Behavioural Aspects of the Neonicotinoid Resistance of *Myzus persicae*

Lucy Fray

Imperial College London

Ecology and Evolution Department

PhD

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Declaration of originality

All of the work contained in this PhD thesis is my own, as described in my own words. All references to the works of others have been appropriately distinguished from my own work and referenced.

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“Aphids, known as greenfly, are very friendly bugs,
They live in great big colonies and give each other hugs,
They all get on most famously until one needs more space,
And then like other sisters, kick each other in the face.

Aphids like to feed on plants, they drink up all the sap,
Hanging underneath the leaves into the phloem they tap,
Their mouth is like a needle, the cuticle it pierces,
It travels to the vessels via intercellular spaces.

As they try to find the phloem, the other cells they puncture,
Sometimes they transmit viruses but sap feeding's their juncture.
When they finally reach the phloem they first will salivate,
This unblocks all the forisomes and seals the poor plant's fate.

Aphids will feed for hours on the phloem and just think,
And when they tire of thinking from the xylem they will drink.
As a pest of plants they do one other thing of point,
They fire out lots of honeydew and fungus up the joint.

They reproduce asexually, they don't go in for dates,
80 daughters born from each no wonder they need space!
The babies are born naturally, for aphids give birth live,
And inside the newborn young there's more daughters inside!

When it gets to winter, the aphids they grow wings,
They fly off to their winter home, where they now mate and things.
They like to overwinter as tiny small black eggs,
And when it's spring they rise again, as horrible plant-based cleggs!”

-Lucy Fray

Abstract

The peach potato aphid *Myzus persicae* (Sulzer) is an agricultural pest of high economic importance distributed across much of the globe. The main method of control of this pest is the application of neonicotinoid insecticides such as imidacloprid and thiamethoxam (TMX). Until recently no significant resistance to neonicotinoids was known in *M. persicae.* In 2009 however, a clone exhibiting severe resistance to neonicotinoids was collected from peach orchards in France. This resistant clone was designated 'FRC'.

Published literature has shown the high levels of neonicotinoid resistance exhibited by the FRC clone to be due to a combination of adaptations including enhanced overexpression of the detoxifying cytochrome P450 *CYP6CY3*, a target site mutation in the nicotinic acetylcholine receptor and reduced cuticular penetration. No studies however have investigated the possibility of a behavioural neonicotinoid resistance mechanism in *M. persicae.* This study describes an investigation into the presence or absence of a behavioural component to the neonicotinoid resistance of the FRC clone.

Two different aspects of the behaviour of the FRC clone, feeding and dispersal, were compared to that of a clone of low neonicotinoid resistance (5191A) and a clone of complete neonicotinoid susceptibility (US1L). A significant difference was found in the dispersal behaviour of the FRC clone which could potentially contribute to its high levels of neonicotinoid resistance. Further investigation was then undertaken to determine whether this altered behaviour influenced the efficiency of the FRC clone as a vector of the persistently transmitted *Turnip Yellows virus*. No difference in vector ability was detected between the US1L and FRC clones. This study concludes that there is significant evidence of a potential behavioural aspect to the neonicotinoid resistance of the *M. persicae* FRC clone.

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Chapter 1: Introduction

There are over 4400 known species of aphid, approximately one hundred of which are of agricultural importance (Blackman & Eastop, 2007). One of the most serious pest species is *Myzus persicae* (Sulzer), a generalist able to transmit over 100 plant viruses to an extremely wide range of crop plants (Blackman & Eastop, 2007). In addition to being a highly efficient virus vector, the widespread distribution, extreme polyphagy, high reproductive rate, parthenogenic ability and genetic variability in colour, life cycle, host-plant relationships and insecticide resistance all contribute to the major pest status of this species (Vucetic *et al.*, 2008).

1.1 Lifecycle and formation of clones

Myzus persicae exhibits an annual heteroecious lifecycle. The primary host on which the species overwinters is peach *Prunus persica* (L.) (Blackman, 1974). Different populations exhibit different overwintering strategies (Margaritopoulos *et al.,* 2002). Holocyclic genotypes overwinter as eggs, laid by sexually reproducing oviparae. Anholocyclic genotypes do not produce a sexual morph in autumn and overwinter as pathenogenetic virginoparous females on alternative hosts to *P. persica*. Androcyclic genotypes produce sexual males, which mate with the sexual females of holocyclic and intermediate clones, but only produce parthenogenic females over winter. Finally, intermediate clones produce both parthenogenic virginoparous females, but also sexual males and alate (winged) virginoparae which give birth to both virginoparae and oviparae (Blackman, 1971; 1972).

In spring the eggs hatch into parthenogenic females known as fundatrices (Dixon, 1985), which eventually produce virginoparous alate offspring in response to seasonal changes in environmental conditions. These parthenogenic alates migrate to summer host plants, which may be of a wide range of species including many crops (Blackman & Eastop, 2007). Reproduction on the summer host is purely parthenogenetic, although both alate and apterous females may be produced in response to a range of environmental conditions including plant nutritional quality, temperature, predation and overcrowding (Muller *et al.,* 2001). The young, known as nymphs, are born with nymphs already formed inside their own reproductive organs, a phenomenon known as telescoping of generations (El Din, 1976; Kindlmann & Dixon, 1989). Nymphs take approximately 8 days to reach maturity, although developmental time varies according to environmental temperature as is common in many aphid species (Campbell *et al.,* 1974). During this process nymphs go through a series of

moults, passing through four instars in total before reaching adulthood. In the autumn adult females of holocyclic genotypes once again begin producing virginoparous winged female and winged sexual males. This occurs in response to changes in environmental conditions including temperature (Dixon & Glen, 1971), night length (Lees, 1963) and host plant changes in response to season (Forrest, 1970).

Because of the lack of sexual reproduction in the parthenogenic summer morph of *M. persicae,* it is possible to develop different genetic lines (otherwise known as clones) by taking a single asexual female and allowing her to reproduce parthenogenetically to produce a genetically identical asexual population. Different clones may be kept under summer environmental conditions in the laboratory to prevent sexual reproduction and so they can be stably maintained for many years. Such clones may exhibit different phenotypic traits, including colour, feeding and reproductive behaviour. Comparing different clones allows for investigation into a wide range of such traits, and can be highly useful for studies into insecticide resistance.

1.2 Control and resistance of *Myzus persicae*

Control of *M. persicae* currently relies upon the application of chemical insecticides, as other control methods have proved unsatisfactory (Denholm *et al.*, 2002). This species has, however, an extensive history of evolving mechanisms of resistance to different insecticide classes (Vucetic *et al.*, 2008). Three genetically distinct mutations conferring resistance to insecticides are currently known in *M. persicae* populations (Devonshire *et al.*, 1998).

The first of these is a detoxification mechanism known as E-Carb which confers resistance to the majority of ester-containing insecticides, including organophosphates and some carbamates and pyrethroids (Devonshire & Moores, 1982). Resistance is caused by the amplification of the E4 gene which increases synthesis of the E4 and FE4 carboxylesterase enzymes (Field *et al.*, 1988). Resistant aphids are classed as moderately resistant (R_1), highly resistant (R_2) or extremely resistant (R_3), according to the levels of carboxylesterase production (Devonshire *et al.*, 1986).

The other resistance mechanisms involve modification of the target site of the active ingredients (AIs) of various insecticides. The substitution of a single serine for a phenylalanine residue within the active site of acetylcholinesterase results in the production

of modified acetylcholinesterase (MACE) (Moores *et al.*, 1994). It is therefore known as the MACE phenotype, and confers resistance to di-methyl carbamates, pirimicarb and triazamate.

The third mechanism is known as knockdown resistance (*kdr*) and confers resistance to pyrethroids and DDT. The main mutation involves substitution of a leucine for phenylalanine in the gene L1014F, which encodes part of the voltage-gated sodium channels (Martinez-Torres *et al.*, 1999). However, a section of the population also possesses a second mutation in which a methionine is substituted with a threonine at the M918T locus. This second mutation is known as super-*kdr* (*s-kdr*) and results in an even greater level of resistance (Eleftherianos *et al.*, 2002).

1.3 Neonicotinoids

It is due to the widespread resistance of many *M. persicae* populations to a variety of methods of chemical control that this species is now commonly controlled using the largest selling commercial insecticides globally known as neonicotinoids, the action of which remains unaffected by the other resistance mechanisms (Foster *et al.*, 2002). The neonicotinoid group includes imidacloprid, thiamethoxam (TMX), thiacloprid, acetamprid, nitenpyram, clothianidin and dinotefuran, all of which are related to the natural product nicotine in their mode of biological action, which also affects vertebrates (Stumpf *et al.*, 2007) (Figure 1.1).

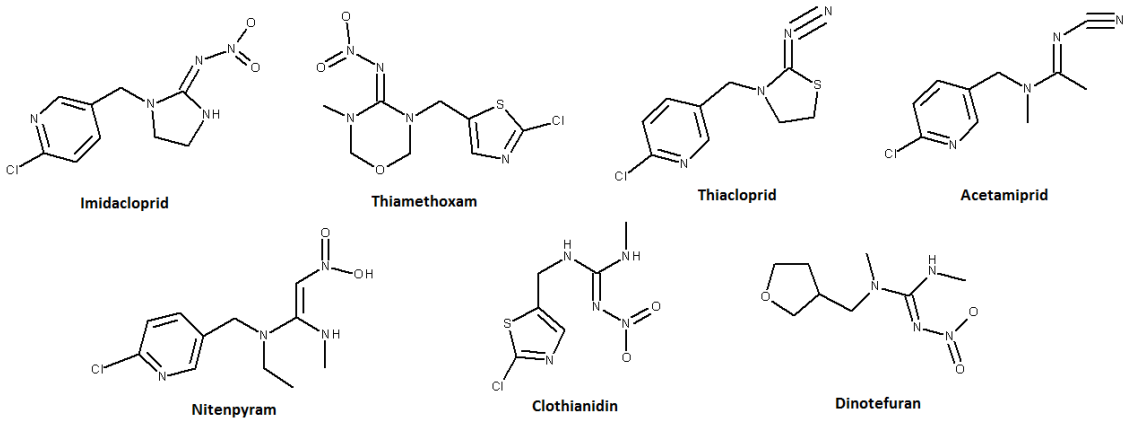


Figure 1.1 The chemical structures of the seven currently known neonicotinoid insecticides.

Neonicotinoids function as agonists of the nicotinic acetylcholine receptors (nAChRs) (Matsuda *et al.*, 2009). By binding with high affinity to the nAChRs neonicotinoid molecules act as agonists at the receptor to produce prolonged nervous signals, blocking sensitivity to

the native neurotransmitters (Brown *et al.,* 2006). Prolonged disruption of these receptors has a toxic effect, leading to the death of affected individuals (Matsuda *et al.,* 2005). In addition to this, neonicotinoids bind preferentially with high affinity to insect as opposed to vertebrate nAChRs (Liu & Casida, 1993; Yamamoto *et al.*, 1995). This increases their appeal and value as insecticides, as they present no direct risk to vertebrates (Tomizawa & Casida, 2003).

1.4 Neonicotinoid Resistance

Until extremely recently, all known neonicotinoid-resistant *M. persicae* strains were only apparent within the laboratory and exhibited no noticeable resistance at field application rates (Nauen & Denholm, 2005). Clones exhibiting low levels of tolerance to imidacloprid (three to ten-fold) had been obtained from Europe and Japan (Devine *et al.*, 1996, Nauen *et al.*, 1996), while clones exhibiting greater tolerances of up to 18-fold had been discovered in regions as widespread as Zimbabwe, the United States and Europe (Foster *et al.*, 2002, Cox *et al.*, 2004). In 2007 the clone '5191A' which exhibits 20 to 60-fold resistance to imidacloprid when compared with the standard susceptible clone '4106A' was collected from tobacco plants in Greece (Puinean *et al.*, 2010). This clone had the greatest known resistance factor to imidacloprid of its time, but this level of resistance was still too low to present a problem within the field using neonicotinoids at recommended field rates and was still only detectable within the laboratory.

In 2009, however, a highly neonicotinoid resistant strain, designated FRC, was discovered in peach orchards in France exhibiting a resistance factor of 1679 to imidacloprid and 225 to TMX (Bass *et al,* 2011). This strain presents a highly significant problem to the agricultural industry, as the majority of crop plants are currently protected from *M. persicae* infestation by the application of neonicotinoids (Denholm *et al.*, 2002). Although the FRC strain is not descended from the asexual reproduction of a single founding individual and so is not a true clone, all individuals of the strain carry the same mutation within the nAChR as described below and exhibit the same levels of neonicotinoid resistance, hence for the purposes of this study they will be referred to as a clone.

Imidacloprid-resistant *M. persicae* clones discovered prior to the FRC clone have exhibited similar levels of resistance to all other neonicotinoids (Foster *et al.*, 2002). In addition, many growers continue to spray crops with mixtures of older compounds such as organophosphates and pyrethroids alongside neonicotinoids as such chemicals are still commonly used for the control of additional invertebrate pests, including other aphid species (van Toor *et al.*, 2008).

This exerts further selective pressure upon *M. persicae* populations to produce further resistant strains. It is therefore essential that alternative chemistries are obtained for the control of *M. persicae*.

Several other chemical compounds have been suggested for this role, including the antifeedants pymetrozine (Harrewijn & Kayser, 1997) and flonicamid (Morita *et al.*, 2007). Although the modes of action of these compounds are thought to be different to that of the neonicotinoids (Harrewijn & Kayser, 1997; Kaufmann *et al.,* 2004; Ausborn *et al.,* 2005; Morita *et al.,* 2007), it largely remains to be seen how effective such compounds will prove against resistant populations within the field.

1.5 Molecular understanding of neonicotinoid resistance

Much progress has been made recently towards understanding the molecular basis of neonicotinoid resistance, especially with the discovery of the FRC strain, which carries a target site mutation within the nAChR (Bass *et al.,* 2011). It is currently impossible however, to produce a native pentameric nAChR *in vitro*, and although much research has been directed towards this achievement (See Millar & Lansdell (2010) for a review) a full understanding of the molecular aspects of neonicotinoid resistance is yet to be obtained.

In order to explain current work on the molecular basis of neonicotinoid resistance, a brief summary of the insect nAChR must be offered. All nAChRs consist of five subunits, each containing a dicysteine loop with a 13-residue N-terminal extracellular domain (Matsuda *et al.*, 2009). The subunits are divided into two categories, based on the presence or absence of two cysteine residues within loop C. Subunits containing the residues are referred to as α subunits, while those from which the residues are absent are termed non-α subunits or β subunits. The majority of heteromeric nAChRs consist of two α and three non-α subunits, with the ligand binding domain (LBD) located at the interface between these two types of subunit (Figure 1.2, B *and* C). Receptors consisting entirely of alpha subunits are also known of within vertebrate systems (Couturier *et al.,* 1990). The LBD itself is made up of six extracellular loops (A-F). Loops A-C are located on the α-subunits and loops D-F on the non-α subunits. Different ‘subtypes’ of nAChR have different subunit combinations. Studies so far suggest insect nAChRs have an equivalent of both α and non-α subunits (Matsuda *et al.*, 2005).

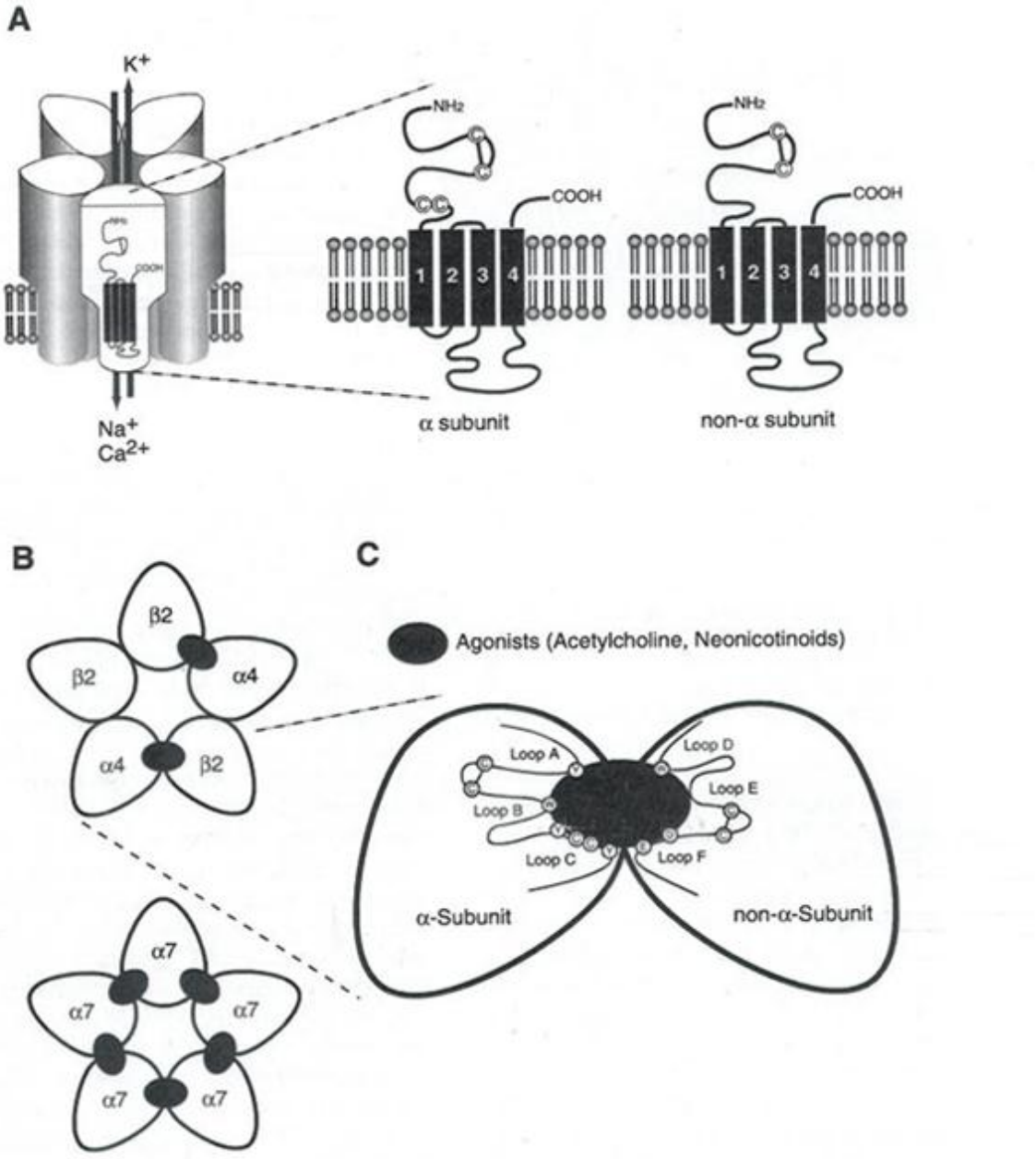


Figure 1.2 The mammalian nicotinic acetylcholine receptor (nAChR) (A, side view; B, top view). Section C shows the ligand binding domain (LBD), which may be located between an α and a non-α subunit or at the interface between two α subunits, depending on the nAChR subtype. Reproduced from Matsuda *et al.* (2005).

Under normal conditions the neurotransmitter acetylcholine (ACh) is released into the synapse by the presynaptic neurone. ACh temporarily binds to the nAChRs at the LBD, allowing Na^+ ions to flow into the postsynaptic neurone causing depolarisation and generating an action potential. ACh is then broken down by the enzyme acetylcholine esterase as it associates and dissociates from the LBD thus ending the action potential. Neonicotinoids act as agonists by competing with ACh to bind to the LBD but at much higher

binding efficacy. Neonicotinoids have a higher binding affinity than endogenous neurotransmitters and hence an action potential continues to be generated for a large period of time. This interferes with nervous signalling by overstimulation of the nerve cells and may eventually lead to the death of the insect (Liu & Casida, 1993).

Myzus persicae of the FRC strain carry a single point mutation in the loop D region of the β1 subunit of the nAChR, known as R81T (Bass *et al.,* 2011). This arginine to threonine substitution significantly reduces the binding affinity of imidacloprid to the nAChR. While threonine is present at the same position in vertebrates such as *Homo sapiens* and *Rattus norvegicus* (Berkenhout), which remain immune to the effects of imidacloprid, all other insect species characterised to date have a positively charge arginine at this position (Bass *et al.,* 2011). Although the FRC is a strain and not a clone, the entire strain remains homozygous for the R81T mutation and so can be treated as a clone in terms of target site based neonicotinoid resistance. For greater simplicity, this study will therefore often refer to the FRC strain as a clone.

Before the discovery of the FRC population, literature concerning the possibility of a binding site mutation within neonicotinoid resistant insects had been contradictory. Ligand competition experiments using [^{3}H] imidacloprid showed no difference in binding to the nAChRs of various resistant and susceptible *M. persicae* clones, suggesting that there was no change in target site sensitivity to imidacloprid within previously known resistant *M. persicae* (Nauen *et al.*, 1998a). The only previously recorded case of a target site mutation conferring resistance to imidacloprid occured in the brown planthopper *Nilaparvata lugens* (Stal) (Liu *et al.*, 2005). Matsuda *et al.* (2009) successfully modelled reduced target-site affinity in the α2β1 nAChR of *M. persicae* based on this mutation. The mutation carried by the resistant *N. lugens* population however, occurred in an artificially selected laboratory strain and no equivalent population has been found to date in the field. Although nerve recordings have suggested that target site insensitivity may be one of the methods of imidacloprid resistance in the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Tan *et al.,* 2005) no mutation has been reported in the α-subunits of the target site in this species (Alyokhin *et al.,* 2008). In addition to this, the majority of the residues in nAChR subunits are highly conserved in both insects and vertebrates (Matsuda *et al.*, 2005), making a mutation at the target site seem an unlikely mode of neonicotinoid resistance. Despite this, the FRC clone exhibits strong resistance to neonicotinoids conferred at least partially via a target site mutation (Bass *et al.,* 2011).

Although as of yet functional expression of heterologous insect nAChRs has not been successful, chimeric nAChRs made up of combinations of vertebrate and insect subunits have been successfully expressed (Bertrand *et al.*, 1994). For example the α2β1 nAChR consists of the α-subunits of *M. persicae* and the β-subunits of a chick. Such chimeric nAChRs however are not fully analogous to the nAChR of *M. persicae*, and thus until further progress is achieved in this area future work on the R81T mutation of the FRC and its contribution to neonicotinoid resistance will be impeded.

In light of the inability to produce stable insect nAChRs *in vitro*, electrophysiological studies combined with site-directed mutagenesis and homology modelling have been carried out by Matsuda *et al.* (2009) to further understand the binding of imidacloprid to the nAChR LBD. This involved the study of structures formed by crystallisation of the molluscan *Lymnaea stagnalis* L. (*Ls*) acetylcholine binding protein (AChBP) in complex with the neonicotinoids imidacloprid and clothianidin (Ihara *et al.*, 2008), and *Aplysia californica* Cooper (*Ac*) AChBP with imidacloprid and thiacloprid (Talley *et al.*, 2008). These water soluble proteins are located in the glial cells which modulate the synaptic acetylcholine concentration of the snails. Both *Ac*-AChBP and *Ls*-AChBP show structural similarities to the extracellular domain of α-7 neuronal nAChRs, particularly in the region of the ligand-binding site (Ihara *et al.*, 2008). Modelling of wild-type and mutant α2β1 *M. persicae* nAChRs, based on the crystal structure of *Ls*-AChBP in complex with imidacloprid (Figure 1.3), has shown that the imidazolidine ring of imidacloprid stacks with the tyrosine residue Tyr176 in loop C, while two protons in the CH_2-CH_2 bridge form CH-π bonds with a tryptophan residue in loop B. Tyr176 is located in a hydrophobic groove, stabilising the position of the loop B tryptophan. Substituting the tyrosine residue for a serine caused the tryptophan to wobble. This reduced the proximity of the tryptophan residue to the imidazolidine ring and so reduced the CH-π bonds, in turn reducing the affinity of the mutated nAChRs to imidacloprid (Matsuda *et al.*, 2009). It must be highlighted that these complexes are not fully analogous to a true insect nAChR. However such studies have shown that when binding to the nAChR, imidacloprid is in close proximal contact with the amino acids involved in the R81T mutation (Talley *et al.*, 2008).

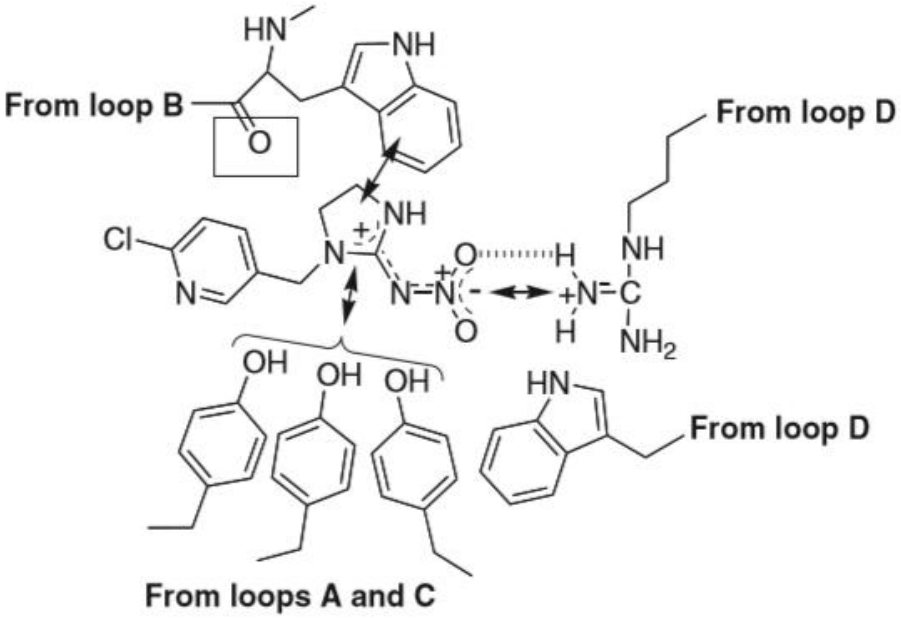


Figure 1.3 Schematic of the binding site of insect nicotinic acetylcholine receptors with an imidacloprid molecule, as modelled on *LS*-AChBP (Matsuda *et al.,* 2005). The nitro group of imidacloprid electrostatically interacts with basuc residues in loop D, increasing the positive charge of the imidazolidine moiety and strengthening the cation-π interactions with the tryptophan residue in loop B. A tryptophan residue in loop D may also interact with the nitro group. One tyrosine residue in loop A and two in loop C surround the imidazolidine ring of imidacloprid. Imidacloprid can also interact with the backbone carbonyl in tryptophan in muscle nAChR, shown boxed. Dotted lines depict hydrogen bonds.

Tomizawa & Casida (2003) have shown that substituting the threonine residue for an arginine at position 77 on the chicken β2 subunit, which corresponds with subunit position 81 on the *M. persicae* subunit Mpβ1, significantly increased the affinity of recombinant chimera *in vitro* nAChRs for imidacloprid. These hybridised nAChRs contained chicken β2 and *Drosophila melanogaster* Meigen α2 subunits. An extra substitution at position E79V of the chicken β2 subunit was however, required to produce this effect, a substitution which caused the chicken β2 subunit to more closely mimic insect β1 subunits. Such work validates current models of imidacloprid binding at the nAChR and supports the theory that the R81T mutation of the FRC clone discovered by Bass *et al.* (2011) was responsible for conferring at least part of the neonicotinoid resistance exhibited by this clone.

1.6 Neonicotinoid metabolism and resistance

Another possible method of neonicotinoid resistance involves the metabolic breakdown of the AI to metabolites of lower insecticidal activity within the aphid. Cytochrome P450s are enzymes often involved in the breakdown of toxic substances within both vertebrates and invertebrates, and are often involved in mechanisms of resistance to insecticides (Feyereisen, 2006). Such is the case in resistant populations of the whitefly *Bemisia tabaci* (Genn.) (Nauen *et al.*; 2002, Rauch & Nauen, 2003), in which exposure to piperonyl butoxide (PBO), which inhibits the action of cytochrome P450 monooxygenases, increased the activity of imidacloprid to previously resistant strains. Further investigation showed a close correlation between monooxygenase activity and the extent of imidacloprid resistance.

Studies involving plant and vertebrate model systems have shown that imidacloprid undergoes oxidative degradation to both insecticidal and non-toxic metabolites (Nauen *et al.*, 1999, Schulz-Jander & Casida, 2002). Rauch & Nauen (2003) have shown that neonicotinoid resistant strains of the invertebrate *B. tabaci* produce relatively large amounts of the metabolite 5-hydroxy-imidacloprid compared to susceptible strains. This metabolite has a lower binding affinity for *B. tabaci* nAChRs than imidacloprid. In addition to this, the recombinant cytochrome P450 isozyme *CYP3A4* from human liver tissue also metabolises imidacloprid to 5-hydroxy-imidacloprid (Schulz-Jander & Casida, 2002), supporting the theory that this detoxification mechanism was responsible for imidacloprid resistance in whitefly. There is also evidence to suggest that detoxification by monooxygenases plays a role in neonicotinoid resistance in *N. lugens* (Zewen *et al.*, 2003), while a study by Daborn *et al.* (2001) showed that imidacloprid-resistant *D. melanogaster* populations showed overexpression of the P450 gene *CYP6G1*.

It now seems that a similar mechanism is responsible for neonicotinoid resistance in *M. persicae.* Philippou *et al.* (2010) have shown that partial susceptibility to neonicotinoids could be restored to the resistant Greek clone 5191A when piperonyl butoxide (PBO) was topically applied. Since PBO is a P450 inhibitor these results suggest that neonicotinoid resistance in 5191A is at least partially due to increased amplification of P450s. This theory was then supported by Puinean *et al.* (2010), who with microarray analysis and quantitative PCR showed that there is 22-fold constitutive over-expression of the P450 gene *CYP6CY3* in 5191A, and that this was due at least partially to gene amplification. The FRC clone has also been shown to overexpress this same gene 9-14 fold, with an increase in gene copy number

similar to that of 5191A (Bass *et al.,* 2011). In addition the FRC also overexpresses seven other P450s compared with both 5191A and the neonicotinoid susceptible reference clone 4106A, although at relatively low levels of 2-4 fold. These changes in the gene expression are accompanied by increased rates of metabolism of imidacloprid into the metabolite 4/5-hydroxy-imidacloprid (Bass *et al.,* 2011). This suggests that the overexpression of cytochrome P450s does indeed play a role in the increased metabolism of imidacloprid and so contributes to the neonicotinoid resistance of *M. persicae.* It does not, however, appear to explain the differences in resistance factor between 5191A and FRC as P450 overexpression does not differ significantly between them. This difference is more likely due to the target site mutation carried by the FRC clone, but not the 5191A clone.

Recently, Ramsey *et al.* (2010) compared *M. persicae* cDNA with the recently sequenced genome of the pea aphid *Acyrthosiphon pisum* (Harris) and established that *M. persicae* appears to contain 115 cytochrome P450 genes, 40% greater than the number found in *A. pisum.* Large numbers of genes encoding glutathione S-transferases and carboxy/choline esterases, enzymes involved in the metabolic breakdown of other toxic substances found in both insecticides and certain host plant species, were also shown to be present. This increased emphasis on cytochrome P450s in the genome of *M. persicae* supports the theory of metabolic P450-mediated neonicotinoid resistance in this species, as it suggests the presence and upregulation of P450s may be more important in *M. persicae* than in *A. pisum*, hence we could expect the probability of insecticide resistance arising by this mechanism in *M. persicae* to be high. The increased number of genes encoding cytochrome P450s in *M. persicae* may be linked to the generalist feeding habits of this species. Since *M. persicae* feeds from a wider range of hosts than *A. pisum* it may also encounter a wider range of plant defence compounds, hence the greater emphasis on detoxification enzymes in the *M. persicae* genome.

Over-expression of several genes encoding cuticular proteins also occurs in 5191A and it has been suggested that this may also offer a mode of resistance via reduced cuticular penetration by imidacloprid (Puinean *et al.,* 2010). There is no evidence of this mode of action in the FRC strain to date.

Recent sequencing of the *A. pisum* genome has brought much insight into insecticide resistance within aphids (Ramsey *et al.,* 2010). Although the *M. persicae* genome is not sequenced at present, an EST cDNA library is currently available, along with a DNA

microarray to study gene expression (Ramsey *et al.*, 2007). Future sequencing of the *M. persicae* genome will allow further advances to be made in understanding the molecular aspects of neonicotinoid resistance in *M. persicae.*

1.7 Behavioural effects of insecticides

It is also potentially possible for behavioural effects to contribute to the neonicotinoid resistance of *M. persicae*. Nauen & Elbert (1997) found that differences in imidacloprid tolerance between a resistant and susceptible strain of the tobacco aphid *Myzus nicotianae* (Blackman), a species closely related to *M. persicae*, were only apparent when the insecticide was administered orally as opposed to a contact assay. The authors concluded that the resistance was due to an increased antifeedant response in resistant as opposed to susceptible aphids. This prevented resistant individuals from ingesting lethal amounts of imidacloprid, allowing them to survive and reproduce at greater AI concentrations.

