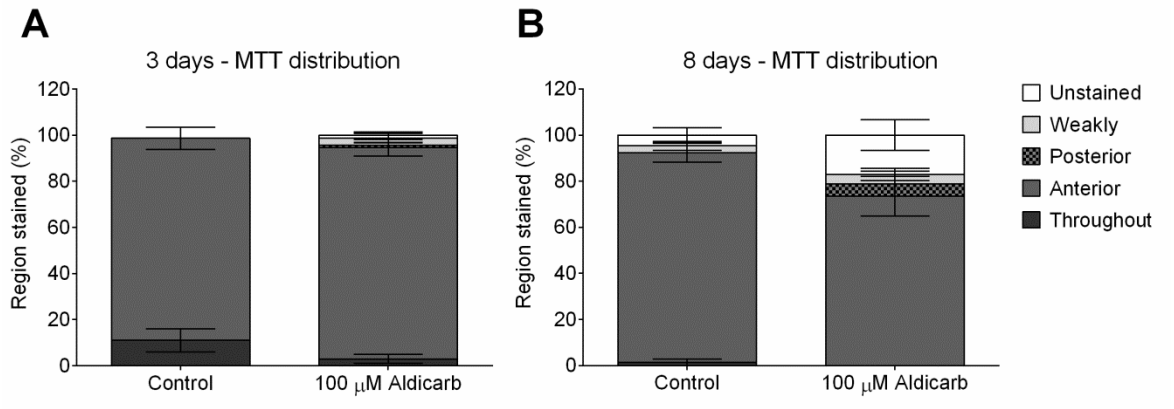

Figure 5.8. Prolonged exposure to aldicarb does not affect the distribution of MTT staining. J2 *G. pallida* were soaked in a control solution (ddH_2O) or 100 µM aldicarb. Worms were removed at 3 days (**A**) and 9 days (**B**), stained with MTT and the distribution of staining was noted. Aldicarb did not alter the distribution of staining at either 3 days or 8 days relative to the control (for A+B n=7 groups of worms from 1 experiment, mean ± s.e mean, student's T-test, P=0.34 for A+B).

5.2.7 The low concentration nematicidal effects of fluensulfone result from interaction with a distinct target from that which mediates the effects of fluensulfone on the stylet

The data presented in chapter 4 (see sections 4.2.11, 4.2.12, 4.2.13) indicate that the effects of high concentrations of fluensulfone on the *G. pallida* stylet and *C. elegans* pharynx result from interaction with serotonergic signalling pathways, as exemplified by the inhibition of fluensulfone-stimulated stylet activity by methiothepin.

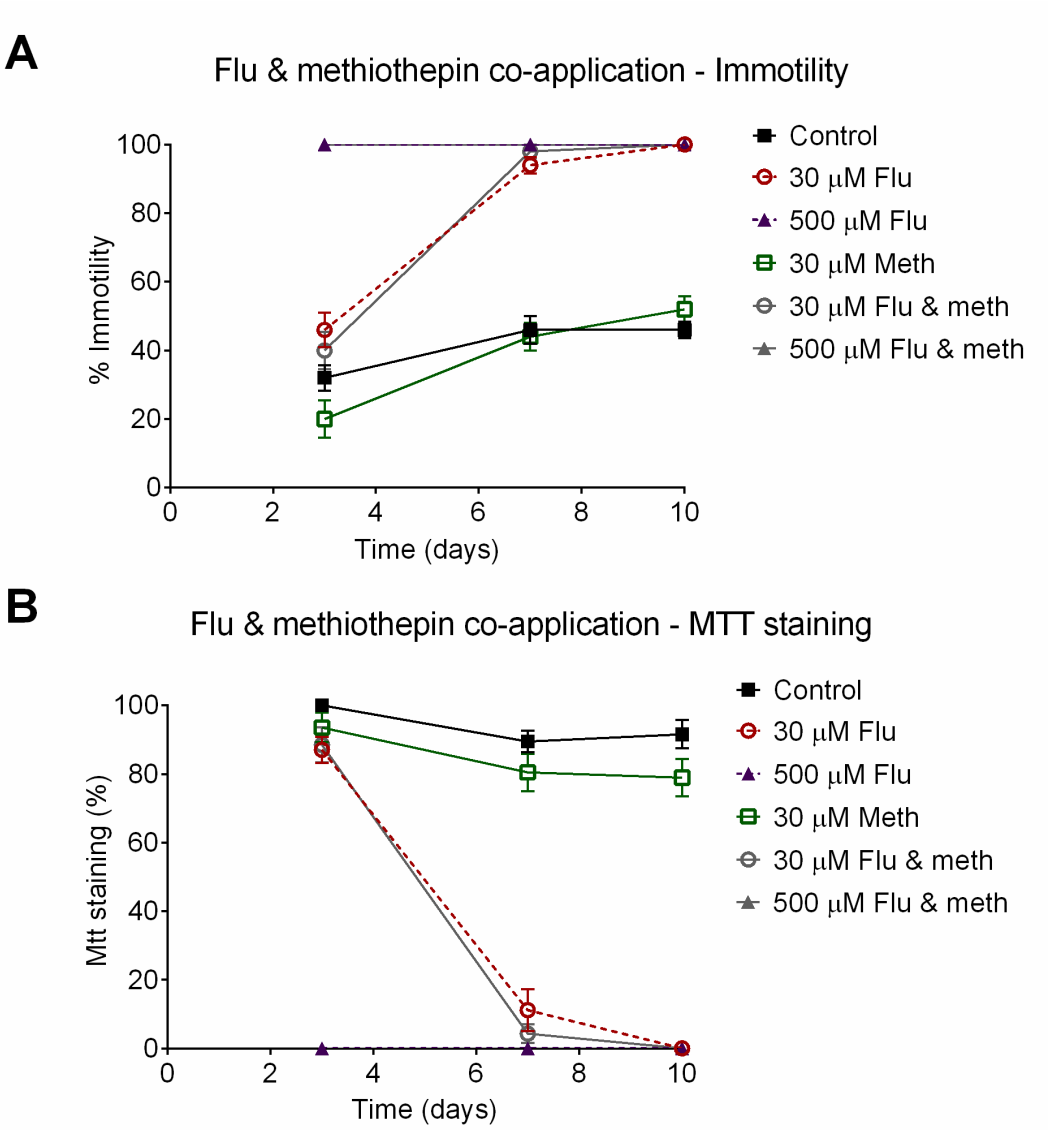

Figure 5.9. Methiothepin does not block the nematicidal activity of fluensulfone against *G. pallida*. J2s were exposed to the indicated concentrations of fluensulfone in the presence and absence of 30 µM methiothepin and were scored for immotility and subsequently stained with MTT. **A**) There was no difference in percentage immotility between J2s exposed to 30 and 500 µM fluensulfone relative to worms exposed to fluensulfone in the presence of methiothepin (mean % immotility ± s.e mean, n=5 dishes with ~10 worms per dish, two-way ANOVA with Tukey post-hoc tests, P>0.05). **B**) There was no difference in percentage exhibiting MTT staining between J2s exposed to 30 and 500 µM fluensulfone relative to worms exposed to fluensulfone in the presence of methiothepin (mean % showing MTT staining ± s.e mean, n=5 dishes with ~10 worms per dish, two-way ANOVA with Tukey post-hoc tests, P>0.05).

Here, methiothepin was used to assess whether the nematicidal activity of fluensulfone against *G. pallida* is dependent on serotonergic signalling. J2 *G. pallida* were exposed to 30 µM and 500 µM fluensulfone and immotility and MTT staining were scored and compared to J2s exposed to 30 µM and 500 µM fluensulfone in the presence of 30 µM methiothepin (Figure 5.9). 30 µM was found to block fluensulfone stimulated stylet activity (see section 4.2.12). The presence of methiothepin did not alter the time course

or level of immotility observed in the presence of either 30 µM or 500 µM methiothepin. The presence of methiothepin also did not alter the time course of the reduction of MTT staining observed in the presence of either 30 µM or 500 µM methiothepin. This indicates that whilst the effects of fluensulfone on the stylet have a serotonergic component, the nematicidal activity is not due to manipulation of 5-HT signalling.

5.2.8 Nile red staining indicates that fluensulfone blocks *G. pallida* lipid consumption

The reduction of MTT staining suggests that fluensulfone affects *G. pallida* metabolism and the shift in the distribution of staining may reflect shifts in regional metabolic activity. The shift in staining to the tail region suggests a shift in metabolic activity to the tail of the worm. The tail is the principle location for lipid stores in *G. pallida* and other PPNs juveniles (*personal communication, Justyna Pertek*). Furthermore, in a previous study it was noted that that *M. javanica* treated in a control solution for 5 days exhibit depleted body contents whereas those treated with fluensulfone do not show this depletion (Oka et al., 2009). This may mean that *M. javanica* in the control solution deplete their lipid reserves whilst those treated with fluensulfone do not. It was therefore hypothesised that the shift in staining could indicate alterations in *G. pallida* lipid metabolism.

To address this, a modified Nile red lipid staining technique was utilised as a means of determining worm lipid content and how this may be affected by fluensulfone treatment (Pono et al., 2013). This technique has been used in *C. elegans* yet has not been used with PPNs. Therefore, the technique was tested with starved and freshly hatched J2 *G. pallida* to verify it as a means of detecting lipid content (Figure 5.10). Two groups of worms were stained: freshly hatched (<24 hours) worms and worms starved for 14 days in ddH_2O. The Nile red fluorescence was predominately in the tail region (Figure 5.10A), which is consistent with previous results using CARS (coherent anti-strokes Raman spectroscopy) (*personal communication, Justyna Pertek*). The intensity of Nile red fluorescence was reduced by >60% in starved worms relative to freshly hatched worms (Figure 5.10B), indicating that the staining technique is capable of detecting changes in the internal lipid content of *G. pallida*.

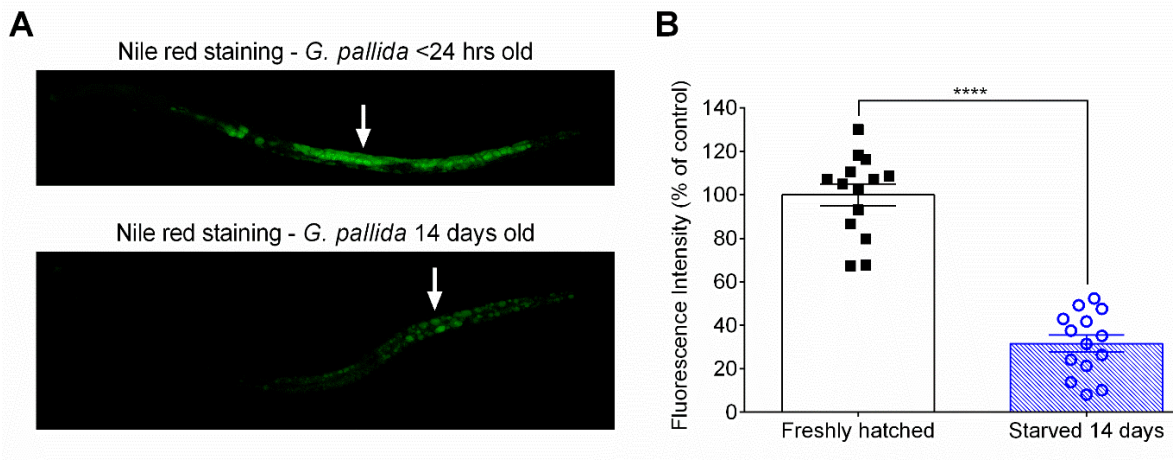

Figure 5.10. The lipophilic dye Nile Red can potentially detect changes in *G. pallida* lipid content. To assess the potential for using Nile Red and a protocol modified from (Pino et al., 2013) as a means for detecting changes in the lipid content of *G. pallida* J2s, 2 groups of worms were stained: worms hatched within 24 hrs and worms starved for 14 days in ddH_2O. Worms from both groups were imaged under a FITC filter with the same exposure time and microscope settings and fluorescence intensity was quantified using ImageJ software. **A**) Representative images of freshly hatched and starved J2s stained with Nile Red and visualised under a FITC filter. The white arrows indicate putative lipids droplets. **B**) Starved worms showed a reduction in fluorescence intensity relative to freshly hatched worms (n=14 worms, data were normalised as a percentage of the control (freshly hatched), individual data points and mean ± s.e mean shown, unpaired student's T-test, P<0.0001).

After the above experiment, which suggested that this Nile red staining technique may detect changes in lipid content, the technique was used to map the stainable content of *G. pallida* J2s treated with fluensulfone (Figure 5.11). In the Nile red experiments, worms were soaked in ddH_2O in the presence of vehicle (control, 0.5% acetone), 10 µM fluensulfone, 30 µM fluensulfone, 10 mM 5-HT or 30 µM aldicarb. Worms treated with vehicle, 30 µM fluensulfone, 5-HT and aldicarb were removed at 5 days to stain for lipids. Worms treated with vehicle, 10 µM fluensulfone, 5-HT and aldicarb were also stained at 10 days. Worms treated with 30 µM fluensulfone were not stained at 10 days due to the high mortality that occurs with this treatment (Figure 5.4). 5-HT has been found to be an important regulator of β-oxidation of lipids in *C. elegans* (Srinivasan et al., 2008, Noble et al., 2013) and therefore J2s were incubated in 10 mM 5-HT to determine if it plays a similar role in *G. pallida*. It would be predicted that 5-HT would increase lipid metabolism if it plays a similar role in *G. pallida*. Cholinesterase inhibitors, such as oxamyl, have been found to reduce lipid consumption in PPNs by reducing movement and thus conserving energy and so aldicarb provided a useful comparison for fluensulfone (Wright et al., 1989).

In both experiments, treatment with 30 µM fluensulfone for 5 days had no effect on *G. pallida* lipid content relative to the vehicle control (Figure 5.11A,C). Treatment with 10 µM fluensulfone for 10 days however resulted in a reduced reduction of fluorescence intensity, relative to the vehicle control as fluorescence intensity was 50% higher in worms treated with 10 µM fluensulfone relative to the control. This indicates that treatment with 10 µM fluensulfone results in elevated lipid levels relative to control-treated worms and suggests that lipid consumption is reduced in the presence of fluensulfone.

Aldicarb had no effect on *G. pallida* lipid content relative to the control, contrary to what has been reported in the literature for other cholinesterase inhibitors (Wright et al., 1989). Lipid content in J2s treated with 10 µM fluensulfone for 10 days was also significantly higher than lipid content in aldicarb-treated worms. Fluorescence intensity was reduced after 5 days treatment with 10 mM 5-HT relative to control-treated worms yet 10 day treatment with 5-HT had no effect on the lipid content of the J2s.

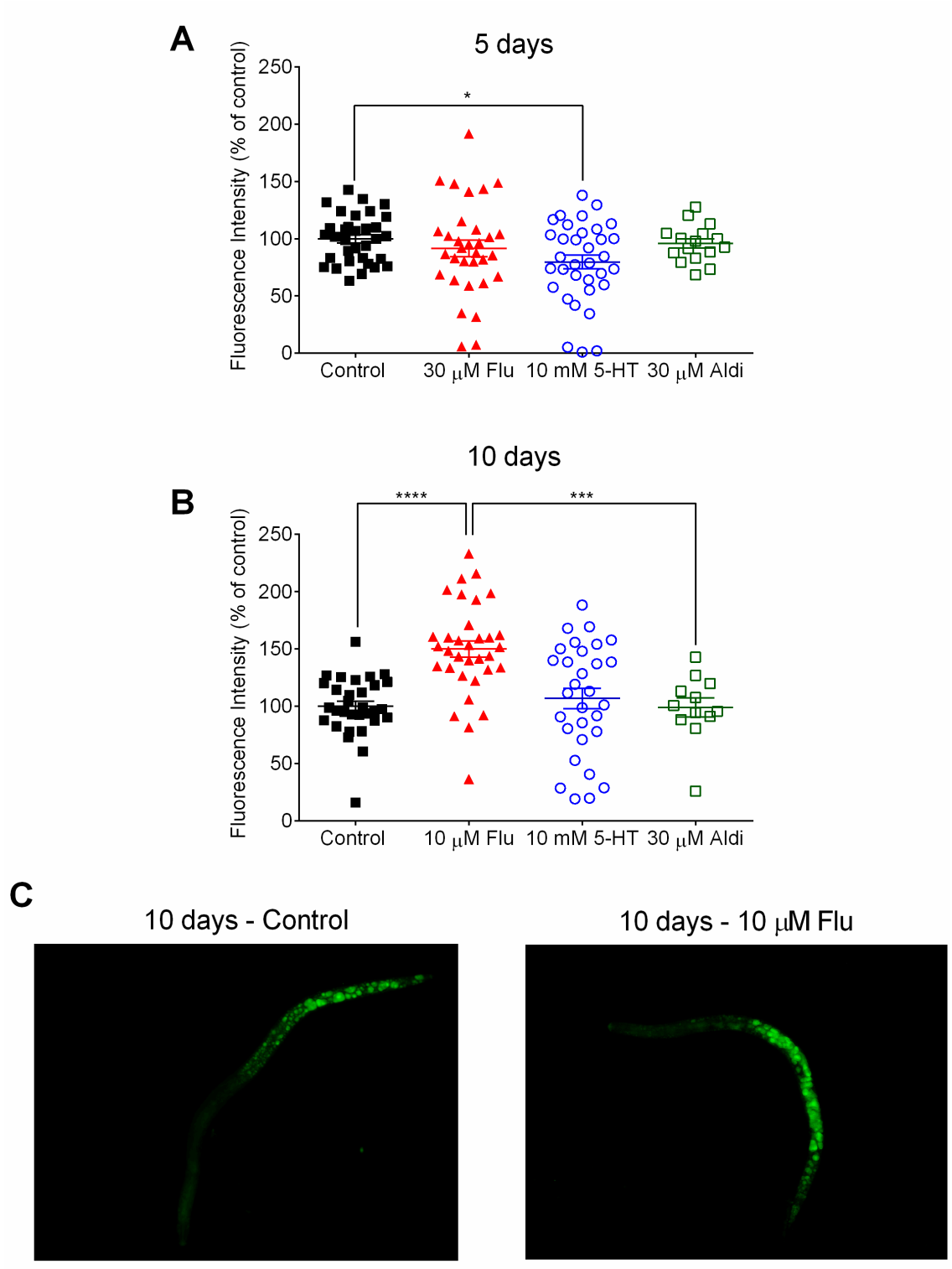

Figure 5.11. Prolonged treatment with fluensulfone results in elevated lipid levels. J2 *G. pallida* were soaked in either ddH_2O (control), 10 µM fluensulfone, 30 µM fluensulfone, 10 mM 5-HT or 30 µM aldicarb for up to 10 days. At 5 days (**A**) and 10 days (**B**), worms were removed from the drug solutions, washed, fixed and then stained with Nile Red to assess lipid content. At 10 days worms soaked in 10 µM fluensulfone had elevated lipid levels relative to the control group and worms treated with aldicarb (n=12-18 worms, data were normalised as a % of the control, individual data points and mean ± s.e mean shown, one-way ANOVA with Dunnett post-hoc tests, P<0.0001). **C**) Representative images of Nile red-stained control- and 10 µM fluensulfone-treated *G. pallida* at 10 days exposure. Images were taken under a FITC filter block.

5.2.9 The effects of fluensulfone on the hatching of *G. pallida* from cysts

The previous sections show that fluensulfone has effects on *G. pallida* motility and metabolic capability with protracted exposure at 1-30 µM (Figure 5.4, Figure 5.6). In the literature, it has been reported that fluensulfone can inhibit *M. javanica* egg hatching at comparable concentrations (Oka et al., 2009). The effects of fluensulfone on *G. pallida* cyst hatching were therefore investigated.

G. pallida cysts were soaked in a solution of 1 part PRD to 3 parts ddH_2O in the presence of either vehicle (0.5% acetone) or fluensulfone and hatching of J2s from the cysts was scored. It has been found that the half-life of fluensulfone in most soils is around 24-25 days (Norshie, 2014). Therefore, once 24 days had elapsed in the presence of vehicle or fluensulfone, the cysts were transferred to 1:3 PRD diffusate alone to assess the reversibility of any effects on hatching.