Many insecticides are known to affect insect behaviour, particularly feeding, locomotion and reproduction. Pymetrozine, when applied orally within artificial diet, inhibits stylet penetration and causes abnormal salivation and feeding patterns in *M. persicae* (Harrewijn & Kayser, 1997), as well as causing spontaneous contractions of the foregut in *Locusta migratoria* (Ussure) (Kaufmann *et al.*, 2004). It appears however, to be of relatively low toxicity and exerts no effect upon aphid locomotion (Harrewijn and Kayser, 1997). Drenching grapevines with pymetrozine also reduced plant contact and increased the incidence of dispersal from the stem of the glassy-winged sharpshooter *Xylella fastidiosa* (Wells) (Bextine *et al.*, 2004). Similar to pymetrozine, flonicamid rapidly inhibits feeding in aphids without causing convulsion or producing any other effect upon locomotion (Sadeghi *et al.*, 2009). Again, the time spent salivating into the phloem and ingesting sap are strongly reduced in directly treated aphids (Morita *et al.*, 2007). Azadirachtin, a limonoid insecticide extracted from the Neem tree, reduces plant penetration and the percentage of probes which enter the vascular tissues, but increases the overall frequency of probing in *M. persicae* (Nisbet *et al.*, 1993).

In terms of neonicotinoids, it is well established that imidacloprid produces an antifeedant response in *M. persicae* and reduces reproduction (Nauen, 1995, Nauen & Elbert, 1997, Nauen *et al.*, 1998a). This effect is also seen in other Hemiptera such as *B. tabaci* (Nauen *et al.*, 1998b, Nauen *et al.*, 2002) and the Asian citrus psyllid *Diaphorina citri* Kuwayama (Boina *et al.*, 2009). Additionally, TMX has been found to decrease the amount of time spent

ingesting xylem sap as opposed to phloem sap, although it remains unclear whether this is due to an antifeedant or neurotoxic effect (Daniels *et al.*, 2009). It is therefore possible that neonicotinoid resistance in certain clones of *M. persicae* may be due to modified behavioural effects, such as a more sensitive, rapid antifeedant response, preventing resistant individuals from ingesting a lethal amount of insecticide. Such a behavioural response would not be the sole cause of neonicotinoid resistance, but could potentially contribute in combination with the already characterised mechanisms of target site mutation and enhanced metabolism.

In addition to this, certain mutations within aphid populations which confer resistance to insecticides have already been found to affect aphid behaviour, most noticeably in terms of fitness trade-offs. For example, several *M. persicae* clones with the *kdr* mutation have been reported as having a markedly reduced response to aphid alarm pheromone (Foster *et al.*, 2005). Since this mutation is located within the sodium-gated voltage channels, it is not unlikely that another mutation affecting the nervous system, such as one in the nAChR may also affect aphid behaviour. The main focus of this study is to investigate any differences in feeding and dispersal behaviour between the neonicotinoid-susceptible *M. persicae* clone US1L and the neonicotinoid resistant clones 5191A and FRC. Any such differences in behaviour could potentially contribute to the neonicotinoid resistance of these clones.

1.8 Hemipteran feeding physiology

Aphid feeding behaviour is highly conserved and performed in a strict sequence. Before inserting the mouthparts into a host plant, known as 'probing', aphids secrete a small droplet of watery saliva onto the plant surface (Miles, 1999). They then penetrate this initial saliva droplet, known as the flange, at the same time as they penetrate the host plant epidermis (Walker, 2000). Progression deeper into the plant tissues is done in a series of short stages (Figure 1.4). First the stylets are advanced slightly so they protrude from the initial saliva droplet. A droplet of gelling saliva is then secreted in a watery, liquid form. The droplet merges with the existing salivary sheath, completely re-encasing them. After a short time the saliva becomes more viscous and gels. The aphid then advances the stylets further so that they protrude from the new sheath tip, and the process repeats. This pattern of saliva secretion and extension of the stylets forms a salivary sheath which completely encases the stylets within the plant tissue (Kunkel, 1977).

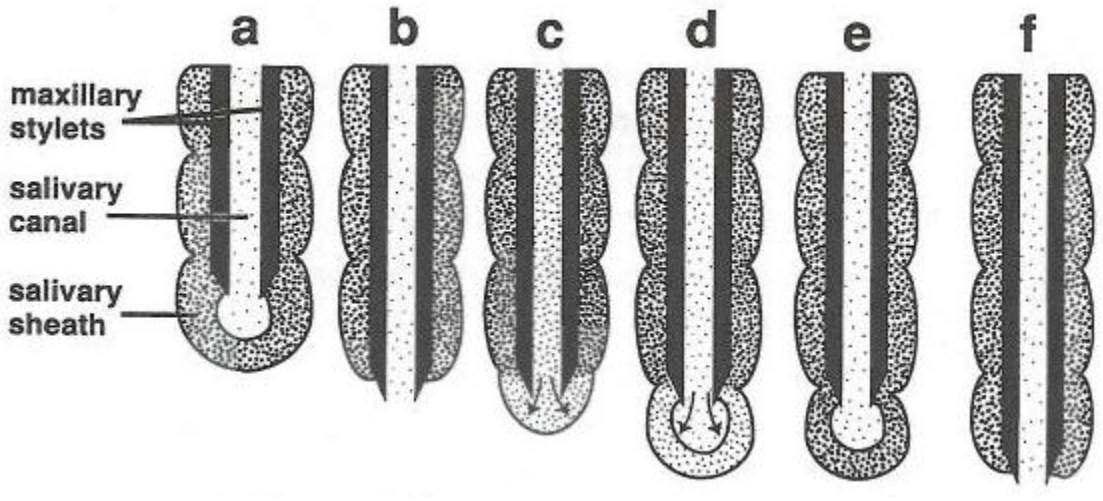


Figure 1.4 The progression of the aphid stylets into the tissues of the host plant. Stages a to f follow on from each other as a time sequence. Arrows indicate the direction of flow of gelling saliva secreted by the aphid. (Adapted from Walker (2000)).

Initially a feeding aphid inserts its stylets into the plant epidermis and mesophyll. The stylets must travel within the intercellular space of these tissues to reach the phloem and xylem vessels (Tjallingii & Hogen Esch, 1993). Throughout the journey towards the vascular tissues, the aphid repeatedly penetrates the cells of the host plant with the stylet tips for short periods of time (Pettersson *et al.,* 2007). During these brief cell punctures a small amount of cell contents are ingested and a small amount of saliva is expelled into the cell. Ingestion of cell contents during these brief punctures is thought to aid in assessing host quality (Pettersson *et al.,* 2007). The function of the salivation remains unknown, but the behaviour plays a role in virus transmission (Martín *et al.,* 1997). All feeding behaviours from initial entry of the stylets into the plant until just before entry into the phloem are known as the 'pathway phase' or pathway activities.

Once the phloem is located, the aphid typically performs an increased number of brief cell punctures, before entering the phloem. The beginning of the phloem phase is characterised by the secretion of watery saliva into the sieve element. This behaviour usually lasts from 5 s to 30 min (Tjallingii & Hogen Esch, 1993) and is usually followed by sustained sap ingestion, which may last for many hours. In some cases however the aphid may return to the pathway phase after phloem salivation rather than entering phloem feeding. Phloem feeding (sap ingestion) never occurs without preceding phloem salivation behaviour (Pettersson *et al.,* 2007). The stimuli which induce aphids to initiate phloem salivation and feeding are currently unknown (Fereres & Moreno, 2009).

Studies have suggested that the watery saliva secreted into the phloem before feeding may repress sieve tube occlusion (Will & van Bel, 2006; Will *et al.*, 2009), a defensive response performed by the plant in response to aphid feeding or other types of physical damage. Plants of the Fabaceae family contain a crystal-like protein body within the sieve elements, known as the 'forisome'. Upon damage to the phloem, calcium levels elevate and the forisome rapidly expands, blocking the sieve elements and preventing transport within the phloem (Knoblauch & Peters, 2004). Reversing expansion of the forisome and 'unblocking' the phloem would allow aphids to continue feeding. When forisomes are artificially induced to expand by the application of physical damage to the plant, aphids already engaged in phloem feeding switch to phloem salivation (Will & van Bel, 2006). At least seven different aphid species have been shown to switch from phloem feeding to phloem salivation in response to plant wounding (Will *et al.*, 2009). After a period of time equal to that needed for forisome contraction they will then return to phloem feeding once more. Watery saliva has been shown to contain Ca^{2+} binding proteins, although the exact protein composition varies significantly between aphid species (Will *et al.*, 2009). This suggests that aphid saliva may remove calcium from the phloem local to the stylet entry point, reversing or preventing the expansion of the forisomes. A very recent study however, has shown that phloem penetrations by *A. pisum* do not trigger forisome dispersal in faba bean *Vicia faba* L. (Walker & Medina-Ortega, 2012); hence a greater understanding of this interaction is required.

In addition to feeding from the phloem, aphids will occasionally feed from the xylem (Spiller *et al.*, 1990). The frequency of xylem feeding appears to depend upon both the aphid species (Gabrys *et al.*, 1997; Prado & Tjallingii, 1997) and the host plant species (Tjallingii, 1986). There is also evidence that alate morphs show differential xylem feeding behaviour to apterous aphids (Powell & Hardie, 2002). It is thought that xylem feeding may represent an attempt by the aphid to avoid dehydration (Daniels *et al.*, 2009). The majority of aphid feeding is however directed towards the nutritious phloem.

1.9 Hemipteran feeding terminology

Hemipteran feeding behaviour has traditionally been studied using the electrical penetration graph (EPG) technique (McLean & Kinsey, 1964; Tjallingii, 1978). In brief this method involves attaching electrodes to both an aphid and a host plant, effectively wiring them into an electrical circuit of very low voltage. When the aphid feeds on the plant, the stylets pierce the waxy insulating cuticle and complete the circuit, allowing an electrical signal to be

recorded. Analysis of this electrical signal allows the number and duration of different feeding behaviours being performed by the aphid to be analysed. Due to the historical use of the EPG technique to study Hemipteran feeding, all feeding behaviours performed by aphids are often referred to by the name of the electrical waveform that corresponds to that behaviour when using EPG (Table 1.1). Such terms occur frequently within literature and will also be used throughout this study. Terminology referring to waveforms and feeding behaviours exhibited by aphids are consistent across all aphid species. Feeding behaviours exhibited by different types of Hemiptera however, such as whitefly, planthoppers and psyllids, are referred to by different nomenclature since each family produces unique waveforms during EPG recordings (Joost *et al.,* 2006; Kindt *et al.,* 2003; Stafford & Walker, 2009; Bonani *et al.,* 2010).

Table 1.1 The classification of feeding behaviours performed by aphids as recorded during the electrical penetration graph (EPG) technique, as originally described in Tjallingii (1978).

Behaviour	EPG waveform terminology
Pathway (stylets in intracellular spaces)	C
Short cell puncture (during pathway)	pd
Phloem salivation	E1
Phloem ingestion	E2
Disrupted stylet mechanics	F
Xylem ingestion	G

In addition, the pd waveform performed during a short cell puncture as part of the pathway phase, otherwise known as a 'potential drop', can be separated into three distinct phases known as phases I, II And III. Phase II can be further subdivided into three subphases, II-1, II-2 and II-3. Sub-phase II-1 represents the injection of watery saliva into the punctured cell, while II-3 represents the ingestion of cell contents (Fereres & Moreno, 2009). The function of phase II-2 remains unknown (Pettersson *et al.,* 2007).

While it is important to understand a brief overview of these terms in order to discuss the ability of *M. persicae* to act as a vector of plant viruses, a more in-depth explanation of the EPG technique and the waveforms produced will be given in chapter 4 when relevant.

1.10 Hemipteran feeding and plant virus transmission

There is an additional concern with the emerging neonicotinoid resistance of *M. persicae* in the form of the high performance of this species as a virus vector (Blackman & Eastop, 2007) and the implications that this has for the control of plant viruses.

Plant viruses are divided into three main groups; non-persistent, semi-persistent and persistent. Non-persistent viruses only require a brief stylet penetration of less than 1 minute for acquisition and inoculation. Aphids are only capable of transmitting these viruses for a very short period following acquisition from an infected plant. Sometimes these viruses are referred to as 'stylet-borne' viruses, due to the theory that such viruses are mechanically transmitted via the stylet tips (Watson & Roberts, 1939; Kennedy *et al.,* 1962). Examples of non-persistent viruses include *Potato virus Y* (PVY) and *Cucumber mosaic virus* (CMV) (Martin *et al.*, 1997).

Semi-persistent viruses require a longer acquisition access period of at least 15 minutes and aphids are capable of transmitting the virus for a greater period of up to 2 days after access to a source plant. *Citrus tristeza virus* (CTV) is a semi-persistent virus (Katis *et al.*, 2007).

Persistent viruses require longer periods for acquisition and inoculation, often of several hours. After initially ingesting the virus the aphid enters a latent period, during which it is unable to transmit the virus and act as a vector. Once the latent period has ended however, the aphid may remain infectious for life. An example of a persistent virus is *Cereal yellow dwarf virus*-RVP (CYDV-RPV) (Katis *et al.*, 2007).

Until recently, it was assumed that non-persistent plant viruses were retained in the stylets, while semi persistent viruses were retained in the foregut (Fereres & Moreno, 2009) and persistent circulative viruses in the hindgut, hemocoel and salivary glands (Gray & Gildow, 2003). Recent work by Uzest *et al.*, (2007) has however shown that the semi-persistent *Cauliflower mosaic virus* (CaMV) is retained at specific receptors at the extreme tip of the maxillary stylets; in the common duct caused by the fusing of the salivary and food canals 2-4 µm from the stylet tips. In addition to this, aphid infections by persistent viruses may take the form of two different modes, propagative and circulative (Sylvester, 1980). After ingestion circulative viruses are actively moved through the gut wall into the hemocoele and then into the heamolymph. This allows them to circulate around the body of the aphid to reach the accessory salivary glands before transmission (Gray & Gildow, 2003, Hogenhout *et*

al., 2008). Propagative viruses also follow this mode of circulation around the aphid, but in addition are capable of multiplying in the aphid vector as well as the plant. Aphid infection by propagative viruses therefore involves the slow spread of viral infection through the aphids tissues and organs before the virus reaches the salivary system, hence there is a long latent period before the aphid becomes infectious to plants (Sylvester, 1980). Virus transmission by aphids is therefore highly complex and further work is required for a full understanding of these interactions.

There are three competing theories for how aphids transmit non-persistent plant viruses. The first, the stylet-borne hypothesis, maintains that viral particles attaches to the tip of the stylets, either on the outside or within the end of the maxillary food canal. Virus acquisition and inoculation is mechanical as the stylets are inserted into host plants (Kennedy *et al.*, 1962). This theory is however no longer widely accepted.

The second theory is known as the ingestion-egestion hypothesis. This theory postulates that aphids acquire viral particles by ingesting the sap of infected plants, retain the viral particles within the foregut, and then transmit the virus to fresh hosts via the regurgitation of this previously ingested sap during typical feeding behaviour (Harris, 1977).

More recent evidence has led to a widely accepted modified version of the ingestion-egestion hypothesis, the 'ingestion-salivation' hypothesis, which takes into account aphid salivary secretions (Powell, 1991, Powell *et al.*, 1995a, Martin *et al.*, 1997). It is now well established that viral particles are ingested along with sap from virus-infected plants and retained within the food canal of the maxillary stylets (Pirone & Perry, 2002) or the foregut. In the case of potyviruses this is achieved with the help of a virally encoded helper component (HC) protein, the presence of which causes viral particles to adhere to the lining of the maxillary food canal and the common duct (Wang *et al.*, 1998).

In contrast to the ingestion-egestion hypothesis, the ingestion-salivation hypothesis suggests that viral particles are inoculated into host plants by the secretion of watery saliva (Powell, 2005). Viral particles adhering to the cuticular lining at the common duct shared by the food and salivary canals may therefore be transported into the plant tissue during saliva secretion (Figure 1.5).

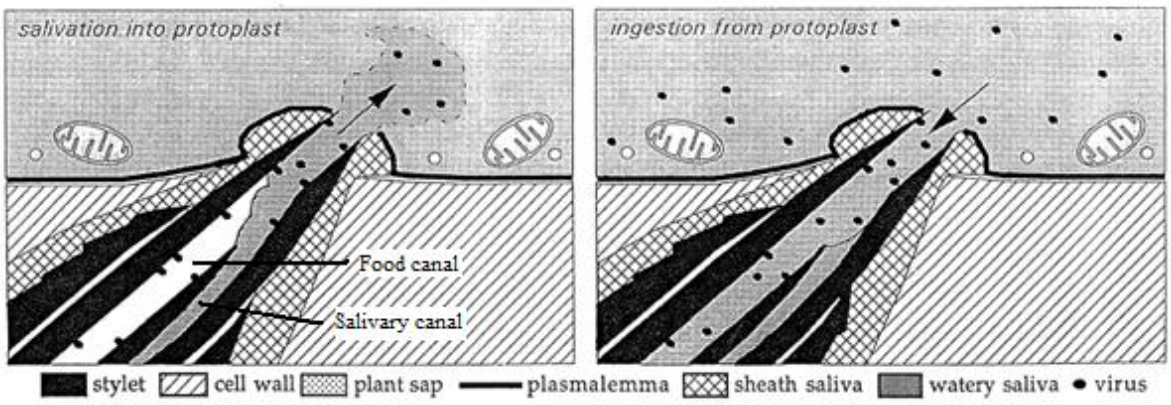


Figure 1.5 Acquisition and inoculation of a non-persistent virus by a Hemipteran vector during a short probe into a plant cell. The arrows represent the flow of both liquid and virions. a) Secretion of watery saliva into plant cell, along with viral particles. b) Ingestion of contents of plant cell, along with viral particles. Note how the salivary and food canals merge before the end of the aphid stylets. Adapted from Martín *et al.* (1997).

This theory is now widely supported. Several studies have shown that subphase II-3 is essential for the acquisition of non-persistent viruses by the aphid vector; hence it must represent the ingestion of cell contents (Powell, 1991, Powell *et al.*, 1995a, Martín *et al.*, 1997, Powell & Hardie, 2000). In addition to this, the duration of subphase II-3 is strongly related to the acquisition efficiency, with those of reduced duration associated with a significantly decreased probability of virus acquisition (Collar *et al.*, 1998, Palacios *et al.*, 2002, Moreno *et al.*, 2005, Symmes *et al.*, 2008).

Martín *et al.* (1997) showed that only subphase II-1 was required for the inoculation of PVY and CMV by *M. persicae and Aphis gossypii* Glover. This was supported by work from Powell (2005) which showed that interrupting aphids at the end of subphase II-1 still allowed them to inoculate plants with *Pea enation mosaic virus* (PEMV). Since this virus is retained in the accessory salivary glands rather than the stylets, this work also proved that watery salivation occurs during subphase II-1. Symmes *et al.* (2008) showed that the duration of subphase II-1 was associated with an increased transmission efficiency of *Zucchini yellow mosaic virus* (ZYMV). They also showed increased inoculation efficiency to be associated with a decreased duration of the overall intracellular puncture. Moreno *et al.* (2005) showed that the semi-persistent virus CaMV can also be inoculated during the first intracellular puncture; hence this mode of transmission is not restricted to potyviruses. It should be noted however, that recent work by Fereres & Moreno (2009) showed that this same virus was transmitted during subphase II-2 rather than subphase II-1; hence an additional feeding behaviour or additional salivation is needed for the transmission of caulimoviruses.

Certain viruses are only able to exist within the phloem tissue, such as those of the genera *Luteovirus* (Fereres & Moreno, 2009). These viruses are therefore reliant on phloem-feeding vectors to transmit them from host to host. It is probable that the inoculation of such viruses occurs during salivation into the phloem. Transmission of *Barley yellow dwarf virus* (BYDV) has been shown to occur during E1, supporting this theory (Prado & Tjallingii, 1994). In addition to this, the acquisition of BYDV has been shown to be associated with entry into the phloem-feeding E2 phase (Limburg *et al.*, 1997).

There is also some evidence that differences in feeding behaviour between vectors account for differences in virus transmission efficiency. The aphid *Brachycaudus helichrysi* (Kaltenbach) is a more efficient vector of PVY than *Drepanosiphum platanoidis* (Schrank). This difference in transmission ability has been linked to the greater number of short cell punctures performed by *B. helichrysi* (Powell *et al.*, 1992). Collar *et al.*, (1998) showed that *M. persicae* and *A. gossypii*, two efficient vector species of PVY, had greater subphase II-3 durations than *Rhopalosiphum padi* (L.) and *Sitobion avenae* (Fabricius), two less efficient vectors of PVY.

Several aspects of feeding behaviour have therefore been linked to the uptake and transmission of plant viruses by aphid vectors, including the number and duration of probes into the plant tissue, the durations of ingestion and salivation during short cell punctures and the length of phloem salivation and phloem feeding (Powell *et al.*, 1995a, Martin *et al.*, 1997, Powell, 2005, Fereres & Moreno, 2009). Since many plant viruses require phloem feeding for successful inoculation and transmission by the vector (Katis *et al.*, 2007), insecticides which alter the frequency and duration of phloem feeding such as TMX (Daniels *et al.*, 2009) may also have important implications for the efficiency of viral transmission. Insecticides which reduce the frequency and duration of both epidermal probes and phloem feeding have often been shown to reduce virus transmission (Perring *et al.*, 1999, Mowry & Ophus, 2002, Margaritopoulos *et al.*, 2010).

In addition, it can be argued that any insecticide which affects the dispersal of an aphid population may exert an effect on viral spread within a crop field or glasshouse environment. While imidacloprid exerts an antifeedant response in *M. persicae*, it also appears to increase dispersal from treated plants (Nauen *et al.*, 1998a, Nauen *et al.*, 1998c). It has also been shown to increase the production of alate aphids (Wang *et al.*, 2008), which have the potential to disperse much more rapidly over a far greater area than apterous aphids. Such an

effect may potentially increase the rate of virus transmission within a densely planted crop field, particularly in the case of non-persistent viruses where only brief initial probes may be required for virus acquisition and inoculation. Dispersing aphids searching for suitable hosts may probe greater numbers of potential host plants under conditions where an antifeedant has been applied, as opposed to insecticide-free conditions in which they may be more likely to enter a sustained period of feeding upon a single plant host.

Despite this, literature generally demonstrates the application of insecticides to be beneficial in the reduction of plant virus transmission by aphid vectors. Imidacloprid and TMX have been shown to decrease the transmission of *Potato leafroll virus* (PLRV) by *M. persicae* (Boiteau & Singh, 1999, Mowry & Ophus, 2002, Qi *et al.*, 2004, Mowry, 2005) either due to their toxicity or antifeedant effects. Pymetrozine has been found to decrease the rate of transmission of *Tomato yellow leaf curl virus* (TYLCV) by the whitefly *Bemisia tabaci* (Polston & Sherwood, 2003) and PLRV by *M. persicae* (Mowry, 2005). This insecticide also reduced the uptake of CaMV by *M. persicae*, though it did not prevent transmission to turnip plants by already viruliferous aphids (Bedford *et al.*, 1998). It is possible that any negative effects of insecticides on vector dispersal are therefore compensated for by a greater mortality rate and the antifeedant properties of such chemistries.

Since viral transmission and Hemipteran feeding and dispersal behaviours are closely linked, any alterations in these behaviours exhibited by the FRC or 5191A clones are likely to affect the dynamics and rate of plant virus transmission. No studies have yet been carried out upon the viral transmission capabilities of neonicotinoid resistant as opposed to susceptible aphid clones. Further work is therefore required to improve the understanding of the vector dynamics of neonicotinoid resistant as opposed to susceptible *M. persicae,* and the effects of the application of neonicotinoids to such clones on viral transmission in crop systems. This study aims to provide a starting point for addressing such questions.

1.11 Aims and objectives

This study involves several hypotheses:

1) The FRC clone will exhibit a higher lethal dose value than the other two *M. persicae* clones US1L and 5191A when exposed to the neonicotinoid insecticide TMX. This hypothesis was investigated during the work described in chapter two.

2) The FRC clone will exhibit differences in dispersal behaviour compared to the *M. persicae* clones US1L and 5191A when exposed to a sublethal dose of TMX. This altered dispersal behaviour will contribute to the greater neonicotinoid resistance of the FRC clone. This hypothesis was investigated during the work described in chapter three.

3) The FRC clone will exhibit differences in feeding behaviour compared to the *M. persicae* clones US1L and 5191A when exposed to a sublethal dose of TMX. This altered feeding behaviour will contribute to the greater neonicotinoid resistance of the FRC clone. This hypothesis was investigated during the work described in chapter four.

4) The differential feeding and dispersal behaviour of the FRC clone will lead to this clone exhibiting an increased efficiency as a vector of a persistent virus, compared to the *M. persicae* clones US1L and 5191A, when exposed to a sublethal dose of TMX. This hypothesis was investigated during the work described in chapter five.

The overall aim of this study was to investigate whether the neonicotinoid resistant FRC clone exhibits differences in feeding and dispersal behaviour compared to the susceptible US1L and weakly resistant 5191A clones when exposed to a sublethal dose of TMX, and whether these differences in behaviour could potentially contribute to the neonicotinoid resistance of this clone. In addition, the effect of any changes in these behaviours was predicted to increase the efficiency of the FRC clone as a vector of a persistent virus.

Chapter 2: Discerning appropriate sublethal doses

2.1 Introduction

In order to investigate the behaviour of insects under insecticide treatment it is necessary to treat individuals with a dose of chemical which, while of a great enough concentration to significantly affect behaviour, does not cause high levels of mortality. The concentration which kills 15% of a population, denoted the $LD_{15,}$ has been recommended for such work in many studies (Nauen, 1995; Mowry & Ophus; 2002, Daniels *et al.*, 2009). In order to analyse the behaviour of the three *M. persicae* clones under the influence of different AIs, it was therefore necessary to first calculate the appropriate LD_{15} values.

Lethal and sublethal doses of insecticides are generally calculated using dose-response assays, which can involve a wide range of application techniques. Spraying, drenching, oral administration via artificial diet, residual contact with treated surfaces or host plants, topical application directly to the insect and microinjection have all been successfully utilised in various studies (Devine *et al.*, 1996, Harrewijn & Kayser, 1997, Bedford *et al.*, 1998, Nauen *et al.*, 1998c, Nauen *et al.*, 2001, Kaufmann *et al.*, 2004, Morita *et al.*, 2007, Daniels *et al.*, 2009, Gharalari *et al.*, 2009, Sadeghi *et al.*, 2009; Philippou *et al.*, 2010; Shi *et al.*, 2011). A major limitation of such research is that different methods of insecticide application cause the insect to receive a different dose of the AI over different time periods at a variety of sites around the body, including the alimentary canal, cuticle and haemolymph. This hinders accurate comparison of studies which have used differing methods and thus the application of such research to the field.

Of the application methods mentioned above, spraying and root drenching are arguably the most comparable to the field situation. Root drenching involves the systemic uptake of insecticide from the soil by the host plant prior to ingestion of the active ingredient (AI) by the target pest and mimics a direct application to the soil or a seed treatment. This process may reduce the amount of AI which finally reaches the aphid due to several factors, the most important being AI remaining trapped or metabolised in the soil. Once root uptake has occurred, the insecticide will concentrate in different tissues of a host plant according to their physical characteristics. For example, TMX concentrates in the xylem (Maienfisch *et al.*, 2001a). Since aphids feed primarily from the phloem (Dedryver *et al.*, 2010), if an insecticide becomes more concentrated in a tissue they do not feed from such as the cuticle or roots it may limit the amount of AI they are exposed to. Many insecticides are also metabolised by

the host plant into a range of metabolites, which may or may not exert a detrimental effect upon target pests. An example of this would be TMX being converted into the metabolite clothianidin (Casida, 2011), which also exerts a potent insecticidal action.

Oral administration via artificial diet allows a more accurate measurement of the amount of AI presented to the aphid, as there is no breakdown and conversion of the AI prior to ingestion. Root drenching, however, allows for investigation into the effects of the AI upon a greater range of feeding behaviours than oral ingestion from a parafilm sachet, due to the presence of stimulatory chemical and physiological signals provided by the host plant. Both methods are therefore of great practical interest and are represented in recent literature (Harrewijn & Kayser, 1997, Daniels *et al.*, 2009, Sadeghi *et al.*, 2009). The current study will incorporate a comparison of the effects of two different methods of insecticide application upon *M. persicae*; root drenching and oral ingestion via an artificial diet. It will be necessary to calculate LD_{15} values separately for these two methods due to the differences in the amount of AI reaching the target aphid in each case.

Due to the differing levels of neonicotinoid resistance exhibited by the three clones it is expected that they would require different concentrations of neonicotinoid compounds to reach the $LD_{15.}$ It is also possible that the three clones would exhibit different LD_{15} values for non-neonicotinoid chemistries, even though any slight differences in tolerance towards these insecticides may not lead to notable differences in resistance within the field. It is common for different susceptible clones of *M. persicae* to vary in tolerance towards various insecticides (Devine *et al.*, 1996; Nauen *et al.*, 1998a; Foster *et al.*, 2008; Philippou *et al.*, 2009; Bass *et al.*, 2011) and it would therefore be important to perform separate dose-response assays on the three clones for non-neonicotinoid as well as neonicotinoid insecticides.

2.2 Methods

2.2.1 Insects and plant material

The susceptible *M. persicae* clone US1L was obtained from Imperial College London, while 5191A and FRC were obtained from Syngenta, with the permission of Rothamsted Research, UK. All three clones were reared on Chinese cabbage (*Brassica chinensis* L. *cv* Apex) in separate temperature control cabinets (22 ± 1 ºC, 16:8 h L:D photoperiod, 50 % r.h.). The 5191A and FRC clones were subject to DEFRA regulations whilst in culture in the UK. Chinese cabbage plants for the FRC culture were sprayed to run off with 10 ppm formulated TMX (Actara, provided by Syngenta) made up with Milli-Q water. Such pressuring was required to retain the neonicotinoid resistance of the clone (Table 2.1). To obtain adults of known age, aphids were removed from the main culture and reared on leaf discs. Three and a half centimetre discs were cut from the tissue either side of the central vein of two-week old true Chinese cabbage leaves, inverted, and set into 2% agar within the wells of a six-well plate. Five adult aphids were placed on the disc and allowed to reproduce for 24 h before removal. The remaining nymphs were raised to the first day of adulthood before being used in experiments. Aphids were starved in a Petri dish for 1.5 h prior to use.

Table 2.1 The insecticide resistance profiles of the three *Myzus persicae* clones US1L, 5191A and FRC.

Clone	Esterase phenotype	MACE genotype	kdr genotype	skdr genotype	Neonicotinoid resistance
US1L	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
5191A	R3	R/S	S/S	S/S	Low level
FRC	R3	S/S	R/S	R/S	High level

Chinese cabbage plants were obtained as seed from Tozier and grown individually in 3 inch pots filled with 'Levington F2 + sand' compost in a growth room (20 ± 1 ºC, 16:8 h L:D photoperiod and 50% r.h.). Plants for all experiments were used at the seedling stage, when two to three true leaves had formed.

2.2.2 Plant drench dose-response

Chinese cabbage plants as described earlier were not watered for 24 h to allow the compost to dry. They were then root drenched with either 50 ml of formulated TMX (Actara) or pymetrozine (Plenum), as provided by Syngenta, and left for a further 24 h to allow uptake of the insecticide. Ten adult aphids of the same clone were then placed on each plant, which was covered with a 1240 cm^3 cylindrical plastic ventilated cage and placed into a temperature control cabinet under conditions of 22 ± 1 ºC, 16:8 h L:D photoperiod, 50 % r.h.. After a further 96 h mortality of the aphids was assessed.

Plants were treated with a range of at least five different TMX or pymetrozine concentrations, diluted with Milli-Q water. Control mortality was assessed from aphids on plants root drenched with pure Milli-Q water. Ten separate repeats were taken at each concentration using ten separate plants for the susceptible clones. Only four repeats at each concentration could be taken for the resistant clones, due to space limitations within the DEFRA-licenced facility. The entire dose response test was repeated three times for each insecticide.

2.2.3 Preparation of artificial diet

The artificial diet was modified from the *M. persicae* diet of Dadd & Mittler (1966). A glass beaker containing 750 ml nanopure water and a magnetic stirrer was placed upon a hotplate and the ingredients in Table 2.2 fully dissolved into the solution.

25 mg of pyridoxine hydrochloride and 0.1 mg of crystalline D-biotin were also dissolved within the solution. KOH was then added to the diet until it reached pH 7, as measured with a pH meter, and the solution made up to 1000 ml by the addition of further nanopure water.

The diet was then sterilised by being passed through a 0.2 μm Nalgene sterile filter in 10 ml batches. The sterile filter was replaced after every ten uses. During sterilisation the diet was decanted into clean Falcon tubes, and then frozen for later use.

Table 2.2 The ingredients forming the artificial diet for *Myzus persicae* as modified from Dadd and Mittler (1966). It is important that the ingredients are dissolved in the order shown, starting with the left-hand column.

Chemical	**mg**	**Chemical**	**mg**
sucrose	150000.0	L-methionine	400.0
di-potassium hydrogen orthophosphate	7500.0	L-phenylalanine	400.0
Magnesium Sulphate	1230.0	L-proline	800.0
L-tyrosine	400.0	L-serine	800.0
L-asparagine	5500.0	L-threonine	1400.0
L-aspartic acid	1400.0	L-valine	800.0
L-tryptophan	800.0	ascorbic acid	1000.0
L-alanine	1000.0	aneurine hydrochloride	25.0
L-arginine	2700.0	riboflavin	5.0
L-cysteine	400.0	nicotinic acid	100.0
L-glutamic acid	1400.0	folic acid	5.0
L-glutamine	1500.0	inositol (meso) active	500.0
L-glycine	800.0	Choline chloride	500.0
L-histidine	800.0	$FeCl_3 \cdot 6H_2O$	9.1
L-isoleucine	800.0	$ZnSO_4 \cdot 6H_2O$	9.6
L-leucine	800.0	$MnSO_4 \cdot H_2O$	6.1
L-lycine monohydrochloride	1200.0	$CuSO_4 \cdot H_2O$	5.6

All insecticides used in artificial diet were supplied by Syngenta in the form of 1 mg of technical pure AI in glass tubes. The neonicotinoids imidacloprid (IMD) and TMX, two non-neonicotinoid antifeedants pymetrozine (PYM) and flonicamid (FLN) and the unclassified pesticide sulfoxaflor (SFX) were included. All insecticides were initially prepared by pipetting 1 ml of acetone into each glass tube and sonicating for 1 minute in a water bath to ensure the AI was completely dissolved within solution.