The results from three independent experiments are shown in Figure 5.12. In the first experiment only 24 ± 9 J2s had hatched from each vehicle-treated cyst after 24 days (Figure 5.12A). The rate of hatching increased however after removal from the presence of vehicle and transfer to 1:3 PRD alone, with the number hatched per cyst increasing to 63 ± 20 J2s, suggesting an inhibitory effect of acetone on hatching. No increase in the rate of hatching occurred after removal from the presence of vehicle in the second experiment however (Figure 5.12B). Between the three separate experiments, there was significant variability in the number of worms hatching per cyst in the control solution. For example, in the second experiment after 41 days 113 ± 29 J2s had hatched per cyst as compared to only 35 ± 15 J2s in the third experiment. It is clear therefore that hatching varies considerably between experiments, potentially because of differing populations of cysts and different batches of PRD. It is therefore difficult to make comparisons between different cyst hatching experiments. Within each experiment, there was also high variability in the number of juveniles hatching from different vehicle-treated cysts. In the third experiment for example, whilst 131 J2s hatched from one cyst in the control treatment group, just 1 J2 hatched from another cyst.

In the first experiment 500 µM fluensulfone completely inhibited *G. pallida* hatching from cysts and this inhibition was not reversible, with no hatching occurring on removal to PRD alone (Figure 5.12A). Indeed, eggs from cysts treated with 500 µM fluensulfone

were analysed via DIC microscopy and were found to be darkened in appearance relative to vehicle-treated cysts and the J2s inside appeared "granular" (Figure 5.14). This suggests that 500 µM fluensulfone is nematicidal to the J2 within the eggshell. In the same experiment, 30 µM fluensulfone also inhibited hatching relative to the vehicle control, with no worms hatching over a 24 day period, as compared to 24 J2s per vehicle-treated cyst. This inhibition was however partially reversible on transfer to 1:3 PRD alone, as worm hatching was initiated. At 72 days, cumulative hatch of cysts treated with 30 µM fluensulfone reached 52 worms per cyst, as compared to 106 worms per cyst in the control treatment group. At the termination of the experiment at 72 days worms were still hatching from cysts treated with 30 µM fluensulfone and so it is possible that cumulative hatch per cyst could recover to levels comparable to control-treated cysts if observed for longer. These observations were reproduced in a second experiment with greatly reduced hatching in the presence of 30 µM fluensulfone that was reversible on transfer away from drug, with hatching resuming. In the second experiment, 5 µM fluensulfone had a similar effect to 30 µM fluensulfone with near-complete inhibition of hatching whilst the drug was present and a recovery of hatching on removal to PRD alone.

In the third experiment, hatching was inhibited relative to the vehicle control by both 5 and 10 µM fluensulfone. No recovery of hatching on removal from drug occurred in the third experiment after treatment with 5 and 10 µM fluensulfone and cumulative hatch per cyst at 41 days reached 5 worms and 1 worm, respectively, as compared to 35 worms for control-treated cysts. Few J2s hatched from the control-treated cysts after transfer to PRD in the recovery stage, which may explain the lack of recovery seen after fluensulfone treatment (Figure 5.12C). 250 nM and 1 µM fluensulfone did not significantly reduce hatching relative to vehicle, although hatching was lower than the control in the presence of 1 µM. This indicates that the threshold for fluensulfone inhibition of hatching is around 1 µM. These results also suggest that the concentration-dependence of fluensulfone inhibition of *G. pallida* cyst hatching is similar to that seen for the inhibition of worm motility and the inhibition of MTT staining (Figure 5.4, Figure 5.6).

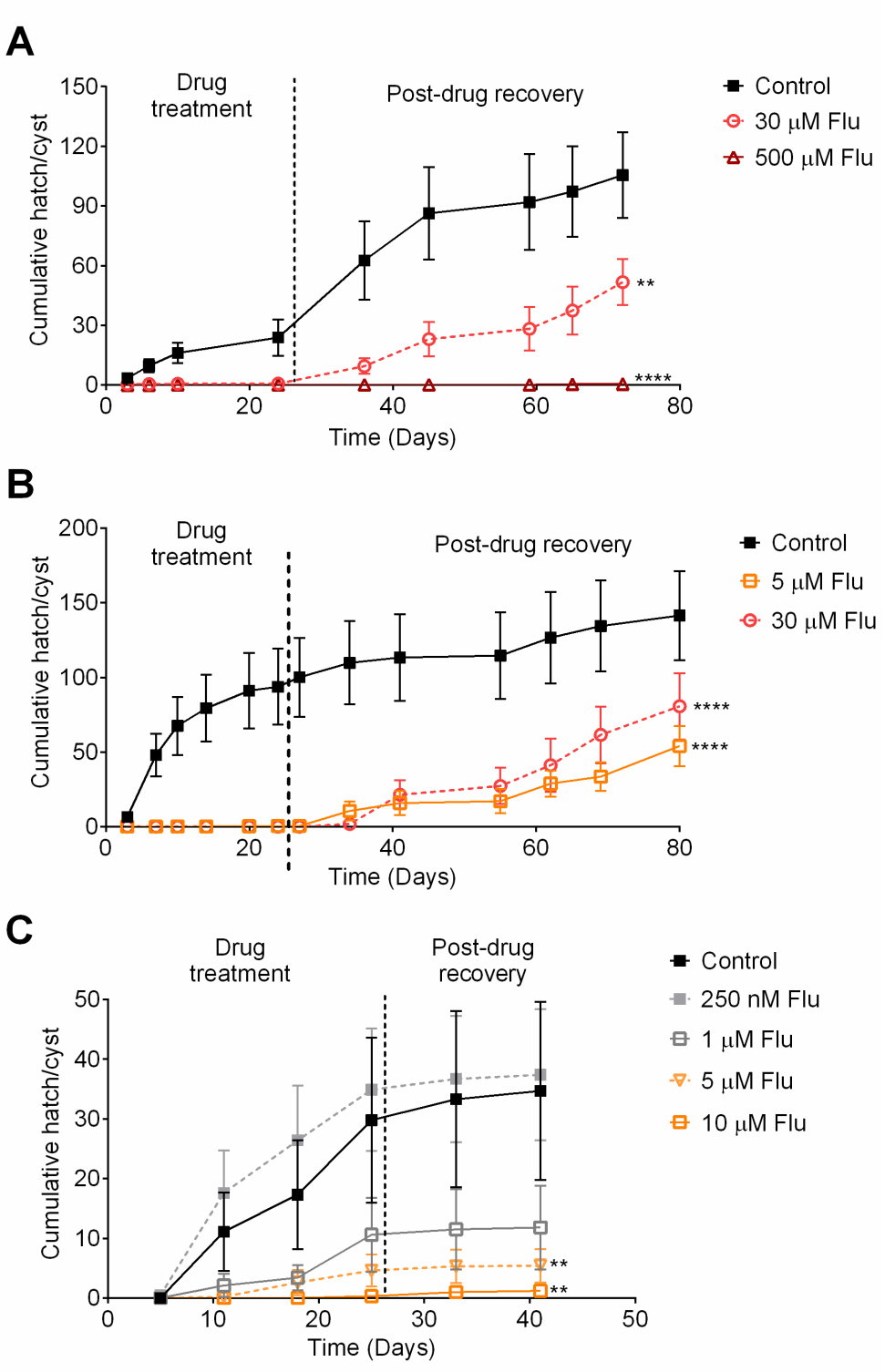

Figure 5.12. Fluensulfone inhibits *G. pallida* hatching from cysts in a dose-dependent manner. A, **B**, **C**) *G. pallida* cysts were exposed to vehicle (control) or the indicated concentration of fluensulfone (flu) in the presence of potato root diffusate (PRD) and the number of hatched J2s per cyst was scored. The root diffusate solution was replaced after each count. At 24 days (A+B) or 25 days (C) the cysts were removed from fluensulfone, washed and placed into root diffusate alone to assess recovery. ≥ 5 μM fluensulfone inhibited hatching from cysts (For all 3 experiments n=10 cysts per treatment, data shown are mean ± s.e mean cumulative hatch per cyst, A) P<0.0001, B) P<0.0001, C) P<0.05, two-way ANOVA with Dunnett post-hoc tests).

5.2.10 Analysing unhatched eggs from fluensulfone-treated cysts

On the conclusion of counting hatching from the cysts in the experiments seen in Figure 5.12B and C, each cyst was broken open and the number of unhatched eggs per cyst was scored (Figure 5.13). It was predicted that as fewer J2s hatched from fluensulfone-treated cysts they would have more unhatched eggs than vehicle-treated cysts. In the second experiment, control-treated cysts had 150 ± 20 unhatched eggs per cyst. Contrary to expectations, there was no difference in the number of unhatched eggs per cyst between fluensulfone-treated and vehicle-treated cysts, with 148 ± 22 and 110 ± 27 unhatched eggs per cyst for cysts treated with 5 and 30 µM fluensulfone, respectively (Figure 5.13A).

In the third experiment, there were actually fewer unhatched eggs in cysts treated with 5 and 10 µM fluensulfone relative to cysts treated with vehicle. Cysts in the control treatment group had 113 ± 19 unhatched eggs per cyst, whereas cysts treated with 5 and 10 µM fluensulfone had 47 ± 7 and 56 ± 17 unhatched eggs per cyst, respectively. There was no significant difference in the number of unhatched eggs per cyst between the 250 nM and 1 µM treatment groups and the control group. Therefore, despite fewer worms hatching in the presence of fluensulfone, the number of unhatched eggs in fluensulfone-treated cysts is also reduced.

It must be noted that even though far fewer J2s hatched from control-treated cysts in the third experiment (Figure 5.12C) relative to the second experiment (Figure 5.12B), there were fewer unhatched eggs in the cysts from the third experiment (Figure 5.13B). This indicates that there is considerable variability in the number of unhatched eggs per cyst, regardless of the treatment. This also indicates that differences in the number of unhatched eggs per cyst between treatments may not necessarily indicate drug effects and may reflect differences in egg number per cyst before experiment initiation.

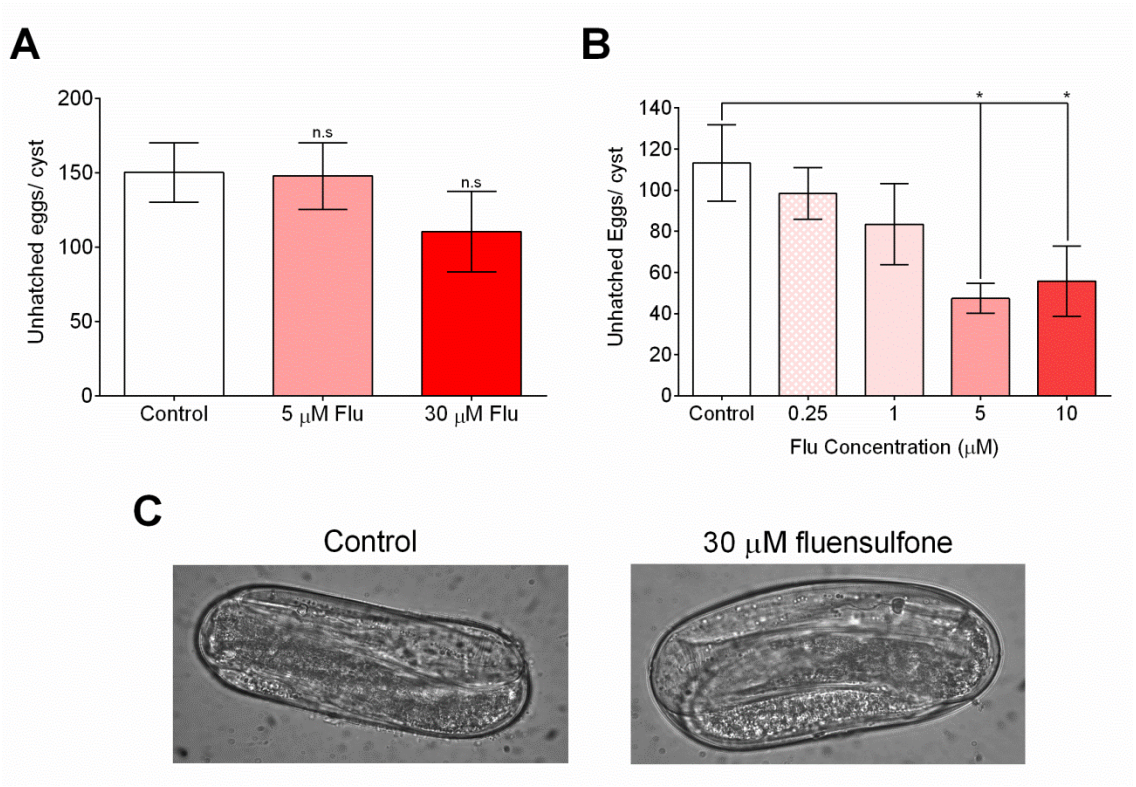

Figure 5.13. The effects of fluensulfone treatment on unhatched *G. pallida* eggs. Following measurements of hatching in the experiment seen in Figure **5.12**, cysts were cracked open and unhatched eggs per cyst were counted and imaged. **A**) In the experiment shown in Figure **5.12**B there was no difference in unhatched egg number between fluensulfone-treated (flu) cysts and vehicle treated cysts (data are mean ± s.e mean number unhatched eggs per cyst, n=10 cysts, one-way ANOVA with Dunnett post-hoc tests, P=0.36). **B**) In the experiment shown in Figure **5.12**C there were fewer unhatched eggs in cysts soaked in 5 and 10 μM fluensulfone as compared to the vehicle control (P<0.05, data are mean ± s.e mean number unhatched eggs per cyst, n=10 cysts, one-way ANOVA with Dunnett post-hoc tests, P<0.05). **C**) Representative images of unhatched eggs from cysts soaked in PRD and vehicle (control) or PRD and 30 μM fluensulfone. There were no obvious differences in fluensulfone-treated cysts relative to control-treated cysts.

Examination of the unhatched eggs via DIC microscopy indicated that unhatched eggs from ≤30 μM fluensulfone-treated cysts were similar to eggs from vehicle treated cyst, with the J2 inside appearing undamaged and unaffected by fluensulfone treatment (Figure 5.13C). This suggests that the *G. pallida* eggshell protects the unhatched J2 from the nematicidal activity fluensulfone, as earlier experiments indicated fluensulfone treatment for >7days would kill hatched J2s (Figure 5.4). As the unhatched eggs look undamaged and hatching can recover after exposure to ≤30 μM fluensulfone this suggests that whilst the concentration-dependence of the inhibition of hatching is similar to that for the inhibition of motility, they may occur via different mechanisms.

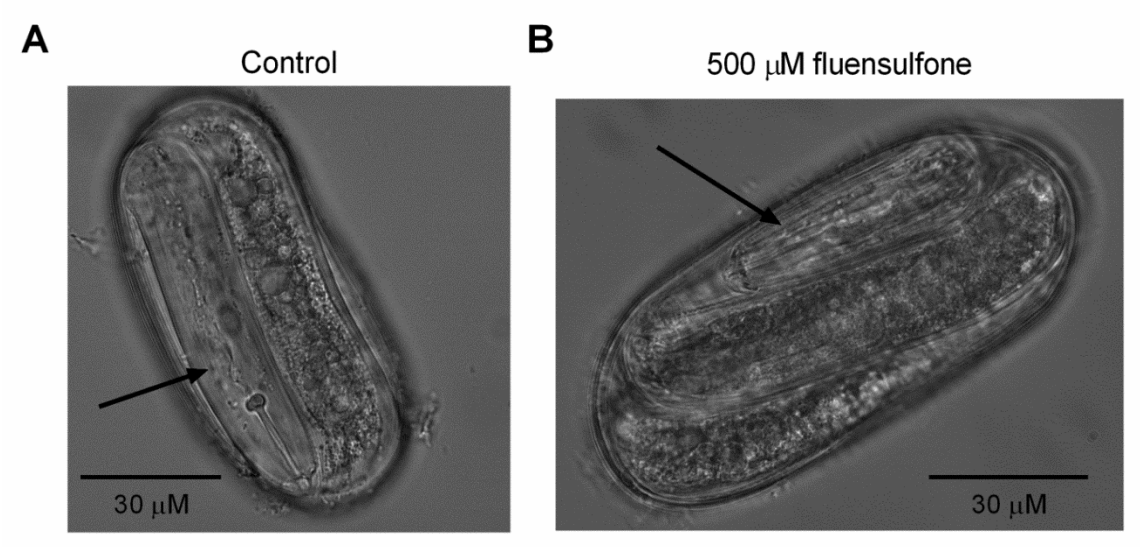

Figure 5.14. 500 µM fluensulfone has nematicidal activity against J2 *G. pallida* within the egg. Following exposure of cysts to either controls or 500 µM fluensulfone solutions, cysts were cracked open and the eggs were imaged. In eggs treated with a control solution (**A**), the J2 appears intact, with the structures of the head including the stylet and the pharynx clearly visible (as indicated by the arrow). The J2 inside eggs treated with 500 µM fluensulfone (**B**) appears "granular" with the structures of the head no longer visible and the appearance of tissue necrosis.

5.2.11 Pharmacological manipulation of serotonergic signalling influences *G. pallida* hatching from cysts

In a further series of experiments, the role of 5-HT signalling in cyst hatching was investigated. When hatching from the egg, PPN juveniles have been found to exhibit vigorous stylet thrusting (Perry and Moens, 2013). It has been suggested that this stylet thrusting is of importance in allowing the juvenile to break the eggshell and thus is important in allowing the emergence of the juvenile during hatching. Given the suggested role of 5-HT signalling in the regulation of stylet thrusting in *G. pallida* (see Chapter 4), it was speculated that pharmacological agents that interfere with 5-HT signalling may reduce or prevent *G. pallida* J2 hatching from cysts.