The insecticide solution was then diluted down to the required concentration using artificial diet and refrigerated within 10 ml glass tubes until required. Pure diet with an acetone concentration equal to that within the strongest insecticide concentration utilised acted as a control for each insecticide.

2.2.4 Formation of diet rings

Solutions containing a mixture of artificial diet and AI were presented to *M. persicae* in the form of diet rings. Diet rings were produced by stretching a layer of Parafilm 'M' across a brass curtain ring of diameter 25 mm. The Parafilm had to be stretched both horizontally and vertically in order to render it permeable to the stylets of *M. persicae.* A 150 µl droplet of artificial diet and insecticide solution was deposited upon the Parafilm. A second layer of

Parafilm was then stretched across the top of the brass ring, trapping the droplet between the two layers of Parafilm and suspending it in a sachet. This formed a single diet ring (Figure 2.1).

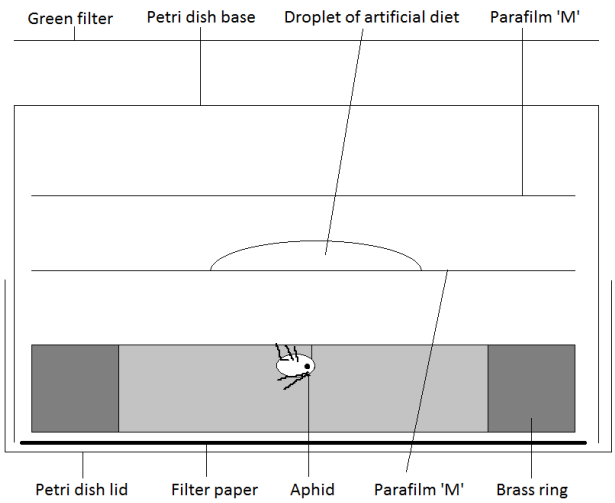


Figure 2.1 The components of a diet ring system for providing a mixture of artificial diet and pesticide to *Myzus persicae*.

An inverted Petri dish lid of diameter 55 mm was lined with a layer of moist filter paper. *Myzus persicae* were then placed into the small space formed between the lower layer of Parafilm and the moist filter paper, encircled by the brass ring. The base of the Petri dish was inverted and used as a lid. An opaque green plastic filter was placed over the top of the Petri dish to encourage the *M. persicae* to probe upwards and feed upon the liquid diet by puncturing the Parafilm with their stylets.

2.2.5 Artificial diet dose-response setup

Diet rings containing artificial diet and pure AI at a range of concentrations were produced according to the method described in section 2.2.4 above. Ten diet rings were made up for each concentration of the insecticides IMD, TMX, PYM, FLN and SFX. In addition to this, ten control diet rings containing only artificial diet and acetone, equivalent to the concentration of acetone used to make up an insecticide solution of concentration 50 ppm, were also produced. The diet rings were placed in separate Petri dishes as described above. Ten *M. persicae* of the first day of adulthood were placed upon each diet ring. The *M.*

persicae were then cultured for 96 h under conditions of 19 ºC ± 1 ºC, 16:8 h L:D and 50% r.h. The numbers of surviving *M. persicae* were recorded each day. Dead aphids were classed as those which moved no more than one leg upon poking with a fine paintbrush. The entire assay was repeated three times.

2.2.6 Wide range artificial diet dose-response

The experimental setup was as in section 2.2.5 above. Insecticide concentrations of 50, 5, 0.5, 0.05 and 0.005 ppm were used, as well as a control solution of artificial diet and acetone. Probit analysis was performed upon the data in Minitab, as at this stage the Excel solver application was not available, and an approximate LD_{50} value calculated for each insecticide.

2.2.7 Narrow range artificial diet dose-response

The experimental setup was as in section 2.2.5 above. The concentrations used for each insecticide were based upon the corresponding LD_{50} value as calculated by probit analysis of the wide dose-response data (Table 2.3). Some insecticides, however, required further dose response tests to be carried out at modified concentration ranges before data suitable for the calculation of an LD_{15} value could be obtained.

Table 2.3 The LD_{50} values for five insecticides administered orally to the *Myzus persicae* clone US1L as calculated in Minitab by probit analysis of a wide ranged dose-response experiment for each insecticide. The Excel solver application was not obtained until a later date; hence the narrow dose-response concentrations were originally based upon the Minitab values rather than Excel solver.

	LD_{50} (ppm)
Imidacloprid	0.09
Thiamethoxam	0.006
Sulfoxaflor	0.13
Pymetrozine	4.64
Flonicamid	4.46

2.2.8 Statistical analysis

The results from all dose-response tests were plotted in Microsoft Excel separately for each clone and treatment. The solver model described by Bowen & Jerman (1995), provided by Rob Lind, was then used to fit dose-response curves from which the LD_{15} values for each insecticide, clone and treatment method combination could be calculated. LD_{20} doses were also calculated from the data on root drenching with TMX, for use in EPG experiments described in a later chapter.

2.3 Results

When treated with TMX the susceptible clone US1L exhibited the lowest LD_{15} out of the three clones, with the LD_{15} of 5191A being nine times greater (Table 2.4). The FRC clone exhibited a substantially greater LD_{15} for TMX than the other two clones (Figure 2.2). When treated with PYM, however 5191A exhibited the greatest LD_{15} value, over twice that of the FRC clone (Table 2.4). US1L still exhibited the lowest LD_{15} value of the three clones when treated with PYM (Figure 2.3). All three clones required greater doses of TMX than PYM to produce mortality in 15% of the population.

Table 2.4 LD_{15} doses in ppm ± standard error for three *Myzus persicae* clones of differing neonicotinoid resistance, when either thiamethoxam or pymetrozine was applied to a host plant by root drench.

	Thiamethoxam LD_{15} (ppm)	**Pymetrozine LD_{15} (ppm)**
US1L	0.1 ± 0.05	0.005 ± 0.004
5191A	0.9 ± 0.08	0.07 ± 0.04
FRC	42.5 ± 1.01	0.03 ± 0.03

The LD_{15} values for US1L were much lower when either TMX or PYM was applied orally in artificial diet as opposed to when applied by root drench of a host plant (Figure 2.4). TMX produced a lower LD_{15} value when applied in artificial diet than IMD (Table 2.5). US1L exhibited lower LD_{15} values to the two neonicotinoid chemicals TMX and IMD than to the antifeedant chemicals PYM and FLN. SFX produced an LD_{15} value for the susceptible aphids closer to that of the neonicotinoid chemistries than that of the antifeedants.

Table 2.5 LD_{15} doses in ppm ± standard error for the susceptible *Myzus persicae* clone US1L when five different insecticides were applied orally within artificial diet.

	LD_{15} (ppm)
Imidacloprid	0.005 ± 0.007
Thiamethoxam	0.013 ± 0.006
Pymetrozine	0.125 ± 0.074
Flonicamid	0.368 ± 0.183
Sulfoxaflor	0.015 ± 0.01

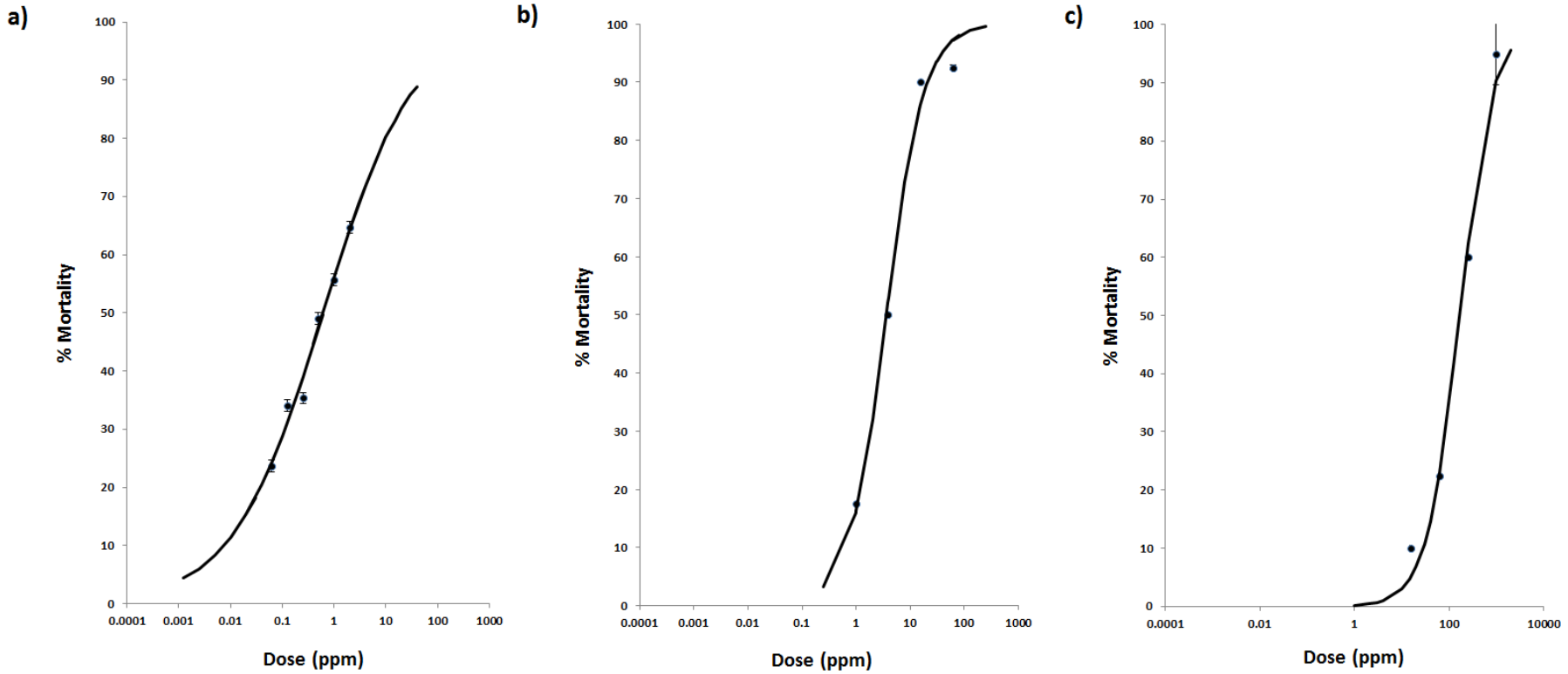


Figure 2.2 The mortality of three different *Myzus persicae* clones when formulated thiamethoxam (Actara) was applied by root drench to a Chinese cabbage host plant. a) US1L b) 5191A c) FRC. Error bars represent ± the standard error.

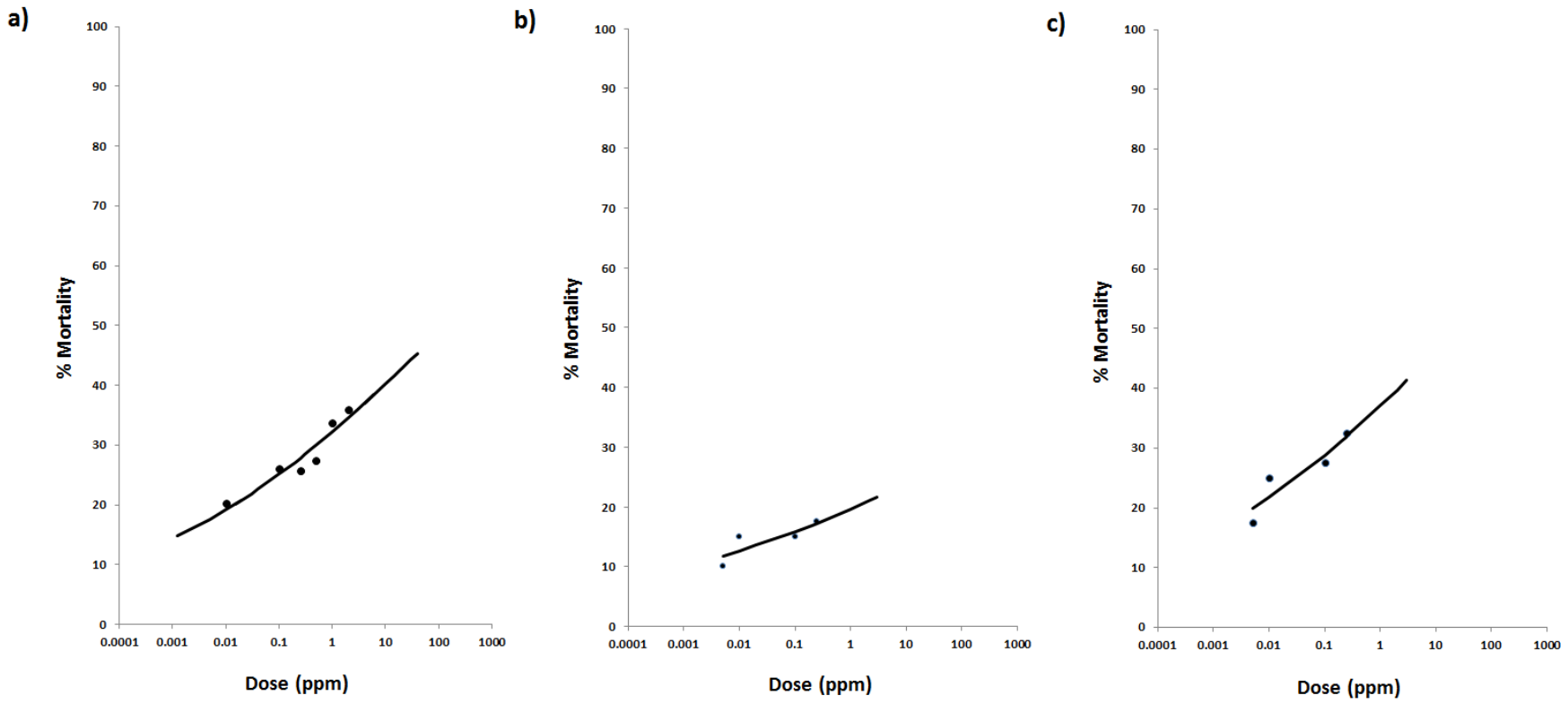


Figure 2.3 The mortality of three different *Myzus persicae* clones when formulated pymetrozine (Plenum) was applied by root drench to a Chinese cabbage host plant. a) US1L b) 5191A c) FRC. Error bars represent ± the standard error. Note the very small standard errors.

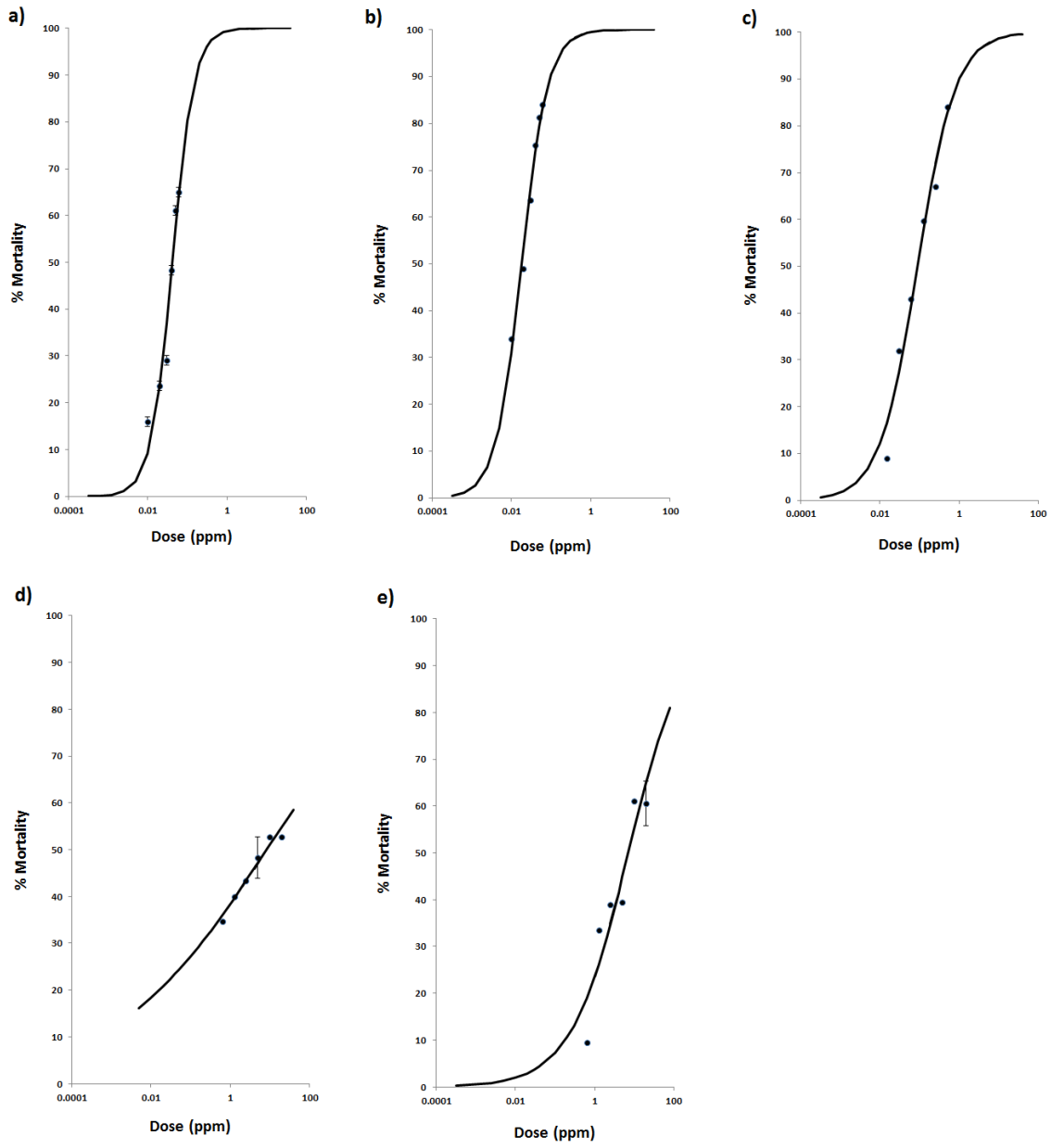


Figure 2.4 The mortality of the *Myzus persicae* clone US1L when five different insecticides in the form of pure AI were offered within artificial diet a) thiamethoxam b) imidacloprid c) sulfoxaflor d) pymetrozine e) flonicamid. Error bars represent ± the standard error.

The LD_{20} values for TMX followed the same pattern as the LD_{15} values (Table 2.6), in that US1L exhibited the lowest value and FRC the greatest.

Table 2.6 LD_{20} doses in ppm ± standard error for three *Myzus persicae* clones of differing neonicotinoid resistance, when thiamethoxam was applied to a host plant by root drench

	LD_{20}
US1L	0.2 ± 0.08
5191A	1 ± 0.11
FRC	53.1 ± 1.49

2.4 Discussion

The greater LD_{15} value produced when aphids of the FRC clone as opposed to US1L were offered TMX treated host plants is consistent with the increased level of neonicotinoid resistance of the FRC clone already described in literature (Bass *et al.,* 2011). When exposed to TMX, 5191A exhibited a LD_{15} value between that of US1L and FRC, which correlates with the low level of neonicotinoid resistance already described in this clone (Philippou *et al.,* 2010; Puinean *et al.,* 2010; Bass *et al.,* 2011).

Lower doses of PYM than TMX were required to reach the LD_{15} for all three clones, indicating that significant resistance to PYM, unlike resistance to neonicotinoids, is not present in these clones. This is also consistent with literature, in which no significant resistance of *M. persicae* to PYM has yet been detected (Margaritopoulos *et al.,* 2010). Interestingly, the LD_{15} value exhibited by 5191A towards PYM was greater than that exhibited by the FRC clone towards this compound, suggesting that 5191A may originally have received greater exposure to PYM than the FRC clone before removal from the field. It is possible that this may be due to the host plant of origin of the two clones. The FRC clone was originally collected from peach in France (Bass *et al.,* 2011), while 5191A came from tobacco in Greece (Philippou *et al.,* 2009). Plenum is not recommended for use on peach (Syngenta Crop Protection, 2011) so it is unlikely that the FRC clone would have been strongly exposed to this insecticide previous to collection from the field. PYM is often used on tobacco in Greece however (Margaritopoulos *et al.,* 2010) so it is likely the 5191A clone was originally exposed to PYM before collection and removal to the laboratory, supporting this hypothesis.

It is difficult to compare the LD_{15} and LD_{50} values obtained by this study with those in literature, due to the wide variation in application techniques and aphid species utilised (Tables 2.6 & 2.7). Sadeghi *et al.* (2009) obtained a lower LD_{50} value than our study detected when applying PYM by oral ingestion in artificial diet to the pea aphid *A. pisum* while Daniels *et al.* (2009) produced a greater LD_{15} value when applying TMX to *R. padi* by root drench on wheat. It is well documented however that different aphid species and even strains of the same species may respond very differently to the same insecticides (Devine *et al.,* 1996; Nauen *et al.,* 1998a; Nauen *et al.,* 1998c; Morita *et al.,* 2007; Sadeghi *et al.,* 2009) so the LD_{15} values obtained by our study do seem consistent with previously observed values.

The differences in the LD_{15} values produced by the artificial diet and root drench assays highlight an interesting comparison between the two classes of compound included; the acutely toxic neonicotinoid TMX and the antifeedant PYM. When offered in artificial diet, the neonicotinoid TMX produced lower LD_{15} values than those of PYM, while the converse effect was seen when treated host plants were used.

The greater amount of PYM required to achieve the LD_{15} when offered in artificial diet as opposed to treated host plants may be due to the mode of action of this insecticide, which is still being fully characterised. PYM and FLN are antifeedants but exert no acute toxic effect (Harrewijn & Kayser, 1997; Morita *et al.,* 2007; He *et al.,* 2011). PYM prevents penetration of the host plant tissues by the aphid, inhibiting feeding and causing death by starvation (Harrewijn & Kayser, 1997; Kaufmann *et al.,* 2004). The mode of action remains unknown, but the mechanism appears linked to the signalling pathway of serotonin, causing contractions of the foregut (Kaufmann *et al.,* 2004) and extension of the femur-tibia joints in the migratory locust *Locusta migratoria* L. (Ausborn *et al.,* 2005). FLN, conversely, appears to act by blocking of the A-type potassium channel (Joost *et al.,* 2006). Treated aphids attach the proboscis to the leaf surface, but phloem feeding and phloem salivation are strongly inhibited (Morita *et al.,* 2007). It is possible that in the absence of the stimulatory chemical and physical signals normally provided by the host plant the mode of action of PYM is somehow compromised and becomes less efficient, hence greater concentrations must be applied to achieve the same level of mortality in artificial diet as in host plant assays.

It is also possible that the differences in LD_{15} values produced by the two application methods, oral ingestion from either an artificial diet or a treated host plant, are due to differences in how the insecticides achieve uptake into or become distributed within the host plant. IMD and TMX have both been shown to move systemically through host plants via the xylem, where they concentrate (Buchholz & Nauen, 2002; Maienfisch *et al.,* 2001a). Pymetrozine has also been shown to be highly systemic, travelling through both the xylem and phloem (Wyss & Bolsinger, 1997). When feeding on artificial diet the compounds are readily available to the aphid rather than spread throughout the host plant tissues and soil. The aphids may therefore have received greater concentrations of TMX when feeding from artificial diet than when feeding from a host plant, hence lower concentrations of IMD and TMX were needed to achieve 15% mortality. This effect would not have been seen for PYM if it required metabolising to more active metabolites to achieve normal levels of aphid control, as again no metabolism would have occurred in artificial diet. It is documented both

that PYM and FLN are metabolised within the host plant and converted into further metabolites (Maienfisch *et al.*, 2001a; Hengel & Miller, 2007), as are TMX and IMD (Casida, 2011).

It is notable that the five compounds tested in artificial diet segregate by both the LD_{15} values produced and by their mode of action. This supports the theory that MOA may account for the differences in LD_{15} values between the artificial diet and host plant assays. It may take a greater amount of time for insects to succumb to death by chronic starvation than acute poisoning, hence the greater LD_{15} values exhibited by the antifeedant insecticides PYM and FLN than the neonicotinoids IMD and TMX after a 96 h observation period. Extending the length of the dose response assay would possibly reduce the difference in LD_{15} values produced by the antifeedant and neonicotinoid compounds. A 96 h assay however is already slightly longer in length than those commonly used (Tables 2.7 & 2.8), so such an increase would be inadvisable.

Although originally it was intended for all five compounds to be utilised in the experiments described in later chapters, ultimately time, cost and other practical limitations made it necessary to be limit this study to a single compound applied by one method. The neonicotinoid TMX was chosen for this purpose as it was readily obtainable in large quantities and produced a clear effect on *M. persicae* at low concentrations. It is also commercially relevant, with neonicotinoids including TMX currently being used for protection of a wide variety of crops (Nauen & Denholm, 2005; Foster *et al.*, 2008; Jeschke *et al.*, 2010). The LD_{15} values for US1L remained lower than those of 5191A and FRC for TMX regardless of application method, highlighting the greater susceptibility of US1L to this insecticide and proving US1L to be useful as a susceptible clone for comparisons of behaviour with the resistant *M. persicae* clones 5191A and FRC.

Overall I accept hypothesis one, that "the FRC clone will exhibit a higher lethal dose value than the other two *M. persicae* clones US1L and 5191A when exposed to the neonicotinoid insecticide TMX". This hypothesis is clearly supported by the dose response data described in this chapter.

Table 2.7 Review of current literature concerning the calculation of LD_{50} results for the neonicotinoid insecticides imidacloprid (IMD) and thiamethoxam (TMX) for a range of Hemipteran species and a variety of different application methods. Note how different application methods produce different LD_{50} doses, even when using the same insecticide upon the same species. All values shown were produced using susceptible strains.

Insecticide	Species	Application method	Time of assessment	LD_{50} (ppm)	Reference
IMD	*Myzus persicae*	Contact	72 h	0.4	Foster *et al.* (2008)
IMD	*Myzus persicae*	Contact	72 h	0.4	Foster *et al.* (2008)
IMD	*Aphis glycines*	Leaf dip	168 h	0.03	Magalhaes *et al.* (2008)
IMD	*Myzus persicae*	Aphid dip	48 h	0.47	Nauen *et al.* (1998a)
IMD	*Bemisia tabaci*	Leaf dip	48 h	50.00	Nauen *et al.* (1998b)
IMD	*Bemisia tabaci*	Systemic	48 h	23.00	Nauen *et al.* (1998b)
IMD	*Aphis gossypi*	Oral	48 h	0.11	Nauen *et al.* (1998c)
IMD	*Myzus persicae*	Oral	48 h	0.07	Nauen *et al.* (1998c)
IMD	*Myzus persicae*	Contact	48 h	0.22	Nauen *et al.* (1998c)
IMD	*Apis mellifera*	Oral	48 h	0.21 – 0.41	Nauen *et al.* (2001)
IMD	*Apis mellifera*	Contact	48 h	0.05-102.00	Nauen *et al.* (2001)
IMD	*Myzus persicae*	Topical application	72 h	1.60	Philippou *et al.* (2010)
IMD	*Myzus persicae*	Topical application	72 h	1.13	Puinean *et al.* (2010)
IMD	*Myzus persicae*	Oral	48 h	0.32	Puinean *et al.* (2010)
IMD	*Acyrthosiphum pisum*	Oral	24 h	100.00	Sadeghi *et al.* (2009)
IMD	*Acyrthosiphum pisum*	Oral	48 h	0.14	Sadeghi *et al.* (2009)
IMD	*Acyrthosiphum pisum*	Oral	72 h	0.03	Sadeghi *et al.* (2009)
IMD	*Aphis gossypii*	Leaf dip	48 h	0.32	Shi *et al.* (2011)
TMX	*Myzus persicae*	Leaf disc dip	48 h	4.02	Cho *et al.,* (2011)
TMX	*Rhopalosiphum padi*	Drench	24 h	0.80 (LC_{15})	Daniels *et al.* (2009)
TMX	*Myzus persicae*	Contact	72 h	0.2	Foster *et al.* (2008)
TMX	*Myzus persicae*	Contact	72 h	0.4	Foster *et al.* (2008)
TMX	*Aphis glycines*	Leaf dip	168 h	0.02	Magalhaes *et al.* (2008)
TMX	*Myzus persicae*	Topical application	72 h	0.65	Puinean *et al.* (2010)
TMX	*Myzus persicae*	Oral	48 h	0.08	Puinean *et al.* (2010)
TMX	*Aphis gossypii*	Leaf dip	48 h	16.00	Shi *et al.* (2011)

Table 2.8 Review of current literature concerning the calculation of LD_{50} results for the antifeedant insecticides pymetrozine (PYM) and flonicamid (FLN) for a range of Hemipteran species and a variety of different application methods. Note how different application methods produce different LD_{50} doses, even when using the same insecticide upon the same species. All values shown were produced using susceptible strains.

Insecticide	Species	Application method	Time of assessment	LD_{50} (ppm)	Reference
PYM	*Myzus persicae*	Plant Contact assay	24 h	200.00	Harrewijin & Kayser (1997)
PYM	*Myzus persicae*	Aphid dip	48 h	0.66	Nauen *et al.* (1998a)
PYM	*Acyrthosiphum pisum*	Oral	24 h	100.00	Sadeghi *et al.* (2009)
PYM	*Acyrthosiphum pisum*	Oral	48 h	0.24	Sadeghi *et al.* (2009)
PYM	*Acyrthosiphum pisum*	Oral	72 h	0.01	Sadeghi *et al.* (2009)
FLN	*Myzus persicae*	Leaf disc dip	96 h	2.56	Cho *et al.,* (2011)
FLN	*Myzus persicae*	Leaf spray	120h	0.70	Morita *et al.* (2007)
FLN	*Acryrthosiphum pisum*	Oral	24 h	0.02	Sadeghi *et al.* (2009)
FLN	*Acryrthosiphum pisum*	Oral	48 h	0.0004	Sadeghi *et al.* (2009)
FLN	*Acryrthosiphum pisum*	Oral	72 h	0.0002	Sadeghi *et al.* (2009)

Chapter 3: Dispersal behaviour of neonicotinoid resistant and susceptible *Myzus persicae*

3.1 Introduction

Much literature on the behaviour-altering effects of insecticides and behavioural insecticide resistance has concentrated on changes in feeding behaviour (Nauen & Elbert, 1997; Rose *et al.,* 2005; Brunissen *et al.,* 2009; Daniels *et al.,* 2009; Cui *et al.,* 2010). Changes in dispersal behaviour may, however, also potentially contribute to neonicotinoid resistance. An insect which shows an increased tendency to quickly disperse from an insecticide-treated plant to an untreated plant may ingest a lower dose of insecticide and so be more likely to survive.

Changes in dispersal behaviour between clones which lead to differences in insecticide resistance must be distinguished from the general repellency effect of insecticides. Insects exhibit repellency when the application of an insecticide to a host plant or food substance causes them to avoid and disperse away from a treated area. Repellency is exhibited by all individuals of a species in response to that insecticide and so all individuals have the same probability of surviving exposure to the chemical. Such behaviours are often considered beneficial in terms of control. For example, the large pine weevil *Hylobius abietis* (L.) will avoid feeding on Scots pine twigs treated with lambda-cyhalothrin but will feed from untreated areas on the same twig (Rose *et al.,* 2005; 2006) and exposure to sublethal doses of imidacloprid significantly increases the general rate of movement of *M. persicae* (Roditakis *et al.,* 2000).

Alterations in dispersal behaviour are only considered to be a form of insecticide resistance when one strain or clone of a species exhibits an alteration in dispersal behaviour in response to an insecticide that makes it more likely to survive exposure to that insecticide than another clone or strain that does not exhibit the altered dispersal behaviour. For example, a resistant strain of the German cockroach *Blattella germanica* (L.) showed increased aversion to agar containing fructose, glucose, maltose and sucrose, all ingredients commonly contained within commercial gel baits, compared with a susceptible strain (Wang *et al.,* 2004). Topical application of abamectin or fipronil reduced the difference in mortality between resistant and susceptible strains. This indicated that the behaviour contributed significantly to the resistance of the resistant strain. Hoy and Head (1995) showed that Colorado potato beetle *L. decemlineata* larvae of Bt resistant strains were more likely to move away from Bt treated

foliage than larvae of a Bt susceptible strain. Aphids may also show behavioural resistance, but most work to date has concentrated on either changes in feeding behaviour (Nauen & Elbert, 1997), differences in dispersal between aphid species (Walters & Dixon, 1983; Hodgson, 1991; Boiteau, 1997; Alyokhin & Sewell, 2003), differences between aphid lineages that do not differ in insecticide resistance (Najar-Rodriguez *et al.,* 2009) or else repellency effects of insecticides (Roditakis *et al.*, 2000). There is currently no literature comparing the dispersal behaviour of a neonicotinoid resistant and susceptible aphid strain.

Two strains of pyrethroid-resistant *M. persicae* have been shown to walk or fly at greater rates from leaves treated with deltamethrin than untreated leaves (Rice *et al.,* 1983), with the moderately resistant strain walking from leaves sooner than the highly resistant strain. No comparison however was made with a susceptible strain so it is difficult to determine whether the aphids were exhibiting behavioural resistance or repellency.