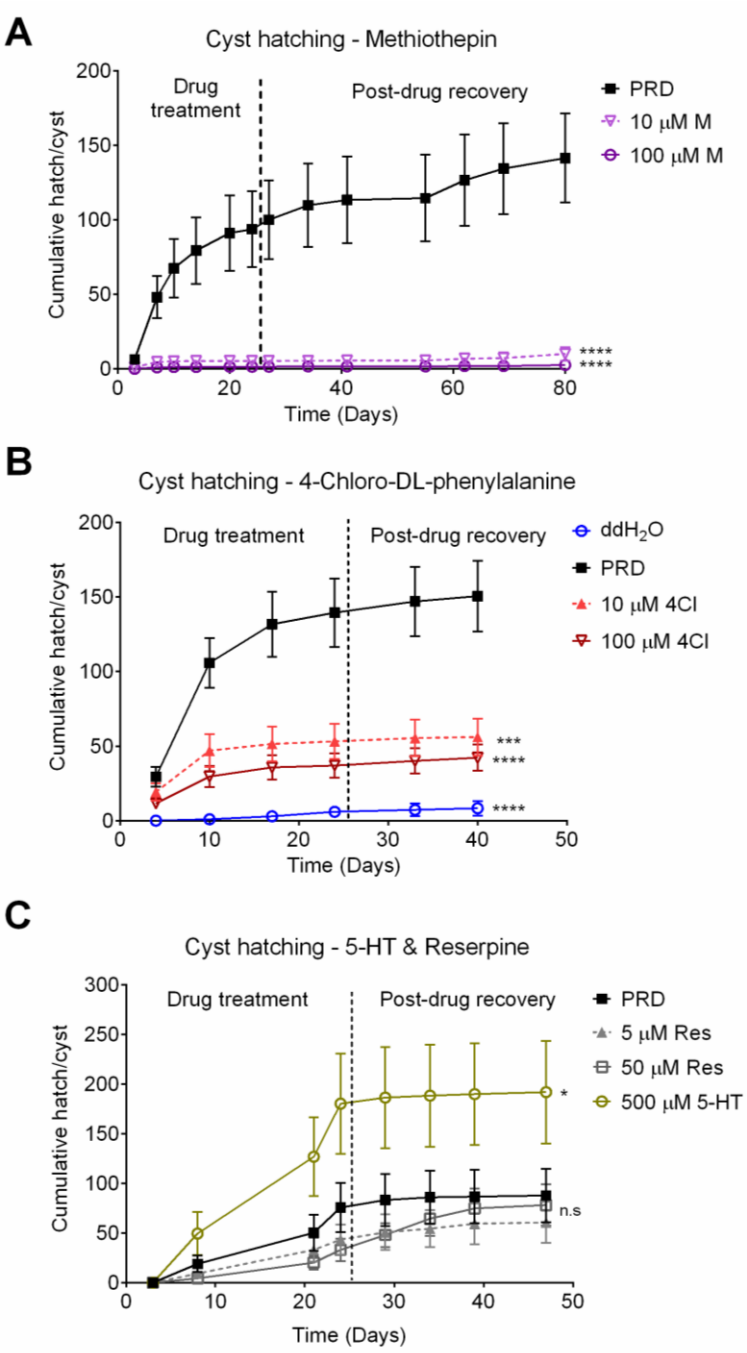

Figure 5.15. The role of 5-HT signalling in *G. pallida* cyst hatching. A) *G. pallida* cysts were soaked in a 24-well plate with 1 cyst per well in the presence PRD modified with either vehicle (PRD), 10 µM methiothepin (M) or 100 µM methiothepin and hatching of J2 juveniles was scored. At 24 days, the cysts were placed into PRD alone to assess recovery of hatching. Methiothepin inhibited hatching and hatching did not recover on removal from drug ($P<0.0001$) **B**) *G. pallida* cysts were soaked in either ddH_2O, PRD (control) or PRD with 10 µM or 100 µM 4-chloro-DL-phenylalanine (4Cl) and hatching was scored. At 24 days PRD-treated cysts were placed in PRD alone to assess recovery of hatching. 4Cl reduced hatching and hatching was reduced in the presence of ddH_2O ($P<0.0001$) **C**) *G. pallida* cysts were soaked in either PRD, or PRD with 5 & 50 µM reserpine (res) or 500 µM 5-HT and hatching was scored. At 24 days cysts were placed in PRD alone to assess recovery of hatching. Reserpine had no effect on hatching. 5-HT stimulated hatching ($P<0.05$, for A,B & C mean cumulative hatch per cyst ± s.e mean shown, A & B n=10 cysts, C n=20 cysts, two-way ANOVA with Tukey post-hoc tests).

To investigate this, hatching assays were conducted in the presence of the 5-HT antagonist methiothepin, the tryptophan hydroxylase inhibitor 4-chloro-DL-phenylalanine, and the vesicular monoamine transporter blocker reserpine (Figure 5.15), all of which can block stylet thrusting (see chapter 4). Soaking in 10 and 100 µM methiothepin strongly reduced hatching and no recovery of hatching occurred on transfer to PRD, indicating an irreversible block of hatching. At the conclusion of the experiment 142 ± 30 J2s had hatched per cyst in the control treatment group. In comparison, 10 ± 5 J2s had hatched per cyst in the presence of 10 µM methiothepin and 3 ± 1 J2s had hatched per cyst treated with 100 µM methiothepin. As with fluensulfone-treated cysts, the unhatched eggs appeared normal and the unhatched J2 within the egg appeared unaffected by methiothepin treatment relative to treatment with vehicle (Figure 5.15).

Hatching was also reduced in cysts exposed to 10 and 100 µM 4-chloro-DL-phenylalanine (Figure 5.15B), suggesting a role for endogenous 5-HT in the regulation of stylet thrusting within the egg. In the presence of PRD alone, 151 ± 24 J2s had hatched per cyst after 40 days, whereas 56 ± 13 J2s had hatched in the presence of 10 µM 4-chloro-DL-phenylalanine and 42 ± 9 J2s had hatched per cyst in the presence of 100 µM 4-chloro-DL-phenylalanine. Whilst hatching was reduced by 4-chloro-DL-phenylalanine soaking, hatching was not completely blocked as with methiothepin and cumulative hatch per cyst was still greater than seen in cysts treated with ddH_2O, where 8 ± 5 J2s had hatched per cyst. On transfer of 4-chloro-DL-phenylalanine –treated cysts to PRD alone, no recovery of hatching was observed.

Treatment of cysts with reserpine did not affect cumulative hatching relative to the control (Figure 5.15C). In the same experiment cysts were also treated with 500 µM 5-HT and this was found to stimulate hatching relative to control-treated cysts, with a total cumulative hatch of 192 ± 52 J2s per cyst, as compared to 88 ± 27 J2s for the control. Interestingly, in experiments described in Chapter 4, 500 µM 5-HT had no significant effect on stylet activity in hatched J2s, with 5 mM the lowest concentration to significantly increase stylet thrusting. This may reflect the treatment time, as in the stylet thrusting assays worms were only exposed for 1 hour as compared to 24 days in the cyst hatching assay. The J2s that hatched in the presence of 500 µM 5-HT were not seen to stylet thrust and moved normally, without adopting the stereotypical s-shaped posture and reduced movement that occurred in the stylet thrusting assays (see chapter

4). This may indicate differences in the sensitivity of unhatched and hatched J2s to 5-HT.

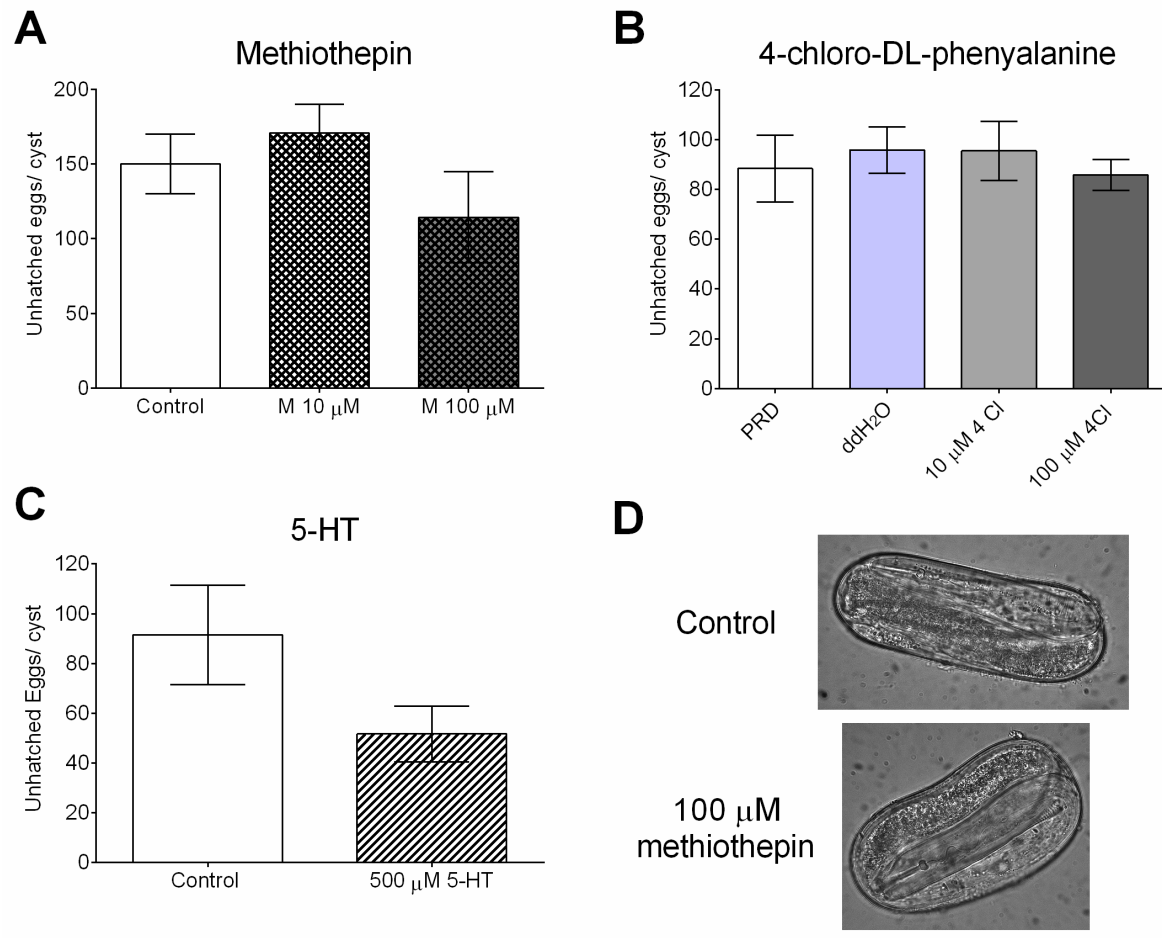

Figure 5.16. The effects of methiothepin, 4-chloro-DL-phenylalanine and 5-HT on unhatched eggs from cysts after prolonged incubation. Following measurements of hatching in the experiments seen in Figure 13, cysts were cracked open and unhatched eggs per cyst were counted and imaged. **A**, **B**, **C**) There was no difference in unhatched egg number between cysts treated with methiothepin (M), 4-chloro-DL-phenylalanine (4Cl) or 5-HT and control-treated cysts (data are mean ± s.e mean number unhatched eggs per cyst, A+C n=10 cysts, B n=20 cysts per treatment, A+B one-way ANOVA with Dunnett post-hoc tests, C student's T-test, A P=0.26, B P=0.88, C P=0.10). **D**) Representative DIC images of unhatched eggs from cysts treated with a control solution and 100 μM methiothepin.

There was no difference in the number of unhatched eggs per cyst relative to the control after treatment with either methiothepin, 4-chloro-DL-phenylalanine, reserpine or 5-HT (Figure 5.16). This is despite the inhibition of hatching by both methiothepin and 4-chloro-DL-phenylalanine and the stimulation of hatching by 5-HT. There was also no significant difference in the number of unhatched eggs per cyst between the ddH_2O and PRD treatment groups, despite the very low hatching of J2s in the cysts treated with ddH_2O. Unhatched eggs for all treatments also appeared normal and undamaged after examination via DIC microscopy (Figure 5.16D).

5.2.12 Summary

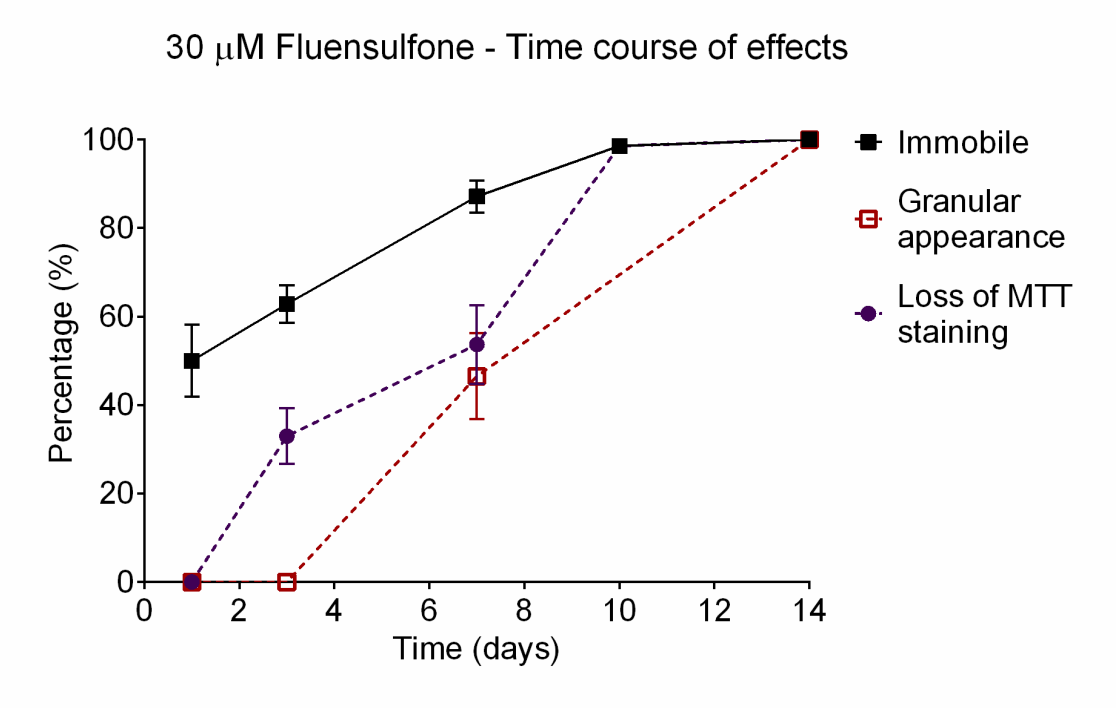

Figure 5.17. A comparison of the time course of the effects of 30 µM fluensulfone on *G. pallida* J2s. Immotility progressively increases with time in the presence of 30 µM fluensulfone, concomitant with a weakening and loss of MTT staining. This is followed by an increase in J2s with a granular appearance, indicating worm mortality. These data are taken from Figure 5.4 and Figure 5.6.

Assay	Fluensulfone concentration (µM)	Time (days)	Effect
Immotility	200-500	≥ 3	100% immotility
	30	> 7	100% immotility
	1-10	≥ 10	40-50% increase in immotility
Granular appearance	200-500	3-7	100% death
	30	14	100% death
	1-10	14	No effect
MTT - % stained	200-500	3	100% loss of staining
	30	10	100% loss of staining
	1-10	14	15-50% loss of staining
MTT – Shift in staining to tail	200	1	50% increase in tail staining
	1-10	10	30-40% increase in tail staining
Nile red – Lipid staining	30	5	No effect
	10	10	50% increase in fluorescent intensity – Reduced lipid consumption
Cyst hatching	500	1-80	100% hatch inhibition
	5-30	1-80	Inhibition with some recovery

Table 5.1. A summary of the effects of fluensulfone on *G. pallida* presented in this chapter.

5.3 Discussion

5.3.1 Fluensulfone has distinct high and low concentration effects on *G. pallida* behaviour

Fluensulfone has been shown to have nematicidal activity towards *M. javanica* at concentrations of ≥3.4 µM (Oka et al., 2009), whilst nematicidal activity towards *C. elegans* is only seen at ≥300 µM (Kearn et al., 2014) (see chapter 3 discussion). In the previous chapter, the effects of fluensulfone on the stylet system of the PPN *G. pallida* were characterised, with overt effects only seen at ≥500 µM. In chapter 4, *G. pallida* were only exposed to fluensulfone for short time periods, whereas the paralytic effects of fluensulfone on *M. javanica* become evident at more than 24 hours (Oka et al., 2009). One of the aims in this chapter was to determine the effects of fluensulfone on *G. pallida* stylet behaviour, motility and mortality with a more protracted incubation in fluensulfone.

Whilst investigating the effects of protracted incubation in fluensulfone on stylet activity, it became evident that 5-HT-stimulated stylet activity became reduced in the presence of fluensulfone in a concentration-dependent manner, with an acute block seen at 500 µM and a more gradual reduction in the response to 5-HT at lower concentrations. Aside from the acute block by 500 µM fluensulfone, the reduction of the response to 5-HT was concomitant with an increase in immotility. It seems likely that the reduction in the 5-HT response at lower concentrations reflects paralytic and/or nematicidal activity and is not a direct effect of fluensulfone on 5-HT-induced stylet thrusting.

200 and 500 µM fluensulfone had acute effects on *G. pallida* behaviour, inducing coiling and stylet thrusting (see chapter 4), whilst ≤30 µM fluensulfone had no overt effects on behaviour with acute exposure. ≥500 µM fluensulfone also acutely blocked 5-HT-induced stylet activity, whereas 30 µM had no effect. Complete paralysis of J2 *G. pallida* exposed to ≥200 µM fluensulfone occurred at 2-3 days, with a granular appearance becoming evident at 3 days. 30 µM fluensulfone on the other hand, caused a slower onset paralysis, with full paralysis achieved at 7-10 days. The data presented in this chapter, along with the data in chapter 4, suggest distinct high and low concentration effects. High concentrations have acute effects that may reflect the

interaction of fluensulfone with neural targets (see Chapter 4, discussion), whilst no such effects are seen at lower concentrations. This suggests that at higher concentrations fluensulfone interacts with molecular targets that are unaffected at lower concentrations. All concentrations of fluensulfone seem to lead to a similar endpoint, characterised by a rod-shaped paralysis, the loss of MTT staining and death, albeit over a different time course. This suggests that the nematicidal activity of fluensulfone at low and high concentrations may occur through interaction with the same target yet occur more rapidly at high concentrations. The neurobiological effects of high concentrations probably result from interactions with targets that have a lower affinity for fluensulfone and are not related to its nematicidal activity in the field.

5.3.2 The sensitivity of *G. pallida* to fluensulfone is similar to *M. javanica,* but not to *C. elegans*

Whilst 30 µM was the lowest concentration of fluensulfone that elicited full immotility and death, some effect on motility was observed at 1 µM, which indicates that *G. pallida* is susceptible to fluensulfone at similar concentrations to *M. javanica*. Immotility only became evident in lower concentrations of fluensulfone at ≥3 days, whereas the paralytic effects of fluensulfone on *M. javanica* are seen after 24 hours (Oka et al., 2009). This difference may reflect difference in cuticle permeability between the two species. Alternatively, differences in the physiology or metabolism of *G. pallida* and *M. javanica* may account for the differences in the time course of fluensulfone activity. The molecular target of fluensulfone may also be differentially expressed between the two species, which could account for some of this difference. Oka (2014) found that some species of migratory nematode, such as *D. dispaci*, were unaffected after 48 hour exposure to 54.4 µM fluensulfone. It would be interesting to see if, as with *G. pallida*, the nematicidal activity of fluensulfone towards these species became evident with a more protracted exposure.

Whilst the time course of the nematicidal activity of fluensulfone may differ between *G. pallida* and *M. javanica*, *C. elegans* were relatively unaffected by prolonged exposure to 50 and 100 µM fluensulfone. 50 and 100 µM fluensulfone did not shorten the lifespan or affect the survival of *C. elegans* adults over the course of 20 days and only 500 µM had nematicidal activity towards as nematicidal activity towards *C. elegans*. Likewise, *C. elegans* dauers were only killed by 500 µM fluensulfone. This indicates that there is

around a 10-50 fold difference in the concentrations that have nematicidal activity against *G. pallida* and *C. elegans*. It appears however that bagging behaviour was increased in the presence of 50 and 100 µM fluensulfone relative to the control. This was not seen at 500 µM, although any effects on bagging would be masked by the nematicidal effects at this concentration. This suggests that fluensulfone does have some effects on *C. elegans* at these lower concentrations, although this is far less profound than the paralytic and nematicidal effects seen in *G. pallida* at these concentrations. Bagging is caused by starvation (Chen and Caswell-Chen, 2004), pathogenic bacteria (Aballay et al., 2000, O'Quinn et al., 2001) and toxic compounds (Calafato et al., 2008, Van Raamsdonk and Hekimi, 2009). Other compounds that induce bagging include paraquat (Van Raamsdonk and Hekimi, 2009), which is believed to increase lipid peroxidation in animals (Bus et al., 1974), and the SLO-1 activator emodepside (Bull et al., 2007). Bagging is also caused by mutations that affect or impair the egg laying process (Trent et al., 1983). It is possible that lower concentrations of fluensulfone cause a slight reduction in egg laying that increases the likelihood of hermaphrodites bagging. There were no other overt behavioural effects at these lower concentrations, although it would be interesting to score egg laying under these conditions, as increased retention of eggs could lead to bagging.

It is possible that differences in the assays used to score the nematicidal activity of fluensulfone in *G. pallida* and *C. elegans* may account for some of the difference between the two species. The lifespan assays with *C. elegans* were conducted on agar in the presence of a food source whereas the immotility assays with *G. pallida* were conducted in ddH_2O. One disadvantage of the use of agar plates is that the bioavailability of chemicals in the agar cannot be quantified. *C. elegans* will remain on the film of moisture on the surface of the agar and may not be exposed to the same concentration of chemical that is added to the plate. It is also possible that chemicals such as fluensulfone could bind to constituents of the NGM agar (Meyer and Williams, 2014). It has for example been shown that 5-HT stimulates *C. elegans* pharyngeal pumping on agar plates at concentrations that are ≥20-fold lower than those required on NGM plates (Law et al., 2015). The *C. elegans* in the lifespan assays may not therefore be exposed to the same concentrations as *G. pallida* in the immotility assays. It would be interesting to try prolonged exposure to fluensulfone in a liquid medium, although some media used for *C. elegans* are also known to reduce the efficacy of nematicides

(Ma et al., 2009). Assays with dauer *C. elegans* were conducted in liquid, yet it is known that dauers are resistant to many chemicals (Cassada and Russell, 1975).
Another difference between the assays used for *G. pallida* and *C. elegans* is that a food source was always present with the *C. elegans* adults yet no food was present for *G. pallida* assays. This reflects differences in their biology, as *C. elegans* must feed yet *G. pallida* J2s are non-feeding. It is possible that the physiological and metabolic state of the two species differs (see section 1.17). It is possible that the susceptibility of *C. elegans* to fluensulfone is similar to the migratory nematodes tested by Oka (2014).

5.3.3 The MTT assay suggests that fluensulfone causes metabolic impairment prior to death

With prolonged exposure to fluensulfone, the percentage of *G. pallida* that showed purple MTT staining decreased. 200 and 500 µM fluensulfone reduced the number of *G. pallida* with visible staining within 24 hours and no worms stained after 3 days. 30 µM fluensulfone treatment caused a more gradual reduction in the percentage of worms staining, with no worms staining after 8-10 days exposure. The decline in percentage staining occurred in tandem with the increase in percentage immotility at all the tested concentrations. This suggests that the increase in immotility and the decrease in MTT staining may be linked.

The conversion of MTT from a yellow salt to a purple formazan product is dependent upon oxidoreductase enzymes and electron donors, predominately NAD(P)H (Berridge et al., 2005) (see section 5.1). There is evidence that MTT reduction can be catalysed by succinate dehydrogenase and the NAD(P)H-dependent oxidoreductase enzymes NQO1 and cytochrome p450 (Stoward and Pearse, 1991, Berridge et al., 2005). Whilst NAD(P)H is the main reducing agent in the MTT reduction reaction, succinate, pyruvate and glutathione are also known to act as electron donors (Berridge et al., 1996). There is also evidence that superoxide contributes to MTT reduction within cells (Burdon et al., 1993). The majority of reduction of MTT is known to occur intracellularly (Bernas and Dobrucki, 2002, Bernas and Dobrucki, 2000), unlike some other tetrazolium dyes (Berridge et al., 2005). There is some evidence that a small percentage of MTT reduction occurs at the cell surface as a result of electron transport at the cell membrane (Burdon et al., 1993). Therefore, not all MTT reduction reflects metabolic capability.

The exact site of MTT reduction within the cell is not known, the enzymes that catalyse the reaction are not known and several compounds have been found to act as reducing agents in MTT reduction (Berridge et al., 2005). The compounds that act as reducing agents are all however produced during normal metabolism and the enzymes involved in catalysis are linked to various aspects of metabolism. MTT can therefore function as a general marker of metabolic function, yet cannot be used to identify anything more specific. The reduction or lack of MTT staining may result from a number of metabolic effects. The reducing agents that are required for the generation of MTT formazan are generated by the citric acid cycle, glycolysis, β-oxidation and the glyoxylate cycle (Berg et al., 2007), and impairment or inhibition of any of these pathways could contribute to a lessening of MTT staining. As there is evidence that some MTT reduction does not result from metabolic products, the MTT results presented in this chapter must be verified with other measures of metabolic capability, for example measurement of oxygen consumption or ATP production (Schouest et al., 2009, Schaffer et al., 2011, Mouchiroud et al., 2013).

The reduction of MTT staining, and its correlation with the rise in immotility suggests that the metabolism of *G. pallida* J2s is impaired following fluensulfone treatment. The MTT staining technique cannot however indicate whether metabolic impairment directly precedes *G. pallida* paralysis or occurs afterwards. Knowing this is important, as it would indicate whether metabolic impairment is causative in the paralysis or whether metabolic impairment results from an unrelated insult. Other measurements of metabolic capability are needed to determine this.

Interestingly, the cholinesterase inhibitor aldicarb had no effect on MTT staining, whilst the mitochondrial inhibitor fluopyram rapidly prevented MTT staining. The aldicarb observation confirms that fluensulfone has a mode of action that is distinct from previously used nematicides and that fluensulfone is nematicidal, results in worm death, and is not a nematostatic like the organophosphates and carbamates. The inhibition of MTT staining by fluopyram was far more rapid than the inhibition of staining by fluensulfone. Fluopyram also caused paralysis of *G. pallida* substantially more rapidly than fluensulfone. Whilst the distinct time course of the action of fluensulfone and fluopyram on *G. pallida* may be due to factors such as cuticle permeability and target sensitivity, it is also possible that this distinction suggests that the compounds have

different modes of action. Fluopyram is known to act through inhibition of succinate dehydrogenase (complex II) of the electron transport chain when used as a fungicide (Veloukas and Karaoglanidis, 2012), although this mode of action has not been verified in nematodes. Mitochondrial inhibition is consistent with the rapid effects of fluopyram on *G. pallida*, and the effects of fluopyram are similar to the classic mitochondrial inhibitor sodium azide, which inhibits complex IV of the electron transport chain (Duncan and Mackler, 1966). The different time course of the activity of fluensulfone and fluopyram on *G. pallida* may suggest that if fluensulfone does inhibit or impair aspects of nematode metabolism, this is unlikely to occur through inhibition of components of the electron transport chain. It cannot however be ruled out that the different time course of fluopyram and fluensulfone may have a pharmacokinetic explanation or that the drugs have differing affinity for their targets.

An interesting observation that was made during MTT staining experiments with *G. pallida* was that the purple staining seemed to shift from the anterior region around the head and pharynx to the tail region after protracted exposure to fluensulfone. The purple MTT staining also became weaker before staining disappeared altogether. This suggests that the metabolic impairment that occurs after fluensulfone treatment is progressive, building from a shift in staining, to a weaker stain and finally to the complete absence of staining. No such weakening or shifting of staining was observed following fluopyram treatment. This may reflect reduced capability to generate reducing agents such as NAD(P)H during metabolism, resulting in a diminished ability to convert MTT to its formazan product.

It is unclear what the shift in staining to the tail region following fluensulfone treatment means, given the uncertainty relating to some aspects of MTT reduction. CARS and Nile red staining suggests that the tail region of *G. pallida* has a higher density of lipid stores than the head region (*personal communication, Justyna Pertek*). The shift in staining may therefore be due to some alteration in lipid metabolism that results in a greater level of staining in the tail region. As mentioned, superoxide can act as a reducing agent in the MTT reduction reaction (Burdon et al., 1993). It is possible that fluensulfone treatment increases superoxide production, predominately in the region of lipid storage.

It is also unclear why untreated *G. pallida* J2s would stain predominately in the head region. *G. pallida* stained with the mitochondrial dyes $DiSC_3(3)$ showed fluorescence predominately in the head region, which suggests that there is a high density of mitochondria in the head (Appendix 2). This may explain the strong purple staining at the anterior in untreated *G. pallida*.

5.3.4 Fluensulfone treatment may reduce *G. pallida* lipid consumption

It has been observed in experiments with *M. javanica* that J2s treated with a control solution exhibit depleted internal contents when soaked for several days yet this was not observed in the presence of fluensulfone (Oka et al., 2009). It can be speculated that this depletion reflects the consumption of lipid stores in the non-feeding J2s that occurs over time (Reversat, 1981). This suggests that fluensulfone treatment reduces lipid consumption in *M. javanica*. When this observation is considered with the shift in the distribution of MTT staining to the tail following fluensulfone treatment, it is possible that fluensulfone impairs or alters lipid consumption in PPNs.

Nile Red is a solvatochromatic dye, in that the intensity of its fluorescence and its excitation and emission wavelengths are highly dependent on solution polarity (Greenspan and Fowler, 1985). Nile Red exhibits strong fluorescence, but only when the dye is in a hydrophobic environment. As such, Nile Red has been found to fluoresce in the presence of triglycerides, cholesterols and phospholipids (Greenspan et al., 1985). Nile Red has been used extensively in the past as a stain for lipids in *C. elegans* (Ashrafi et al., 2003, Srinivasan et al., 2008). Nile Red was predominately used to stain live *C. elegans* by adding the dye to an OP50 *E.coli* bacterial lawn and allowing the worms to feed. *G. pallida* are non-feeding and thus cannot take up Nile Red by ingestion. Furthermore, subsequent studies have demonstrated that Nile Red taken up by feeding *C. elegans* preferentially stains lysosome-related organelles and that these are not the major fat stores in *C. elegans* (O'Rourke et al., 2009). It has been hypothesised that stains such as Nile Red may be treated as xenobiotics and are thus portioned into lysosome-related organelles to allow degradation (O'Rourke et al., 2009).

The technique used here to stain *G. pallida* for lipids using Nile Red was modified from a protocol devised by Pino et al. (2013) for staining *C. elegans* lipid stores. This method involves the use of a fixation step and thus bypasses the need for worms to ingest the

dye, which can be impractical and is potentially misleading (O'Rourke et al., 2009). Pino et al. (2013) found that Nile Red staining using this technique allowed quantification of lipid levels in *C. elegans* that correlated with estimation of lipid levels by solid phase extraction and gas-chromatography mass spectrometry. The authors did however suggest that estimation of lipid levels using this staining method should be verified using other techniques. The Nile Red staining technique used here indicated that the lipid stores of *G. pallida* are predominately in the tail and this was also found using CARS (*personal communication, Justyna Pertek*). Nevertheless, the fluensulfone results must be confirmed through CARS or other techniques for measuring lipid levels.

Here, J2 *G. pallida* treated with 10 µM fluensulfone exhibited greater fluorescence intensity following staining with Nile Red when compared to controls exposed to vehicle. This suggests that fluensulfone treatment does reduce lipid consumption in *G. pallida* relative to untreated worms and this may occur in other PPNs as well. Treatment with 30 µM fluensulfone for 5 days was not found to affect Nile red fluorescence after 5 days. This may be because the Nile Red staining technique used is only capable of detecting larger changes in lipid levels and as such cannot detect differences over 5 days. J2 *G. pallida* begin to die after more than 5 days in the presence of 30 µM fluensulfone and therefore the staining technique could not be used beyond this point.

A parsimonious explanation for reduced lipid consumption in the presence of fluensulfone would be the reduced movement that occurs in the presence of fluensulfone relative to J2s in a control solution. Indeed, it has been found that cholinesterase inhibitors also reduce lipid consumption in PPNs and it was hypothesised that this resulted from reduced movement (Perry and Moens, 2013). In the experiments with fluensulfone however, J2 *G. pallida* were exposed to aldicarb in parallel and did not exhibit greater fluorescence intensity than control-treated J2s, despite reduced motility. This suggests that aldicarb treatment over the course of 10 days did not result in reduced lipid consumption. It is possible that the technique used is of insufficient sensitivity to detect smaller changes in lipid levels. This does however suggest that the reduction of lipid consumption in the presence of fluensulfone is greater than that which occurs in the presence of cholinesterase inhibitors such as aldicarb. This further suggests that the reduced lipid consumption in the presence of fluensulfone may not solely result from the reduction in motility. Fluensulfone could for example affect or impair β-oxidation, which would prevent lipid consumption (Berg et al., 2007). Such an

effect could contribute to the nematicidal effects of fluensulfone against the non-feeding *M. javanica* and *G. pallida* J2s. In addition, if impairment of β-oxidation does contribute to the nematicidal activity of fluensulfone this may explain the reduced susceptibility of *C. elegans* to fluensulfone. It would be interesting to see whether the nematicidal efficacy of fluensulfone is shifted against *C. elegans* deprived of food. It would also be informative to measure β-oxidation in nematodes treated with fluensulfone. Fatty acid oxidation can be measured in *C. elegans* through examining oxidation of radiolabelled fatty acids to H_2O (Elle et al., 2012) and could potentially be used to further investigate the effects of fluensulfone on lipid consumption in nematodes.

5.3.5 Fluensulfone and inhibitors of serotonergic signalling reduce *G. pallida* cyst hatching

Fluensulfone was found to reduce the hatching of J2 *G. pallida* from cysts, and this inhibition occurred at similar concentrations to those found to inhibit motility and reduce MTT staining in hatched J2s. This is consistent with previous studies, where fluensulfone was found to inhibit the hatching of *M. javanica* (Oka et al., 2009).

Whilst fluensulfone was present, hatching was almost completely inhibited over a concentration range of 5-500 µM. When fluensulfone was removed, there was some recovery in two of the three experiments. This suggests that the eggshell protects the unhatched J2 from the nematicidal effects of fluensulfone, as hatched J2s would die if exposed for a similar length of time. This may mean that the nematicidal activity of fluensulfone against hatched J2s occurs through different mechanisms than the inhibition of hatching. On the other hand, the concentration-dependence of both effects is similar, which may suggest that they occur through the same mechanisms. 500 µM fluensulfone did permanently inhibit *G. pallida* hatching and the unhatched eggs showed signs of tissue damage and necrosis, which suggests that fluensulfone can penetrate the eggshell when applied at high concentrations.

The organophosphate cadusafos has been shown to reversibly inhibit *G. pallida* hatching, whilst aldicarb and oxamyl have been found to inhibit *G. rostochiensis* (Osborne, 1973, Evans and Wright, 1982, Ibrahim and Haydock, 1999). The muscarinic acetylcholine receptor antagonist atropine has also been shown to inhibit hatching of *G.*

pallida (Palomares-Rius et al., 2013). The biofumigant 2-propenyl-isothiocyanate has also been found to inhibit *G. pallida* hatching from cysts (Brolsma et al., 2014). This suggests that hatching can be inhibited through several mechanisms.

On breaking open the cysts at the termination of the experiments, fluensulfone-treated cysts either did not have a significantly different number of unhatched eggs or actually had fewer unhatched eggs. It might be expected that if fewer eggs had hatched from fluensulfone treated cysts then they would have a greater number of unhatched eggs per cyst. This could suggest that fluensulfone affects the number of unhatched eggs. In subsequent experiments however, cysts treated in ddH_2O did not have a significantly different number of unhatched eggs per cyst relative to cyst treated with PRD, yet far more J2s hatched from PRD-treated cysts. This indicates that alterations must be made in the experimental procedure to more fully understand the effects of fluensulfone and other compounds on *G. pallida* cyst hatching. In the assays conducted here, hatching was scored and then unhatched eggs were counted at the conclusion of the experiment. This does not take into account the large variation in egg number per cyst, which could skew the results obtained. Indeed, prior to any treatment, egg number per cyst varied between less than 100 to more than 300 (data not shown). Previous studies have also counted the number of egg cases per cyst to allow estimation of the total number of eggs per cyst prior to hatching and this should be attempted on repeating these cyst hatching assays (Twomey et al., 2000).

During these assays, it was observed that there was considerable variation in the number of J2s that hatched per cyst in the presence of PRD. There was also variation in the number of J2s hatching per cyst between experiments. As with the variable stylet thrusting response to 5-HT and other drugs (see chapter 4), this may reflect genetic variation between different cysts. It has been described previously that the speed of juvenile emergence from cysts varies between different nematode populations (Whitehead, 1992). This variation does mean that these results need to be verified and repeated.

As stylet thrusting is known to be involved in the hatching of PPNs from their eggs, it was postulated that compounds that inhibit stylet thrusting could also inhibit hatching. The results presented in chapter 4 show that 5-HT is a key regulator of *G. pallida* stylet thrusting. Therefore, methiothepin, reserpine and 4-chloro-DL-phenylalanine were

tested for effects on cyst hatching. Hatching was almost completely inhibited by ≥10 µM methiothepin and was partially inhibited by ≥10 µM 4-chloro-DL-phenylalanine, although reserpine had no significant effect on the rate of hatching. These observations, along with the inhibition of stylet thrusting in hatched J2s, suggest that inhibition of serotonergic signalling is a potential route to control PPNs through inhibition of both hatching and host invasion. Whilst the inhibition of stylet thrusting by these compounds in hatched J2s suggests that blocking stylet thrusting may cause the reduced hatching in the presence of methiothepin and 4-chloro-DL-phenylalanine, they may block hatching via other means, for example non-specific toxicity. The concentration-dependence of hatching inhibition by these compounds should be established.

In stylet thrusting assays with hatched J2s, 5 µM reserpine completely blocked fluoxetine-induced activity, whilst 10 µM 4-chloro-DL-phenylalanine only partially blocked fluoxetine-induced activity. It might therefore be expected that reserpine would be a more efficacious blocker of hatching than 4-chloro-DL-phenylalanine. The non-effect of reserpine on cyst hatching may reflect the inability of the compound to diffuse across the eggshell (Perry and Moens, 2011). There was considerable variation in the number of J2s that hatched from cysts soaked in PRD alone between the experiments with reserpine and with 4-chloro-DL-phenylalanine. The final cumulative hatch per PRD-treated cyst in the 4-chloro-DL-phenylalanine experiment was 151 ± 24, as compared to 88 ± 27 in the reserpine experiment. It may be that a lower level of hatching in the control cysts masks drug effects, whereas a difference is more evident when hatching is higher in the control cysts. These experiments must be repeated to verify these observations.

500 µM 5-HT was found to stimulate hatching relative to control-treated cysts. As 5-HT stimulates stylet thrusting, this suggests that interference with stylet behaviour is likely to be the means through which methiothepin and 4-chloro-DL-phenylalanine inhibit hatching. 5-HT has been shown previously to inhibit hatching of *H. glycines* at 5-50 mM (Masler, 2007). This may indicate that 5-HT signalling has different roles in the control of hatching in different species. This could also be due to the higher concentrations of 5-HT used. It would be interesting to test whether higher concentrations of 5-HT inhibit *G. pallida* hatching.

Stimulation of cyst nematode hatching could be beneficial in chemical control of nematode infestations. Cyst nematodes can remain dormant within cysts for many years and can emerge when a suitable host and favourable conditions present themselves (Turner and Subbotin, 2013). As a result, fields in which cyst nematodes have been present can become non-viable for the growth of susceptible host crops. Nematodes within cysts are also protected from many nematicidal compounds (Bird and Mcclure, 1976, Perry and Moens, 2011). If serotonergic agonists or other compounds that increase serotonergic signalling can stimulate hatching, the hatched J2s can be treated with nematicides or alternatively can be left to starve. With this aim in mind, it would be useful to see how 5-HT influences hatching in the absence of PRD and how the hatching of other species is affected by 5-HT treatment. Establishing the concentration-dependence of 5-HT on cyst hatching would also be important. Furthermore, specific 5-HT receptor agonists could be used to delineate the receptors involved in mediating this increased hatching.

It is interesting that 500 µM 5-HT stimulates *G. pallida* hatching but did not significantly stimulate stylet thrusting (see chapter 4). This may be due to the length of exposure, with 60 minutes the longest time that hatched J2s were exposed to 5-HT. It is possible that a longer exposure to lower concentrations of 5-HT could stimulate thrusting in hatched J2s. It is also possible that 5-HT does not stimulate hatching through increasing stylet activity. It is conceivable that 5-HT may control or regulate other behaviours that are important in the hatching process or 5-HT signalling could be a part of the biological cascade that leads to hatching. The increased hatching in the presence of 5-HT may reflect hatching of J2s that would not hatch in the presence of PRD alone. It has been suggested that in the presence of PRD, only 60-80% of *G. rostochiensis* hatch, with some J2s remaining in diapause within the egg to hatch out in later growing seasons (Perry and Wharton, 2011). The increased hatching in 5-HT may result from J2s in diapause emerging which would usually remain unhatched.