This study examined whether neonicotinoid-resistant clones of *M. persicae* exhibit any alterations in their dispersal behaviour, either inherently or else in response to the presence of neonicotinoids, that could potentially contribute to their resistance. It is also the first study to compare the effect of an insecticide on the dispersal behaviour of both a resistant and susceptible apterous clone of the same aphid species.

3.2 Methods

3.2.1 Insects and Chinese cabbage cultures

All aphids and plants were cultured as described in chapter 2. All aphids utilised were apterous adults of 8 days of age ± 24 h. Aphids were starved in a Petri dish for 1.5 h prior to use.

Chinese cabbage plants (*B. chinensis cv* Apex) were grown individually in 3 inch pots filled with compost (Levington sand) in a growth room (20 ± 1 ºC, 16:8 h L:D photoperiod and 50% relative humidity). Plants for all experiments were used at the seedling stage, when two to three true leaves had formed.

3.2.2 Experimental Setup

Chinese cabbage plants of the seedling stage were left to dry for 24 h and then root drenched with 50 ml formulated thiamethoxam (TMX) (Actara). Plants were drenched with a TMX concentration equal to the LD_{15} of one of the three clones, as calculated in chapter 2 (Table 2.4). Control plants were root drenched with 50 ml Milli-Q water instead. All plants were left to stand for 24 h at conditions of 22 ± 1 ºC, 16:8 h L:D photoperiod, 50 % r.h. to take up the TMX or Milli-Q water.

A pipette was used to transfer 20 ml of 2% agar into a 5 cm diameter Petri dish. A 3.5 cm diameter hole was then punched into the middle of a leaf, freshly removed from the plant. The leaf was placed abaxial surface uppermost within the agar before it fully set, ensuring that the outer edges protruded underneath the agar to prevent aphids from accessing beneath. A 3.5 cm diameter disc punched from the same location on a different leaf was also placed into the partially set agar, positioned within the vacant hole left in the original leaf (Figure 3.1). The agar was then left for 1 h to set. Ten adult apterous *M. persicae* of the same clone were placed onto the centre of the disc. A second Petri dish in which holes had been punched and covered by netting was inverted and placed over the first Petri dish to form a ventilated lid, and the edges sealed together using parafilm ‘M’.

The setup was then placed on a light box underneath the lens of a time lapse camera. A total of 12 microcosms could be recorded under the camera at the same time, each representing a different treatment/clone combination. Four different combinations of disc and leaf TMX treatments were offered separately to each aphid clone. These were both the leaf and disc

coming from separate control plants treated with Milli-Q water, both the leaf and the disc coming from separate plants treated with TMX, the disc treated with TMX and the leaf from a control plant and the disc from a control plant and the leaf treated with TMX. In each case, when an arena contained either a leaf or a leaf disc treated with TMX, the plant had been treated with a dose of TMX equivalent to the LD_{15} value for the corresponding aphid clone contained within the arena.

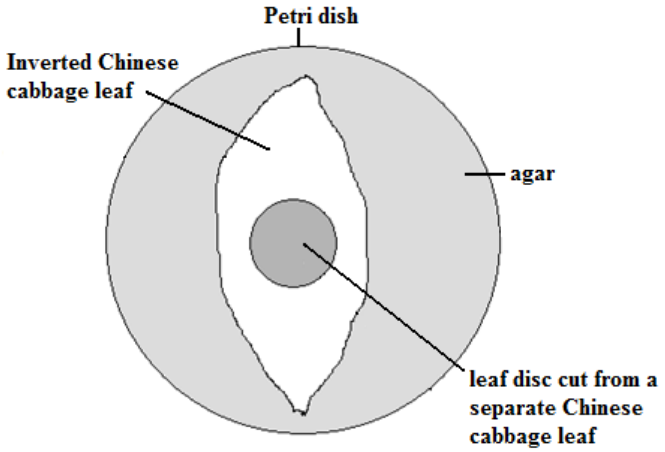


Figure 3.1 The setup of the arenas used to test aphid dispersal as viewed from above. Both the leaf and the leaf disc were inverted before being set into agar. Aphids were therefore exposed to the undersides of the leaves.

The camera was set up to record an image of all twelve dishes every five minutes for a total of 24 h. The position of the treatments under the camera was randomised for each of the ten repeats.

3.2.3 Statistical analysis

Image analysis was carried out using a macro written in the program ImageJ (Rasband, http://imagej.nih.gov/ij/) which was capable of calculating the change in pixel intensity between two successive images. Since leaf shrinkage over a 5 minute period was minimal, change in pixel intensity was considered equivalent to aphid movement between frames. The macro was capable of detecting the change in pixels within two different regions of interest in the dish, with the central disc forming one region and the leaf/agar/dish sides forming the other. The number of aphids located on the leaf, disc and agar were manually recorded for each treatment at hourly intervals from the still images. Dividing the change in number of pixels in a region of the dish by the number of aphids located there gave the rate of aphid movement. The proportion of aphids on the disc out of the number found on both the leaf and disc in each treatment, the proportion of aphids on the agar out of the total number of aphids in the dish, and the proportion of movement occurring on the disc out of the rate of movement across each entire arena was then calculated.

Differences in the proportion of aphid movement on the disc, the proportion of aphids located on the disc and the proportion of aphids located on the agar were compared between treatments by a series of t tests at each hourly time point. Proportions were arcsine transformed prior to analysis. All analyses were carried out using SAS software, version 9.2.

3.3 Results

3.3.1 Rate of movement

When under completely control treated conditions, the resistant clone 5191A exhibited significantly higher rates of movement over the entire arena than the susceptible clone US1L at 18 out of the 24 hourly time points (Figure 3.2). There was no evidence of any consistent pattern of difference in rate of movement between any other combination of clones in a completely control treated environment (Table 3.1). US1L exhibited a slight decrease in rate of movement in the completely TMX treated environment towards the end of the experiment (Figure 3.2). There was no evidence of any other consistent patterns of difference in rate of movement between clones in a completely TMX treated environment (Table 3.2).

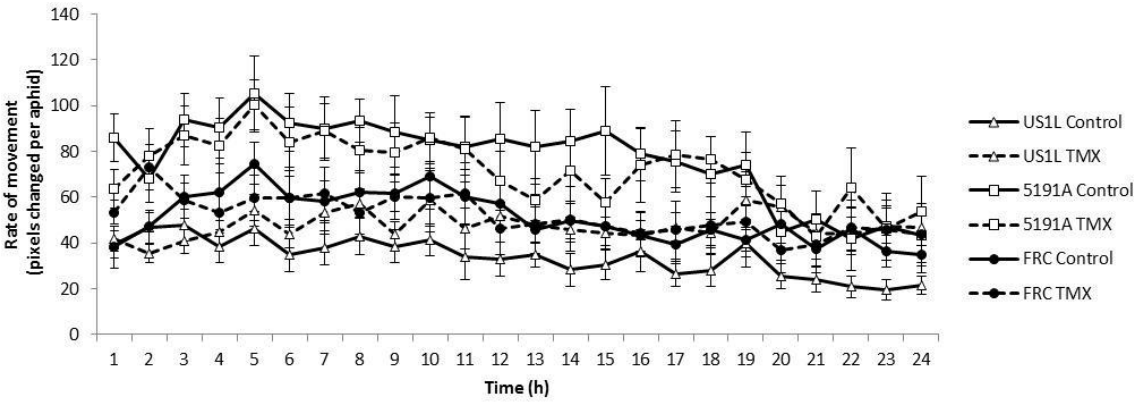


Figure 3.2 The rate of movement of the three *Myzus persicae* clones US1L, 5191A and FRC within arenas containing either completely control treated or completely thiamethoxam (TMX) treated plant material. Error bars represent ± standard error.

There was no consistent pattern of significant difference in the proportion of movement on the disc between the completely control treated and completely TMX treated environments for any clone (Figures 3.3a, b and c). There were also no consistent patterns of significant difference in the proportion of movement on the disc of each clone between the complete control experiment and the control treated disc, TMX treated leaf and the TMX treated disc, control treated leaf experiments (Tables 3.3, 3.4 and 3.5).

Table 3.1 Comparison of the total rate of movement of three *Myzus persicae* clones, US1L, 5191A and FRC, in pixels changed per aphid ± standard error, across an entire arena in which all plant tissue was control treated with Milli-Q water. Values marked with differing superscript letters indicate a significant difference between clones at that time point.

Time	US1L	5191A	FRC
1	39.29 ± 6.07^{a}	86.03 ± 10.38^{b}	38.15 ± 8.99^{a}
2	46.63 ± 6.73	68.34 ± 10.62	47.23 ± 7.24
3	47.67 ± 6.90^{a}	93.79 ± 11.56^{b}	60.36 ± 9.41^{a}
4	38.55 ± 7.29^{a}	90.24 ± 13.24^{b}	62.26 ± 12.34^{ab}
5	46.17 ± 7.51^{a}	105.18 ± 16.58^{b}	74.8 ± 9.33^{b}
6	34.67 ± 7.06^{a}	92.57 ± 12.67^{b}	59.60 ± 11.75^{ab}
7	37.84 ± 7.22^{a}	89.87 ± 13.82^{b}	57.95 ± 8.94^{ab}
8	42.99 ± 7.88^{a}	93.31 ± 9.37^{b}	62.26 ± 9.53^{ab}
9	38.41 ± 7.15^{a}	88.63 ± 15.75^{b}	61.57 ± 8.05^{ab}
10	41.41 ± 6.86^{a}	85.14 ± 9.71^{b}	68.86 ± 13.96^{ab}
11	33.91 ± 10.16^{a}	82.21 ± 12.53^{b}	60.07 ± 12.21^{ab}
12	32.90 ± 7.28^{a}	85.32 ± 16.16^{b}	57.41 ± 11.57^{ab}
13	34.67 ± 5.39^{ab}	81.78 ± 16.14^{a}	45.76 ± 11.02^{b}
14	28.26 ± 7.27^{a}	84.37 ± 14.17^{b}	49.98 ± 8.16^{ab}
15	30.36 ± 6.38^{a}	88.86 ± 19.48^{b}	47.12 ± 9.51^{ab}
16	36.53 ± 9.22^{a}	78.78 ± 11.48^{b}	43.47 ± 9.82^{ab}
17	26.52 ± 5.34^{a}	75.33 ± 13.36^{b}	39.17 ± 8.33^{ab}
18	27.97 ± 6.88^{a}	69.92 ± 16.54^{b}	46.00 ± 7.01^{ab}
19	39.77 ± 10.32^{a}	74.17 ± 14.19^{b}	41.52 ± 5.33^{ab}
20	25.42 ± 5.53^{a}	44.8 ± 12.26^{ab}	48.40 ± 8.42^{b}
21	23.92 ± 5.49^{a}	50.29 ± 12.34^{b}	37.36 ± 7.30^{ab}
22	20.77 ± 4.58	41.6 ± 13.69	46.07 ± 9.81
23	19.52 ± 4.66	47.09 ± 14.46	36.39 ± 6.96
24	21.56 ± 4.11	43.38 ± 11.89	34.83 ± 8.05

Table 3.2 Comparison of the total rate of movement of three *Myzus persicae* clones, US1L, 5191A and FRC, in pixels changed per aphid ± standard error, across an entire arena in which all plant tissue was treated with thiamethoxam. Values marked with differing superscript letters indicate a significant difference between clones at that time point.

Time	US1L	5191A	FRC
1	41.98 ± 5.38	63.79 ± 8.49	53.41 ± 5.08
2	35.41 ± 4.00	77.95 ± 11.76	73.16 ± 9.83
3	40.79 ± 5.46	86.97 ± 13.03	58.45 ± 7.80
4	44.75 ± 8.13	82.62 ± 11.93	53.29 ± 8.92
5	54.29 ± 6.95	100.25 ± 10.83	59.70 ± 10.00
6	43.98 ± 6.29	84.20 ± 15.26	59.89 ± 13.21
7	53.01 ± 8.97	88.98 ± 12.02	61.48 ± 11.68
8	57.38 ± 6.03	80.49 ± 9.68	53.39 ± 9.83
9	43.91 ± 5.19	79.67 ± 12.93	60.12 ± 10.03
10	58.70 ± 13.70	85.86 ± 11.18	59.50 ± 12.03
11	46.11 ± 8.89	81.00 ± 14.43	61.63 ± 13.45
12	51.87 ± 5.94	67.26 ± 12.81	46.16 ± 11.52
13	48.00 ± 7.60	58.92 ± 8.96	48.22 ± 12.77
14	45.85 ± 5.44	71.65 ± 14.26	50.50 ± 14.29
15	44.30 ± 7.04	57.55 ± 10.65	47.18 ± 8.48
16	43.10 ± 6.68	73.83 ± 16.00	44.11 ± 9.44
17	46.95 ± 6.17	78.76 ± 14.55	45.61 ± 12.68
18	44.09 ± 6.32	76.38 ± 10.24	47.76 ± 12.47
19	58.62 ± 12.66	67.85 ± 11.77	49.16 ± 15.44
20	55.61 ± 9.54^{a}	57.39 ± 11.48^{a}	36.66 ± 9.51^{b}
21	44.53 ± 7.16	42.73 ± 9.79	39.57 ± 12.83^{b}
22	43.98 ± 7.09^{a}	64.08 ± 17.32^{a}	46.72 ± 11.79^{b}
23	47.50 ± 7.54^{a}	46.23 ± 10.07^{a}	45.70 ± 13.12
24	47.00 ± 8.32	53.81 ± 15.18	43.69 ± 13.64

Table 3.3 Comparison of the proportion of movement on the disc ± standard error of the *Myzus persicae* clone US1L when placed into arenas containing one of three treatments, a control treated leaf and disc, a thiamethoxam (TMX) treated leaf and control treated disc, and a control treated leaf and TMX treated disc. Values marked with differing superscript letters indicate a significant difference between clones at that time point.

Time	Control disc, Control leaf	TMX leaf, Control disc	Control leaf, TMX disc
1	0.10 ± 0.06^{ab}	0.36 ± 0.08^{a}	0.17 ± 0.07^{b}
2	0.19 ± 0.06	0.29 ± 0.06	0.23 ± 0.07
3	0.22 ± 0.07	0.27 ± 0.09	0.29 ± 0.07
4	0.17 ± 0.04	0.17 ± 0.08	0.16 ± 0.09
5	0.08 ± 0.05	0.19 ± 0.07	0.22 ± 0.07
6	0.12 ± 0.05	0.21 ± 0.09	0.17 ± 0.07
7	0.16 ± 0.06	0.26 ± 0.08	0.23 ± 0.05
8	0.13 ± 0.05	0.34 ± 0.09	0.32 ± 0.10
9	0.11 ± 0.06	0.17 ± 0.03	0.24 ± 0.08
10	0.11 ± 0.06	0.21 ± 0.07	0.21 ± 0.07
11	0.03 ± 0.01^{a}	0.19 ± 0.09^{b}	0.31 ± 0.10^{ab}
12	0.17 ± 0.07	0.18 ± 0.06	0.09 ± 0.04
13	0.35 ± 0.11	0.13 ± 0.04	0.25 ± 0.06
14	0.20 ± 0.09	0.19 ± 0.06	0.32 ± 0.10
15	0.10 ± 0.05	0.19 ± 0.04	0.26 ± 0.10
16	0.14 ± 0.07	0.14 ± 0.06	0.08 ± 0.04
17	0.09 ± 0.03	0.07 ± 0.03	0.26 ± 0.09
18	0.07 ± 0.03^{ab}	0.19 ± 0.05^{a}	0.19 ± 0.08^{b}
19	0.06 ± 0.02	0.19 ± 0.07	0.14 ± 0.07
20	0.17 ± 0.05	0.21 ± 0.08	0.14 ± 0.05
21	0.16 ± 0.08	0.18 ± 0.05	0.09 ± 0.06
22	0.07 ± 0.02	0.23 ± 0.09	0.14 ± 0.09
23	0.08 ± 0.04	0.26 ± 0.08	0.19 ± 0.07
24	0.20 ± 0.05	0.28 ± 0.10	0.10 ± 0.05

Table 3.4 Comparison of the proportion of movement on the disc ± standard error of the *Myzus persicae* clone 5191A when placed into arenas containing one of three treatments, a control treated leaf and disc, a thiamethoxam (TMX) treated leaf and control treated disc, and a control treated leaf and TMX treated disc. Values marked with differing superscript letters indicate a significant difference between clones at that time point.

Time	Control disc, Control leaf	TMX leaf, Control disc	Control leaf, TMX disc
1	0.29 ± 0.09	0.37 ± 0.08	0.20 ± 0.06
2	0.17 ± 0.08^{a}	0.18 ± 0.09^{a}	0.41 ± 0.10^{b}
3	0.28 ± 0.11	0.27 ± 0.08	0.27 ± 0.11
4	0.11 ± 0.05^{a}	0.28 ± 0.09^{b}	0.41 ± 0.07^{b}
5	0.13 ± 0.05^{a}	0.33 ± 0.08^{b}	0.31 ± 0.09^{b}
6	0.19 ± 0.09	0.26 ± 0.08	0.24 ± 0.10
7	0.32 ± 0.09	0.23 ± 0.07	0.40 ± 0.11
8	0.21 ± 0.09^{a}	0.22 ± 0.08^{ab}	0.46 ± 0.11^{b}
9	0.27 ± 0.08	0.35 ± 0.09	0.23 ± 0.07
10	0.39 ± 0.10	0.20 ± 0.08	0.39 ± 0.09
11	0.36 ± 0.10	0.21 ± 0.10	0.39 ± 0.10
12	0.16 ± 0.06^{a}	0.32 ± 0.07^{b}	0.42 ± 0.13^{ab}
13	0.21 ± 0.09	0.28 ± 0.10	0.25 ± 0.08
14	0.12 ± 0.06^{a}	0.12 ± 0.07^{a}	0.48 ± 0.09^{b}
15	0.17 ± 0.10^{a}	0.36 ± 0.09^{b}	0.30 ± 0.10^{b}
16	0.19 ± 0.09	0.31 ± 0.07	0.19 ± 0.06
17	0.21 ± 0.08	0.34 ± 0.10	0.40 ± 0.11
18	0.20 ± 0.08^{a}	0.18 ± 0.05^{a}	0.42 ± 0.09^{b}
19	0.25 ± 0.10	0.19 ± 0.04	0.30 ± 0.09
20	0.07 ± 0.03^{a}	0.34 ± 0.11^{b}	0.30 ± 0.08^{b}
21	0.26 ± 0.03^{a}	0.24 ± 0.10^{ab}	0.44 ± 0.11^{b}
22	0.17 ± 0.12	0.17 ± 0.06	0.31 ± 0.12
23	0.27 ± 0.09	0.27 ± 0.11	0.33 ± 0.11
24	0.09 ± 0.05	0.24 ± 0.09	0.31 ± 0.12

Table 3.5 Comparison of the proportion of movement on the disc ± standard error of the *Myzus persicae* clone FRC when placed into arenas containing one of three treatments, a control treated leaf and disc, a thiamethoxam (TMX) treated leaf and control treated disc, and a control treated leaf and TMX treated disc. Values marked with differing superscript letters indicate a significant difference between clones at that time point.

Time	Control disc, Control leaf	TMX leaf, Control disc	Control leaf, TMX disc
1	0.31 ± 0.09	0.28 ± 0.09	0.17 ± 0.05
2	0.18 ± 0.08	0.15 ± 0.04	0.32 ± 0.09
3	0.09 ± 0.04	0.08 ± 0.03	0.27 ± 0.11
4	0.26 ± 0.06	0.07 ± 0.02	0.29 ± 0.09
5	0.27 ± 0.10	0.12 ± 0.05	0.17 ± 0.09
6	0.06 ± 0.03^{a}	0.15 ± 0.05^{ab}	0.25 ± 0.09^{b}
7	0.07 ± 0.03^{a}	0.07 ± 0.02^{ab}	0.28 ± 0.10^{b}
8	0.13 ± 0.05	0.13 ± 0.04	0.29 ± 0.12
9	0.11 ± 0.04	0.05 ± 0.02	0.25 ± 0.11
10	0.13 ± 0.05	0.13 ± 0.06	0.11 ± 0.07
11	0.19 ± 0.06	0.12 ± 0.04	0.18 ± 0.09
12	0.18 ± 0.07	0.10 ± 0.04	0.08 ± 0.04
13	0.11 ± 0.06	0.08 ± 0.04	0.08 ± 0.03
14	0.16 ± 0.09	0.06 ± 0.03	0.04 ± 0.02
15	0.05 ± 0.03	0.20 ± 0.10	0.13 ± 0.08
16	0.06 ± 0.05^{a}	0.18 ± 0.05^{b}	0.37 ± 0.12^{b}
17	0.20 ± 0.08	0.08 ± 0.03	0.11 ± 0.07
18	0.09 ± 0.05^{a}	0.10 ± 0.04^{b}	0.04 ± 0.03^{ab}
19	0.12 ± 0.05	0.13 ± 0.06	0.06 ± 0.05
20	0.18 ± 0.08	0.04 ± 0.01	0.10 ± 0.07
21	0.24 ± 0.09	0.03 ± 0.01	0.04 ± 0.04
22	0.23 ± 0.07	0.08 ± 0.02	0.18 ± 0.09
23	0.22 ± 0.10	0.07 ± 0.03	0.05 ± 0.02
24	0.13 ± 0.05	0.17 ± 0.08	0.08 ± 0.06

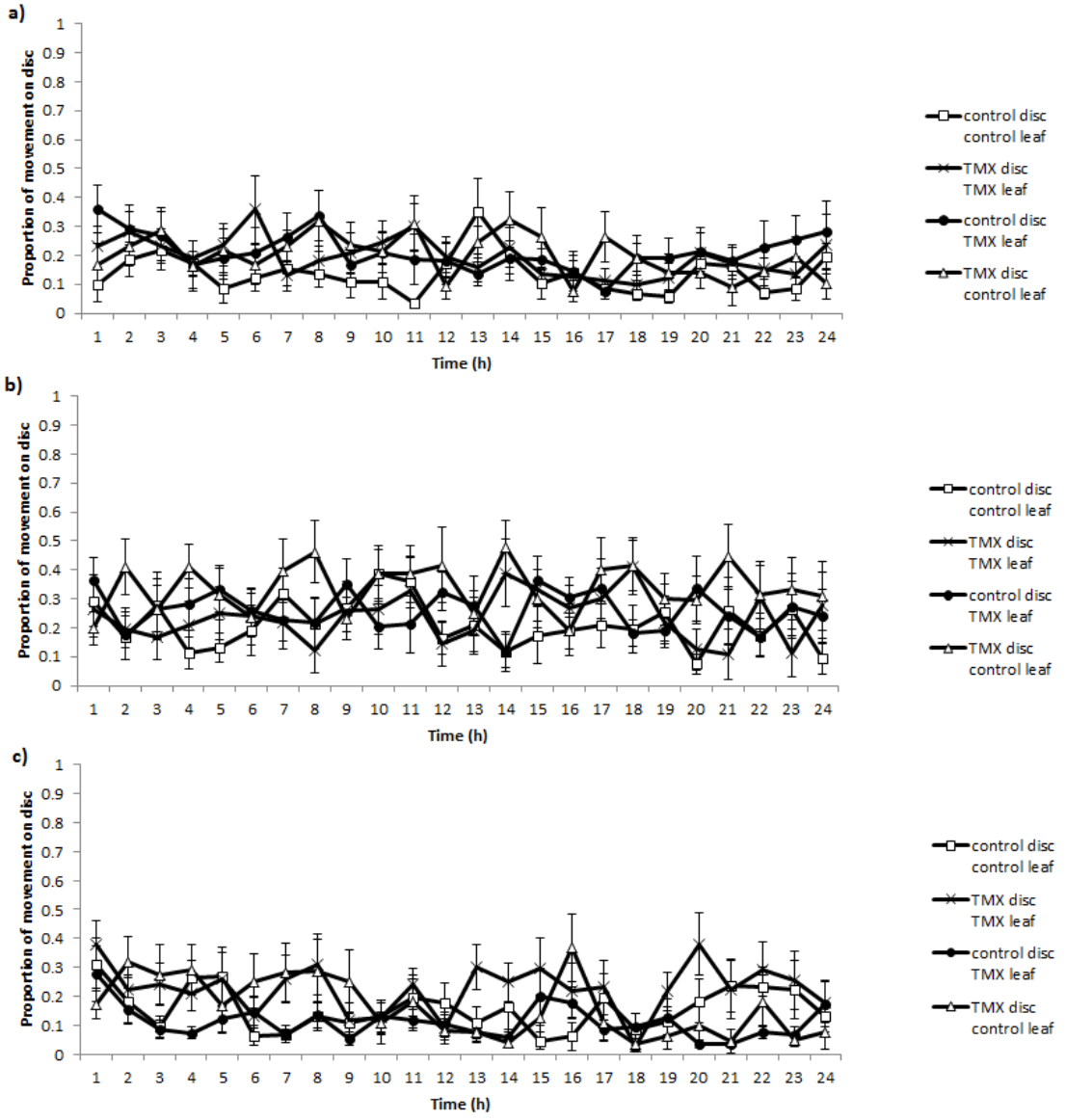


Figure 3.3 The proportion of movement on the disc of the three *Myzus persicae* clones a) US1L b) 5191A and c) FRC within arenas containing four possible treatments, control treated leaf and disc, thiamethoxam (TMX) treated leaf and disc, TMX treated leaf and control treated disc and control treated leaf and TMX treated disc. Error bars show ± the standard error.

3.3.2 Aphid location: leaf vs. disc

There were no consistent patterns of significant differences in the proportions of US1L on the disc between any treatments (Figure 3.4a) (Table 3.6). The proportions of 5191A upon the disc were significantly greater in the control disc TMX leaf treatment than the control leaf and disc treatment for 11 time points (Figure 3.4b), and in the control disc TMX leaf treatment rather than the TMX leaf and disc treatment for 19 time points, with greater proportions of 5191A upon the disc in the control disc TMX leaf treatment than all other possible treatments for the last 3 h of the experiment (Table 3.7). There were significantly greater proportions of FRC upon the disc in the control disk TMX leaf treatment than all three other treatments for a total of 18 time points, more than any other clone (Figure 3.4c). This significant difference between treatments began 4 h into the experiment and lasted until the end (Table 3.8).

Comparing the proportion of aphids on the disc between clones rather than between treatments revealed no consistent patterns of significant difference (Tables 3.9, 3.10, 3.11 and 3.12).

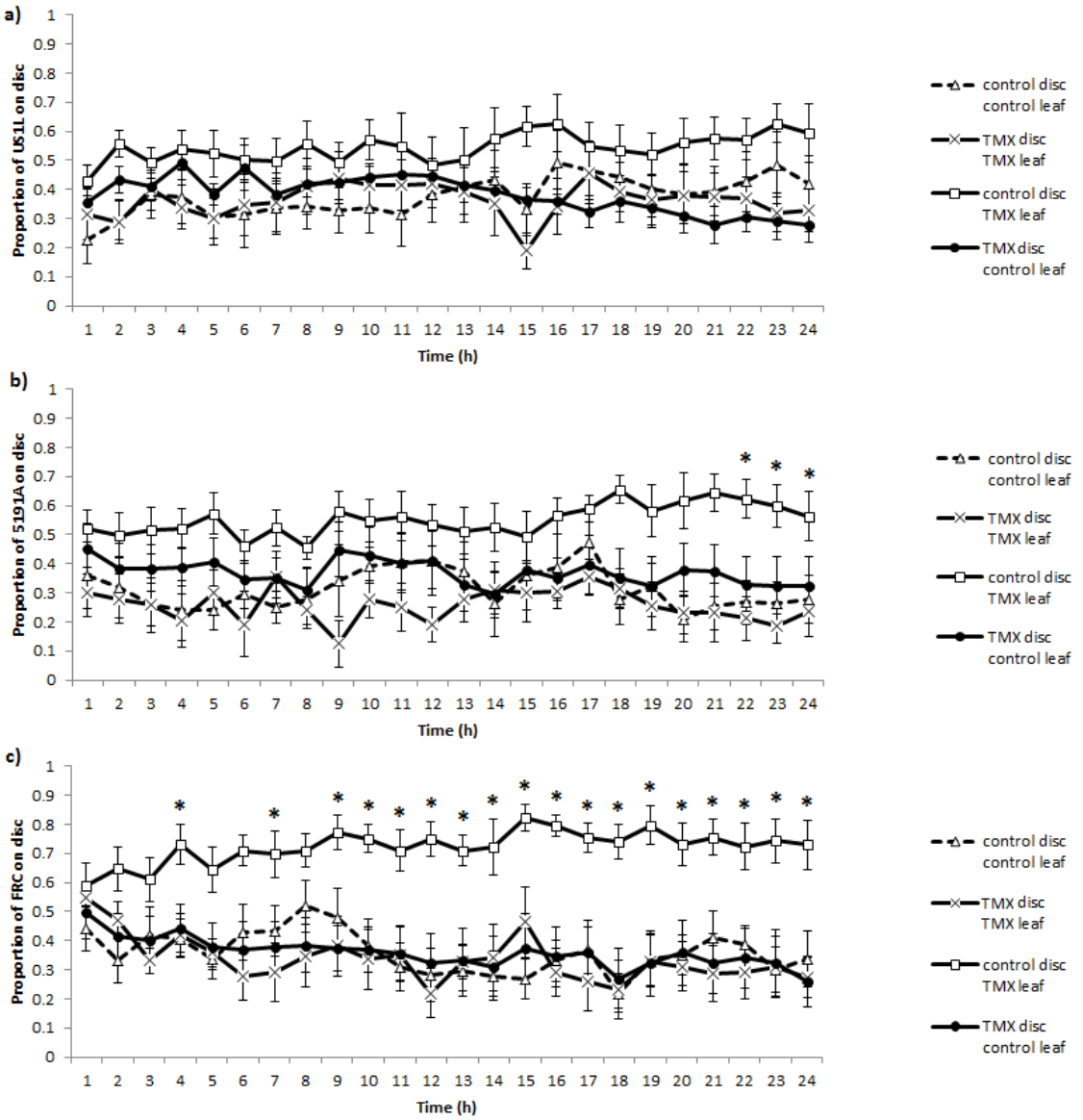


Figure 3.4 The proportion of a) US1L b) 5191A c) FRC on the disc out of the total number of aphids on leaf tissue in Petri dish arenas containing four different possible thiamethoxam (TMX) treatments, control treated leaf and disc, TMX treated leaf and disc, TMX treated leaf and control treated disc and control treated leaf and TMX treated disc. Asterisks indicate that the proportion of aphids on the disc in the control disc TMX leaf treatment was significantly greater than that for all other treatments at that time point. Error bars represent ± the standard error.

Table 3.6 The proportions of *Myzus persicae* of the clone US1L upon the disc out of the entire number of aphids upon both the leaf and disc, when placed in a Petri dish containing one of four possible treatments, a control leaf and disc treated with Milli-Q water, a control treated leaf and thiamethoxam (TMX) treated disc, a TMX treated leaf and a control treated disc, or a TMX treated leaf and disc. Error bars show ± the standard error. Values marked with differing superscript letters indicate a significant difference between treatments at that time point.

Time	Control leaf, Control disc	Control leaf, TMX disc	TMX leaf, Control disc	TMX leaf, TMX disc
1	0.23 ± 0.08^{a}	0.35 ± 0.05^{ab}	0.43 ± 0.05^{b}	0.32 ± 0.08^{ab}
2	0.29 ± 0.08^{a}	0.43 ± 0.05^{ab}	0.56 ± 0.05^{b}	0.29 ± 0.07^{ab}
3	0.38 ± 0.07	0.41 ± 0.05	0.49 ± 0.05	0.40 ± 0.08
4	0.37 ± 0.08^{ab}	0.49 ± 0.06^{ab}	0.54 ± 0.07^{a}	0.34 ± 0.09^{b}
5	0.30 ± 0.09^{a}	0.38 ± 0.04^{ab}	0.52 ± 0.08^{b}	0.30 ± 0.10^{ab}
6	0.31 ± 0.10	0.47 ± 0.08	0.50 ± 0.07	0.35 ± 0.11
7	0.34 ± 0.11	0.38 ± 0.05	0.50 ± 0.08	0.36 ± 0.09
8	0.34 ± 0.09	0.42 ± 0.05	0.56 ± 0.08	0.41 ± 0.08
9	0.33 ± 0.08	0.43 ± 0.06	0.49 ± 0.07	0.44 ± 0.08
10	0.34 ± 0.08^{a}	0.44 ± 0.04^{ab}	0.57 ± 0.07^{b}	0.41 ± 0.09^{ab}
11	0.32 ± 0.09	0.45 ± 0.05	0.55 ± 0.11	0.41 ± 0.11
12	0.38 ± 0.11	0.45 ± 0.03	0.48 ± 0.10	0.42 ± 0.10
13	0.41 ± 0.10	0.42 ± 0.06	0.50 ± 0.11	0.39 ± 0.10
14	0.44 ± 0.10	0.40 ± 0.05	0.58 ± 0.10	0.35 ± 0.10
15	0.33 ± 0.10^{a}	0.36 ± 0.04^{ab}	0.61 ± 0.07^{b}	0.19 ± 0.09^{a}
16	0.50 ± 0.09^{ab}	0.36 ± 0.04^{ab}	0.63 ± 0.10^{a}	0.34 ± 0.11^{b}
17	0.47 ± 0.11	0.33 ± 0.05	0.55 ± 0.08	0.46 ± 0.10
18	0.44 ± 0.09	0.36 ± 0.04	0.53 ± 0.09	0.39 ± 0.09
19	0.40 ± 0.10	0.34 ± 0.07	0.52 ± 0.07	0.36 ± 0.10
20	0.38 ± 0.10	0.31 ± 0.06	0.56 ± 0.08	0.38 ± 0.10
21	0.39 ± 0.08^{ab}	0.28 ± 0.07^{a}	0.57 ± 0.07^{b}	0.38 ± 0.08^{ab}
22	0.43 ± 0.10^{ab}	0.31 ± 0.05^{a}	0.57 ± 0.07^{b}	0.37 ± 0.10^{ab}
23	0.49 ± 0.11^{ab}	0.29 ± 0.06^{a}	0.63 ± 0.07^{b}	0.32 ± 0.11^{a}
24	0.42 ± 0.10^{ab}	0.28 ± 0.06^{a}	0.59 ± 0.10^{b}	0.33 ± 0.10^{a}

Table 3.7 The proportions of *Myzus persicae* of the clone 5191A upon the disc out of the entire number of aphids upon both the leaf and disc, when placed in a Petri dish containing one of four possible treatments, a control leaf and disc treated with Milli-Q water, a control treated leaf and thiamethoxam (TMX) treated disc, a TMX treated leaf and a control treated disc, or a TMX treated leaf and disc. Error bars show ± the standard error. Values marked with differing superscript letters indicate a significant difference between treatments at that time point. Time points where the control disc TMX leaf treatment was significantly different from all other treatments are highlighted in bold.