To summarise, 1-30 µM fluensulfone causes a progressive reduction in *G. pallida* motility over 14 days resulting in eventual death. MTT staining indicates that this activity is nematicidal and may reflect metabolic inhibition. *C. elegans* is not susceptible to the nematicidal activity at fluensulfone at these concentrations. Fluensulfone treatment also reduces *G. pallida* lipid consumption relative to untreated worms. Similar concentrations of fluensulfone inhibit *G. pallida* J2 hatching from cysts.

5.3.6 Summary

In this chapter, the effects of lower concentrations of fluensulfone on *G. pallida* were investigated. The nematicidal action of lower concentrations of fluensulfone are revealed following protracted incubation in fluensulfone, with 30 µM exhibiting full nematicidal activity at 10 days. No overt behavioural effects occur at lower concentrations, other than a progressively increasing paralysis leading to death that is evident from extensive tissue necrosis. Prolonged exposure of *C. elegans* adults and dauers to fluensulfone confirmed the reduced sensitivity of this free-living species relative to PPNs. Staining with the metabolic marker MTT revealed that fluensulfone is a "true" nematicide and suggested that fluensulfone might affect nematode metabolism prior to death. Low concentrations of fluensulfone also inhibit *G. pallida* hatching from cysts. Studies with pharmacological agents showed that 5-HT signalling is likely to be an important regulator of the hatching process.

Further studies are needed to elucidate the mode of action of fluensulfone, focussing on further characterisation of nematode metabolism following fluensulfone exposure.

Chapter 6: General Discussion

6.1 Mode of action studies on fluensulfone

The central aim of this project was to study the effects of the newly-registered nematicide fluensulfone (Nimitz®) on nematodes and to gain an insight into its mode of action. Fluensulfone has a generally favourable toxicity profile when compared to previously used non-fumigant nematicides such as the carbamates and the organophosphates (see general introduction). Furthermore, evidence from past studies, reinforced in this thesis, suggests that the mode of action of fluensulfone is novel and that it does not act as an anticholinesterase like other non-fumigant nematicides (Oka et al., 2009, Kearn et al., 2014). Finding the target(s) with which fluensulfone interacts is desirable due to its relatively selective toxicity. Identifying the target(s) of fluensulfone could aid in the development of other nematicides that have a favourable toxicity profile and could further research into anthelmintics for treatment of animal parasites. Knowing the mode of action of nematicides should also contribute to the safe use of these compounds in crop protection.

In this chapter, the effects of fluensulfone on both PPNs and *C. elegans* will be discussed in relation to potential modes of action and routes for further investigation will be discussed. The use of *C. elegans* as a model for mode of action studies on nematicides will also be addressed, with this work highlighting some of the benefits and limitations of this approach.

6.2 The effects of high and low concentrations of fluensulfone on nematodes

In the work presented in this thesis, *C. elegans* was initially used with a view to characterising the behavioural effects of fluensulfone on a well-understood organism and using the knowledge gained to inform genetic studies into potential mechanisms of action. Fluensulfone did have nematicidal activity towards *C. elegans* but only at concentrations ≥50-fold higher than those reported to irreversibly paralyse and kill the PPN *M. javanica* (Oka et al., 2009). Despite this concentration discrepancy, the effects of fluensulfone on *C. elegans* were similar to those reported for PPNs, including a non-spastic paralysis and an inhibition of egg laying (Oka et al., 2009, Kearn et al., 2014).

Fluensulfone was found to inhibit *C. elegans* motility, feeding, egg laying, egg hatching and development. EMS mutagenesis was attempted to identify targets that conferred altered sensitivity to fluensulfone, yet no strains with high-level resistance were identified (data not shown/ appendix 1). This may be due to the high concentrations (1 mM) required to kill *C. elegans*, which may lead to interaction with multiple, lower affinity targets. Fluensulfone is known to be phytotoxic at high concentrations in the field (Oka et al., 2012) and this suggests that selective toxicity is lost at these concentrations. If fluensulfone is interacting with several targets at these concentrations, finding strains with high-level resistance is likely to be difficult. The anthelmintic ivermectin, for example, is known to interact with multiple targets at higher concentrations, though the sensitive targets with which it interacts to paralyse nematodes are glutamate-gated chloride channels (Dent et al., 2000, Ardelli and Prichard, 2013, Hernando and Bouzat, 2014). A reverse genetic approach using known *C. elegans* mutants also failed to identify altered susceptibility to fluensulfone, most likely due to non-selective toxicity. These mutants were selected based on the acute effects of high concentrations of fluensulfone on *C. elegans*, which are consistent with a neuroactive target. This approach did however indicate that fluensulfone does not have anticholinesterase activity and thus does not act like previously used soluble nematicides (Kearn et al., 2014).

It was found that similar concentrations of fluensulfone have acute effects on the PPN *G. pallida*, stimulating stylet activity and a distinct, coiled posture. Fluensulfone also inhibited stylet activity in the presence of 5-HT and other serotonergic compounds such as fluoxetine, indicating a block of stylet activity stimulated by both exogenous and endogenous 5-HT. As with stylet thrusting, fluensulfone stimulated pharyngeal pumping in *C. elegans* cut heads and blocked the stimulatory effects of 5-HT. As higher concentrations of fluensulfone have qualitatively similar effects on *C. elegans* pumping and *G. pallida* stylet activity, this suggested that these effects may result from a common mechanism. Investigations into this stimulatory activity with a 5-HT antagonist and other compounds suggested that both effects might result from interaction with 5-HT signalling pathways.

The concentrations of fluensulfone that had acute, overt behavioural effects were still substantially higher than those reported to be nematicidal against *M. javanica* (see section 3.1.1) (Oka et al., 2009). Experiments in which *G. pallida* were exposed to

fluensulfone for up to 14 days showed that *G. pallida* are susceptible to fluensulfone at similar concentrations to *M. javanica*, yet a more protracted incubation is required for the nematicidal activity to become manifest. ≤30 µM fluensulfone did not have any obvious behavioural effects on initial exposure but with longer incubation a progressive increase in percentage immotility occurred, followed by apparent tissue necrosis. The metabolic viability dye MTT showed that fluensulfone is a true nematicide. Prior to *G. pallida* death fluensulfone exposure resulted in a re-distribution of MTT staining from the head region to the tail and a weakening of the staining, which suggests that fluensulfone may have effects on PPN metabolism. Experiments with the lipid stain Nile red also suggested that fluensulfone treatment might prevent *G. pallida* lipid consumption. In contrast, extended exposure of *C. elegans* adults and dauers to fluensulfone indicated that *C. elegans* are less susceptible to fluensulfone than these PPNs.

These results show that fluensulfone has acute effects on both *G. pallida* and *C. elegans* at ≥300 µM and some of these effects, particularly those on the stylet and pharynx might result from interaction with neural signalling pathways (Table 6.1). The stimulatory effects on the stylet and pharyngeal systems appear, at least in part, to result from interaction with serotonergic signalling, with fluensulfone potentially acting as a partial agonist (see sections 4.2.11, 4.2.12 & 4.2.13). The effects at these concentrations are likely to result from interactions with multiple targets. For example, the stimulatory effect of fluensulfone on the stylet is blocked by methiothepin, yet methiothepin does not block the coiled posture that occurs in the presence of fluensulfone, indicating that fluensulfone is interacting with both methiothepin-sensitive and methiothepin-insensitive pathways at these concentrations. Methiothepin also failed to block the nematicidal activity of fluensulfone against *G. pallida* (see section 5.2.7), indicating distinct targets for the high and low concentration effects. 1-50 µM fluensulfone is only nematicidal towards *G. pallida* and this might result from a slowly building metabolic insult. The contrast with the speed of action of the electron transport chain inhibitor fluopyram (Veloukas and Karaoglanidis, 2012) suggests that fluensulfone might not be a classic mitochondrial toxin and has effects on metabolic pathways that result in a slower death.

The highest concentration of fluensulfone that *G. pallida* are likely to be exposed to in the field on initial application is around 30 µM (Norshie, 2014). This means that the

high concentration neural effects are unlikely to be relevant to the selective toxicity of fluensulfone and it might be effects on PPN metabolism that allow this selectivity.

<table>
<tr><td colspan="2">High concentration (≥200 μM)</td><td>Low concentration (≤30 μM)</td></tr>
<tr><td>C. elegans</td><td>G. pallida</td><td>G. pallida</td></tr>
<tr><td>Nematicidal (24 hrs)</td><td>Nematicidal (48 hrs)</td><td>Nematicidal (7-14 days)</td></tr>
<tr><td>Motility ↓</td><td>Motility ↓</td><td>Motility ↓</td></tr>
<tr><td>Stimulated pumping ↓</td><td>Stimulated stylet ↓</td><td rowspan="2">Stylet – No effect</td></tr>
<tr><td>Cut head pumping ↑</td><td>Basal stylet ↑</td></tr>
<tr><td>Egg laying ↓</td><td>Cyst hatching ↓</td><td>Cyst hatching ↓ (Partially reversible)</td></tr>
<tr><td colspan="3"></td></tr>
<tr><td colspan="2">Acute behavioural effects neurobiological?
e.g. Stylet & pharyngeal effects 5-HT dependent</td><td rowspan="2">Nematicidal action metabolic impairment?
e.g. Slow time course, reduced & weakened MTT staining</td></tr>
<tr><td colspan="2">Nematicidal action metabolic impairment?
e.g. G. pallida endpoint similar to low concentration (rod-shaped paralysis, tissue necrosis, reduced MTT staining)</td></tr>
</table>

Table 6.1. A summary of the effects of fluensulfone on *G. pallida* and *C. elegans*. High concentrations of fluensulfone acutely affect both *G. pallida* and *C. elegans* behaviour and are nematicidal at 24-48 hours. The acute behavioural effects may reflect non-specific interaction with neural targets, as exemplified by the 5-HT-dependent stimulation of stylet thrusting and pharyngeal pumping. The nematicidal activity at both high and low concentrations may result from metabolic impairment as both shame a similar endpoint, characterised by a rod-shaped paralysis, tissue necrosis and reduced MTT staining.

6.3 The use of *C. elegans* as a model organism for studies into nematicides

C. elegans has been used in the past for mechanism of action studies with several anthelmintics, including ivermectin (Dent et al., 2000, James and Davey, 2009, Ardelli and Prichard, 2013), moxidectin (Ardelli et al., 2009), emodepside (Bull et al., 2007,

Guest et al., 2007), tribendimidine (Hu et al., 2009), abigenin (Kawasaki et al., 2010) and the spiroindolines (Schaeffer et al., 1992). *C. elegans* has also been used to further knowledge of anthelmintics with known modes of action, including levamisole (Lewis et al., 1980, Qian et al., 2008) and the benzimidazoles (Driscoll et al., 1989). *C. elegans* is well established for studies into chemicals for the treatment of animal parasitic nematodes but has not been used as extensively for studies into nematicides for PPN treatment. In recent years *C. elegans* has been used for some studies into the organophosphates and carbamates, nematicides with a known mode of action (Nguyen et al., 1995, Lewis et al., 2013). It has also been suggested that *C. elegans* can be useful as a model for studies into PPN biology (Costa et al., 2007, Jones et al., 2011).

In this study however, it appears that whilst *C. elegans* is susceptible to fluensulfone, high concentrations are required relative to PPNs and the effects seen are likely to be non-selective and may not result from interaction with the "true" target that results in fluensulfone selectivity. This highlights the importance of comparative studies with target species when using *C. elegans* as a model for nematicide and anthelmintic studies. It is possible that *C. elegans* will still be of use in further studies into the mode of action of fluensulfone. Whilst there is a concentration and time discrepancy between the nematicidal activity of high concentrations against *C. elegans* and low concentrations against *G. pallida*, the actual killing effect may still result from interaction with the same target(s). A larger forward genetic screen with *C. elegans* could still provide information regarding the molecular mediators of sensitivity to fluensulfone that may be relevant to its activity in the field. *C. elegans* could also be useful for further studies in PPNs, for example through heterologous expression of putative PPN targets for further characterisation of fluensulfone activity (Cotton et al., 2014). Such an approach would allow investigation of the specificity of fluensulfone toxicity towards *G. pallida* and *M. javanica* over other nematodes such as *C. elegans* and non-nematode species and could inform investigations into new nematode control agents.

Due to differences between *C. elegans* and PPNs, mortality assays with *C. elegans* could not be conducted in the same fashion as those with *G. pallida* and *M. javanica* (Oka et al., 2009). Out of necessity, protracted experiments with *C. elegans* must be conducted in the presence of food to avoid death from starvation or bagging (Chen and Caswell-Chen, 2004). It seems likely that the metabolic and physiological status of

feeding *C. elegans* may be different to that of the non-feeding J2 *G. pallida*. For example, *C. elegans* only upregulate β-oxidation when starved (Elle et al., 2012). Starved *C. elegans* might be more sensitive to fluensulfone. Dauer *C. elegans* were not found to be any more susceptible to fluensulfone than other life cycle stages but it is well established that dauers are highly resistant to most chemical insults (Cassada and Russell, 1975). Assays with *G. pallida* were also conducted in ddH_2O, whilst *C. elegans* assays were conducted on solid NGM media. *G. pallida* were left for prolonged periods in water and the levels of available oxygen may be far lower than in the *C. elegans* assays. This may also result in differences in metabolic or physiological status that could alter susceptibility to fluensulfone. Neutral lipid metabolism in nematodes is known to require oxygen (Green and Wakil, 1960, Fairbairn, 1969) and this may render non-feeding J2 PPNs more susceptible to fluensulfone as they are reliant on their lipid reserves. *C. elegans* are known to be tolerant of low oxygen conditions however (Van Voorhies and Ward, 2000), and it seems likely that PPNs would also be capable of withstanding low oxygen. Exposing *G. pallida* on agar plates in abundant oxygen would reveal whether oxygen levels were a factor determining fluensulfone susceptibility. This difficulty conducting equivalent assays between *C. elegans* and PPNs is a caveat to using *C. elegans* for nematicide mode of action studies.

It seems unlikely however that experimental differences account for the considerable difference in the concentrations that are nematicidal towards *C. elegans* and *G. pallida*. It may be that the target that fluensulfone interacts with at low concentrations in *G. pallida* and *Meloidogyne spp*. is sufficiently different in *C. elegans* and thus far higher concentrations of fluensulfone are required to bind to a lower affinity target. Alternatively, due to physiological or biological differences between *C. elegans* and *G. pallida*, the low concentration target plays a different role in *C. elegans* or a less crucial role. For example, genomic sequencing indicates that *G. pallida* has far fewer predicted genes that encode transporters and enzymes that allow the metabolism and detoxification of xenobiotics, secondary metabolites and other toxins (Lindblom and Dodd, 2006, Cotton et al., 2014).

6.4 The implications of this work regarding the mechanism of action of fluensulfone

During the course of this work, the definitive mode of action of fluensulfone was not identified. The effects of fluensulfone were however carefully characterised, and the data collected provides some indication of avenues to explore in further studies as a route to understanding its nematicidal mode of action.

At 1-30 µM, the paralytic and killing effects of fluensulfone on *G. pallida* were slow, requiring >7 days exposure. Whilst this is a longer exposure than that required to paralyse *M. javanica* (Oka et al., 2009), high immotility only became evident in *M. javanica* at 48 hours. With *G. pallida*, the decline in MTT staining also occurred slowly. As mentioned, fluopyram acts far more rapidly than fluensulfone. This may suggest that fluensulfone is a prodrug and must be metabolised by the target organism to generate the nematicidal metabolite. Alternatively, fluensulfone may cause a slowly building metabolic insult that builds to paralysis and ultimately death. It is possible that inhibition of metabolism could explain the selective toxicity of fluensulfone towards some PPNs. The metabolic requirements of PPNs may be considerably different to other organisms, for example due to the non-feeding nature of sedentary endoparasitic J2s. This may render them more sensitive to a particular metabolic insult. The mechanisms and pathways involved in intermediary metabolism are relatively conserved however throughout the animal, plant and fungi kingdoms (Morowitz, 1992) which could explain the non-selective toxicity of fluensulfone at higher concentrations. The succinate dehydrogenase inhibitor fluopyram, for example has relatively low toxicity towards mammals (*Velum Prime technical bulletin, Bayer Crop Science*), despite this target being conserved across all animal phyla (Scheffler, 1998). It may be that nematodes are more sensitive to such metabolic toxins, yet at higher concentrations these toxins are harmful to other organisms.

The potential block of *G. pallida* lipid consumption by fluensulfone may indicate that the target is involved in β-oxidation. This could explain the slow death that is observed, as other reserves, for example glycogen, could sustain J2s if lipids are inaccessible (Dropkin and Acedo, 1974). As lipids are their primary energy source (Van Gundy et al., 1967, Storey, 1983, Storey, 1984, Robinson et al., 1987a, Robinson et al., 1987b, Perry and Moens, 2013), inhibition of β-oxidation would lead to greatly reduced ATP

levels, which could further impair other metabolic pathways, such as gluconeogenesis (Fromenty and Pessayre, 1995). Reduced acetyl-CoA generation because of β-oxidation inhibition would also adversely affect other metabolisms (Fromenty and Pessayre, 1995). Inhibition of β-oxidation as a mechanism of action could also explain the reduced susceptibility of *C. elegans* and other migratory PPNs to fluensulfone as these nematodes can feed and may therefore be less reliant on lipid consumption. Such inhibition could occur through several mechanisms, including inhibition of β-oxidation enzymes, sequestering of acetyl-CoA or impairment of mitochondrial DNA transcription, translation or replication (Fromenty and Pessayre, 1995).

The slow onset of fluensulfone-induced paralysis could also reflect the metabolic status of the J2. For example, it has been found that the respiration of *M. javanica* declines over the course of 8 days (Fairbairn, 1960). Perhaps as metabolic capability declines with time in the absence of a host plant, the J2 is rendered more susceptible to metabolic insult. The slow onset of fluensulfone-induced death could also result from a form of genotoxicity that is not immediately nematicidal and that indirectly impairs metabolism.

The weakening and subsequent decline in MTT staining that occurs in fluensulfone-exposed *G. pallida* suggests impaired metabolism in the presence of fluensulfone. The fall in MTT staining occurs in parallel with increased percentage immotility however and thus it cannot be known whether fluensulfone directly inhibits nematode metabolism, whether this is an indirect consequence of another mode of action or if the reduced percentage staining merely reflects death. Whether fluensulfone does impair *G. pallida* metabolic capability and whether this causes the ensuing paralysis must therefore be confirmed through other, more selective and sensitive measurements of metabolic activity such as measurement of ATP levels following fluensulfone exposure or measurement of oxygen consumption (see Appendix C) (Schouest et al., 2009, Schaffer et al., 2011, Mouchiroud et al., 2013). This would also have to be confirmed with other PPNs, such as *Meloidogyne spp*. It could also be interesting to measure hydrogen peroxide to assay oxidative stress, which can result from metabolic insults (Gruber et al., 2011). Measures for cytotoxicity or genotoxicity would also be important, as metabolic impairment could potentially result from other mechanisms of toxicity and may not be the underlying reason for the nematicidal activity of fluensulfone. It would be interesting to determine cell viability during exposure to fluensulfone and to compare the time course of this with metabolic impairment. Dyes

for determining cell viability such as trypan blue, Sytox or propidium iodide could be used to this aim (Stoddart, 2011, Ferreira et al., 2015). CARS could also be used to determine whether fluensulfone treatment leads to cellular apoptosis or necrosis through examining effects on DNA, lipids and proteins during exposure (Stoddart, 2011). Confidently determining the fundamental cause of fluensulfone toxicity would be important prior to any further studies and would allow a focussed search for targets. It would also be important to determine whether the cause of fluensulfone toxicity is consistent between *G. pallida* and *Meloidogyne spp*., given the different time course of fluensulfone nematicidal activity in these species.

If metabolic impairment were confirmed as the cause of fluensulfone toxicity, it would be informative to test the effects of known inhibitors of β-oxidation, for example etomoxir (Kruszynska and Sherratt, 1987) on *G. pallida* and to compare their effects to those of fluensulfone. If similar, this might suggest inhibition of β-oxidation as a potential means through which fluensulfone kills *G. pallida*. This could be further investigated through measurement of β-oxidation in *G. pallida* exposed to fluensulfone (Elle et al., 2012). It would also be useful to determine the effects of compounds that inhibit other metabolic pathways, such as the Krebs cycle, glycolysis or glycogen metabolism, on *G. pallida*, and to compare their activity with fluensulfone and inhibitors of β-oxidation.

If fluensulfone were found to affect nematode oxygen consumption or ATP production, metabolomics would also be a route that could shed further light on the effects of fluensulfone on nematode metabolism. Metabolomics would allow measurement of the physiological changes in nematodes that result from fluensulfone exposure, which could shed light on the pathway(s) affected (Ramirez et al., 2013). For example, changing levels of acetyl-coA would indicate alterations in fatty acid oxidation (Li et al., 2015). Metabolomics has been used to elucidate the metabolic effects of compounds *in vitro* and is capable of predicting drug modes of action (Strigun et al., 2011a, Strigun et al., 2011b). Measuring changes in metabolites such as fatty acids, glucose and pyruvate would indicate the metabolic pathway(s) affected by fluensulfone and this could be further investigated through pharmacological manipulation. Such techniques have been used in *C. elegans* to assess changes in metabolites in mutant strains, in response to heat shock or exposure to toxins (Jones et al., 2012, Stupp et al., 2013, Vergano et al., 2014, Pontoizeau et al., 2014). It cannot however be known how feasible or effective such

techniques would be in PPNs. Forward and reverse genetics in *C. elegans* could also still prove useful once the pathways affected are identified. DNA microarrays could also shed light on changes in gene expression following exposure to fluensulfone, which could provide indications of potential targets (Portman, 2006). Such experiments could only however be conducted in *C. elegans*, which is far from ideal given the discrepancies in effects between *C. elegans* and *G. pallida*. Furthermore, drug treatment may result in changes the expression of many genes unrelated to its target.

Other techniques for tracking the location of fluensulfone within cells and nematodes would also contribute to understanding the action of fluensulfone and knowing its cellular localisation could provide indications as to its target. Isotope radiography could be used to this aim, although such techniques can be poorly suited for tracking compounds within living cells or organisms (Ling, 2008). It is not known whether such techniques could be used in PPNs.

The distinct effects of fluensulfone on some PPNs are a considerable obstacle in mechanism of action studies, as PPNs are still poorly understood relative to model organisms such as *C. elegans* and fewer experimental procedures that are useful in determining drug targets have been used in these nematodes. For example, mutagenesis cannot be used in PPNs. Some of the techniques described above have been used in *C. elegans* but have not been used in PPNs before. Much remains unknown about PPN biology, including the metabolic pathways that they employ and the importance of these pathways. Further studies into the mode of action of fluensulfone will have to elucidate the underlying causes for its nematicidal activity in PPNs, for example, whether metabolic impairment leads to death or whether there are other causes. Once this is known, metabolomics, genomic or other techniques would be required to determine the exact effects of fluensulfone exposure on nematode physiology to provide an indication as to the specific processes affected. Narrowing down the pathways affected will enable a more targeted approach, potentially using other model organisms to discover the mechanism of action of fluensulfone.

In conclusion, fluensulfone has distinct high and low concentration effects, with high concentrations affecting *C. elegans* and *G. pallida*, and the low concentrations only affecting *G. pallida*. High concentrations acutely affect multiple *C. elegans* and *G. pallida* behaviours, most likely through non-selective interaction with neuromuscular

targets. Low concentrations do not acutely affect *G. pallida* behaviour but elicit a progressively building paralysis concurrent with metabolic impairment and eventual death. The close parallels between the time course of paralysis and metabolic impairment suggests that fluensulfone is nematicidal through impairment of PPN metabolic capability.

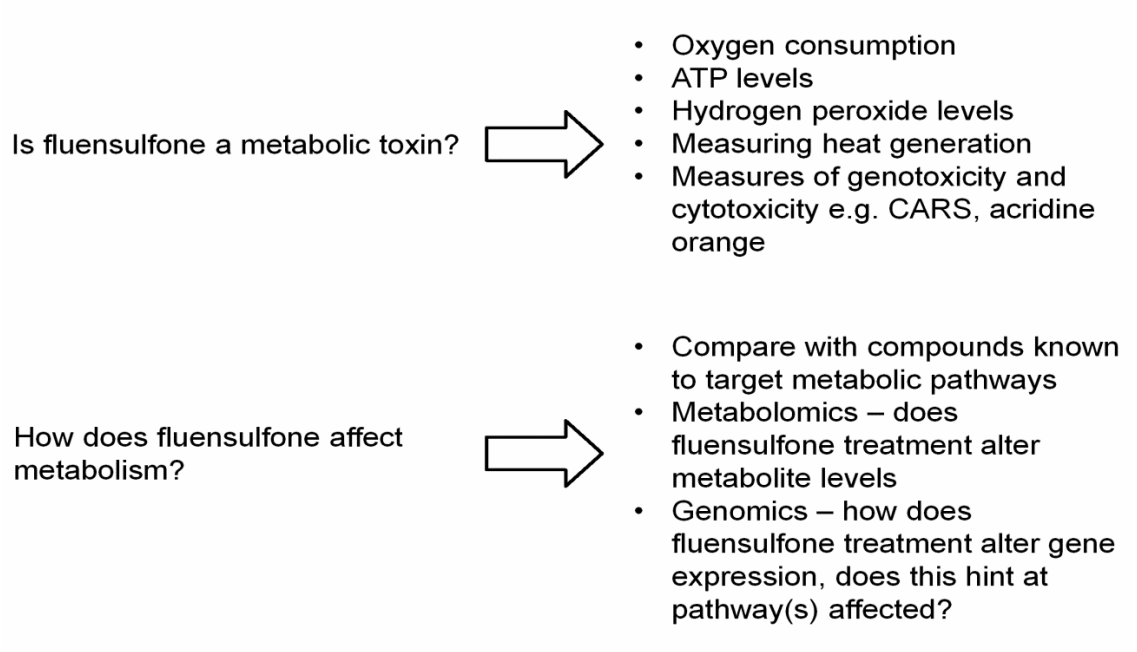

Figure 6.1. A summary of future work for investigating the mode of action of fluensulfone.

Appendix A - A forward genetic screen for mediators of fluensulfone susceptibility

EMS mutagenesis of *C. elegans* to identify targets of fluensulfone

A forward genetic screen was performed for mutants with altered sensitivity to fluensulfone, with a view to identifying putative targets mediating the nematicidal activity of fluensulfone. Susceptibility to the nematicidal activity of 1 mM fluensulfone with 24 hours exposure was used as a selection protocol in an attempt to identify genes that affected susceptibility to fluensulfone. This would allow further investigation of the role of these genes in fluensulfone susceptibility through other genetic, electrophysiological and behavioural analyses.

The EMS mutagenesis was performed as described in chapter 2. 10,000 haploid genomes were screened for resistance to 1 mM fluensulfone. Following exposure to EMS, surviving L4 worms (The F0 generation) were individually picked onto separate plates. These L4s were then allowed mature and to lay eggs, following which the adults were removed. The hatched eggs (The F1 generation) were allowed to develop into gravid adults in which self-fertilisation had occurred and were subsequently bleached to remove the eggs. These eggs (The F2 generation) were transferred to 1 mM fluensulfone plates seeded with OP50, with the progeny of each line on separate plates. After 24 hours, the plates were observed to identify lines that had successfully hatched to L1 worms in the presence of 1 mM fluensulfone. Two F2 lines successfully grew to L3/L4 in the presence of fluensulfone and were moved to separate 1 mM fluensulfone plates seeded with OP50. These worms developed to adults and were able lay eggs. These adults were picked to OP50-seeded NGM plates without drug and allowed to propagate.

These strains were named “Flu1” and “Flu3”. Both strains were able to propagate on OP50-seeded 1 mM fluensulfone plates, whereas N2 could not develop and propagate in the presence of 1 mM fluensulfone. The growth, development and reproduction of both Flu1 and Flu3 in the presence of 1 mM fluensulfone was still however very poor, with the majority of eggs still failing to hatch and develop into adults. As the pharyngeal pumping of L4+1 day N2 worms was rapidly and profoundly inhibited by fluensulfone (see section 3.2.3), L4+1 day Flu1 and Flu3 strains were exposed to 500 µM and 1 mM fluensulfone and pharyngeal pumping was scored over a 24 hour period (Figure A.1). In the absence of fluensulfone, the pump rate of Flu1 worms was not different from the N2 control, whereas the pump rate of Flu3 worms was consistently lower and more variable

(Figure A.1). This suggests that the mutagenesis process may have compromised the physiology of the Flu3 strain. The rate of pharyngeal pumping in the presence of 500 µM was not significantly different between N2, Flu1 and Flu3 worms and pump rate was significantly lower than in the absence of fluensulfone for all three strains (Figure A.1). After 24 hours in the presence of 1 mM fluensulfone, Flu1 worms exhibited more pumping than both N2 and Flu3 worms, in which no pumping was observed (Figure A.1). Despite this, all N2, Flu1 and Flu3 worms died when left for 48 hours in the presence of 1 mM fluensulfone. This indicates that the Flu1 and Flu3 strains had low-level resistance to fluensulfone.

The low-level resistance of these strains to fluensulfone suggests that neither strain had mutations that profoundly altered susceptibility to fluensulfone. It also seems unlikely that either strain had mutations in the genes encoding the major target(s) that mediate the nematicidal activity of fluensulfone on *C. elegans*. This mutagenesis screen may not have identified any strains with high-level resistance due to an insufficient number of haploid genomes screened. It is also possible that fluensulfone interacts with multiple targets at 1 mM and thus multiple mutations would be required to yield high-level resistance. Another possibility is that the target(s) of fluensulfone are integral to *C. elegans* development, growth and physiology and so any mutations in the gene encoding these target(s) of fluensulfone are lethal. It is possible that repeating this mutagenesis screen with a greater number of haploid genomes could identify potential target that mediate the action of fluensulfone.

It was concluded that neither the Flu1 or Flu3 strains had mutations in the major target(s) that mediate the action of fluensulfone on *C. elegans* and therefore a reverse genetic approach was adopted.

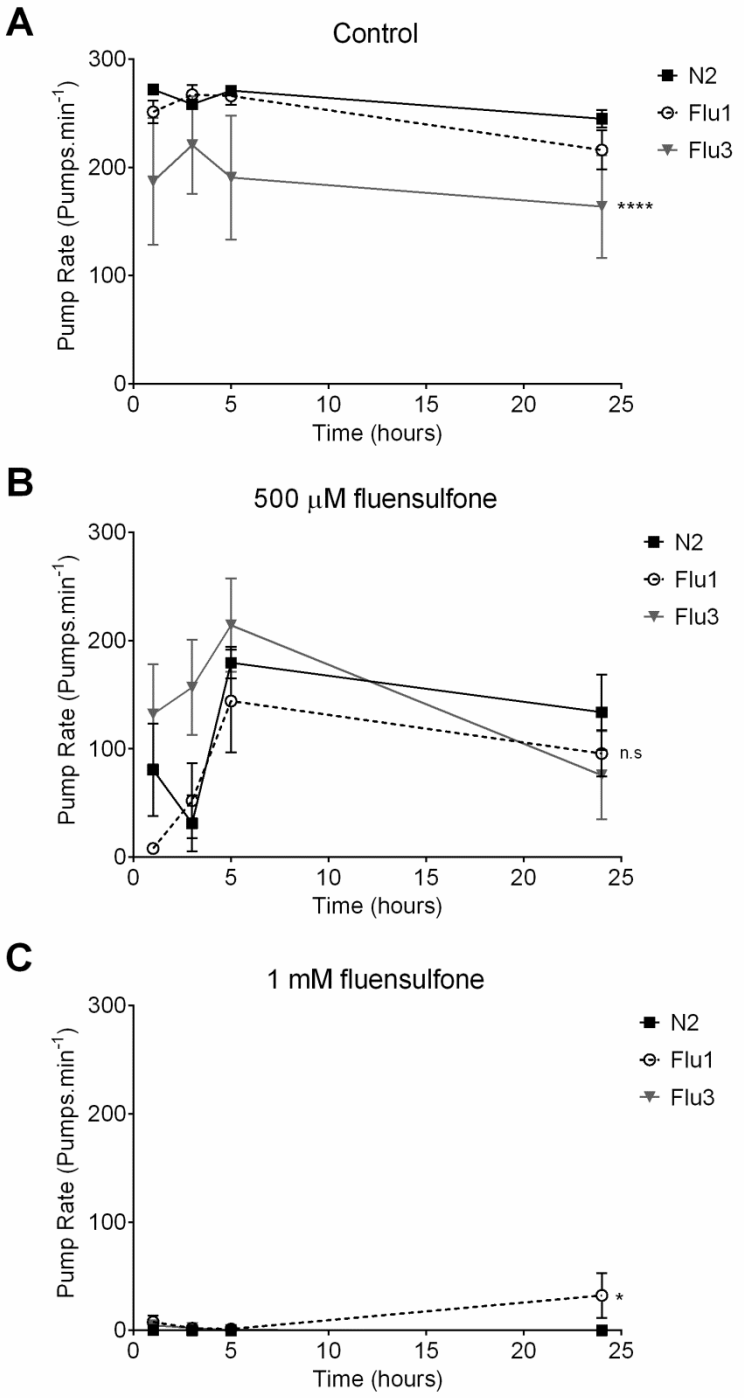

Figure A.1. The effects of fluensulfone on the pharyngeal pumping of mutant strains generated by EMS mutagenesis. The mutant strains generated by mutagenesis, termed Flu1 and Flu3, were placed on food plates in the presence of vehicle (control), 500 µM fluensulfone or 1 mM fluensulfone and pharyngeal pumping was counted at intervals up to 24 hours. An N2 control was also performed. **A**) On the control plates, Flu3 worms had a significantly lower pump rate than N2 and Flu1 worms ($P<0.001$). **B**) There was no difference in pump rate at 500 µM fluensulfone between strains ($P=0.21$). **C**) The pump rate of Flu1 worms was slightly higher after 24 hours relative to N2 ($P<0.05$). (A, B and C represent different drug treatments conducted in the same experiment, n=6 worms per treatment, per strain, two-way ANOVA with Dunnett post-hoc tests).

Appendix B - Staining of *G. pallida* and *C. elegans* mitochondria

Mitochondrial staining with the fluorescent dye DiS-C3(3)

Mitochondrial membrane potential can act as an indicator of metabolic activity and functionality (Chen, 1988, Pendergrass et al., 2004). The electron transport that occurs during oxidative phosphorylation generates a potential difference across the mitochondrial membrane, and changes in this can indicate alterations in metabolic activity (see general introduction, section 1.17). The lipophilic carbocyanine fluorescent dye 3,3'-dipropylthiocarbocyanine iodide (DiS-C3(3)) has been used successfully in *C. elegans* to measure changes in mitochondrial membrane potential in different mutant backgrounds (Gaskova et al., 2007). Gaskova et al. (2007) also showed that DiS-C3(3) can detect changes in mitochondrial membrane potential induced by compound CCCP, which acts to uncouple the electron transport chain (Kasianowicz et al., 1984). In this appendix, the use of DiS-C3(3) for staining mitochondria of *C. elegans* and *G. pallida* will be discussed.

C. elegans adults and *G. pallida* J2s were stained with DiS-C3(3) and imaged under a DsRed filter. *C. elegans* exhibited strong fluorescence in the head and around the pharynx, with weaker fluorescence in the rest of the body (Figure A.2A, B). This is consistent with observations made by Gaskova et al. (2007), who speculated that this distribution of staining results from the dye entering the worm through the mouth and the intestine rather than diffusing across the cuticle. *G. pallida* J2s also fluoresced predominately around the head region (Figure A.2C, D). The strong staining in the head suggests that both *C. elegans* and *G. pallida* have a high density of mitochondria in the head region. For *C. elegans*, this is consistent with past observations that suggest that the tissues in the head and around the pharynx possess a high density of mitochondria (Labrousse et al., 1999, Settivari et al., 2009, Dingley et al., 2010). This may reflect the high energy requirements of the pharyngeal and head muscles during rapid pharyngeal pumping. This may also explain a higher density of mitochondria in the head region of *G. pallida* J2s, as vigorous stylet and pharyngeal activity is likely to require high energy consumption. The strong staining in the head may however be due to diffusion occurring predominately through the mouth.

The ability of DiS-C3(3) to assess mitochondrial membrane potential in *C. elegans* and *G. pallida* was not assessed here. If DiS-C3(3) can measure membrane potential in *C.*

elegans and *G. pallida* it may prove a useful tool for assessing the metabolic impact of nematicides, anthelmintics and other toxins on nematodes.

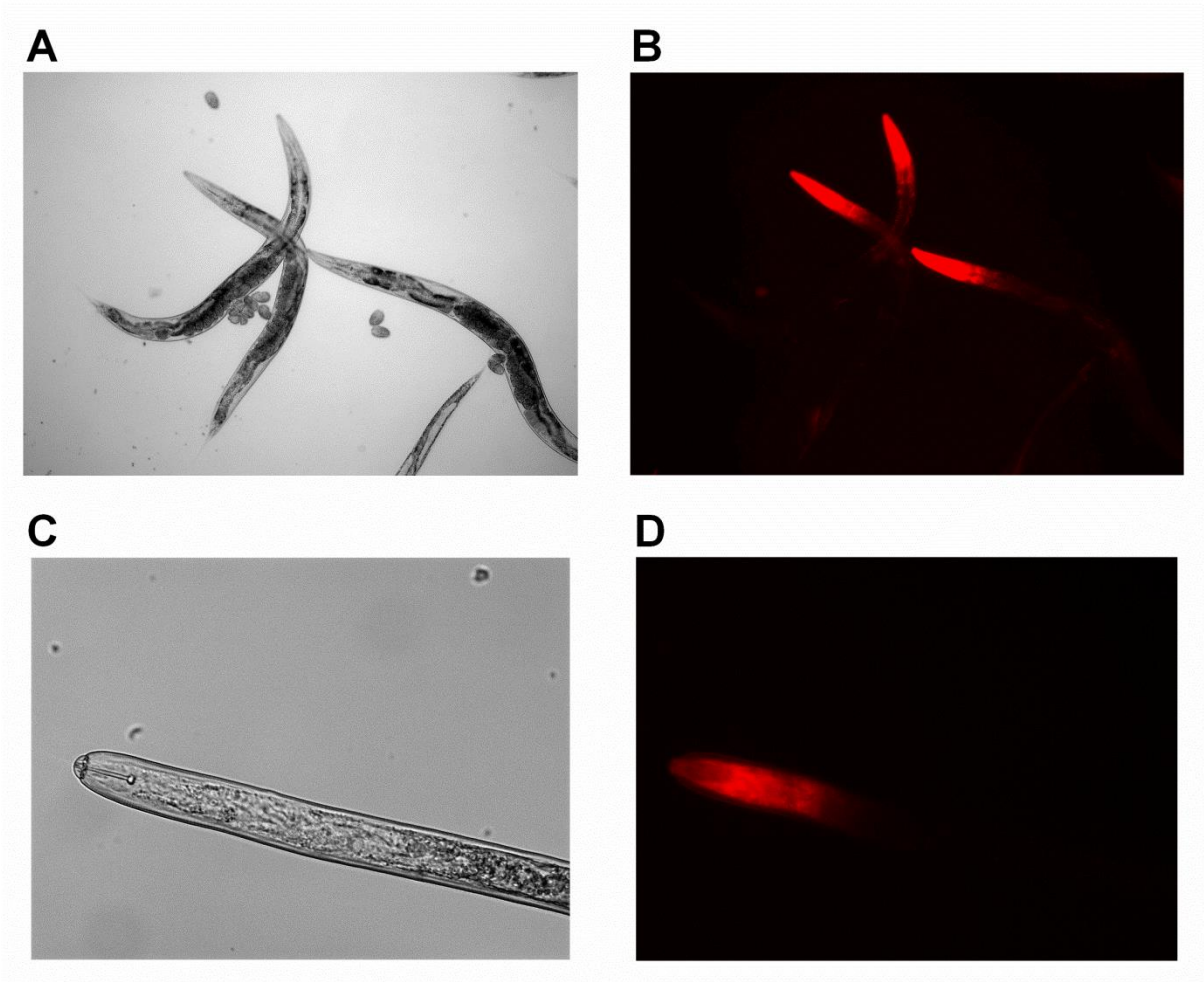

Figure A.2. The mitochondrial dye DiS-C3(3) stains *C. elegans* and *G. pallida* predominately in the head region. A+**B**) *C. elegans* adults were stained with 4 µM DiS-C3(3) and imaged via DIC microscopy (A) and fluorescence microscopy (B) under a DsRed filter. **C**+**D**) *G. pallida* J2s were stained with 4 µM DiS-C3(3) and imaged via DIC microscopy (**C**) and fluorescence microscopy (**D**) under a DsRed filter.