Time	Control leaf, Control disc	Control leaf, TMX disc	TMX leaf, Control disc	TMX leaf, TMX disc
1	0.36 ± 0.11^{ab}	0.45 ± 0.09^{ab}	0.52 ± 0.07^{a}	0.30 ± 0.08^{b}
2	0.32 ± 0.11^{ab}	0.38 ± 0.09^{ab}	0.50 ± 0.08^{a}	0.28 ± 0.08^{b}
3	0.26 ± 0.10^{ab}	0.38 ± 0.08^{b}	0.52 ± 0.08^{bc}	0.26 ± 0.09^{a}
4	0.24 ± 0.07^{a}	0.39 ± 0.06^{ab}	0.52 ± 0.07^{b}	0.21 ± 0.09^{a}
5	0.24 ± 0.10^{a}	0.41 ± 0.08^{b}	0.57 ± 0.07^{b}	0.30 ± 0.08^{a}
6	0.29 ± 0.07	0.35 ± 0.10	0.46 ± 0.05	0.19 ± 0.11
7	0.25 ± 0.11^{a}	0.35 ± 0.07^{ab}	0.52 ± 0.06^{b}	0.35 ± 0.09^{a}
8	0.28 ± 0.05^{ab}	0.31 ± 0.08^{a}	0.45 ± 0.04^{a}	0.24 ± 0.05^{b}
9	0.34 ± 0.10^{ab}	0.45 ± 0.09^{ab}	0.58 ± 0.07^{a}	0.13 ± 0.08^{b}
10	0.39 ± 0.12^{ab}	0.43 ± 0.10^{ab}	0.55 ± 0.07^{a}	0.28 ± 0.06^{b}
11	0.41 ± 0.06^{ab}	0.40 ± 0.10^{ab}	0.56 ± 0.08^{a}	0.25 ± 0.08^{b}
12	0.41 ± 0.10^{a}	0.41 ± 0.10^{a}	0.53 ± 0.07^{a}	0.19 ± 0.06^{b}
13	0.38 ± 0.12^{ab}	0.33 ± 0.09^{ab}	0.51 ± 0.08^{a}	0.28 ± 0.08^{b}
14	0.26 ± 0.10	0.30 ± 0.07	0.53 ± 0.08	0.31 ± 0.10
15	0.36 ± 0.11	0.38 ± 0.10	0.50 ± 0.08	0.30 ± 0.10
16	0.39 ± 0.12^{ab}	0.35 ± 0.07^{ab}	0.57 ± 0.06^{a}	0.31 ± 0.06^{b}
17	0.48 ± 0.12	0.40 ± 0.10	0.59 ± 0.05	0.36 ± 0.06
18	0.28 ± 0.12^{a}	0.35 ± 0.10^{ab}	0.65 ± 0.05^{b}	0.31 ± 0.07^{a}
19	0.32 ± 0.09^{a}	0.32 ± 0.10^{ab}	0.58 ± 0.09^{b}	0.26 ± 0.08^{a}
20	0.21 ± 0.08^{a}	0.38 ± 0.09^{ab}	0.62 ± 0.09^{b}	0.23 ± 0.07^{a}
21	0.25 ± 0.08^{a}	0.38 ± 0.09^{ab}	0.64 ± 0.06^{b}	0.23 ± 0.10^{a}
22	$\mathbf{0.27 \pm 0.08^{a}}$	$\mathbf{0.33 \pm 0.10^{a}}$	$\mathbf{0.62 \pm 0.07^{b}}$	$\mathbf{0.21 \pm 0.08^{a}}$
23	$\mathbf{0.27 \pm 0.08^{a}}$	$\mathbf{0.32 \pm 0.10^{a}}$	$\mathbf{0.60 \pm 0.07^{b}}$	$\mathbf{0.19 \pm 0.06^{a}}$
24	$\mathbf{0.28 \pm 0.08^{a}}$	$\mathbf{0.33 \pm 0.10^{a}}$	$\mathbf{0.56 \pm 0.08^{b}}$	$\mathbf{0.23 \pm 0.09^{a}}$

Table 3.8 The proportions of *Myzus persicae* of the clone FRC upon the disc out of the entire number of aphids upon both the leaf and disc, when placed in a Petri dish containing one of four possible treatments, a control leaf and disc treated with Milli-Q water, a control treated leaf and thiamethoxam (TMX) treated disc, a TMX treated leaf and a control treated disc, or a TMX treated leaf and disc. Error bars show ± the standard error. Values marked with differing superscript letters indicate a significant difference between treatments at that time point. Time points where the control disc TMX leaf treatment was significantly different from all other treatments are highlighted in bold.

Time	Control leaf, Control disc	Control leaf, TMX disc	TMX leaf, Control disc	TMX leaf, TMX disc
1	0.44 ± 0.08	0.50 ± 0.09	0.59 ± 0.08	0.55 ± 0.05
2	0.33 ± 0.08^{a}	0.41 ± 0.08^{ab}	0.65 ± 0.07^{b}	0.47 ± 0.06^{a}
3	0.42 ± 0.08	0.40 ± 0.08	0.61 ± 0.08	0.33 ± 0.04
4	**0.41 ± 0.10^{a}**	**0.44 ± 0.08^{a}**	**0.73 ± 0.07^{b}**	**0.42 ± 0.07^{a}**
5	0.34 ± 0.07^{a}	0.38 ± 0.08^{ab}	0.64 ± 0.08^{b}	0.36 ± 0.05^{a}
6	0.43 ± 0.07^{ab}	0.37 ± 0.10^{a}	0.71 ± 0.05^{b}	0.28 ± 0.08^{a}
7	**0.43 ± 0.10^{a}**	**0.38 ± 0.08^{a}**	**0.70 ± 0.08^{b}**	**0.29 ± 0.10^{a}**
8	0.52 ± 0.09^{ab}	0.39 ± 0.09^{a}	0.71 ± 0.06^{b}	0.35 ± 0.11^{a}
9	**0.48 ± 0.09^{a}**	**0.37 ± 0.08^{a}**	**0.77 ± 0.06^{b}**	**0.38 ± 0.10^{a}**
10	**0.38 ± 0.10^{a}**	**0.37 ± 0.08^{a}**	**0.75 ± 0.05^{b}**	**0.34 ± 0.10^{a}**
11	**0.31 ± 0.09^{a}**	**0.36 ± 0.09^{a}**	**0.71 ± 0.07^{b}**	**0.35 ± 0.09^{a}**
12	**0.28 ± 0.08^{a}**	**0.32 ± 0.10^{a}**	**0.75 ± 0.06^{b}**	**0.22 ± 0.08^{a}**
13	**0.30 ± 0.09^{a}**	**0.33 ± 0.10^{a}**	**0.71 ± 0.05^{b}**	**0.33 ± 0.06^{a}**
14	**0.28 ± 0.09^{a}**	**0.31 ± 0.10^{a}**	**0.72 ± 0.10^{b}**	**0.34 ± 0.12^{a}**
15	**0.27 ± 0.08^{a}**	**0.37 ± 0.11^{a}**	**0.82 ± 0.05^{b}**	**0.46 ± 0.12^{a}**
16	**0.33 ± 0.07^{a}**	**0.34 ± 0.10^{a}**	**0.80 ± 0.04^{b}**	**0.29 ± 0.08^{a}**
17	**0.37 ± 0.07^{a}**	**0.36 ± 0.11^{a}**	**0.75 ± 0.05^{b}**	**0.26 ± 0.10^{a}**
18	**0.22 ± 0.08^{a}**	**0.27 ± 0.10^{a}**	**0.74 ± 0.06^{b}**	**0.23 ± 0.10^{a}**
19	**0.34 ± 0.07^{a}**	**0.32 ± 0.11^{a}**	**0.80 ± 0.07^{b}**	**0.33 ± 0.09^{a}**
20	**0.35 ± 0.09^{a}**	**0.36 ± 0.11^{a}**	**0.73 ± 0.07^{b}**	**0.31 ± 0.09^{a}**
21	**0.41 ± 0.07^{a}**	**0.32 ± 0.10^{a}**	**0.75 ± 0.06^{b}**	**0.28 ± 0.10^{a}**
22	**0.39 ± 0.09^{a}**	**0.34 ± 0.12^{a}**	**0.72 ± 0.08^{b}**	**0.29 ± 0.09^{a}**
23	**0.30 ± 0.05^{a}**	**0.32 ± 0.11^{a}**	**0.74 ± 0.07^{b}**	**0.31 ± 0.10^{a}**
24	**0.34 ± 0.08^{a}**	**0.26 ± 0.09^{a}**	**0.73 ± 0.08^{b}**	**0.27 ± 0.07^{a}**

Table 3.9 Comparison of the proportions of *Myzus persicae* of three different clones upon the disc when placed in a Petri dish arena where all leaf tissue was control treated with Milli-Q water. p values highlighted in bold indicate a significant difference between the respective clones at that time point.

Time	5191A vs. FRC	5191A vs. US1L	FRC vs. US1L
1	0.364	0.187	**0.027**
2	0.783	0.878	0.903
3	0.100	0.165	0.794
4	**0.050**	0.089	0.786
5	0.224	0.408	0.696
6	0.198	0.770	0.318
7	0.119	0.441	0.426
8	**0.014**	0.438	0.088
9	0.588	0.433	0.159
10	0.924	0.553	0.618
11	0.368	0.388	0.969
12	0.228	0.715	0.398
13	0.488	0.703	0.284
14	0.816	0.211	0.308
15	0.509	0.581	0.911
16	0.706	0.382	0.212
17	0.377	0.682	0.634
18	0.665	0.219	0.088
19	0.854	0.772	0.913
20	0.264	0.319	0.898
21	0.235	0.503	0.578
22	0.197	0.291	0.807
23	0.875	0.188	0.232
24	0.728	0.665	0.927

Table 3.10 Comparison of the proportions of *Myzus persicae* of three different clones upon the disc when placed in a Petri dish arena where all leaf tissue was treated with thiamethoxam. p values highlighted in bold indicate a significant difference between the respective clones at that time point.

Time	5191A vs. FRC	5191A vs. US1L	FRC vs. US1L
1	0.748	0.400	0.246
2	0.774	0.776	0.998
3	0.776	0.861	0.913
4	0.583	0.459	0.848
5	0.855	0.748	0.889
6	0.814	0.273	0.388
7	0.751	0.810	0.938
8	0.497	0.385	0.848
9	0.574	0.870	0.690
10	0.510	0.958	0.477
11	0.748	0.772	0.542
12	0.422	0.803	0.293
13	0.986	0.459	0.448
14	0.834	0.582	0.447
15	0.818	0.783	0.964
16	0.794	0.981	0.776
17	0.566	0.436	0.837
18	0.265	0.957	0.288
19	0.649	0.853	0.787
20	0.639	0.507	0.845
21	0.495	0.395	0.867
22	0.912	0.757	0.842
23	0.722	0.694	0.970
24	0.346	0.636	0.638

Table 3.11 Comparison of the proportions of *Myzus persicae* of three different clones upon the disc when placed in a Petri dish arena where the leaf was treated with thiamethoxam and the disc control treated with Milli-Q water. p-values highlighted in bold indicate a significant difference between the respective clones at that time point.

Time	5191A vs. FRC	5191A vs. US1L	FRC vs. US1L
1	0.484	0.508	0.175
2	0.144	0.551	0.384
3	0.307	0.982	0.296
4	0.070	0.925	0.086
5	0.684	0.683	0.415
6	0.126	0.701	0.249
7	0.152	0.978	0.144
8	0.068	0.404	0.316
9	0.156	0.474	**0.034**
10	0.133	0.986	0.129
11	0.262	0.703	0.134
12	0.083	0.738	**0.040**
13	0.193	0.791	0.118
14	0.123	0.510	0.374
15	**0.016**	0.392	0.113
16	0.115	0.790	0.188
17	0.147	0.880	0.110
18	0.472	0.364	0.105
19	0.096	0.471	**0.018**
20	0.365	0.597	0.153
21	0.359	0.640	0.167
22	0.395	0.705	0.220
23	0.220	0.881	0.282
24	0.210	0.913	0.173

Table 3.12 Comparison of the proportions of *Myzus persicae* of three different clones upon the disc when placed in a Petri dish arena where the leaf was control treated with Milli-Q water and the disc treated with thiamethoxam (TMX). p-values highlighted in bold indicate a significant difference between the respective clones at that time point.

Time	5191A vs. FRC	5191A vs. US1L	FRC vs. US1L
1	0.053	0.847	0.081
2	0.362	0.116	0.503
3	**0.010**	**0.047**	0.535
4	0.317	0.857	0.411
5	0.185	0.068	0.610
6	0.624	0.777	0.440
7	0.164	0.054	0.586
8	**0.016**	**0.003**	0.578
9	0.504	0.237	0.604
10	0.329	0.559	0.120
11	0.277	0.150	0.720
12	0.969	**0.047**	0.051
13	0.440	0.377	0.910
14	0.671	0.714	0.953
15	0.092	0.405	**0.013**
16	0.937	0.933	0.995
17	0.318	0.409	0.070
18	0.498	0.523	0.189
19	0.357	0.396	0.942
20	0.260	0.154	0.763
21	0.400	0.116	0.461
22	0.300	0.120	0.598
23	0.274	0.187	0.819
24	0.569	0.344	0.705

3.3.3 Aphid location: leaf tissue vs. agar

The proportions of 5191A on the agar were significantly greater than those of both FRC and US1L for 22 time points in the control disc and leaf treatment (Figure 3.5a) and the last seven time points of the TMX leaf control disc treatment (Figure 3.5b). There were no other consistent patterns of significant difference in the proportions of aphids on the agar between clones for any other treatments (Figures 3.5c & 3.5d) (Tables 3.13, 3.14, 3.15 & 3.16).

3.3.4 Aphid mortality and reproduction

No mortality of aphids occurred during the experiment, although there was a low level of reproduction. Since newborn nymphs move very little initially (personal observation) this was not judged to significantly affect the change in pixels between images as detected by the software.

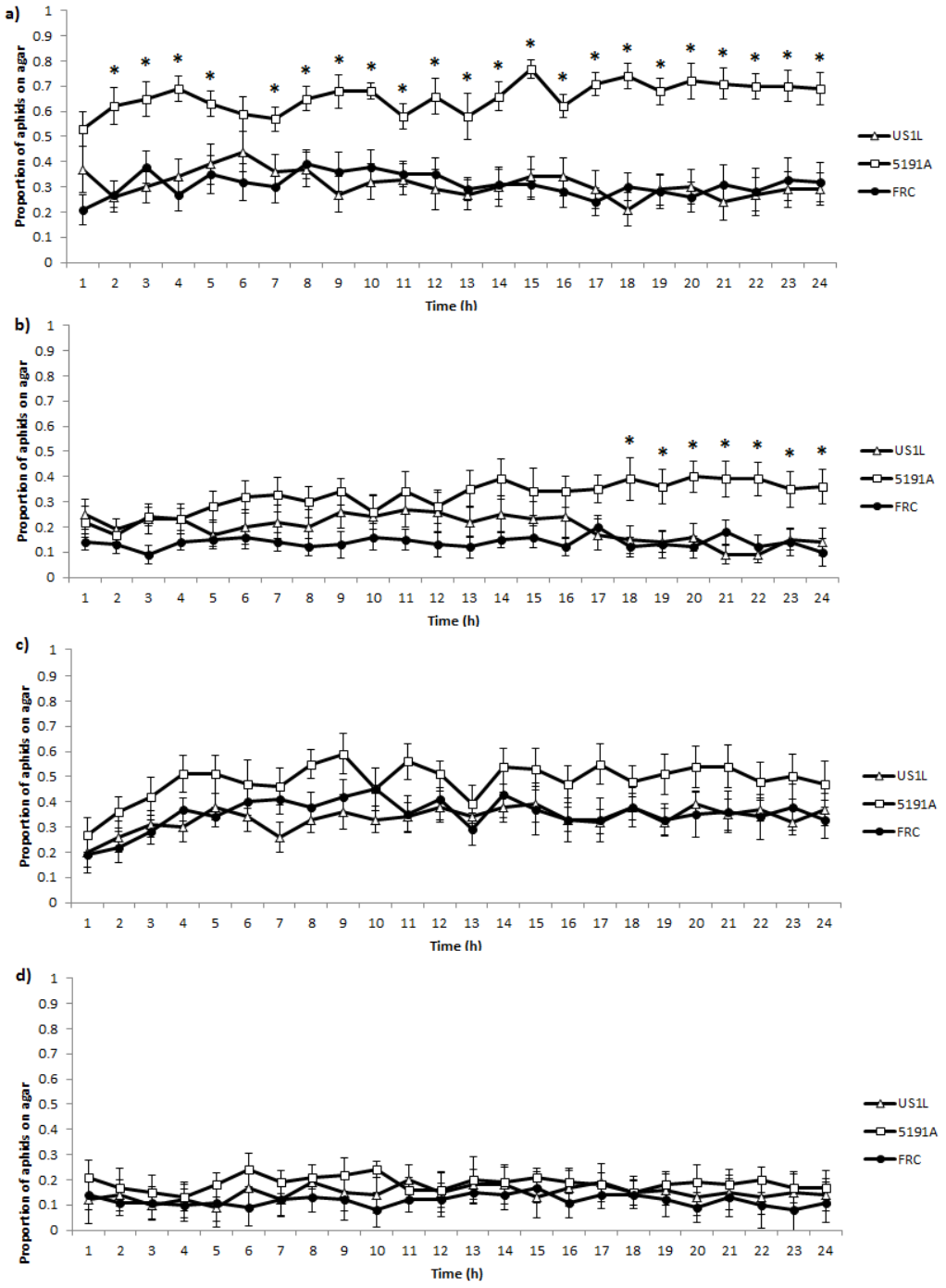


Figure 3.5 The proportion of three *Myzus persicae* clones on the agar in a Petri dish arena where a) all plant tissue is control treated with Milli-Q water b) the disc is control treated and the leaf treated with thiamethoxam (TMX) c) all plant tissue is TMX treated d) The disc is treated with TMX and the leaf control treated. Asterisks indicate that the proportion of 5191A on the agar was significantly greater than all other clones at that time point. Error bars represent ± the standard error.

Table 3.13 The proportions of three *Myzus persicae* clones (US1L, 5191A and FRC) on the agar and dish sides, when placed in a Petri dish arena in which all plant tissue was control treated with Milli-Q water. Values marked with differing superscript letters indicate a significant difference between clones at that time point. Error bars represent ± the standard error.

Time	US1L	5191A	FRC
1	0.37 ± 0.09^{a}	0.53 ± 0.07^{b}	0.21 ± 0.06^{a}
2	0.26 ± 0.06^{ab}	0.62 ± 0.07^{a}	0.27 ± 0.05^{b}
3	0.30 ± 0.06^{a}	0.65 ± 0.07^{b}	0.38 ± 0.06^{a}
4	0.34 ± 0.07^{a}	0.69 ± 0.05^{b}	0.27 ± 0.06^{a}
5	0.39 ± 0.08^{a}	0.63 ± 0.05^{b}	0.35 ± 0.07^{a}
6	0.44 ± 0.08^{ab}	0.59 ± 0.07^{a}	0.32 ± 0.07^{b}
7	0.36 ± 0.07^{a}	0.57 ± 0.05^{b}	0.30 ± 0.06^{a}
8	0.37 ± 0.07^{a}	0.65 ± 0.05^{b}	0.39 ± 0.06^{a}
9	0.27 ± 0.07^{a}	0.68 ± 0.07^{b}	0.36 ± 0.08^{a}
10	0.32 ± 0.07^{a}	0.68 ± 0.03^{b}	0.38 ± 0.07^{a}
11	0.33 ± 0.06^{a}	0.58 ± 0.05^{b}	0.35 ± 0.05^{a}
12	0.29 ± 0.08^{a}	0.66 ± 0.07^{b}	0.35 ± 0.07^{a}
13	0.27 ± 0.06^{a}	0.58 ± 0.09^{b}	0.29 ± 0.05^{a}
14	0.30 ± 0.08^{a}	0.66 ± 0.06^{b}	0.31 ± 0.06^{a}
15	0.34 ± 0.08^{a}	0.77 ± 0.04^{b}	0.31 ± 0.06^{a}
16	0.34 ± 0.08^{a}	0.62 ± 0.05^{b}	0.28 ± 0.06^{a}
17	0.29 ± 0.08^{a}	0.71 ± 0.05^{b}	0.24 ± 0.05^{a}
18	0.21 ± 0.07^{a}	0.74 ± 0.05^{b}	0.30 ± 0.06^{a}
19	0.29 ± 0.06^{a}	0.68 ± 0.05^{b}	0.28 ± 0.06^{a}
20	0.30 ± 0.07^{a}	0.72 ± 0.07^{b}	0.26 ± 0.06^{a}
21	0.24 ± 0.07^{a}	0.71 ± 0.06^{b}	0.31 ± 0.08^{a}
22	0.27 ± 0.07^{a}	0.70 ± 0.05^{b}	0.28 ± 0.09^{a}
23	0.29 ± 0.07^{a}	0.70 ± 0.06^{b}	0.33 ± 0.09^{a}
24	0.29 ± 0.06^{a}	0.69 ± 0.07^{b}	0.32 ± 0.09^{a}

Table 3.14 The proportions of three *Myzus*± *persicae* clones (US1L, 5191A and FRC) on the agar and dish sides, when placed in a Petri dish arena in which the leaf was treated with thiamethoxam and the disc with Milli-Q water. Values marked with differing superscript letters indicate a significant difference between clones at that time point. Error bars represent ± the standard error.

Time	US1L	5191A	FRC
1	0.25 ± 0.06	0.22 ± 0.06	0.14 ± 0.03
2	0.19 ± 0.04	0.17 ± 0.03	0.13 ± 0.03
3	0.23 ± 0.06^{ab}	0.24 ± 0.04^{a}	0.09 ± 0.03^{b}
4	0.23 ± 0.06	0.23 ± 0.04	0.14 ± 0.03
5	0.17 ± 0.06	0.28 ± 0.06	0.15 ± 0.03
6	0.20 ± 0.07	0.32 ± 0.06	0.16 ± 0.05
7	0.22 ± 0.07^{ab}	0.33 ± 0.07^{a}	0.14 ± 0.04^{b}
8	0.20 ± 0.07^{ab}	0.30 ± 0.06^{a}	0.12 ± 0.04^{b}
9	0.26 ± 0.07^{ab}	0.34 ± 0.05^{a}	0.13 ± 0.05^{b}
10	0.24 ± 0.08	0.26 ± 0.07	0.16 ± 0.05
11	0.27 ± 0.07	0.34 ± 0.08	0.15 ± 0.04
12	0.26 ± 0.08	0.28 ± 0.07	0.13 ± 0.05
13	0.22 ± 0.06^{ab}	0.35 ± 0.07^{a}	0.12 ± 0.04^{b}
14	0.25 ± 0.07^{ab}	0.39 ± 0.08^{a}	0.15 ± 0.03^{b}
15	0.23 ± 0.07	0.34 ± 0.09	0.16 ± 0.04
16	0.24 ± 0.08^{ab}	0.34 ± 0.06^{a}	0.12 ± 0.03^{b}
17	0.17 ± 0.06^{a}	0.35 ± 0.06^{b}	0.20 ± 0.05^{ab}
18	0.15 ± 0.06^{a}	0.39 ± 0.09^{b}	0.12 ± 0.04^{a}
19	0.14 ± 0.04^{a}	0.36 ± 0.07^{b}	0.13 ± 0.06^{a}
20	0.16 ± 0.05^{a}	0.40 ± 0.06^{b}	0.12 ± 0.04^{a}
21	0.09 ± 0.04^{a}	0.39 ± 0.07^{b}	0.18 ± 0.05^{a}
22	0.09 ± 0.03^{a}	0.39 ± 0.07^{b}	0.12 ± 0.05^{a}
23	0.15 ± 0.04^{a}	0.35 ± 0.07^{b}	0.14 ± 0.05^{a}
24	0.14 ± 0.06^{a}	0.36 ± 0.07^{b}	0.10 ± 0.05^{a}

Table 3.15 The proportions of three *Myzus persicae* clones (US1L, 5191A and FRC) on the agar and dish sides, when placed in a Petri dish arena in which all plant tissue was treated with a sublethal dose of thiamethoxam. Values marked with differing superscript letters indicate a significant difference between clones at that time point. Error bars represent ± the standard error.

Time	US1L	5191A	FRC
1	0.20 ± 0.06	0.27 ± 0.07	0.19 ± 0.07
2	0.26 ± 0.06	0.36 ± 0.06	0.22 ± 0.06
3	0.31 ± 0.05	0.42 ± 0.08	0.28 ± 0.05
4	0.30 ± 0.06	0.51 ± 0.07	0.37 ± 0.05
5	0.38 ± 0.05	0.51 ± 0.08	0.34 ± 0.04
6	0.34 ± 0.06	0.47 ± 0.10	0.40 ± 0.06
7	0.26 ± 0.06^{a}	0.46 ± 0.07^{b}	0.41 ± 0.06^{ab}
8	0.33 ± 0.05^{a}	0.55 ± 0.06^{b}	0.38 ± 0.06^{ab}
9	0.36 ± 0.07	0.59 ± 0.08	0.42 ± 0.07
10	0.33 ± 0.05	0.45 ± 0.09	0.45 ± 0.08
11	0.34 ± 0.06	0.56 ± 0.07	0.35 ± 0.07
12	0.38 ± 0.06	0.51 ± 0.05	0.41 ± 0.08
13	0.34 ± 0.05	0.39 ± 0.08	0.29 ± 0.06
14	0.38 ± 0.06	0.54 ± 0.07	0.43 ± 0.09
15	0.39 ± 0.07	0.53 ± 0.08	0.37 ± 0.10
16	0.33 ± 0.05	0.47 ± 0.07	0.33 ± 0.09
17	0.32 ± 0.05^{ab}	0.55 ± 0.08^{a}	0.33 ± 0.09^{b}
18	0.38 ± 0.05	0.48 ± 0.06	0.38 ± 0.08
19	0.32 ± 0.06	0.51 ± 0.08	0.33 ± 0.06
20	0.39 ± 0.05	0.54 ± 0.08	0.35 ± 0.09
21	0.35 ± 0.06	0.54 ± 0.09	0.36 ± 0.08
22	0.37 ± 0.05	0.48 ± 0.08	0.34 ± 0.09
23	0.32 ± 0.05	0.50 ± 0.09	0.38 ± 0.09
24	0.37 ± 0.06	0.47 ± 0.09	0.33 ± 0.08

Table 3.16 The proportions of three *Myzus persicae* clones (US1L, 5191A and FRC) on the agar and dish sides, when placed in a Petri dish arena in which the leaf was treated with Milli-Q water and the disc with thiamethoxam. Values marked with differing superscript letters indicate a significant difference between clones at that time point. Error bars represent ± the standard error.

Time	US1L	5191A	FRC
1	0.12 ± 0.09	0.21 ± 0.07	0.14 ± 0.06
2	0.14 ± 0.06	0.17 ± 0.07	0.11 ± 0.05
3	0.10 ± 0.06	0.15 ± 0.07	0.11 ± 0.06
4	0.12 ± 0.07	0.13 ± 0.05	0.10 ± 0.06
5	0.09 ± 0.08	0.18 ± 0.05	0.11 ± 0.07
6	0.17 ± 0.08^{ab}	0.24 ± 0.07^{a}	0.09 ± 0.07^{b}
7	0.12 ± 0.07	0.19 ± 0.05	0.12 ± 0.06
8	0.19 ± 0.07	0.21 ± 0.05	0.13 ± 0.06
9	0.15 ± 0.07	0.22 ± 0.07	0.12 ± 0.08
10	0.14 ± 0.07	0.24 ± 0.03	0.08 ± 0.07
11	0.20 ± 0.06	0.16 ± 0.05	0.12 ± 0.05
12	0.15 ± 0.08	0.16 ± 0.07	0.12 ± 0.07
13	0.18 ± 0.06	0.20 ± 0.09	0.15 ± 0.05
14	0.18 ± 0.08	0.19 ± 0.06	0.14 ± 0.06
15	0.13 ± 0.08	0.21 ± 0.04	0.17 ± 0.06
16	0.17 ± 0.08	0.19 ± 0.05	0.11 ± 0.06
17	0.19 ± 0.07	0.18 ± 0.05	0.14 ± 0.05
18	0.15 ± 0.07	0.15 ± 0.05	0.14 ± 0.06
19	0.16 ± 0.06	0.18 ± 0.05	0.12 ± 0.06
20	0.13 ± 0.07	0.19 ± 0.07	0.09 ± 0.06
21	0.15 ± 0.07	0.18 ± 0.06	0.13 ± 0.08
22	0.13 ± 0.07	0.20 ± 0.05	0.10 ± 0.09
23	0.15 ± 0.07	0.17 ± 0.06	0.08 ± 0.09
24	0.14 ± 0.06	0.17 ± 0.07	0.11 ± 0.08

3.4 Discussion

Two different dispersal behaviours were noted in 5191A and FRC, the two resistant clones in this study. While 5191A showed an increased tendency to disperse away from all plant tissue regardless of insecticide treatment, the FRC clone exhibited an increased ability to locate itself to areas of untreated plant tissue in an environment consisting of mixed insecticide treatment. While these changes in dispersal behaviour should increase the fitness of both clones under TMX treated conditions and so contribute significantly to their neonicotinoid resistance, the enhanced ability of the FRC clone to locate untreated plant tissue and therefore target its dispersal may be more relevant given the high level of resistance already exhibited by this strain (Bass *et al.,* 2011)

The dispersal behaviour of the FRC clone has significant implications for agricultural practices, particularly when spraying infested crops with neonicotinoids, as it is likely that the FRC, if present, will relocate to any untreated areas within a field. This finding therefore emphasises the importance of ensuring that an insecticide is applied evenly throughout a crop. The modified dispersal behaviour exhibited by the FRC clone, however, may be potentially useful in the case of an unsprayed trap crop (Shelton & Badenes-Peres, 2006) as there is potential for the aphid to move into this untreated region for destruction by other means. In addition within-field dispersal by apterae exposes them to additional mortality (Sunderland *et al.,* 1986; Mann *et al.,* 1995).

Alterations in dispersal behaviour could lead to changes in the transmission of aphid-vectored plant viruses. Transmission of plant viruses is the major cause of yield loss and economic damage to crop systems by aphids (Dedryver *et al.,* 2010). *Myzus persicae* is a known vector of at least 100 plant viruses. Previous studies have often shown the application of insecticides to be beneficial in reducing the spread of plant viruses (Bedford *et al.,* 1998; Groves *et al.,* 2001; Mowry & Ophus, 2002;, Castle *et al.,* 2009; Margaritopoulos *et al.,* 2010). From this study it appears that *M. persicae* disperses at the same rate regardless of the presence or absence of neonicotinoid treatment, hence in theory the application of neonicotinoids should exert either a beneficial effect due to increased vector mortality or else no effect on the rates at which plant viruses spread within a crop, supporting such findings.

Of more relevance from a control perspective, the FRC may be more difficult to remove from the field than other *M. persicae* clones due to this combination of behavioural, metabolic and target site resistance. Treating plants with neonicotinoids would probably cause the FRC to

relocate to any untreated or partially sprayed plants. In the case of a persistent virus where feeding must occur for several hours in order for the virus to successfully infect a new host plant, (Hogenhout *et al.,* 2008) this could limit or slow the spread of plant viruses to untreated plants, with treated plants gaining fuller protection from plant viruses. A plant virus however, may be more difficult to remove from a crop when resistant aphids are present due to the persistence of the vector.

Different aphid species have been shown to disperse at different rates (Boiteau *et al.,* 1997; Alyokhin & Sewell, 2003) as do alate individuals of the same clone that differ in ovariole number (Shaw, 1970; Walters & Dixon, 1983). This is the first study however to show differences in dispersal rates between different neonicotinoid resistant aphid clones of the same species with regard to apterous individuals. The greater rate of movement of 5191A compared with the other clones, even under completely control-treated conditions, suggests that different clones may disperse across a field at different rates. It is therefore also possible that the rate of viral spread within a field may vary according to the different aphid clones present. In particular, the presence of 5191A and potentially other resistant clones may lead to the spread of crop disease at a greater rate, although the lack of an enhanced dispersal rate exhibited by the resistant FRC clone should be noted. The effect of the enhanced dispersal of resistant *M. persicae* on the spread of plant viruses however, remains theoretical and further work is required to both determine whether this enhanced dispersal occurs under field conditions, and to link such changes in behaviour to any possible effects on vector ability.