Appendix C – Optical fluorimetry to measure oxygen consumption in nematodes

Using the phosphorescent dye Mito-ID to measure oxygen consumption in *C. elegans*

The reduction and loss of MTT staining following exposure of J2 *G. pallida* to fluensulfone suggested that fluensulfone might impair PPN metabolism and that this may contribute to its nematicidal activity (see Chapter 5). Following these observations, quantitative measures of *G. pallida* metabolism were required to investigate the time course of potential metabolic effects in detail.

Measuring oxygen consumption allows direct analysis of aerobic metabolism and has been used as a measure of metabolism in several organisms, including *C. elegans* (Rosenfeld et al., 2003, Van Voorhies et al., 2008, Schouest et al., 2009, Van Raamsdonk et al., 2010b, Streeter and Cheema, 2011). Oxygen consumption in *C. elegans* can been measured using a Clark electrode, optical tools, a Seahorse analyser and using a phosphorescent dye (Suda et al., 2005, Schouest et al., 2009, Luz et al., 2015).

Here, a technique using a phosphorescent oxygen-sensing probe was adapted from Schouest et al. (2009). This technique was adopted as it was reported that accurate readings could be obtained from fewer *C. elegans* using the phosphorescent oxygen-sensing probe (Schouest et al., 2009, Luz et al., 2015). It is reasonable to assume that *G. pallida* J2s, with their smaller body size and low metabolic activity relative to adult *C. elegans*, would consume less oxygen than *C. elegans*. Therefore, it seems likely that a considerably larger number of *G. pallida* would be required to obtain measurable and accurate readings of oxygen consumption. Ideally, a more sensitive technique will permit the use of fewer *G. pallida*, which may be a limiting factor in experimental design.

Here, the phosphorescent oxygen-sensing probe Mito-ID (Enzo Life sciences) was used to measure oxygen consumption in *C. elegans*, as a prelude to measurements using *G. pallida*. Several of these oxygen-sensing probes have been developed and all share a common mechanism of measuring oxygen consumption (Papkovsky and Dmitriev, 2013). These probes are luminophores and work via quenched phosphorescence, which is a photochemical process in which molecular oxygen collides with the indicator dye molecule when it is in an excited state (Papkovsky and Dmitriev, 2013). Following

excitation by light of an appropriate wavelength, the luminophore indicator molecule relaxes to an excited triplet state, which slowly emits light and phosphoresces. When present, oxygen molecules collide with the luminophore, which reduces the yield and lifetime of this phosphorescence in a concentration-dependent manner. Thus when oxygen is depleted by a respiring organism, there is less oxygen to quench the phosphorescence of the probe and thus the emission signal increases (see Papkovsky and Dmitriev (2013) for detailed review). Schouest et al. (2009) used this phenomenon to measure extracellular oxygen in a 96 well plate containing *C. elegans* and sealed with either oxygen-impermeable tape or mineral oil. A fluorescent plate reader was used to excite the phosphorescent probe and to read the subsequent emission, with emission strength increasing as *C. elegans* consumed oxygen.

Here, a series of experiments with *C. elegans* were performed, in which L4+1 day adults were added to the wells of a 96 well plate in M9 buffer. Drugs were then added to the solution where applicable, following which the Mito-ID probe was added. Each well was then sealed with oxygen impermeable mineral oil and the plate was measured in a fluorescent plate reader using time-resolved fluorescent readings to measure the phosphorescent signal. For each treatment, blanks were performed with no Mito-ID present in the well and all the data shown are corrected to the appropriate blank wells.

In initial experiments, the dye was tested without *C. elegans*, to ensure that a measurable signal was generated (data not shown). The fluorescence intensity from wells in which Mito-ID was present was ~20-fold greater than wells without the probe, indicating that the settings on the plate reader were sufficient to detect the emissions from the probe.

Subsequently, experiments were performed with *C. elegans*, to ascertain whether the probe could detect and measure changes in oxygen levels as a result of *C. elegans* respiration. Firstly, three treatments were compared with 15 µl of the probe present: no *C. elegans* with M9 buffer alone, 500 L4+1 *C. elegans* and 500 L4+1 *C. elegans* in the presence of 20 mM sodium azide (Figure A.3). The fluorescent signal was recorded every minute for 3 hours. The fluorescence intensity for the wells with untreated *C. elegans* increased over the first 15 minutes, at which point the fluorescent signal reached the maximal detection limit for the plate reader used. In contrast, the fluorescent signal for wells with M9 alone and the wells with sodium azide-treated *C.*

elegans fell over the 3 hours. This reduction in signal was not reported by Schouest et al. (2009) or by the manufacturers of the probe (Enzo Life Sciences). This reduction in the fluorescent signal could be due to increased oxygen concentrations in the well because of back diffusion of oxygen into the well through the plastic. A reduction in signal could also result from bleaching of the fluorescent probe following repeated excitation. Another explanation is a reduction in the concentration of the probe in the solution, perhaps due to its degradation.

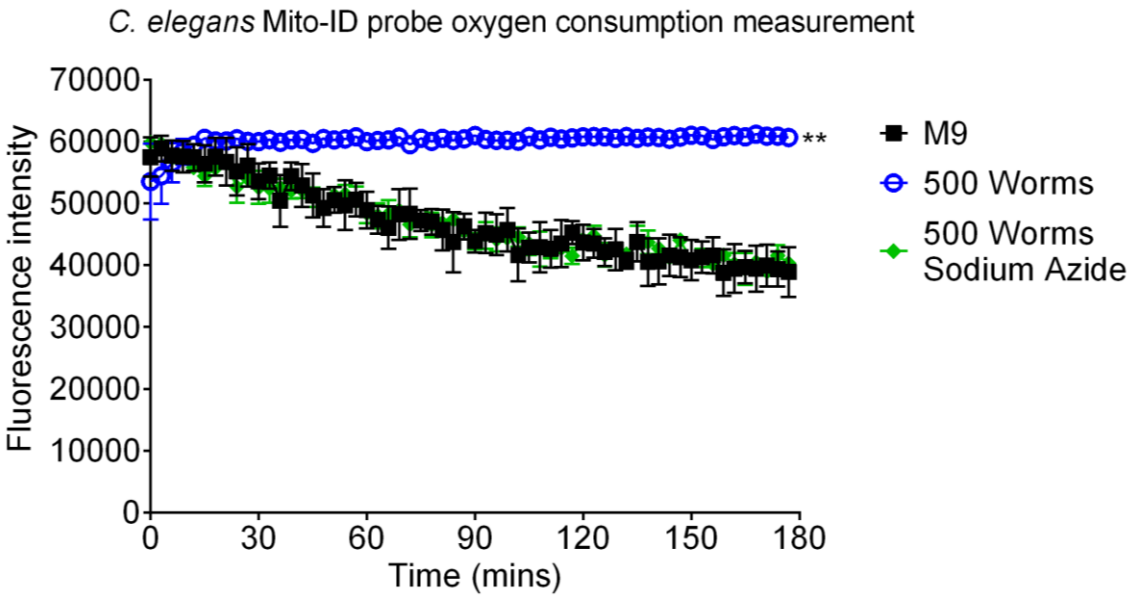

Figure A.3. The Mito-ID oxygen-sensing probe can detect differences in oxygen consumption in *C. elegans*. Mito-ID oxygen measurements were conducted using 96 well plates. Wells contained M9 buffer and 15 µl of the Mito-ID probe. Wells were set up with M9 alone, 500 L4+1 day *C. elegans* or 500 *C. elegans* treated with 20 mM sodium azide. Fluorescence intensity was read over 180 minutes with a gain setting of 4095. The fluorescence intensity per well was higher when untreated *C. elegans* were present relative to wells without *C. elegans* or wells with sodium azide-treated *C. elegans*. The fluorescence intensity for the wells with untreated *C. elegans* reached the detection threshold of the plate reader. The data are corrected with the intensity readings from the appropriate blanks wells (n=3 wells per treatment, mean ± s.e mean, two-way ANOVA with Tukey post-hoc tests, $P<0.0001$).

In spite of this observation, the signal from wells with untreated *C. elegans* did increase and was higher than that of both wells with no *C. elegans* and wells with sodium azide-treated *C. elegans*. Furthermore, there was no difference in the fluorescence signal between sodium azide-treated worms and wells with no worms. This indicates that as untreated *C. elegans* consume oxygen, the fluorescent signal increases relative to wells without worms. In contrast, worms treated with the metabolic inhibitor sodium azide do not consume oxygen and thus the fluorescent signal does not differ from wells without

worms. This shows that the probe is capable of detecting oxygen consumption by *C. elegans*.

In the experiment shown in Figure A.3, the fluorescent signal for 500 untreated *C. elegans* reached the detection threshold of the plate reader used. This could be due to the high number of *C. elegans* used per well. Alternatively, this could result from the concentration of the dye used. Therefore, an experiment was conducted to compare the signal obtained with different number of *C. elegans* and different concentrations of the probe (Figure A.4). Wells were set up with either 10 µl or 15 µl of the Mito-ID probe and with 20, 50, 100 and 200 *C. elegans* per well. As in the experiment described above, the fluorescence intensity fell in wells with no *C. elegans* for both 10 and 15 µl of the probe. At 15 µl of the probe, wells with 50, 100 and 200 *C. elegans* exhibited an increase in fluorescence intensity relative to wells with no worms (Figure A.4). Wells with 20 worms did not exhibit an increased fluorescence signal relative to wells without worms. The fluorescence intensity of wells with 20 worms did however increase over time, whereas the intensity recorded from wells with no worms fell. It seems likely then that 20 worms is a sufficient number to measure oxygen consumption using the Mito-ID probe, and would probably be significantly different from wells with no worms with more repeats. As in the experiment shown in Figure A.3, fluorescence intensity reached the detection limit of the plate reader for wells with 100 and 200 worms.

At 10 µl Mito-ID probe per well, fluorescence intensity was higher in the wells with 100 and 200 worms relative to wells with no worms (Figure A.4B). Wells with 20 and 50 worms did not have significantly higher fluorescence than wells with no worms. Fluorescence intensity did however increase relative to the initial intensity with both 20 and 50 worms per well, whereas the fluorescence intensity fell in wells without worms. As with 15 µl of the probe, the fluorescent signal from wells with 100 and 200 worms per well reached the detection limit for the plate reader.

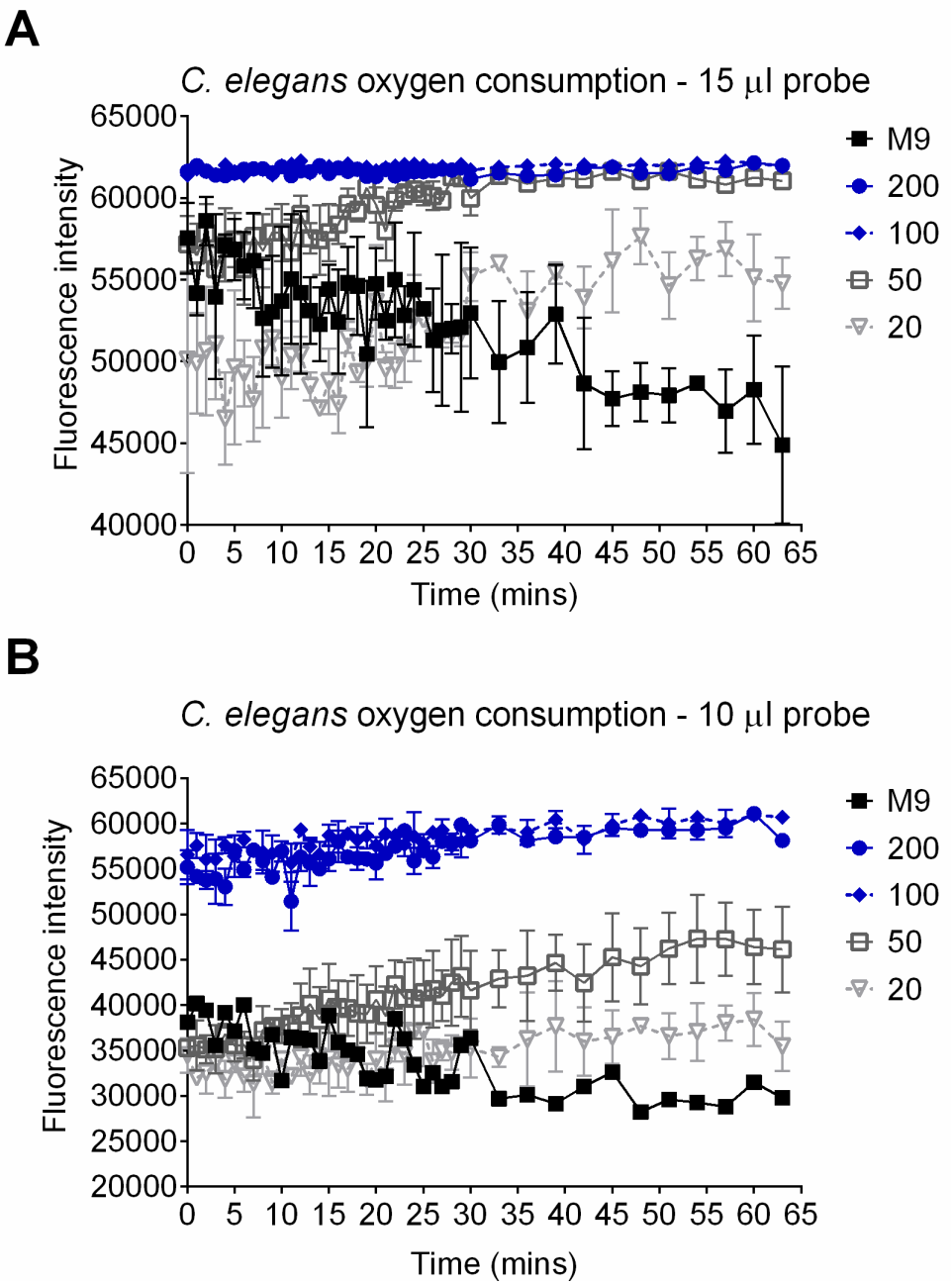

Figure A.4. Optimisation of the number of *C. elegans* required for oxygen consumption measurements using the Mito-ID probe. Mito-ID oxygen measurements were conducted using 96 well plates. Wells contained M9 buffer and 15 µl of the Mito-ID probe (**A**) or 10 µl of the Mito-ID probe (**B**). Wells were set up with M9 alone and 200, 100, 50 and 20 L4+1 day *C. elegans*. Fluorescence intensity was read over 65 minutes with a gain setting of 4095. **A**) Fluorescence intensity was higher in wells with 50, 100 and 200 worms relative to wells with M9 alone n=2-3 wells per treatment, mean ± s.e mean, two-way ANOVA with Tukey post-hoc tests, P<0.0001). **B**) Fluorescence intensity was higher in wells with 100 and 200 worms relative to wells with M9 alone (n=2-3 wells per treatment, mean ± s.e mean, two-way ANOVA with Tukey post-hoc tests, P<0.0001). The fluorescence intensity for the wells with 100 and 200 *C. elegans* reached the detection threshold of the plate reader in both A and B. The data are corrected with the intensity readings from the appropriate blanks wells

In the experiments described above (Figure A.3, Figure A.4), the FLUOstar Optima fluorescence plate reader automatically adjusted its gain settings based on fluorescence intensity following excitation of the Mito-ID probe. This gave the maximal gain value of 4095. The gain value sets the relative sensitivity of the plate reader to the fluorescent signal; thus the greater the gain value the greater the sensitivity. In subsequent experiments, the gain value was adjusted and it was found that 3500 was the optimal value for allowing measurement of oxygen consumption without the fluorescence intensity reaching the sensitivity limit for the plate reader (data not shown).

Having ascertained the optimal settings and worm number for measuring oxygen consumption with the Mito-ID probe, a further experiment was performed to determine whether this technique could measure changes in oxygen consumption resulting from exposure of *C. elegans* to nematicidal compounds (Figure A.5). In this experiment, *C. elegans* (50 per well) were exposed to 1 mM fluensulfone, 50 µM fluopyram or 0.5% ethanol as a vehicle control and fluorescence intensity was read over 3 hours with 15 µl of the Mito-ID probe present. Fluopyram is a succinate dehydrogenase inhibitor (complex II of the electron transport chain) (Veloukas and Karaoglanidis, 2012, Hungenberg et al., 2013) and so should reduce oxygen consumption as sodium azide did in Figure A.3. Whilst *C. elegans* is less sensitive to fluensulfone than PPNs, it does acutely affect behaviour at 1 mM (see Chapter 3). A fluensulfone treatment was included to ascertain whether high concentrations of fluensulfone acutely affect *C. elegans* oxygen consumption.

When expressed as a percentage of the origin fluorescence intensity, wells without any *C. elegans* exhibited the same reduction in fluorescence intensity over time as described above (Figure A.3, Figure A.4). In contrast, the florescence intensity of wells with *C. elegans* treated with vehicle steadily increased over 3 hours, reaching 130% of the origin intensity. The fluorescence intensity of fluopyram wells increased over the initial 50 minutes of exposure and then began to fall to below the origin fluorescence intensity at 3 hours exposure. This indicates that fluopyram does impair *C. elegans* oxygen consumption. The distinct time course to sodium azide, which immediately inhibited oxygen consumption, is likely to reflect differences in concentration, as the sodium azide was applied at 20 mM. The change in fluorescence intensity in fluensulfone wells did not differ from the control, rising gradually over time to 123% origin intensity. This suggests that fluensulfone does not alter *C. elegans* oxygen consumption with acute

exposure. As shown in Chapter 5 however, *C. elegans* is less sensitive to fluensulfone than *G. pallida*. Furthermore, it is possible that changes in oxygen consumption may have become apparent with longer exposure times. This must be repeated over a longer time course before any firm conclusions can be drawn from this data.