TMX has been shown to induce up-regulation of salicylic acid-associated plant defence and stress responses in treated plants (Ford *et al.,* 2010). *Myzus persicae* appears to use phloem constituents in host discrimination (Margaritopoulos *et al.,* 2005) and it is possible that when feeding on treated plant tissue FRC and 5191A detect such stress chemical markers, leading to the induction of the alternative dispersal behaviours shown in this study. *Hylobius abietis* is also capable of detecting and avoiding lambda-cyhalothrin, although the method of detection remains unclear (Rose *et al.,* 2005). Alternatively, the resistant aphids may be responding to olfactory signals produced by the host plant or insecticide. Aphids are capable of detecting plant volatiles and appear to use them when locating host plants (Bruce & Pickett, 2011). It is the blend of different host volatiles, rather than individual volatiles, which appear attractive to aphids (Webster *et al.,* 2010) and certain plant volatiles offered individually may even be repellent. It is therefore possible that treatment of plants with TMX masks or changes such mixes of host plant volatiles and makes them less attractive to

resistant *M. persicae*, while the susceptible clone US1L makes no such distinction. Different lines of the cotton aphid *A. gossypii* show different behavioural responses to the volatiles of the same host plants (Najar-Rodríguez *et al.*, 2009); hence it is possible for different clones of *M. persicae* to also show different behavioural responses to the same volatiles. Further olfactometry work would be needed to determine whether this is truly the case.

Since resistance to one neonicotinoid compound usually confers resistance to all other compounds within that class (Elbert & Nauen, 2000; Foster *et al.*, 2003, 2008) it is not unreasonable to suppose that the FRC and 5191A *M. persicae* clones will exhibit similar behavioural differences in response to all neonicotinoid compounds. Indeed, the resistance factor for the FRC clone is substantially higher for IMD than TMX (Bass *et al.*, 2011). It should be noted that in this study the FRC aphids were treated with a dose greater than the conventional field dose. TMX is not generally applied to Chinese cabbage, but the maximum recommended application rate on potato is 400 mg L^{-1}/ha (Syngenta Crop Protection, 2008). Further study is therefore needed to determine whether the FRC would behave in a similar way at conventional field rates. US1L and 5191A would be unable to survive such rates, and so would present no problem under current control regimes. In addition, the dispersal behaviour of the FRC clone should also be investigated at 0.1 ppm and 0.9 ppm, to verify that any changes in behaviour are due to the resistance of the strain and not due to the increased concentration of TMX applied.

In addition, since formulated TMX was used in this study, it is possible that the altered dispersal behaviour exhibited by the FRC clone is being performed in response to an adjuvant or another ingredient of the formulation, rather than the active compound TMX. The behaviour of the FRC clone on plants treated with these adjuvants and ingredients in the absence of TMX should also be investigated.

It is difficult to accurately predict the behaviour of insects in the field from laboratory results. Aphids in the field are exposed to a range of temperature and light conditions, both of which have been shown to affect host-finding ability of the potato aphid *Macrosiphum euphorbiae* (Thomas) (Narayandas & Alyokhin, 2006) as well as the effects of weather, predation and physical barriers to movement between host plants such as soil (Bailey *et al.*, 1995; Mann *et al.*, 1995). While it has been shown that *M. euphorbiae* will travel up to 1.8 m over bare soil to successfully locate host plants (Boiteau, 1997) this experiment is not comparable with such conditions.

Aphids have been shown to disperse upwards from the older leaves at the bottom of the plant to the younger, newer leaves at the top (Harrington & Taylor, 1990). It is suggested that this is a mechanism for finding suitable feeding spots on a host as the population rapidly grows. While the leaf tissue in each treatment of this study always originated from two separate plants, it is possible that the dispersal behaviour exhibited by these clones is representative of intra-plant movement on the same host rather than inter-plant dispersal throughout a field. Understanding the differences in intra and inter-plant dispersal between these clones would require further work in suitable field conditions.

Finally, much dispersal of aphid populations is mediated by winged (alate) individuals (Boiteau & Parry, 1985). There is however, evidence that dispersal of non-winged apterae can also cause substantially large infestations of plants (Hodgson, 1991; Diaz *et al.,* 2012). While revealing several interesting implications, further work on alates is needed before a full understanding of the dispersal differences between these clones can be obtained. Nevertheless, this study reveals significant differences in the dispersal behaviour of *M. persicae* clones of different neonicotinoid resistance levels, and it would not be unreasonable to suppose that similar behavioural differences might also occur between these clones under field conditions. The lack of literature on the dispersal behaviour of differentially resistant aphid clones makes this an especially interesting study. This may be the first step towards discovering the presence of such behavioural differences in the field between neonicotinoid resistant *M. persicae*.

In summary, this is the first characterisation of a behavioural resistance in aphids that actively disperse away from neonicotinoid treated leaves to untreated plant material. Together with the intrinsic biochemical and target site resistance it represents new challenges in the field for aphid control and the viruses they spread.

Overall I accept hypothesis two, that "the FRC clone will exhibit differences in dispersal behaviour compared to the *M. persicae* clones US1L and 5191A when exposed to a sublethal dose of TMX. This altered dispersal behaviour will contribute to the greater neonicotinoid resistance of the FRC clone". This hypothesis is supported by the enhanced ability to relocate to control treated tissue in an environment of mixed TMX treatment exhibited by the FRC clone during this study, and it is potentially possible that such behaviour could contribute to the neonicotinoid resistance of this clone.

Chapter 4: The feeding behaviours of neonicotinoid-resistant and susceptible *Myzus persicae*

4.1 Introduction

Changes in feeding behaviour have been shown to contribute to insecticide resistance in a range of insects (Hoy & Head, 1995; Nauen & Elbert, 1997; Guedes *et al.*, 2009). The feeding behaviour of aphids is complex and has been shown to vary with a wide range of factors including aphid species, insecticide resistance status, host plant species and variety, application of chemical control and environmental stress (Montllor & Tjallingii, 1989; Cole, 1994; Paul *et al.*, 1996; Sauge *et al*, 1998a; Prado & Tjallingii, 1999; Daniels *et al.*, 2009; Cui *et al.*, 2010; Boquel *et al.*, 2011; Pompon & Pelletier, 2012). It is this plasticity which allows alterations in feeding behaviour to potentially contribute to the insecticide resistance of aphid clones.

When insecticides are applied to a plant they do not distribute evenly, but rather concentrate in specific tissues according to their chemical and physical properties, such as polarity, mass and charge (Buchholz & Nauen, 2002; Maienfisch *et al.*, 2001a). Insects which avoid feeding on tissues in which an insecticide may concentrate and feed preferentially on plant tissues in which insecticides are less likely to sequester will receive lower doses than insects which do not show these behavioural adaptations. Such insects will therefore be more likely to survive. In this manner, alterations in feeding behaviour may contribute to insecticide resistance. In addition, constant exposure of the insect to a lower dose of insecticide may potentially increase the probability of development of other types of resistance mechanisms (Gressel, 2010); hence in theory insecticide resistance and altered feeding behaviour may be expected to be sometimes associated.

Hemipteran feeding can be divided into distinct phases, which can be closely examined using the electrical penetration graph (EPG) technique (Walker, 2000). Different aspects of feeding behaviour have been linked to unique electrical waveforms produced by feeding aphids during EPG experiments. The different phases of Hemipteran feeding behaviour are therefore traditionally classified with reference to EPG waveforms.

The EPG technique itself involves attaching a thin gold wire to an aphid using a conductive paint as glue. This forms the input electrode, which links the aphid to a voltage source and a fixed input resistor contained within the EPG amplifier (Figure 4.1). The voltage source is

also linked to the host plant by the insertion of a copper probe into the damp compost in which the plant grows, forming the output electrode. When the aphid inserts its stylets into the plant, the circuit is completed. A computer records any changes in the resistance and voltages generated within the insect-plant combination as an electrical waveform (Pettersson *et al.*, 2007).

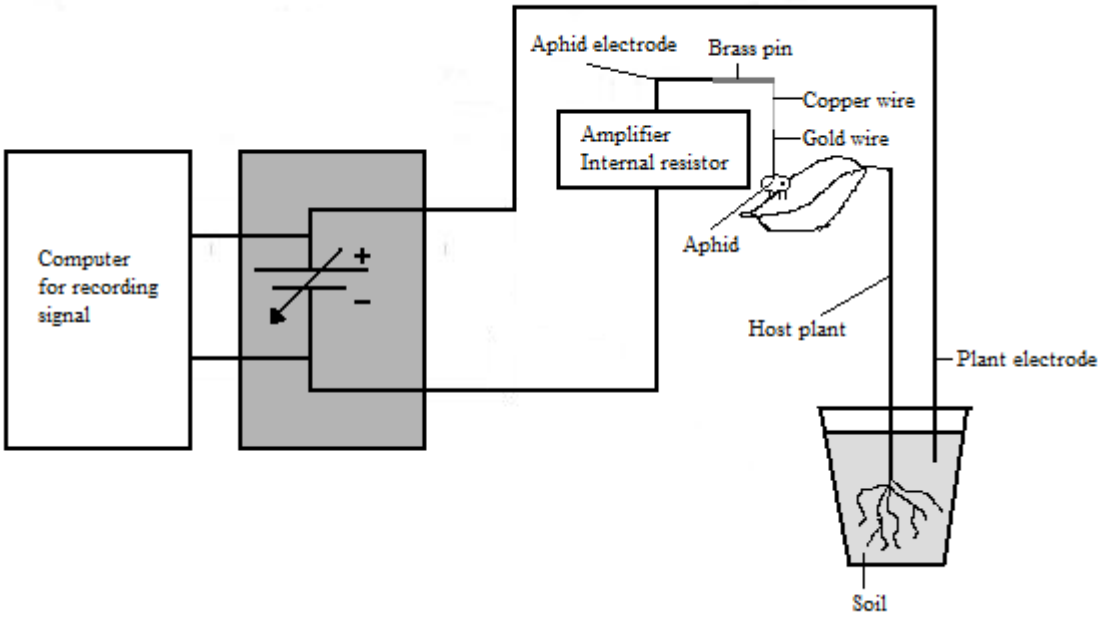


Figure 4.1 The setup for a plant-based EPG experiment. The EPG machine acts as both an amplifier and a resistance source. The computer records all data obtained by the end of the experiment. Up to eight insects on separate host plants can be incorporated into the DATAQ box, each with their own internal variable resistor. Adapted from Walker (2000).

Fluctuations in resistance are measured using an internal fixed resistor within the EPG system. The insect-plant combination acts as a second resistor within the series circuit; hence the voltage across the internal resistor varies according to the changing resistance and therefore voltage of the insect-plant combination. The EPG signal output is therefore a representation of the voltage across the internal resistor (Reese *et al.*, 2000).

There are two types of EPG system, AC and DC. While both types are able to measure this change in resistance, the DC EPG system is also able to measure internal voltage fluctuations generated in the insect and plant, known as the electromotive force (emf). This is due to the greater resistance provided by the internal resistor within DC systems. In DC systems the recorded signal is therefore composed of both the resistance and the emf generated by the

insect-plant combination, while in AC systems the recorded signal is only equivalent to the resistance (Reese *et al.*, 2000).

The resistance may fluctuate for a variety of reasons, including the concentration of solutes such as sap and different types of saliva within the aphid food and salivary canals, as well as the opening and closing of the cibarial and salivary pumps. When an aphid's stylets puncture a plant cell, the negative charge within the cell superimposes a negative voltage drop upon the recorded signal of the EPG. The emf also fluctuates according to the rate of movement of fluids within the aphid stylets, which differs when the insect feeds from different plant tissues such as the highly pressurised phloem. The resistance and emf making up the recorded signal are therefore intimately linked with the feeding behaviour of the insect (Reese *et al.*, 2000).

The waveforms associated with aphid feeding have been described by Tjallingi (1978) but will be reviewed here. When the aphid initially inserts the stylets into the plant epidermis, mesophyll and intercellular spaces three different waveforms known as A, B and C are produced. Since these waveforms are not clearly distinguishable from one another they are generally categorised together as waveform C, the 'pathway' waveform (Reese *et al.*, 2000).

Epidermal, mesophyll and palisade cell punctures performed during 'pathway' cause rapid drops in voltage when the negative cell membrane potential superimposes on the original electrical signal. This produces a distinctive 'potential drop' (pd) waveform which typically lasts for 5-10 s (Tjallingii, 1985).

The pd waveform can be divided into three phases, phases I, II and III, based on distinctive patterns in the waveform produced (Figure 4.2). Of these, phase I appears to reflect a voltage drop due to the transmembrane potential as the stylets puncture the plasmalemma of the host plant cell, while phase III represents withdrawal of the stylets from the host plant cell, hence the voltage returns to the extracellular level (Pettersson *et al.*, 2007).

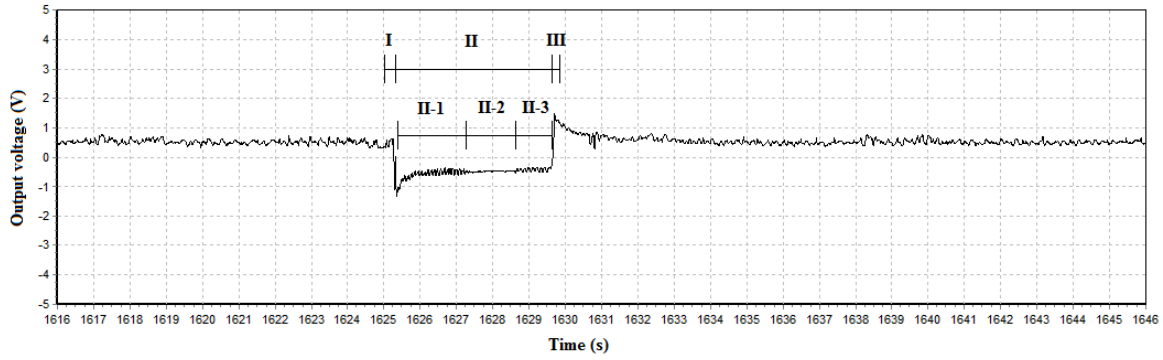


Figure 4.2 A typical potential drop waveform as recorded by the electrical penetration graph (EPG) technique. This waveform was produced by an adult *Myzus persicae* briefly inserting the stylets into tissue of the host plant, otherwise known as carrying out a 'probe'. Note the three phases I, II and III and the three subphases of phase II, II-1, II-2 and II-3.

Phase II can be further subdivided into the three sub-phases II-1, II-2 and II-3. Sub-phase II-1 represents the injection of watery saliva into the punctured cell, while II-3 represents the ingestion of cell contents (Fereres & Moreno, 2009). The function of phase II-2 remains unknown.

Phloem activities are always accompanied by a decrease in the voltage of the electrical signal. Phloem salivation and ingestion from the phloem produce different electrical signals and are easily discerned using the EPG technique. Phloem salivation is labelled 'E1' and phloem feeding 'E2' in EPG terminology (Prado & Tjallingii, 1994).

It is also possible to distinguish phloem feeding from xylem feeding using the EPG technique. The latter is associated with a waveform clearly different to that of E2, known as waveform G (Spiller *et al.*, 1990) (Figure 4.3). The final waveform, known as F, indicates disrupted stylet mechanics.

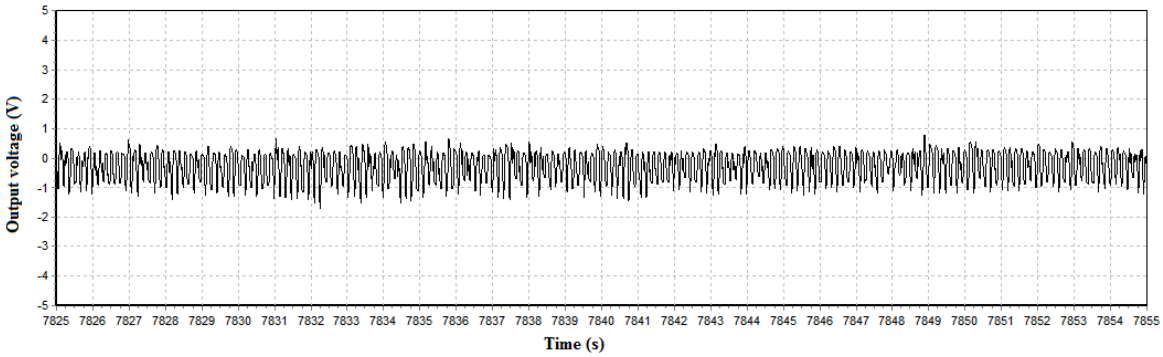


Figure 4.3 The electrical waveform produced by an adult *Myzus persicae* when feeding upon the xylem of a host plant (waveform G), as recorded by the electrical penetration graph (EPG) technique.

The ability of the EPG technique to distinguish how often and for how long hemiptera feed upon a range of different host plant tissues makes it extremely suitable for an investigation into the feeding behaviour of the neonicotinoid resistant FRC and 5191A *M. persicae* clones. If the feeding behaviour of these clones differentiates from the feeding behaviour of the susceptible clone US1L then it is possible that such behaviours may contribute to the neonicotinoid resistance of the FRC and 5191A clones, alongside the already documented metabolic and target site based neonicotinoid resistance.

It is possible to utilise the EPG technique to monitor insect feeding behaviour on both a plant host (Kindt *et al.,* 2003; Symmes *et al.,* 2008; Daniels *et al.,* 2009) and a liquid artificial diet (Powell *et al.,* 1995a; Joost *et al,* 2006). While performing EPG using an artificial diet is not comparable to a field situation, it may allow for a more accurate interpretation of the effect of different concentrations of insecticides such as TMX upon aphid behaviour. This is because insecticides are not metabolised within an artificial diet before reaching the aphid, unlike when an insecticide is applied via a host plant. The concentration and the amount of the active ingredient reaching the aphid can therefore be determined. Any observed changes in behaviour will be due to the presence of the active ingredient rather than metabolites of the insecticide.

While EPG on artificial diet is possible, the range of waveforms produced is significantly limited compared with those produced on host plants. This is due to the absence of different layers of plant tissue in the artificial diet system. Nauen (1995) observed three different waveforms produced by *M. persicae* feeding on artificial diet. These were a ‘B wave’ pattern produced directly after stylet insertion, the ‘salivation’ pattern which always proceeded the B wave and was accompanied by salivation, and ‘pattern G’, which always followed the previous two waves and was thought to represent ingestion of the artificial diet (Figure 4.4).

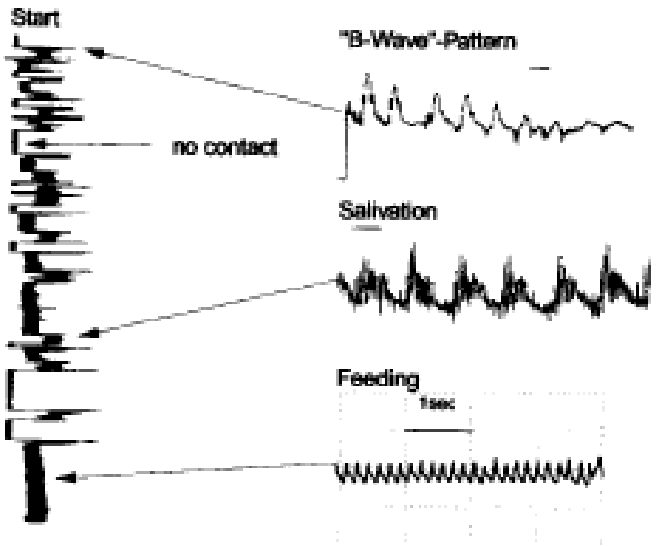


Figure 4.4 The three waveforms consistently observed by Nauen *et al.* (1995) as produced by *Myzus persicae* when feeding on sachets of artificial diet recorded using the EPG technique. The diagram on the left shows a typical entire graph produced by a single aphid.

Early on in the study, it was realised that in the interests of time only one of the two techniques could be used for a full investigation into the feeding behaviours of both neonicotinoid resistant and susceptible *M. persicae* clones, in both the presence and absence of TMX. Feeding behaviour was therefore only compared on both an artificial diet and a host plant using the susceptible clone US1L. This, however, was carried out in both the presence and absence of TMX, and it was decided to include the work in this thesis to give a fuller understanding into the effect of TMX on the feeding behaviour of US1L. Comparisons of the feeding behaviour between the susceptible and neonicotinoid-resistant clones were all carried out within a host plant based system.

4.2 Methods

4.2.1 Aphid and plant cultures

All aphid and plant cultures were as described in chapter 2. All aphids used in experiments were apterous individuals of the first day of adulthood. All plants used were two week old Chinese cabbage (*B. chinensis cv* Apex) of the seedling stage with two to three true leaves.

4.2.2 Plant based EPG Setup

All EPG data acquisition and analysis was carried out using the program Stylet+ for Windows. The setup was similar to the DC system described by Tjallingii (1978). A thin gold wire of diameter 18.5 µm and length 3 cm was attached to a 3 cm length of copper wire using silver conductive paint. The other end of the copper wire was soldered to a 20 mm brass pin. The free end of the gold wire was glued to the dorsal cuticle of an adult *M. persicae* using silver conductive paint. The brass pin was inserted into the input probe of the EPG, which was held within a stand, suspending the aphid from the wire. A two week old two-true leaf seedling stage Chinese cabbage plant in a 180 ml plastic pot was placed upon a plastic weigh boat to achieve electrical isolation. A thin copper probe, connected to the EPG voltage supply, was then inserted into the damp compost within the pot of the plant. The aphid was lowered onto the leaf surface by adjustment of the stand.

The voltage was supplied and EPG recording obtained using a Giga-8 EPG amplifier system with 1 GΩ input resistance. This was connected to a DATAQ box, which was in turn linked to a computer. To reduce electrical interference the entire setup, excluding the computer and DATAQ box, was located within an earthed Faraday cage. There was room for eight individual aphids upon separate host plants to be connected to the EPG system within the Faraday cage, with each aphid occupying a separate ‘channel’. A maximum of eight EPG recordings could therefore be carried out simultaneously. Aphids were starved in an empty Petri dish for two hours prior to the attachment of the wire under conditions of approximately 23 ºC and 30 % r.h.

At the beginning of each trial, Stylet+ was initiated on the computer and the aphids lowered by adjusting the stand until they came into contact with a leaf of their respective host plant. The voltage was adjusted individually for each channel using the amplifier to optimise the recorded electrical signals. The setup was then left for 6 hours under conditions of approximately 23 ºC and 30 % r.h. Since only eight aphids could be analysed at one time, any

variations in humidity or temperature between batches were also recorded. All electrical signals during this time were recorded and stored by the program for analysis.

4.2.3 Artificial diet EPG setup

For EPGs on artificial diet as opposed to a host plant, the setup was identical to that described in section 4.2.2 except for the following changes (Figure 4.5). Instead of the thin copper probe, a plastic sheathed metal wire 100 µm in diameter was connected to the amplifier using a banana plug attachment. The other end of the wire was stripped of insulating plastic for 6 cm.

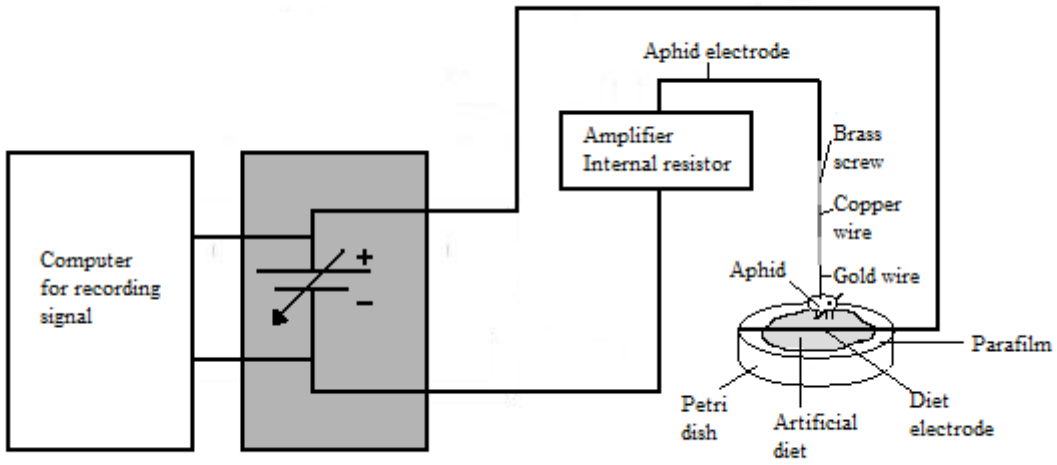


Figure 4.5 The setup for the EPG technique when carrying out analysis of aphid feeding behaviour when fed on artificial diet as opposed to a host plant. Adapted from Walker (2000).

A layer of Parafilm® 'M' was stretched across an inverted plastic Petri dish lid of diameter 55 mm. An artificial diet solution was made up as described in Chapter 2, with any added TMX in the form of pure technical AI dissolved in acetone. A 2 ml droplet of a solution of artificial diet and TMX was then deposited upon the surface of the Parafilm®. The large amount of solution provided reflected the typical feeding behaviour of *M. persicae*, which tends to walk a few steps before attempting to probe. The diet provided an area wide enough for the *M. persicae* to take several steps before probing without travelling beyond the liquid diet.

The stripped part of the wire attachment was stretched across the Parafilm layer and secured at the edges of the Petri dish with Blu-Tack®, with care taken that the wire made contact with the liquid diet. A second layer of Parafilm® 'M' was then stretched across the first layer of

Parafilm®, puddle of liquid diet and the wire, producing a sachet of artificial diet between two layers of Parafilm® with the wire probe running through, supported by the Petri dish lid.

The Petri dish lid was then placed on an inverted plastic cup of height 9 cm within the Faraday cage, in order to ensure electrical isolation of the setup. In addition to this, a filter made of translucent green plastic was placed between the plastic cup and the base of the Petri dish lid to provide a stimulus to induce the aphids to probe downwards into the artificial diet. There was room for eight individual aphids, each with a separate artificial diet sachet to be connected to the EPG machine; hence eight EPG experiments could be carried out simultaneously. When an aphid probed through the Parafilm® into the artificial diet the circuit was completed and the behaviour recorded as a change in the electrical signal.

4.2.4 Comparison of feeding behaviour of US1L upon host plants and artificial diet

This trial only contained the clone US1L, due to DEFRA licensing limitations. The feeding behaviour of US1L was recorded under four different treatments, a TMX treated plant, a control treated plant, TMX treated artificial diet or a sachet of control treated diet. Since a maximum of eight recordings or 'channels' could be used at any one time within the EPG setup, the experiment had to be divided into many trials performed on separate days. Each trial contained eight *M. persicae* of the first day of adulthood. The position of each of the treatments within the Faraday cage was randomly assigned for each trial (Figure 4.6).

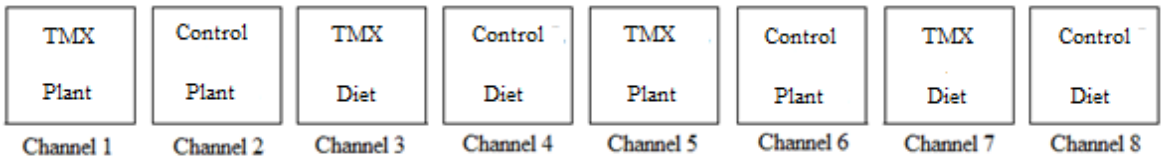


Figure 4.6 Example of the treatment arrangement used to compare the feeding behaviour of the *Myzus persicae* clone US1L on thiamethoxam (TMX) treated and untreated artificial diet and host plants using the EPG technique. The diagram represents a single trial, which would be repeated multiple times. The eight boxes represent the eight channels of the EPG machine, each of which is assigned a treatment within a Faraday cage. The upper line indicates whether the host plant/artificial diet solution was treated with TMX or not. Control plants were treated with Milli-Q water and control artificial diet solutions treated with acetone. The lower line indicates whether the feeding behaviour of the aphid was monitored on a host plant or a sachet of artificial diet. Note that each possible treatment was randomly assigned to a channel for each trial; the positions of treatments were not fixed for all trials in the manner shown above.

Two week old two true leaf seedling stage Chinese cabbage plants (*Brassica chinensis cv* Apex) were stood on blue roll for 24 h under conditions of 20 ± 1 ºC, 16:8 h L:D photoperiod and 50% r.h. for the soil to dry out. 50 ml of 0.1 ppm TMX, equivalent to the LD_{15} concentration of US1L, was then poured onto the soil of each plant. The plants were then left under the same conditions for 24 h to take up the TMX before use in EPG trials. Control plants were root drenched with 50 ml of Milli-Q water instead of TMX. The plant based treatments were set up as in section 4.2.2 above.

Artificial diet was made up as described in chapter 2 section 2.2.3 and frozen until use. Once defrosted, TMX pure AI dissolved in 1 ml acetone was added to the artificial diet to make up a solution of 0.013 ppm TMX, equivalent to the LD_{15} of US1L. Artificial diet containing an equivalent amount of acetone to the TMX treated diet, but with no AI added, was used as a control solution. The artificial diet based treatments were set up as in section 4.2.3 above.

Aphids were removed from culture and starved for 2 h previous to the experiment. A gold wire was then attached to each aphid and they were simultaneously lowered onto their respective host plants or artificial diet sachets. Their feeding behaviour was recorded by the EPG setup for a 6 h period. A minimum of twenty repeats were taken for each treatment. The temperature during each trial was monitored to be included in the analysis to check for the effect of environmental variation.

4.2.5 Comparison of feeding behaviour of resistant and susceptible clones at the LD_{15}

The feeding behaviour of the susceptible clone US1L and the two neonicotinoid resistant clones 5191A and FRC were compared on both TMX and control treated plants using the EPG technique. No artificial diet recordings were utilised in this experiment. Since a maximum of eight recordings or ‘channels’ could be used at any one time within the EPG setup, the experiment had to be divided into many trials performed on separate days. Each trial contained eight *M. persicae* of the first day of adulthood. The position of each treatment-clone combination within the Faraday cage was randomly assigned for each trial (Figure 4.7).

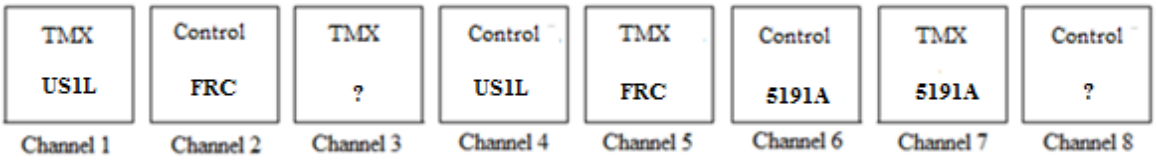


Figure 4.7 Setup to compare the feeding behaviour of the *Myzus persicae* clones US1L, 5191A and FRC on thiamethoxam (TMX) treated and untreated host plants using the EPG technique. The diagram represents a single trial, which would be repeated multiple times. The eight boxes represent the eight channels of the EPG machine, each of which is assigned a treatment within a Faraday cage. The upper line indicates whether the host plant was treated with TMX or control treated with Milli-Q water. The lower line indicates the clone of aphid that was attached to the gold electrode. Note that each possible treatment was randomly assigned to a channel for each trial; the positions of treatments were not fixed for all trials in the manner shown above.

Two week old two true leaf seedling stage Chinese cabbage plants (*B. chinensis cv* Apex) were stood on blue roll for 24 h under conditions of 20 ± 1 ºC, 16:8 h L:D photoperiod and 50% r.h. for the soil to dry out. 50 ml of TMX was then poured onto the soil of each plant, and the plants left under the same conditions for 24 h to take up the insecticide. Plants were treated with TMX at the concentration equivalent to the LD_{15} for each respective clone, as calculated in the experiments described in Chapter 2. Plants offered to US1L were therefore treated with 0.1 ppm TMX, plants offered to 5191A were treated with 0.9 ppm TMX and plants offered to FRC were treated with 42.5 ppm TMX. Control plants were root drenched with 50 ml of Milli-Q water instead of TMX. After allowing uptake of the insecticide, the EPG equipment was set up as in section 4.2.2 above.

Aphids were removed from culture and starved for 2 h previous to the experiment. A gold wire was then attached to each aphid and they were simultaneously lowered onto their respective host plants. Their feeding behaviour was recorded by the EPG setup for a 6 h period. A minimum of twenty repeats were taken for each aphid clone under each treatment. The temperature during each trial was monitored to be included in the analysis to check for the effect of environmental variation.

4.2.6 Comparison of feeding behaviour of resistant and susceptible clones at the LD_{20}

After analysis of the comparison of the feeding behaviour of the three *M. persicae* clones on both plants treated with TMX at the LD_{15} concentration and control treated plants, it was noted that there were several trends of differences in feeding behaviours between clones on TMX treated plants which were consistently seen over many repeats, but were not quite great enough in value to be statistically significant. It was therefore decided to repeat the experiment described in section 4.2.5 above, but to treat plants with TMX at the respective LD_{20} value for each clone, rather than the LD_{15} value. Plants offered to US1L were treated with 50 ml of TMX at a concentration of 0.2 ppm, plants offered to 5191A were treated with 1 ppm TMX and plants offered to FRC were treated with 53.1 ppm. The rest of the setup was kept consistent with that described in section 4.2.5.

4.2.7 Analysis of EPG signals

All analysis of EPG signals was carried out using the ANA34 tool of the Stylet+ program. Electrical signals were analysed and specific waveform components, described as distinct by Reese *et al.* (2000), manually labelled by the investigator. These parameters were then converted into an Excel format, averaged and summated using an Excel workbook provided by Sarria *et al.*, (2009). The output from the Excel workbook was statistically analysed using the program R version 2.1.4.

Since the data were not normally distributed, analyses of the differences in behavioural parameters between sets of possible treatments such as artificial diet versus host plants, TMX treatment versus control treatment of plants and treatment with the LD_{15} as opposed to the LD_{20} concentrations of TMX were carried out using Mann-Whitney U tests separately for each behavioural parameter and *M. persicae* clone. Analyses of the differences in behaviours observed from the three *M. persicae* clones were carried out using Kruskal-Wallis tests separately for both the LD_{15} and LD_{20} experiments. Analyses of the differences in behaviours performed by the three clones on control treated plants were also analysed separately for the LD_{15} and LD_{20} experiments. *Post hoc* analyses were carried out after the Kruskal-Wallis tests to determine which clones performed significantly differently from one another. In total, 21 behavioural parameters were analysed for each experiment, with the exception of studies containing artificial diet fed aphids from which only four behavioural parameters could be analysed. Chi-squared tests were also performed for the LD_{15} and LD_{20} experiments to analyse the numbers of aphids which engaged in E1, E2, G and F during the 6 h period.

4.3 Results

4.3.1 The feeding behaviour of US1L on host plants compared to artificial diet

The waveforms produced by *M. persicae* of the US1L clone on artificial diet in this experiment were not comparable with those described by Nauen (1995). Two different waveforms were, however, consistently recognisable. These included a waveform similar to the 'salivation waveform' and a waveform similar to the 'feeding waveform' described by Nauen (1995) (Figures 4.8 a) and b) respectively). Since the waveforms did not entirely match those of Nauen (1995), this study designated them as waveforms 1 and 2. The frequencies of these waveforms did not match the frequencies described by Nauen (1995). Waveform 1 had a relatively low frequency of 0.5 Hz. Waveform 2 exhibited a combination of three different frequencies of 4, 6 and 8 Hz., but this was not consistent for all individuals. Waveform 1 was always exhibited at the start of a probe into the artificial diet, and was usually followed by waveform 2. Probing of the artificial diet was often accompanied by an initial brief increase in voltage (Figure 4.8c). This did not occur for the probes of all individuals (Figure 4.8d) but was very frequently seen. The waveforms produced by *M. persicae* offered Chinese cabbage host plants were very similar to those described by Tjallingi (1978) (Figures 4.9 and 4.10) and so were designated as representing the same behaviours.

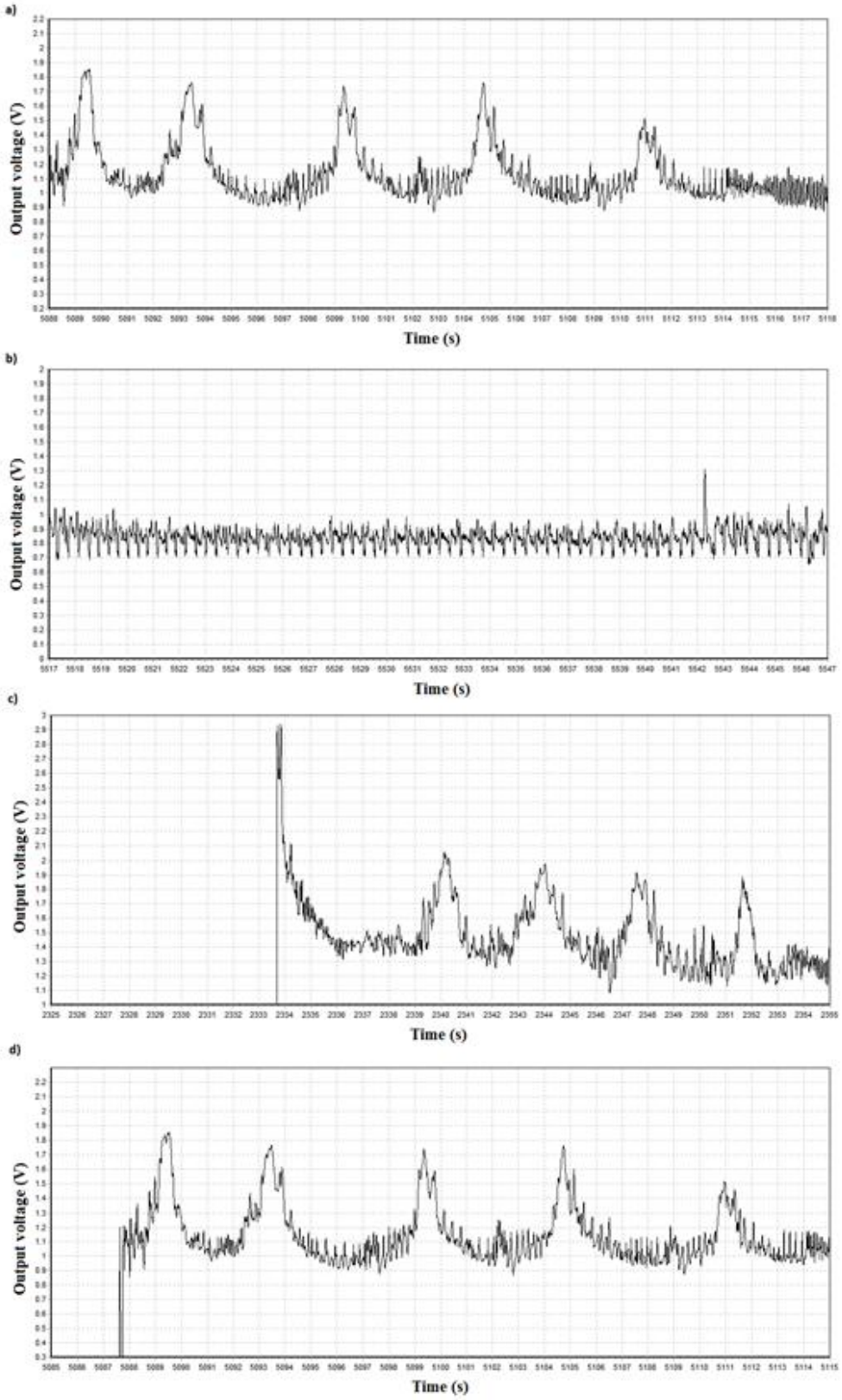


Figure 4.8 Waveforms exhibited by *Myzus persicae* of the US1L clone when fed on artificial diet. a) Waveform 1 b) Waveform 2 c) Typical initial brief increase in voltage exhibited at the start of a probe into artificial diet d) A probe initiated without a brief increase in voltage

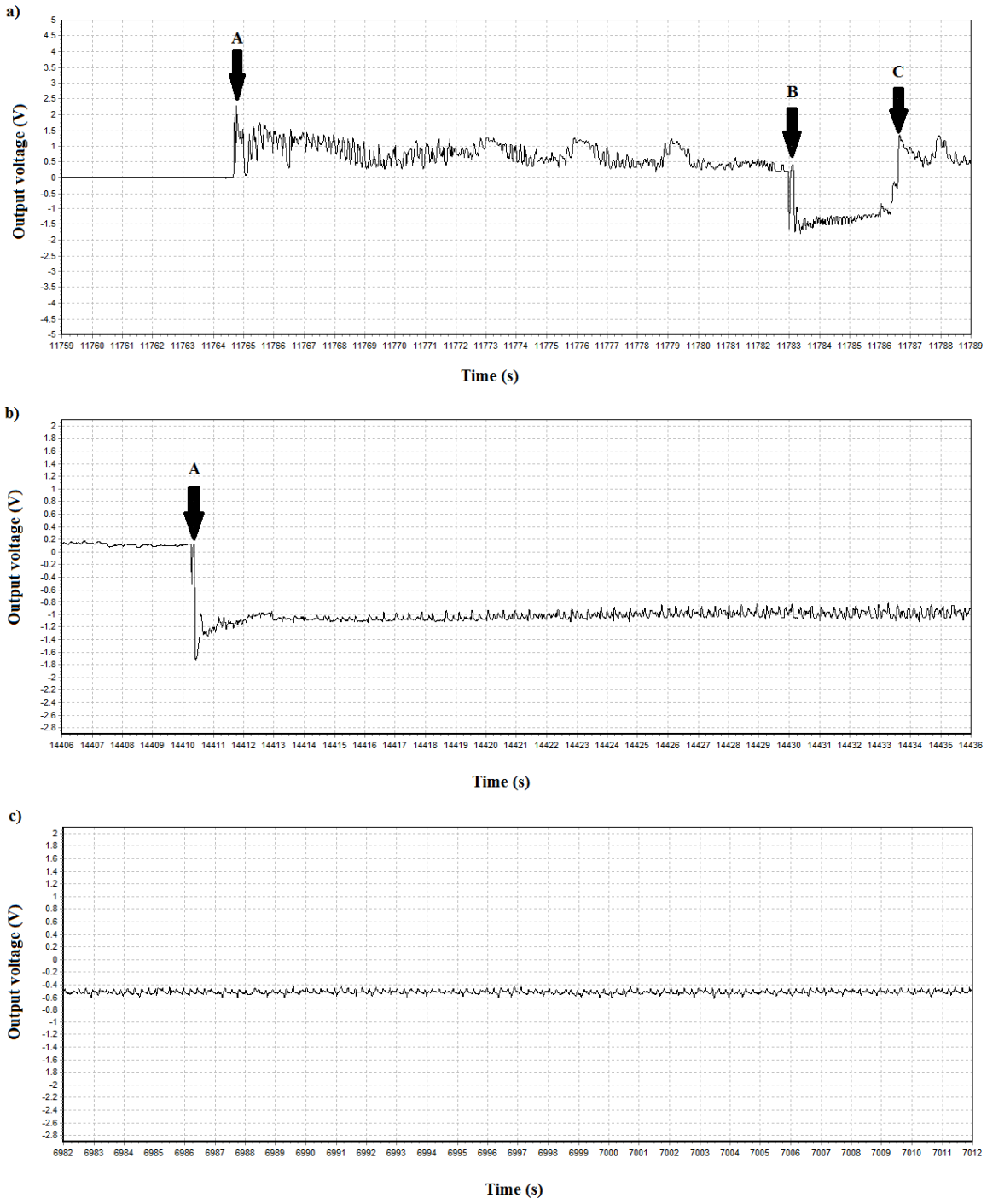


Figure 4.9 Waveforms exhibited by a single individual *Myzus persicae* feeding upon a two week old Chinese cabbage host plant. a) To the left of arrow A shows non-feeding, during which the aphid's proboscis is not attached to the plant and so no electrical signal is produced. Arrow A shows the beginning of pathway phase (waveform C), during which the stylets are located within the intracellular spaces. Arrow B indicates the initiation of a short cell puncture, known as a potential drop. This is characterised by a decrease in voltage. Arrow C indicates the termination of the cell puncture, characterised by an increase in voltage. b) Arrow A indicates entry into phloem salivation (E1 waveform) c) Phloem feeding (E2 waveform)

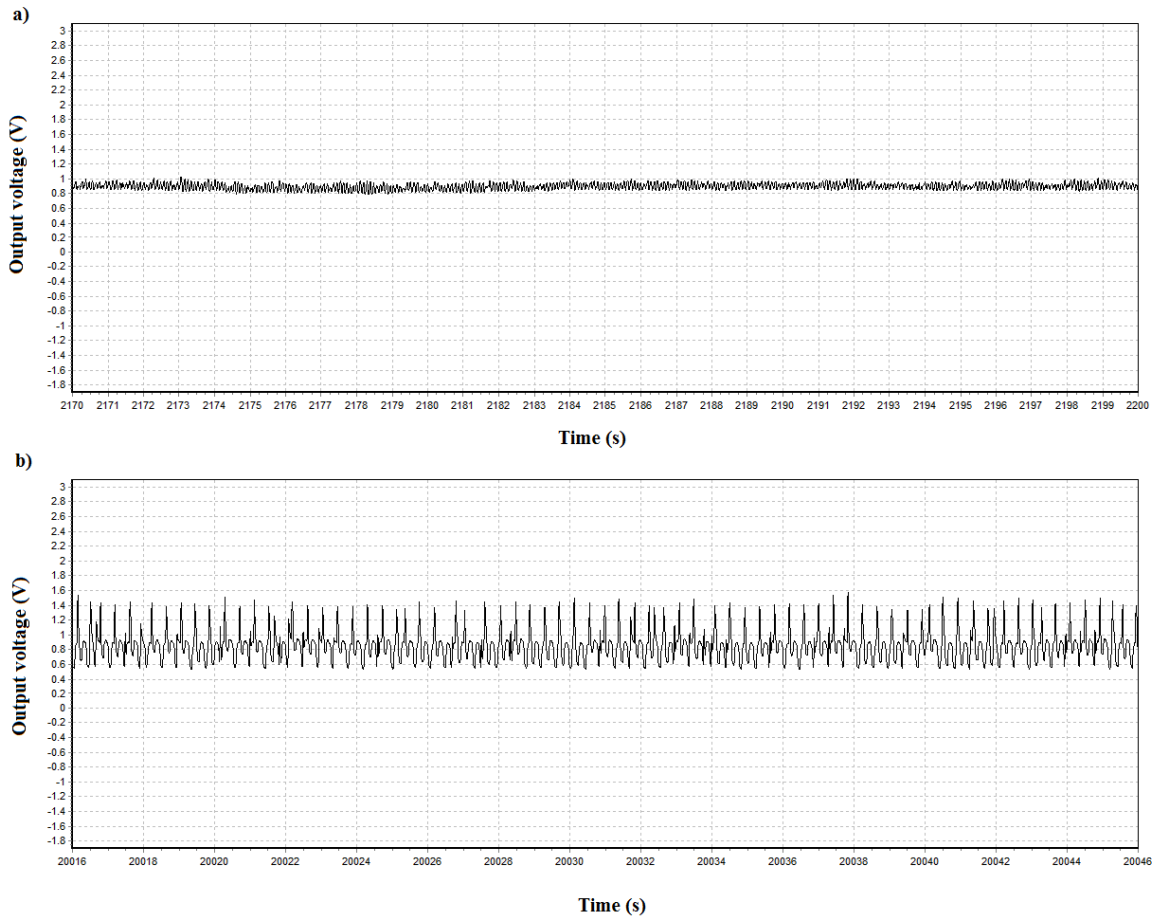


Figure 4.10 Waveforms exhibited by a single individual *Myzus persicae* feeding upon a two week old Chinese cabbage host plant. a) Disrupted stylet mechanics (Waveform F) b) Xylem feeding (Waveform G)

When fed on untreated artificial diet the US1L clone took a significantly greater time to perform the first probe than when offered a control treated host plant (Figure 4.11). There were no significant differences however in the total time spent probing, mean time spent probing and number of probes performed upon either a control treated host plant or untreated artificial diet (Table 4.1).

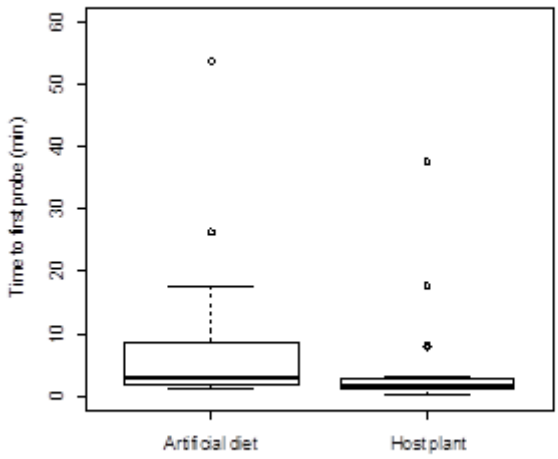


Figure 4.11 The time taken by *Myzus persicae* of the US1L clone to perform the first probe when offered either a control treated sachet of artificial diet or a control treated host plant. Bold line shows the median value, while the upper and lower edge of the box show the upper and lower quartiles of the data respectively. The range of the data is shown by the 'whiskers'. Outlying data points are shown as circles separate to the main body of data.

Table 4.1 Probing behaviours performed by *Myzus persicae* of the US1L clone on either a host plant or a sachet of artificial diet. Host plants were either treated with a sublethal dose of thiamethoxam (TMX) or control treated with Milli-Q water. Artificial diet was either treated with a sublethal dose of TMX dissolved in acetone or else control treated with an equal concentration of acetone. Error bars represent ± the standard error. Differing superscript letters indicate a significant difference between values.

	Control		TMX	
	Host plant	Artificial diet	Host plant	Artificial diet
Number of probes	21.48 ± 3.98	16.20 ± 3.75^{a}	23.46 ± 3.32	23.25 ± 3.42^{b}
Total time probing (min)	285.76 ± 19.94^{a}	292.01 ± 14.54^{a}	235.13 ± 17.53^{b}	299.72 ± 10.53^{a}
Mean time probing (min)	44.53 ± 17.85	67.73 ± 17.74^{a}	14.32 ± 2.08	19.61 ± 3.35^{b}
Time to first probe (min)	4.58 ± 1.87^{a}	7.68 ± 2.28^{b}	12.20 ± 7.67	2.65 ± 0.56^{ac}

In contrast, when offered TMX treated artificial diet and host plants there was no significant difference in the time taken for the US1L clone to reach the first probe (Table 4.1). The

aphids however did exhibit a significantly greater total amount of time probing when offered TMX treated artificial diet as opposed to TMX treated host plants (Figure 4.12). There were no other significant differences in probing behaviour exhibited between the two treatments.

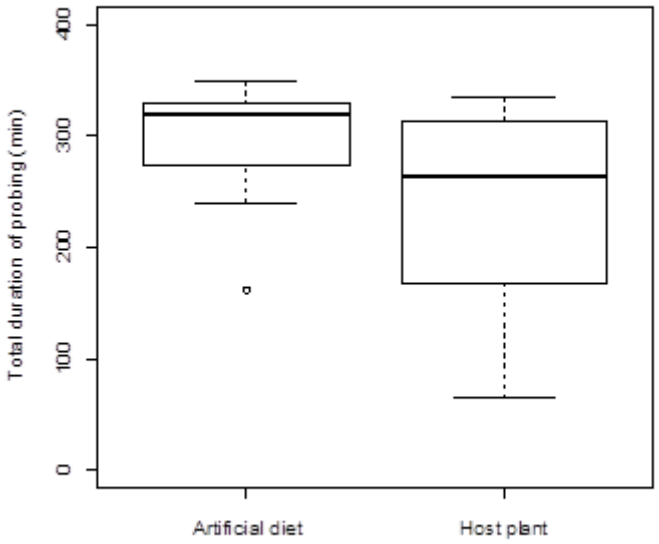


Figure 4.12 The total duration spent probing by *Myzus persicae* of the US1L clone when offered either a thiamethoxam treated host plant or a thiamethoxam treated artificial diet.

4.3.2 The effect of thiamethoxam on the feeding behaviour of individual clones

The application of TMX at the LD_{15} concentration produced no significant effect on the feeding behaviour of any of the three clones, when compared with the feeding behaviour of the same clone on control treated plants (Tables 4.2 & 4.3).

The application of TMX at the LD_{20} concentration did however, significantly increase the mean duration of E2 for 5191A (Figure 4.13). There was no effect on any other behavioural parameter exhibited by 5191A, or any behaviours exhibited by the US1L and FRC clones (Tables 4.4 & 4.5). Since such small amounts of the G and F waveforms occurred, no comparison of the total and mean duration of these two behaviours could be made between treatments for any clone.

There was no significant effect of the application of TMX upon the number of aphids of any clone performing E1, E2, G and F for both the LD_{15} and LD_{20} experiments (Table 4.6).

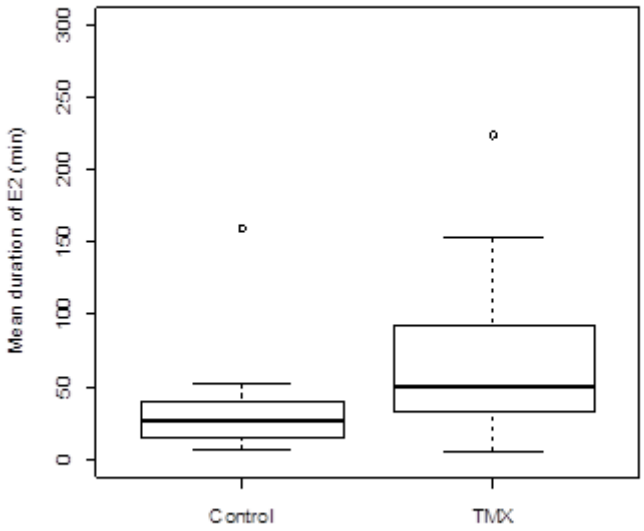


Figure 4.13 The mean duration of E2 performed by *Myzus persicae* of the clone 5191A when offered either host plants root drenched with 1 ppm of thiamethoxam (TMX), equivalent to the LD_{20} of this clone, or else control plants drenched with Milli-Q water.

Table 4.2 The differences in feeding behaviour of three *Myzus persicae* clones when offered thiamethoxam (TMX) treated as opposed to untreated control plants. TMX treated plants were drenched at the LD_{15} concentration. Error bars show ± the standard error.

	US1L		5191A		FRC	
	Control	TMX	Control	TMX	Control	TMX
Total time probing (min)	266.49 ± 16.74^{abcd}	171.49 ± 23.51^{abcd}	316.85 ± 6.59^{abc}	145.81 ± 23.03^{acd}	255.53 ± 16.60^{abd}	239.41 ± 25.24^{bcd}
Number of probes	21.70 ± 2.81^{ab}	22.43 ± 2.65^{ab}	18.14 ± 2.50^{ab}	14.38 ± 1.89^{a}	30.18 ± 4.10^{ab}	31.86 ± 3.65^{b}
Total time in C (min)	155.67 ± 13.53	183.59 ± 15.50	199.57 ± 15.69	179.01 ± 17.00	173.90 ± 12.03	172.40 ± 13.94
Number of C	23.65 ± 2.91^{ab}	24.17 ± 2.72^{ab}	20.14 ± 2.55^{ab}	15.90 ± 1.95^{a}	31.95 ± 3.93^{ab}	33.86 ± 3.62^{b}
Mean duration of C (min)	8.35 ± 1.03^{abc}	9.63 ± 1.32^{abcd}	11.60 ± 1.13 ^{abd}	16.50 ± 3.21^{acd}	6.77 ± 0.66^{abc}	5.96 ± 0.61^{bcd}
Number of E1	1.91 ± 0.32	1.43 ± 0.22	2.27 ± 0.42	1.81 ± 0.30	1.91 ± 0.60	2.00 ± 0.40
Number of E2	1.57 ± 0.29	1.22 ± 0.21	1.95 ± 0.34	1.71 ± 0.29	1.68 ± 0.52	1.52 ± 0.30
Number of sustained E2 (> 10 min)	0.97 ± 0.19	0.65 ± 0.15	1.64 ± 0.32	1.19 ± 0.20	1.14 ± 0.33	0.95 ± 0.23
Time to first probe (min)	2.46 ± 0.52	4.34 ± 1.09	2.89 ± 0.93	4.39 ± 1.11	7.25 ± 2.71	5.37 ± 1.95
Time taken to reach E1 from first probe (min)	180.12 ± 26.69^{ab}	172.69 ± 23.93^{ab}	158.13 ± 24.30^{ab}	146.73 ± 23.44^{a}	210.94 ± 29.87^{ab}	240.58 ± 25.51^{b}
Time taken to reach E2 from first probe (min)	195.89 ± 26.54^{ab}	187.73 ± 25.12^{ab}	159.84 ± 24.19^{ab}	149.20 ± 23.51^{a}	223.71 ± 30.54^{ab}	249.55 ± 22.47^{b}
Total Time in E1 (min)	3.30 ± 1.91^{ab}	3.92 ± 1.87^{a}	7.72 ± 5.14^{ab}	3.16 ± 0.89^{ab}	10.16 ± 7.16^{ab}	11.53 ± 4.95^{b}

Table 4.3 The differences in feeding behaviour of three *Myzus persicae* clones when offered thiamethoxam (TMX) treated as opposed to untreated control plants. TMX treated plants were drenched at the LD_{15} concentration. Error bars show ± the standard error.

	US1L		5191A		FRC	
	Control	TMX	Control	TMX	Control	TMX
Mean duration of E1 (min)	1.52 ± 0.95	2.33 ± 1.02	2.35 ± 1.28	1.52 ± 0.29	8.25 ± 7.26	2.98 ± 1.16
Number of probes to the first E1	12.28 ± 2.14^{ab}	11.05 ± 1.99^{ab}	8.78 ± 1.19^{ab}	7.89 ± 1.85^{a}	12.92 ± 3.06^{ab}	20.54 ± 4.29^{b}
Total time in E2 (min)	76.44 ± 21.04	85.22 ± 20.97	127.72 ± 20.54	118.16 ± 22.16	123.15 ± 27.10	69.18 ± 14.90
Mean duration of E2 (min)	49.96 ± 17.78	73.35 ± 21.67	63.76 ± 12.31	74.21 ± 17.63	53.15 ± 20.50	27.01 ± 5.22
% contribution of E1 to the phloem phase	14.61 ± 5.86	18.94 ± 7.17	7.64 ± 3.20	8.40 ± 3.66	19.45 ± 9.42	18.29 ± 6.28
Total number of pd	3.50 ± 0.62	3.14 ± 0.75	3.59 ± 0.45	4.05 ± 0.62	3.73 ± 0.60	3.62 ± 0.68
Total number of pds in first 10 min	79.83 ± 6.76	84.45 ± 6.45	96.32 ± 8.52	77.68 ± 6.38	98.45 ± 9.46	86.86 ± 10.54
Total duration of G	32.58 ± 8.96	37.86 ± 12.50	30.05 ± 7.65	0.00 ± 0.00	13.62 ± 3.69	26.20 ± 14.09
Number of G	0.30 ± 0.13	0.35 ± 0.13	0.09 ± 0.06	0.00 ± 0.00	0.09 ± 0.06	0.14 ± 0.08
Total duration of F	110.42 ± 21.06	73.92 ± 15.36	40.95 ± 30.89	54.33 ± 18.83	158.71 ± 0.00	132.51 ± 0.00
Number of F	0.30 ± 0.13	1.40 ± 0.40	0.09 ± 0.06	0.33 ± 0.17	0.05 ± 0.05	0.05 ± 0.05

Table 4.4 The differences in feeding behaviour of three *Myzus persicae* clones when offered thiamethoxam (TMX) treated as opposed to untreated control plants. TMX treated plants were drenched at the LD_{20} concentration. Error bars show ± the standard error.

	US1L		5191A		FRC	
	Control	TMX	Control	TMX	Control	TMX
Total time probing (min)	304.15 ± 10.69	298.82 ± 11.37	298.58 ± 14.20	287.72 ± 18.01	294.53 ± 11.49	307.94 ± 9.77
Number of probes	24.65 ± 3.21	19.80 ± 2.78	17.45 ± 1.99	16.09 ± 1.44	27.045 ± 3.32	25.40 ± 3.77
Total time in C (min)	199.73 ± 15.44	222.10 ± 15.12	208.83 ± 17.74	195.21 ± 16.47	214.16 ± 12.01	188.83 ± 19.07
Number of C	27.00 ± 3.25	21.75 ± 2.87	19.82 ± 2.00	17.52 ± 1.44	29.05 ± 3.33	26.90 ± 3.80
Mean duration of C (min)	9.71 ± 1.25	13.84 ± 2.27	13.79 ± 2.48	13.15 ± 2.42	10.61 ± 2.05	8.47 ± 0.88
Number of E1	2.35 ± 0.39	1.60 ± 0.29	2.73 ± 0.39	1.70 ± 0.30	1.91 ± 0.36	1.85 ± 0.32
Number of E2	2.22 ± 0.34	1.45 ± 0.29	2.59 ± 0.40	1.52 ± 0.29	1.86 ± 0.36	1.80 ± 0.30
Number of sustained E2 (> 10 min)	1.22 ± 0.22	0.75 ± 0.19	1.64 ± 0.27	1.00 ± 0.18	1.18 ± 0.21	1.40 ± 0.28
Time to first probe (min)	2.55 ± 0.92	4.22 ± 1.67	11.95 ± 9.80	26.06 ± 15.37	2.46 ± 0.50	7.95 ± 2.92
Time taken to reach E1 from first probe (min)	156.65 ± 26.27	186.86 ± 29.15	172.51 ± 25.28	181.46 ± 23.87	212.02 ± 24.67	191.16 ± 29.94
Time taken to reach E2 from first probe (min)	206.88 ± 27.00	243.82 ± 27.11	196.11 ± 26.51	207.96 ± 23.44	216.92 ± 25.14	200.83 ± 30.84
Total Time in E1 (min)	2.75 ± 0.74^{a}	2.15 ± 0.39^{ab}	3.83 ± 0.50^{b}	2.78 ± 0.50^{ab}	1.82 ± 0.29^{a}	1.72 ± 0.29^{ab}

Table 4.5 The differences in feeding behaviour of three *Myzus persicae* clones when offered thiamethoxam (TMX) treated as opposed to untreated control plants. TMX treated plants were drenched at the LD_{20} concentration. Error bars show ± the standard error.

	US1L		5191A		FRC	
	Control	TMX	Control	TMX	Control	TMX
Mean duration of E1 (min)	0.94 ± 0.^{12abc}	1.12 ± 0.15^{abcd}	1.50 ± 0.^{40abc}	1.48 ± 0.40^{acd}	0.64 ± 0.^{05abd}	0.76 ± 0.08^{bcd}
Number of probes to the first E1	11.70 ± 2.35	10.38 ± 1.29	10.00 ± 1.43	10.22 ± 1.60	14.20 ± 2.52	18.13 ± 3.81
Total time in E2 (min)	89.59 ± 17.18	64.34 ± 14.36	98.32 ± 22.09	110.76 ± 17.84	91.27 ± 17.05	126.0 ± 27.20
Mean duration of E2 (min)	39.84 ± 9.75^{ab}	42.91 ± 10.41^{ab}	33.37 ± 8.11^{a}	69.12 ± 14.43^{b}	43.71 ± 10.34^{ab}	55.57 ± 11.46^{ab}
% contribution of E1 to the phloem phase	5.10 ± 1.10	18.84 ± 6.99	11.21 ± 5.14	8.92 ± 5.43	3.11 ± 0.76	6.01 ± 2.88
Total number of pd	99.70 ± 8.16	94.10 ± 9.82	117.18 ± 9.71	97.30 ± 8.12	101.55 ± 8.50	110.70 ± 11.78
Total number of pds in first 10 min	4.57 ± 0.57	3.90 ± 0.58	5.00 ± 0.46	4.70 ± 0.72	3.55 ± 0.59	3.85 ± 0.49
Total duration of G	9.86 ± 3.47	69.87 ± 37.60	14.79 ± 1.33	41.87 ± 40.32	12.59 ± 4.20	9.49 ± 0.00
Number of G	0.17 ± 0.08	0.15 ± 0.08	0.14 ± 0.10	0.22 ± 0.18	0.14 ± 0.07	0.05 ± 0.05
Total duration of F	85.88 ± 28.70	54.21 ± 19.70	51.13 ± 31.33	110.10 ± 0.00	47.74 ± 15.22	65.82 ± 19.93
Number of F	0.35 ± 0.13	0.50 ± 0.20	0.09 ± 0.06	0.09 ± 0.09	0.36 ± 0.12	0.25 ± 0.10

Table 4.6 The numbers of aphids of three different *Myzus persicae* clones, US1L, 5191A and FRC, performing a range of feeding behaviours when offered host plants treated with thiamethoxam (TMX) at either the LD_{15} or LD_{20} concentration for their respective clone. Control plants were treated with Milli-Q water. P values show whether a significant difference existed between the LD_{15} and LD_{20} treatments. Asterisks indicate that values within the column are significantly different from one another at $p < 0.05$.

Clone	Treatment	Parameter	LD_{15}	LD_{20}	p value
US1L	Control	E1	18	20	n.s.
		E2	17	20	n.s.
		G	5	4	n.s.
		F	5	6	n.s.
	TMX	E1	19	16	n.s.
		E2	17	15	n.s.
		G	6*	3	n.s.
		F	5	6	n.s.
5191A	Control	E1	18	19	n.s.
		E2	18	18	n.s.
		G	2	2	n.s.
		F	2	2	n.s.
	TMX	E1	19	18	n.s.
		E2	19	17	n.s.
		G	0*	2	n.s.
		F	4	1	n.s.
FRC	Control	E1	13	15	n.s.
		E2	12	15	n.s.
		G	2	3	n.s.
		F	1	7	< 0.05
	TMX	E1	13	16	n.s.
		E2	13	16	n.s.
		G	3	1	n.s.
		F	1	5	n.s.

During the artificial diet vs. host plant experiment the US1L clone exhibited a significantly greater number of probes when offered TMX treated artificial diet as opposed to control treated diet (Figure 4.14a). The mean time spent probing and the time taken to reach the first probe however, was greater when offered control treated as opposed to TMX treated artificial diet (Figures 4.14b and 4.14c). There was no significant difference in the total amount of time spent probing (Table 4.1).