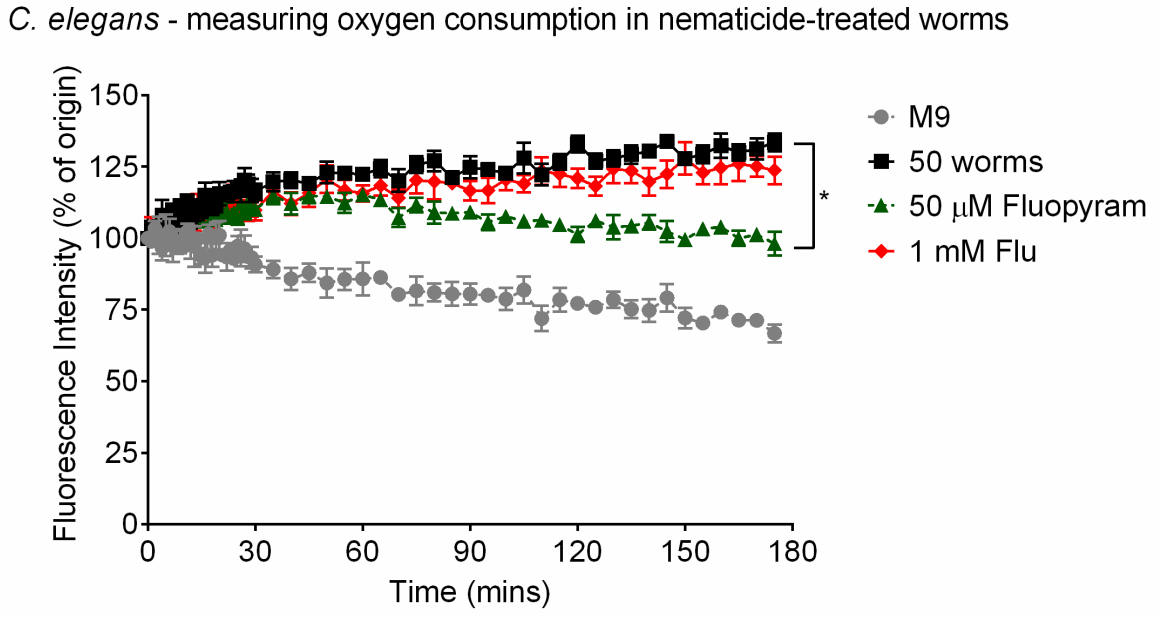

Figure A.5. The Mito-ID probe can detect differences in oxygen consumption of *C. elegans* resulting from treatment with nematicides. Mito-ID oxygen measurements were conducted using 96 well plates. Wells contained M9 buffer and 15 µl of the Mito-ID probe. Wells were set up with M9 alone, 50 L4+1 day *C. elegans* or 50 *C. elegans* treated with 50 µM fluopyram or 1 mM fluensulfone. Fluorescence intensity was read over 180 minutes with a gain setting of 3500. The data are shown as a percentage of the fluorescence intensity at the origin for each treatment (time 0). The fluorescence intensity per well was higher when untreated *C. elegans* were present relative to wells without *C. elegans* or wells with fluopyram-treated *C. elegans*. Fluensulfone did not affect oxygen consumption. The data are corrected with the intensity readings from the appropriate blanks wells (n=3-4 wells per treatment, mean ± s.e mean, two-way ANOVA with Tukey post-hoc tests, $P<0.0001$).

In this experiment, there were substantial differences in the origin fluorescence intensity between treatments, with intensity at 12000 for the wells without worms as compared to 17000 for the control wells with *C. elegans* present (data not shown). The origin intensity in the fluopyram wells was 16000, whilst the fluensulfone origin intensity was 15000. This was also seen in the experiment shown in Figure A.4, in which the origin fluorescence intensity was progressively higher in wells with a greater number of *C. elegans*. This is unlikely to be due to auto-fluorescence of the *C. elegans*, as all the data were blank corrected and the blank wells contained the corresponding number of worms. As the wells with fluopyram-treated worms also exhibited lower fluorescence

intensity at the origin, it is possible that the difference in origin intensity reflects very rapid oxygen consumption that occurs before the initial reading can be conducted. The differences in origin intensity must be investigated in further experiments, as must the fall in fluorescence intensity in wells without worms.

Despite these issues, the data shown here indicate that the Mito-ID dye can measure oxygen consumption by *C. elegans* and can be used to determine differences in oxygen consumption that result from drug treatments and differing worm numbers. Therefore, an experiment was conducted to ascertain the suitability of the Mito-ID dye for measuring oxygen consumption in *G. pallida*. Wells were set up containing 200, 500 and 100 *G. pallida* J2s and fluorescence intensity in the presence of 15 µl Mito-ID was measured over 3 hours (Figure A.6). An additional well, containing 500 *G. pallida* J2s treated with 20 mM sodium azide was included. In all the treatments, fluorescence intensity declined with time. In spite of this, fluorescence intensity was higher in the wells with 500 J2s relative to the wells with 500 J2s treated with sodium azide. Furthermore, fluorescence intensity was higher in wells with greater numbers of worms. This suggests that the Mito-ID dye could still prove useful for measuring oxygen consumption in *G. pallida*.

The difference between the readings made from *G. pallida* and *C. elegans* probably reflects the greater body size of the adult *C. elegans* and a higher metabolic rate relative to the generally inactive J2s. This could be overcome using a greater number of J2s per well, although obtaining more worms may not be feasible for experiments assessing the effects of fluensulfone on *G. pallida* metabolism, given the extended time course of its effects on *G. pallida* motility. Adjusting experimental conditions could also permit measurement of *G. pallida* oxygen consumption using the Mito-ID probe, for example increasing the gain settings or increasing the concentration of Mito-ID present in each well. It seems likely that the decline in fluorescence that occurs in wells without worms may mask potential differences in fluorescence intensity when oxygen consumption is low. This issue must therefore be addressed before attempting any more readings with *G. pallida*. Schouest et al. (2009) investigated how the plate format affects readings of oxygen consumption using a phosphorescent oxygen-sensing probe and found that different plate formats could greatly alter the number of worms required to obtain accurate readings of oxygen consumption. This avenue will need to be explored in

further work using optical oxygen phosphorescence to measure oxygen consumption in PPNs.

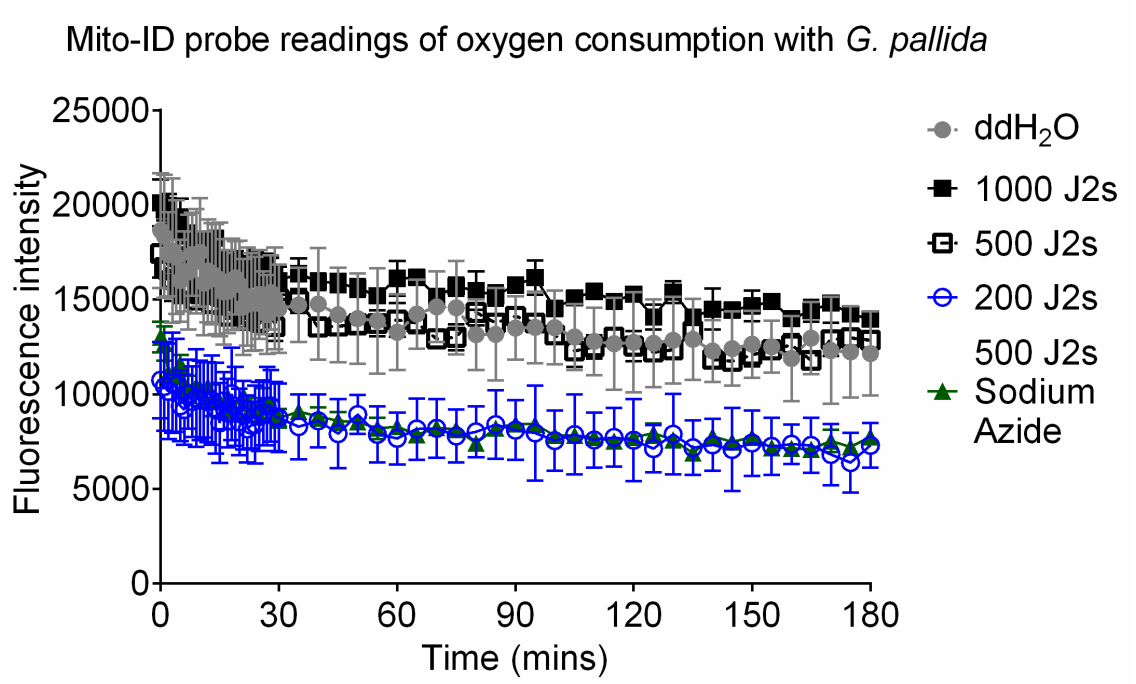

Figure A.6. Measuring *G. pallida* oxygen consumption with the Mito-ID probe. Mito-ID oxygen measurements were conducted using 96 well plates. Wells contained ddH_2O and 15 µl of the Mito-ID probe. Wells were set up with ddH_2O alone and 1000, 500 and 200 J2 *G. pallida*. Fluorescence intensity was read over 180 minutes with a gain setting of 3500. Fluorescence intensity fell in all the wells with time. The data are corrected with the intensity readings from the appropriate blanks wells (n=2 wells per treatment, mean ± s.e mean, two-way ANOVA with Tukey post-hoc tests, P>0.05).

Appendix D – Assessing the sensitivity of *Saccharomyces cerevisiae* to fluensulfone

Assessing the sensitivity of the yeast *Saccharomyces cerevisiae* to fluensulfone

C. elegans is less sensitive to fluensulfone than PPNs, with ≥20-fold higher concentrations required to kill *C. elegans* than *G. pallida* and *M. incognita*. A mutagenesis screen in *C. elegans* failed to identify any high-level resistance against fluensulfone. This approach is not currently feasible in PPNs. Mutagenesis and other genomic approaches are powerful tools for identifying drug targets and have been used in the past to this aim (Kaletta and Hengartner, 2006). If another organism that is sensitive to fluensulfone could be used for forward or reverse genetic approaches, this could allow identification of target(s) that mediate fluensulfone effects.

Mutagenesis is possible in yeasts such as *S. cerevisiae* and could be used to identify drug targets (Kilbey et al., 1978, Alcalde, 2010, Xu et al., 2011). *S. cerevisiae* mutant libraries are also available for screening for sensitivity to drugs (Scherens and Goffeau, 2004). Here, experiments were conducted to ascertain whether *S. cerevisiae* is sensitive to fluensulfone and could be used for mutagenesis experiments for identifying the target(s) which mediate sensitivity to fluensulfone.

In initial experiments, YEPD plates were made up containing either vehicle (0.5% acetone) or fluensulfone. These plates were seeded with serial dilutions of liquid yeast cultures grown to an optical density of OD_{600} 0.6-0.8. The plates were seeded with 1 in 10, 1 in 100, 1 in 1000, 1 in 10,000 and 1 in 100,000 dilutions of the original yeast culture. The YEPD plates were then grown at 30^{o}C for up to 7 days and were imaged. At all dilutions of the liquid yeast culture, colonies grew in the presence of 1-100 µM fluensulfone as well as on the control plates. Colonies expanded from 1 day to 6 days, indicating colony growth. There were no overt differences in the colonies exposed to fluensulfone relative to those on the control plates and colony number did not significantly differ between any of the treatments (Figure A.7). This suggests that fluensulfone treatment does no impair yeast growth or kill yeast at concentrations up to 100 µM. There was however considerable variability in the number of colonies that grew at the 1 in 10,000 dilution concentration. These spot cultures were grown for several further weeks and no change was seen in the cultures exposed to either vehicle or fluensulfone (data not shown).

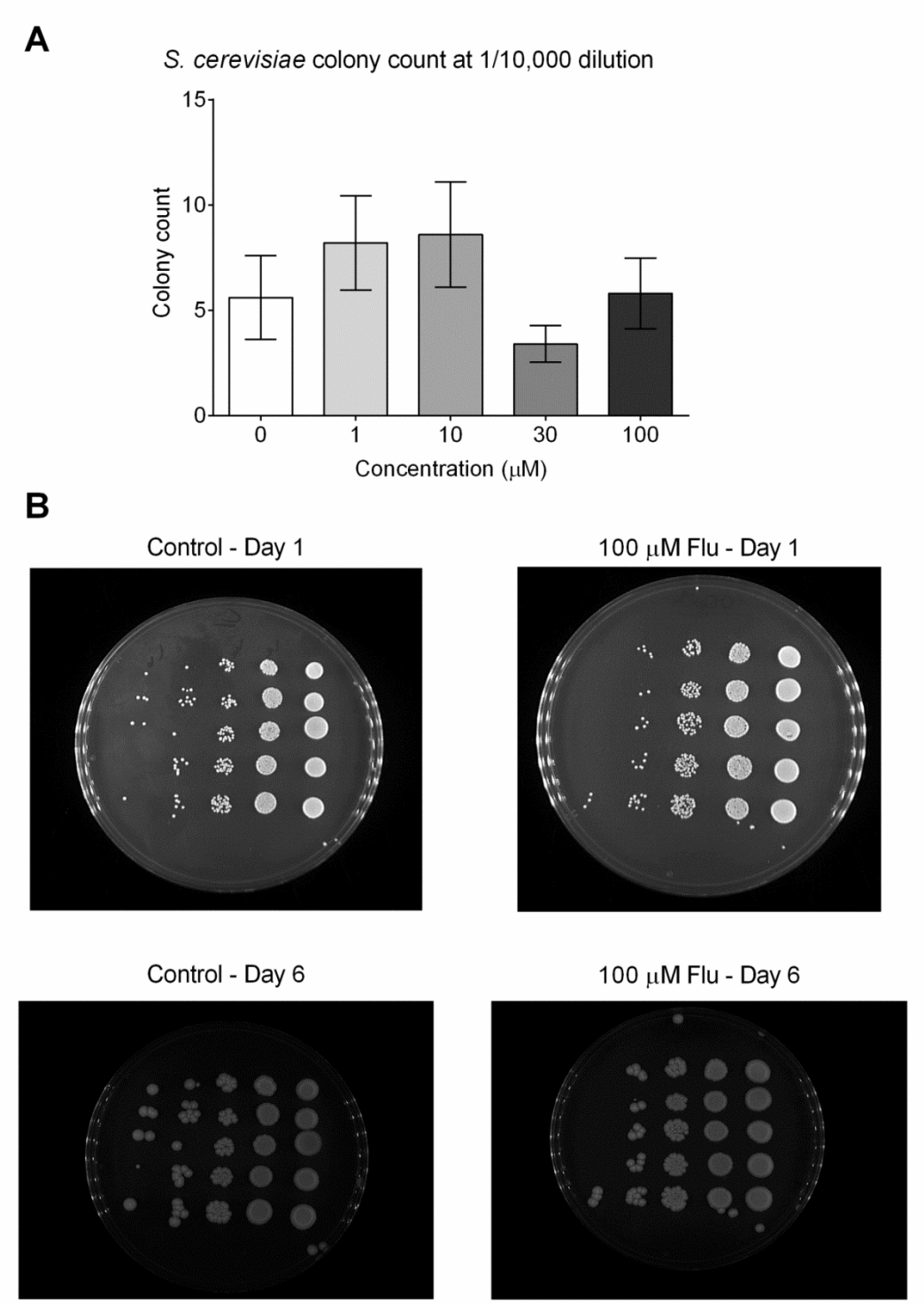

Figure A.7. Fluensulfone does not impair yeast spot culture growth. *S. cerevisiae* liquid cultures, grown in YEPD media were diluted 1/10, 1/100, 1/1000, 1/10,000 and 1,100,000 and pipetted onto plates modified with either vehicle or fluensulfone and grown at 30^{o}C. **A**) Colony number at 1/10,000 dilution was not affected by fluensulfone treatment (n=5 separate yeast cultures spotted per plate, mean ± s.e mean colony number shown, one-way ANOVA, P=0.34). **B**) Representative images of the yeast spot cultures at 1 day and 6 days of growth. The 1/10 dilution is on the far right of each plate; the 1/100,000 is on the far left. The colonies on both the control and 100 μM fluensulfone plates continued to grow and expand from 1 day to 6 days.

To further investigate the sensitivity of *S. cerevisiae* to fluensulfone, liquid yeast cultures were exposed to fluensulfone to determine its effect on their growth (Figure

A.8A). Liquid yeast cultures were grown in YEPD media overnight and subsequently diluted and grown to OD_{600} 0.6-0.8. These cultures were then diluted 1/10 and fluensulfone dissolved in acetone was added to give final concentrations of 30 µM and 500 µM. These cultures were then grown at 30^{o}C and optical density was measured at 1 and 2 hours. Fluensulfone did not affect the increase in optical density that occurred as the yeast grew and there was no significant difference in optical density between fluensulfone cultures and the control cultures. In a subsequent experiment, yeast cultures were exposed to the nematicide fluopyram (Figure A.8B). Fluopyram was originally used as a fungicide (Veloukas and Karaoglanidis, 2012) and therefore may be able to prevent yeast growth. 50 µM and 500 µM fluopyram did not affect yeast growth relative to the control however, even at 24 hours.

As it would be expected that fluopyram would prevent yeast growth, this calls into question the methods used here for determining yeast sensitivity to fluensulfone and fluopyram. Little can be concluded from the data shown here regarding the sensitivity of yeast to fluensulfone and its applicability for mutagenesis studies to determine the target(s) of fluensulfone.

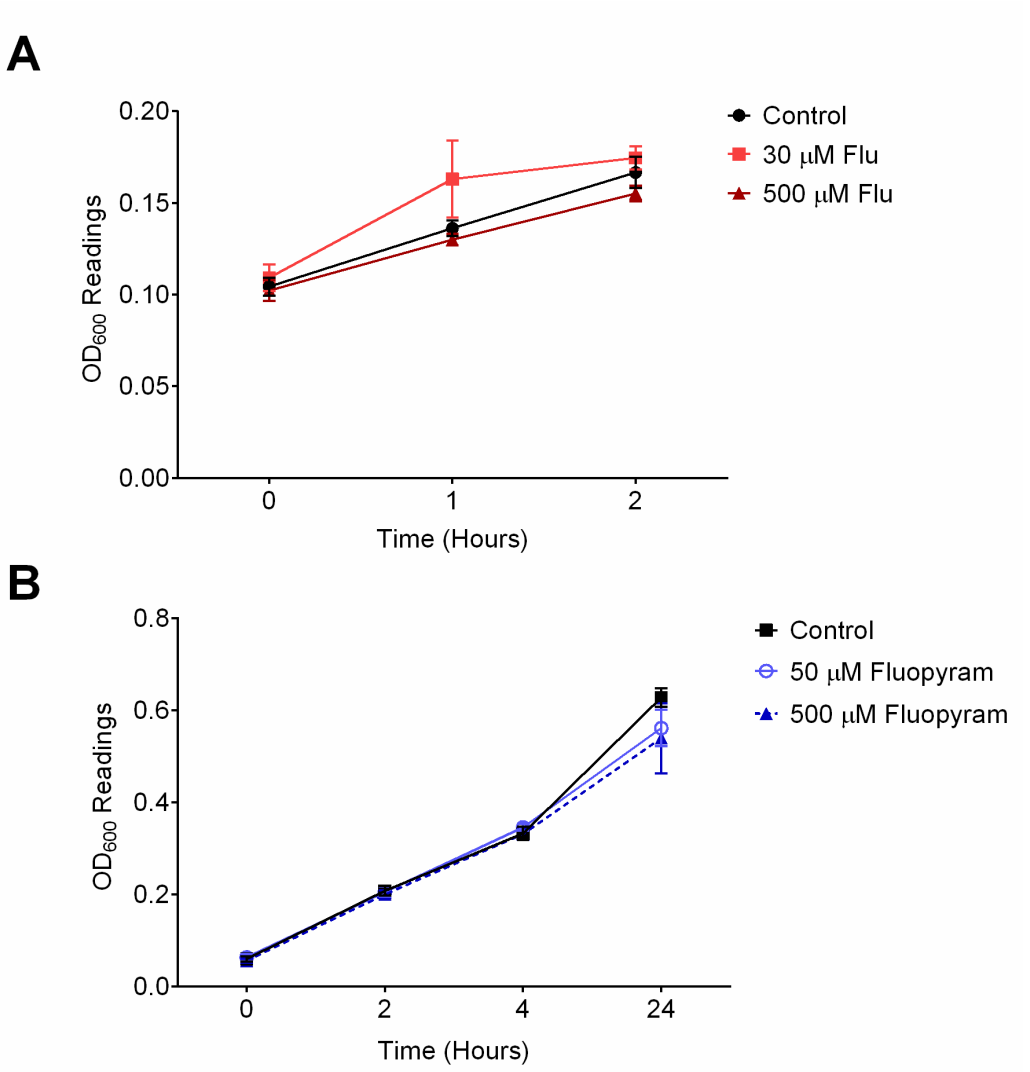

Figure A.8. Fluensulfone and fluopyram do not affect yeast growth in liquid culture. Yeast liquid cultures, grown overnight, were diluted 1/10 and had fluensulfone (**A**) or fluopyram (**B**) added. Growth was then measured by measuring optical density following incubation at 30°C. Fluensulfone and fluopyram did not affect yeast growth relative to the control (n=5 cultures per treatment, mean ± s.e mean optical density of each culture, two-way ANOVA with Bonferroni post-hoc tests, A: P=0.59, B: P=0.67).

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