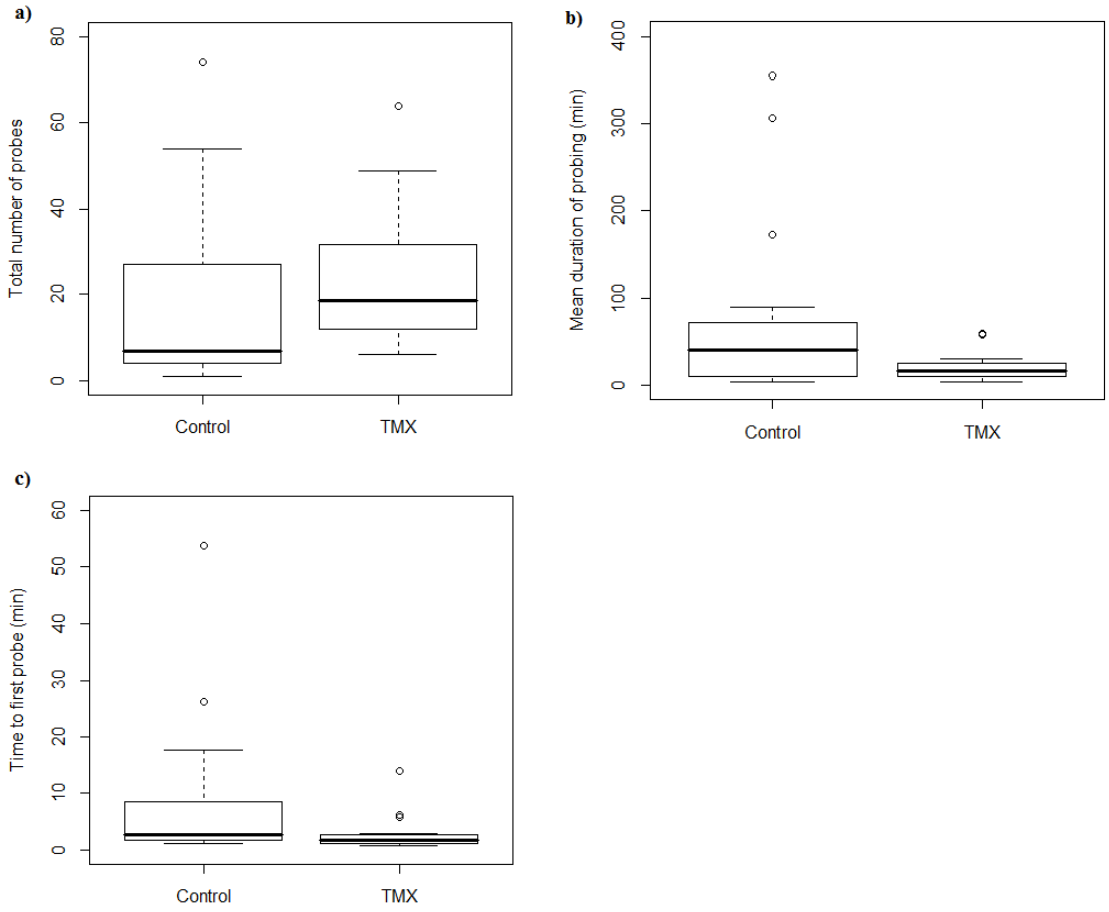


Figure 4.14 Feeding behaviours exhibited differentially by the *Myzus persicae* clone US1L when offered either a thiamethoxam (TMX) treated or control treated sachet of artificial diet. Control treated diet contained an amount of acetone equal to that required to dissolve the TMX AI in the artificial diet. a) Total number of probes performed into the diet b) Mean duration of probing c) Time taken to perform the first probe from the start of the experiment.

The only behavioural parameter which differed between US1L offered TMX and control treated host plants during the artificial diet vs. plant experiment was the total time spent probing (Tables 4.1 & 4.7), which was greater on control treated as opposed to TMX treated plants (Figure 4.15). There was no significant difference in the numbers of US1L performing

E1 ($\chi^2 = 2.09$, d.f = 2, $p > 0.05$) or E2 ($\chi^2 = 2.08$, d.f. = 2, $p > 0.05$) between TMX treated and untreated plants. Note that such low numbers of individuals performed G and F that no statistical comparison of these parameters was possible for this experiment.

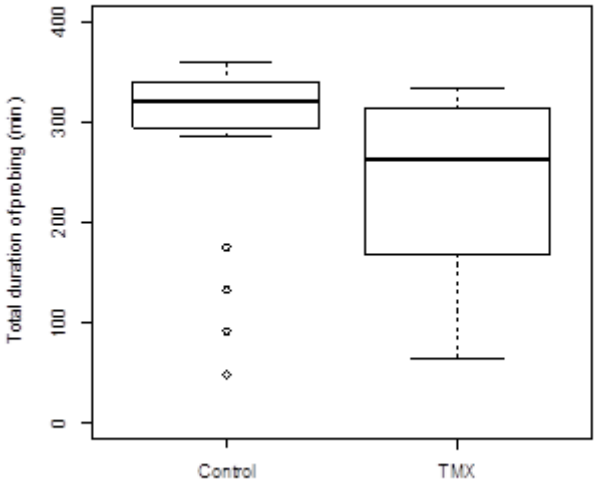


Figure 4.15 The total time spent probing exhibited by *Myzus persicae* of the US1L clone on host plants root drenched with thiamethoxam (TMX) or control treated with Milli-Q water.

Table 4.7 Feeding behaviours performed by the *Myzus persicae* clone US1L on host plants either root drenched with thiamethoxam (TMX) or root drenched with Milli-Q water. Differing superscript letters indicate a significant difference between behavioural parameters. Error bars represent ± the standard error.

	Control	TMX
Total time in C (min)	181.29 ± 80.45	197.13 ± 16.42
Number of C	22.62 ± 16.23	24.46 ± 24.46
Mean duration of C (min)	13.68 ± 5.02	10.12 ± 1.02
Number of E1	1.76 ± 1.13	1.17 ± 0.23
Number of E2	1.29 ± 0.26	0.96 ± 0.24
Number of sustained E2 (> 10 min)	0.81 ± 0.82	0.63 ± 0.17
Time to first probe (min)	4.58 ± 31.32	11.01 ± 6.39
Time taken to reach E1 from first probe (min)	181.63 ± 122.58	206.62 ± 25.02
Time taken to reach E2 from first probe (min)	220.56 ± 128.86	233.62 ± 26.30
Total Time in E1 (min)	10.14 ± 13.33	7.09 ± 3.33
Mean duration of E1 (min)	5.59 ± 12.60	5.37 ± 3.15
Number of probes to the first E1	16.14 ± 15.31	17.71 ± 3.12
Total time in E2 (min)	132.87 ± 53.32	53.74 ± 13.77
Mean duration of E2 (min)	105.35 ± 48.15	37.28 ± 13.36
% contribution of E1 to the phloem phase	22.07 ± 37.48	28.14 ± 9.37
Total number of pd	75.62 ± 39.62	75.83 ± 8.09

4.3.3 Inherent feeding behavioural differences between clones

When the behaviour of the three clones was compared in the absence of TMX treatment, in the initial LD_{15} experiment 5191A exhibited a significantly greater probing time than the FRC clone (Figure 4.16). This difference however, was not present between the same clones in completely control-treated conditions in the LD_{20} experiment.

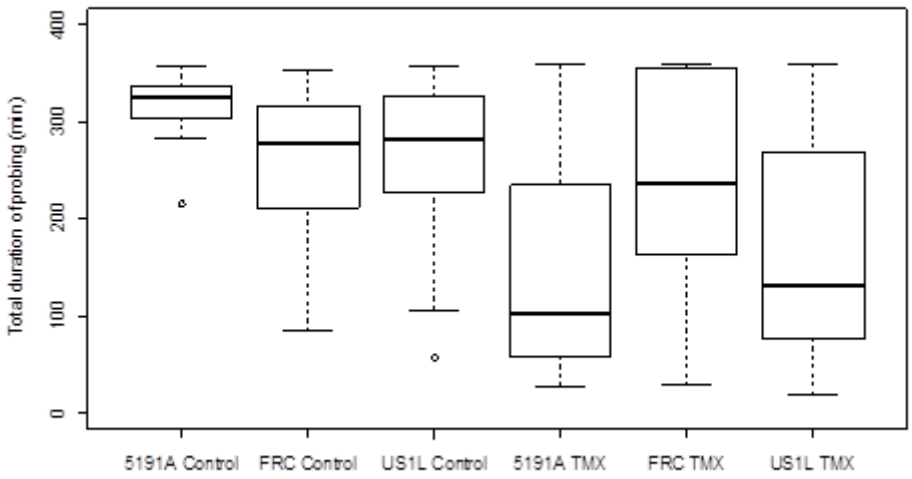


Figure 4.16 The total probing time exhibited by *Myzus persicae* of the clones US1L, 5191A and FRC when offered either host plants root drenched with thiamethoxam (TMX) at the LD_{15} concentration for the respective clone, or else control plants drenched with Milli-Q water.

The mean duration of C shown by 5191A was also greater than that shown by both US1L and FRC (Figure 4.17) when comparing the behaviour of aphids of different clones on completely control treated plants during the LD_{15} experiment. Again, this difference was not seen in the LD_{20} tests.

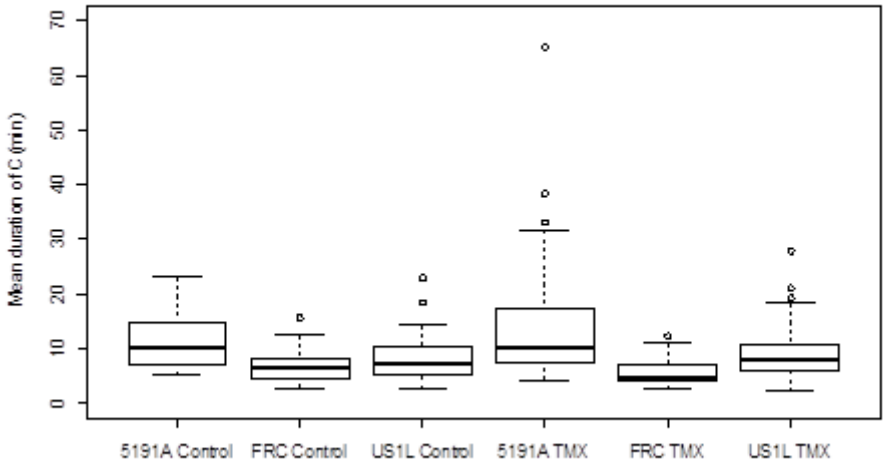


Figure 4.17 The mean duration of C performed by *Myzus persicae* of the clones US1L, 5191A and FRC when offered either host plants root drenched with thiamethoxam (TMX) at the LD_{15} concentration for the respective clone, or else control plants drenched with Milli-Q water.

Conversely, during the LD_{20} experiment 5191A exhibited a significantly greater total duration of E1 than US1L or FRC upon control treated plants (Figure 4.18), but there was no significant difference in this behaviour between the three clones during the LD_{15} experiment.

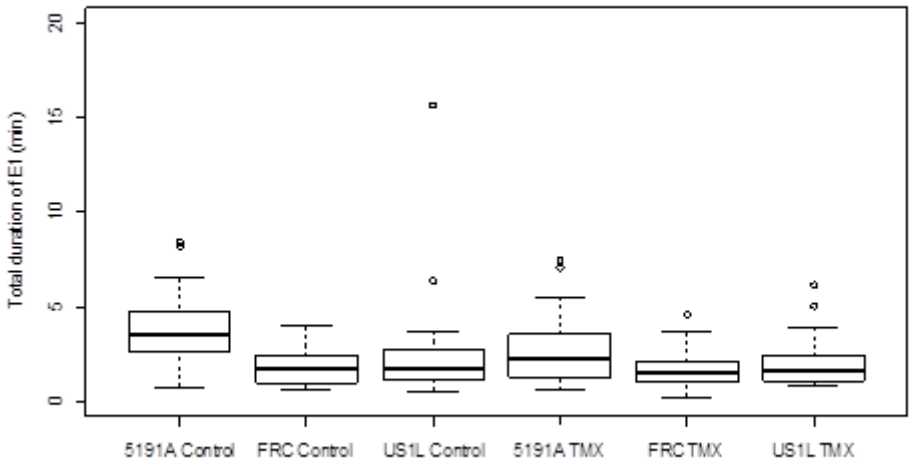


Figure 4.18 The total duration of E1 performed by *Myzus persicae* of the clones US1L, 5191A and FRC when offered either host plants root drenched with thiamethoxam (TMX) at the LD_{20} concentration for the respective clone, or else control plants drenched with Milli- Q water.

The FRC clone exhibited a lower mean duration of E1 upon control treated plants than either US1L or 5191A during the LD_{20} experiments (Figure 4.19). This behavioural difference was not seen when aphids were offered control treated plants during the LD_{15} experiment.

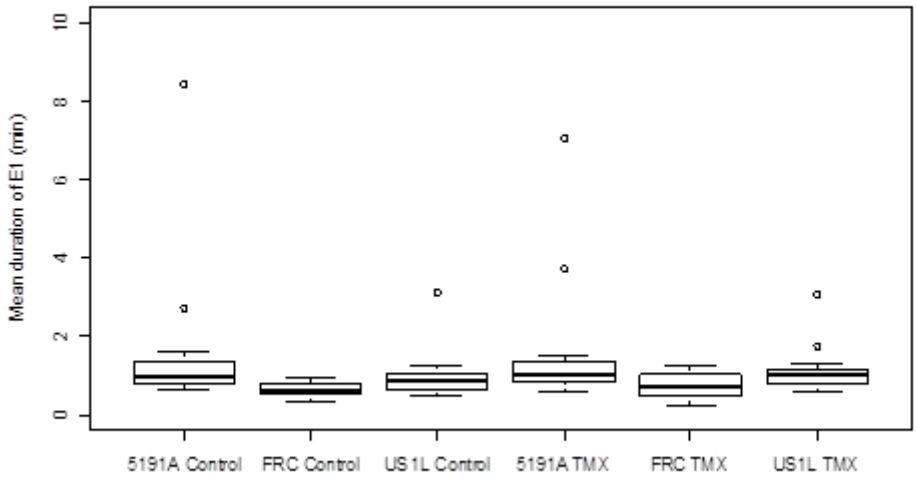


Figure 4.19 The mean duration of E1 exhibited by *Myzus persicae* of the clones US1L, 5191A and FRC when offered either host plants root drenched with thiamethoxam (TMX) at the LD_{20} concentration for the respective clone, or else control plants drenched with Milli-Q water.

There were no significant differences between the numbers of aphids of different clones performing E1, E2, G or F on control treated host plants, either at the LD_{15} or the LD_{20} (Table 4.6).

4.3.4 The effect of thiamethoxam on feeding behaviour between clones

When the feeding behaviour of the three clones was compared on host plants root drenched at the LD_{15} nine behavioural parameters were performed significantly differently between the clones (Tables 4.2 & 4.3). The total number of C (Figure 4.20), total number of probes (Figure 4.21), number of probes to the first E1 (Figure 4.22), time to E1 from the first probe (Figure 4.23) and time to E2 from the first probe (Figure 4.24) were all significantly greater for the FRC clone than 5191A. The total time probing (Figure 4.16) and mean duration of C (Figure 4.17) were greater for the 5191A clone than FRC. The total duration of E1 was greater for the FRC than the susceptible clone US1L (Figure 4.25). None of these behaviours were found to be significantly different between the clones when the LD_{20} dose was applied rather than the LD_{15} (Tables 4.4 & 4.5).

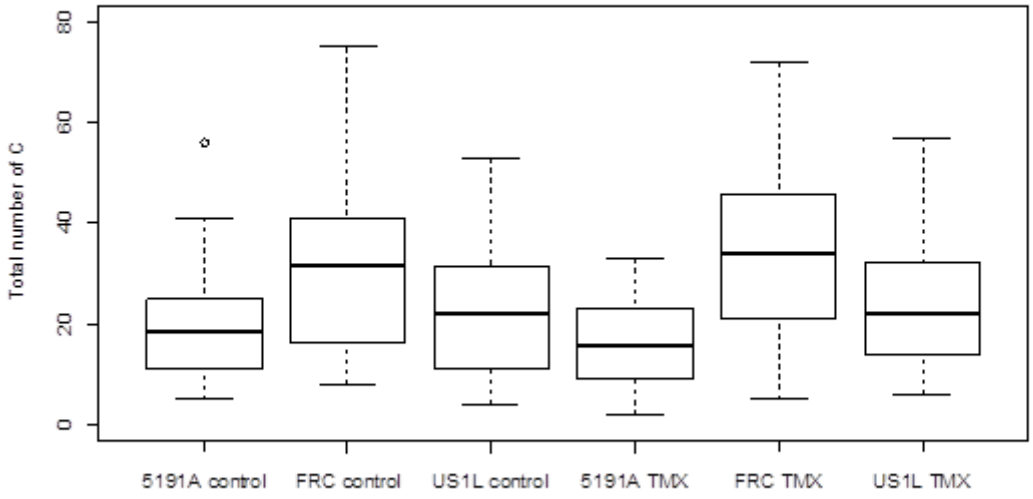


Figure 4.20 The total number of C exhibited by three *Myzus persicae* clones, US1L, 5191A and FRC, on plants root drenched with thiamethoxam (TMX) at the LD_{15} concentration for the respective clone, or else control plants drenched with Milli-Q water.

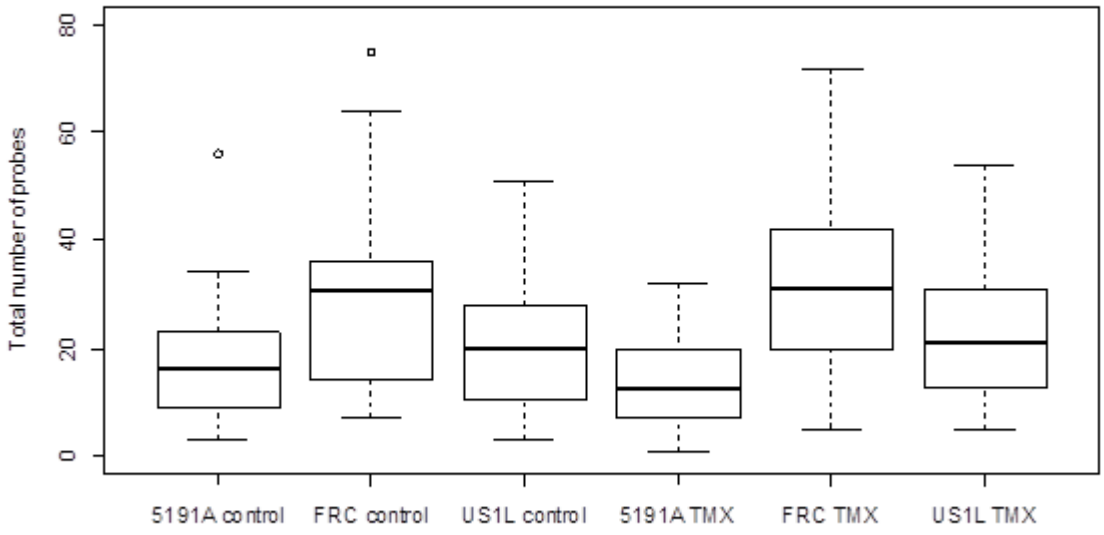


Figure 4.21 The total number of probes exhibited by three *Myzus persicae* clones, US1L, 5191A and FRC, feeding on plants root drenched with thiamethoxam (TMX) at the LD_{15} concentration for the respective clone, or else control plants drenched with Milli-Q water.

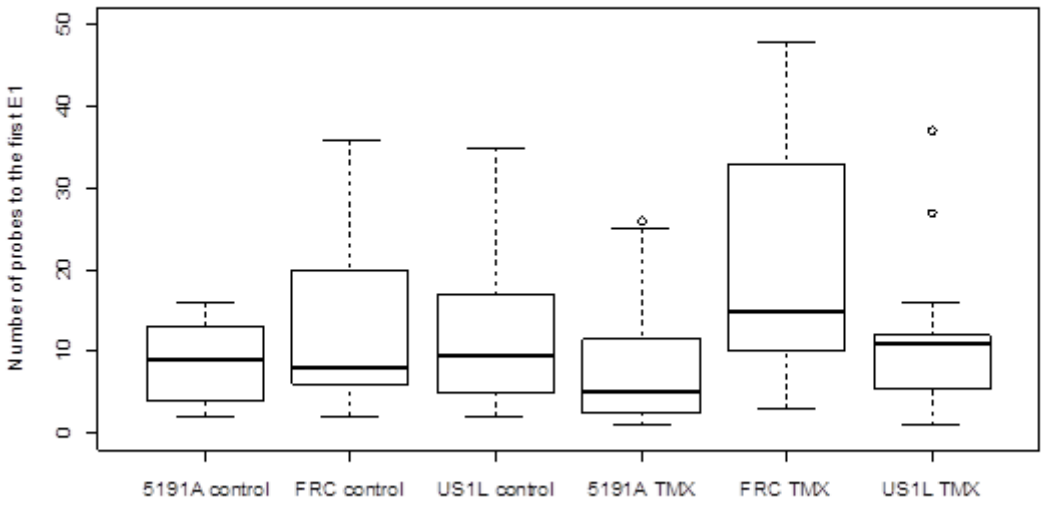


Figure 4.22 The total number of probes to the first E1 exhibited by three *Myzus persicae* clones, US1L, 5191A and FRC, on plants root drenched with thiamethoxam (TMX) at the LD_{15} concentration for the respective clone, or else control plants drenched with Milli-Q water.

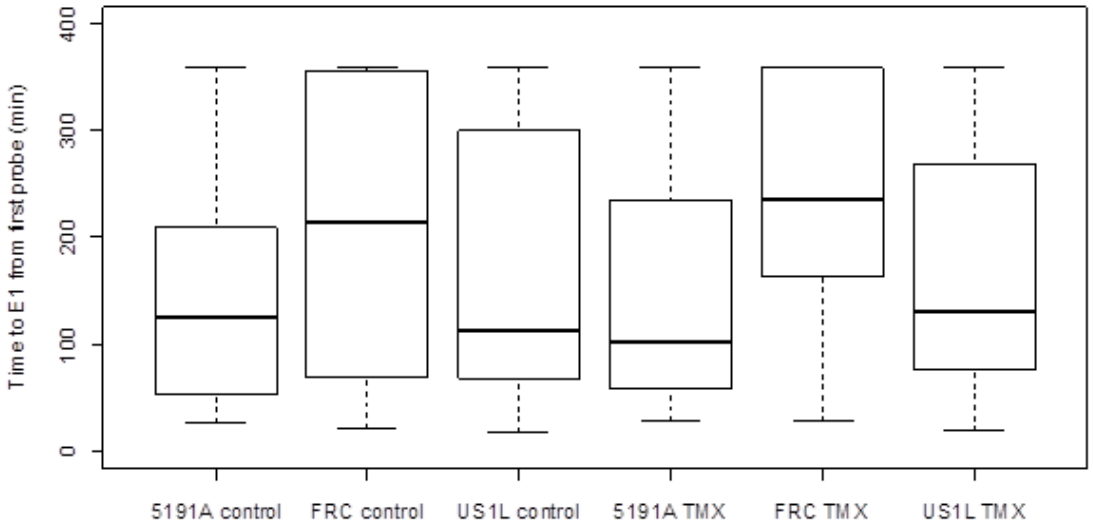


Figure 4.23 The time to E1 from the first probe exhibited by three *Myzus persicae* clones, US1L, 5191A and FRC, on plants root drenched with thiamethoxam (TMX) at the LD_{15} concentration for the respective clone, or else control plants drenched with Milli-Q water.

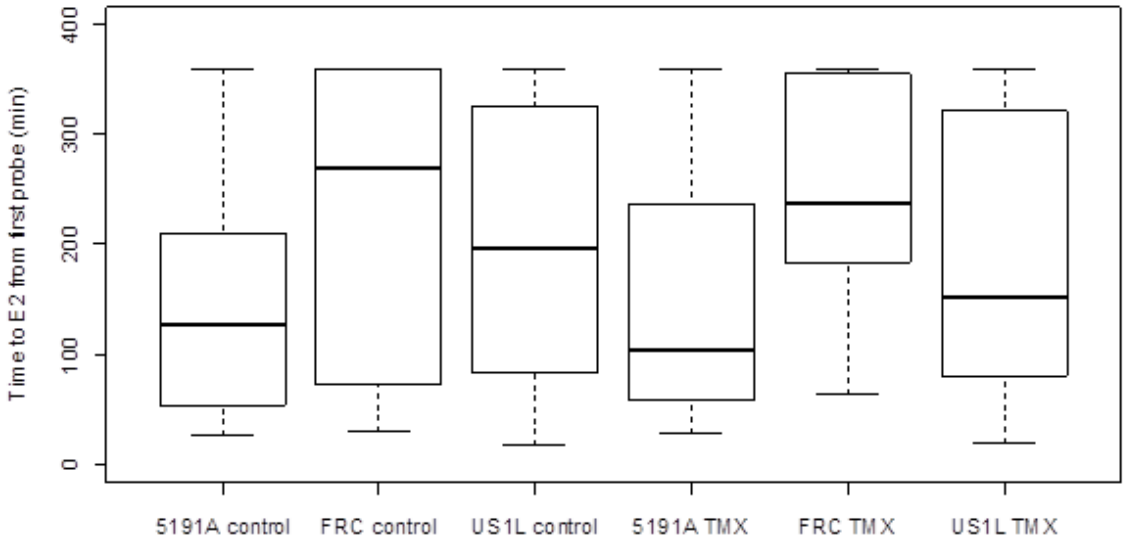


Figure 4.24 The time to E2 from the first probe exhibited by three *Myzus persicae* clones, US1L, 5191A and FRC, on plants root drenched with thiamethoxam (TMX) at the LD_{15} concentration for the respective clone, or else control plants drenched with Milli-Q water.

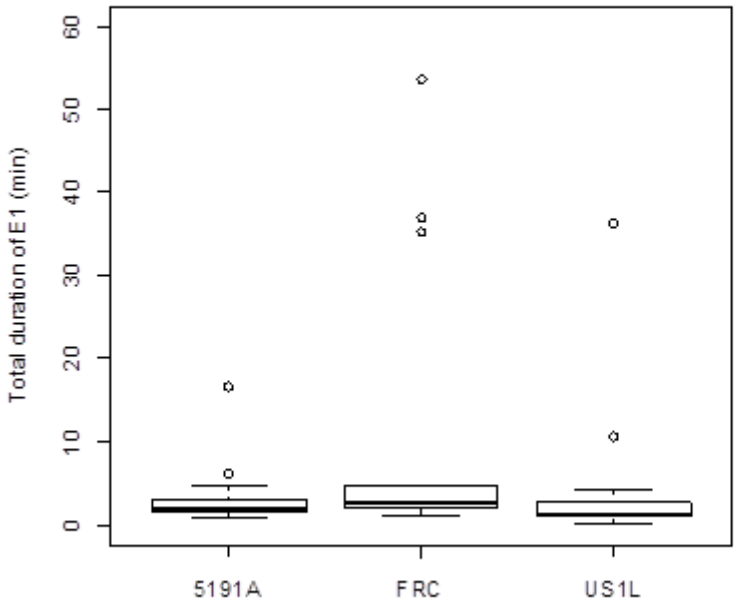


Figure 4.25 The total duration of E1 exhibited by three *Myzus persicae* clones, US1L, 5191A and FRC, on plants root drenched with thiamethoxam (TMX) at the LD_{15} concentration for the respective clone.

When the feeding behaviour of the three clones was compared on host plants root drenched at the LD_{20} the only the mean duration of E1 was found to be significantly different between the clones (Tables 4.4 & 4.5), with the mean duration of E1 performed by the 5191A clone significantly greater than that performed by the FRC clone (Figure 4.19). This behaviour was no longer significantly different between clones when the LD_{15} was applied rather than the LD_{20}.

There were no significant differences between the numbers of aphids of different clones performing E1, E2 or F upon TMX treated host plants, either at the LD_{15} or the LD_{20} (Table 4.6). A significantly greater number of US1L than 5191A performed G upon plants treated with the LD_{15} concentration of TMX (Figure 4.26). However, the numbers of aphids of all three clones performing G remained low. There was no significant difference between the numbers of aphids of different clones performing G on plants treated at the LD_{20} concentration of TMX.

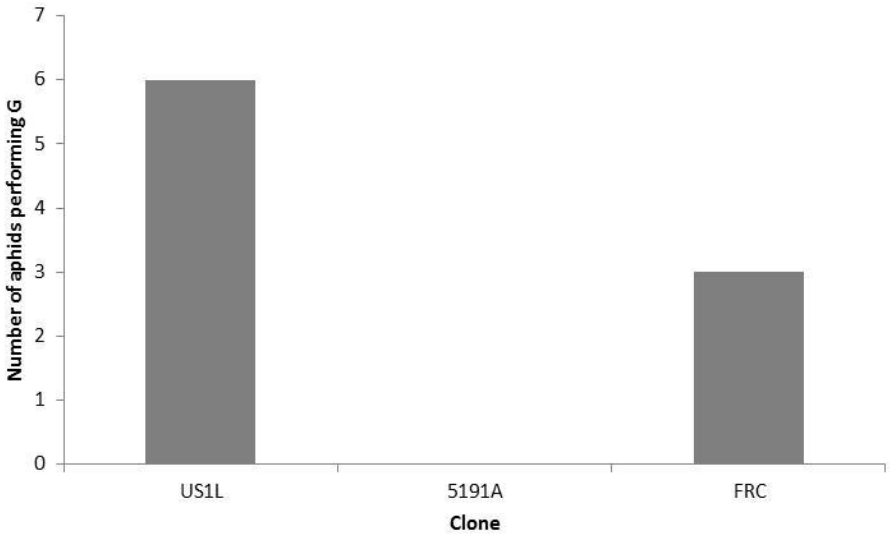


Figure 4.26 The numbers of *Myzus persicae* of different clones performing xylem feeding (EPG waveform G) on plants treated with thiamethoxam (TMX) at the LD_{15} concentrations specific to each clone.

4.3.5 Differences in feeding behaviour with changing sublethal dose

There were several behavioural parameters that differed significantly between the LD_{15} and LD_{20} experiments (Tables 4.8 & 4.9). While the feeding behaviour of the clones under these different concentrations cannot be compared directly, as they were carried out in separate experiments at different times of year, the differences remain of interest.

The majority of parameters that differed between the LD_{15} and LD_{20} experiments were performed for greater amounts of time during the LD_{20} experiment (Figure 4.27). The clones US1L and 5191A spent a greater total amount of time probing under TMX treated conditions at the LD_{20} than the LD_{15}. Although the FRC clone showed no difference in probing duration between the two experiments it did exhibit a greater mean duration of C in the LD_{20} experiment than the LD_{15} experiment on TMX treated plants. The total time spent in C by both the US1L and FRC clones and the total number of probes performed by 5191A within the first ten minutes of an EPG recording, were also all significantly greater in the LD_{20} than the LD_{15} experiments on control treated plants. There was no significant differences in these behaviours between the two experiments on TMX treated plants. The 5191A clone exhibited a greater duration of E1 in the LD_{20} than the LD_{15} experiment upon both TMX and control treated plants (Figure 4.28). In addition, a significantly greater number of FRC performed the F wave upon control plants in the LD_{20} as opposed to the LD_{15} experiment (Figure 4.29).

Table 4.8 p values showing the difference in behavioural parameters between two separate experiments, one designed to observe the effect of applying the LD_{15} concentration of TMX to three different aphid clones, and one designed to observe the effect of applying the LD_{20} concentration of TMX to three different aphid clones. p values < 0.05 indicate a significant difference in behavioural parameters between the two experiments for the relevant aphid clone and host plant treatment. Bolded values represent a significant difference between clones for that behavioural parameter.

	US1L		5191A		FRC	
	Control	TMX	Control	TMX	Control	TMX
Total time probing (min)	0.14	**0.001**	0.60	**0.0003**	0.10	0.24
Number of probes	0.58	0.43	0.85	0.59	0.64	0.21
Total time in C (min)	**0.05**	0.12	0.52	0.35	0.018	0.19
Number of C	0.55	0.52	0.66	0.54	0.66	0.16
Mean duration of C (min)	0.50	0.10	0.81	0.81	0.12	**0.02**
Number of E1	0.49	0.81	0.35	0.70	0.44	0.93
Number of E2	0.16	0.65	0.25	0.54	0.37	0.57
Number of sustained E2 (> 10 min)	0.46	0.83	0.90	0.54	0.49	0.26
Time to first probe (min)	0.40	0.15	1.00	1.00	0.10	0.21
Time taken to reach E1 from first probe (min)	0.55	0.97	0.84	0.29	0.68	0.08
Time taken to reach E2 from first probe (min)	0.52	0.078	0.47	0.47	0.47	0.28

Table 4.9 p values showing the difference in behavioural parameters between two separate experiments, one designed to observe the effect of applying the LD_{15} concentration of TMX to three different aphid clones, and one designed to observe the effect of applying the LD_{20} concentration of TMX to three different aphid clones. p values < 0.05 indicate a significant difference in behavioural parameters between the two experiments for the relevant aphid clone and host plant treatment. Bolded values represent a significant difference between clones for that behavioural parameter.

	US1L		5191A		**FRC**	
	Control	TMX	Control	TMX	Control	TMX
Total Time in E1 (min)	0.53	0.65	**0.05**	**0.05**	0.61	**0.009**
Mean duration of E1 (min)	0.25	0.58	0.06	0.06	0.75	0.08
Number of probes to the first E1	0.60	0.92	0.62	0.62	0.56	0.66
Total time in E2 (min)	0.59	0.65	0.10	0.05	0.48	0.15
Mean duration of E2 (min)	0.42	0.43	0.05	0.10	0.98	0.06
% contribution of E1 to the phloem phase	0.71	0.70	0.08	0.08	0.68	**0.006**
Total number of pd	0.13	0.63	0.11	0.06	0.89	0.12
Total number of pds in first 10 min	0.19	0.20	**0.05**	0.70	0.82	0.67
Total duration of G	0.07	0.52	0.25	0.25	0.77	n.a.
Number of G	0.74	0.50	0.99	0.99	0.81	0.62
Total duration of F	0.24	0.41	0.70	0.70	n.a.	n.a.
Number of F	0.83	0.59	1.00	1.00	0.12	0.27

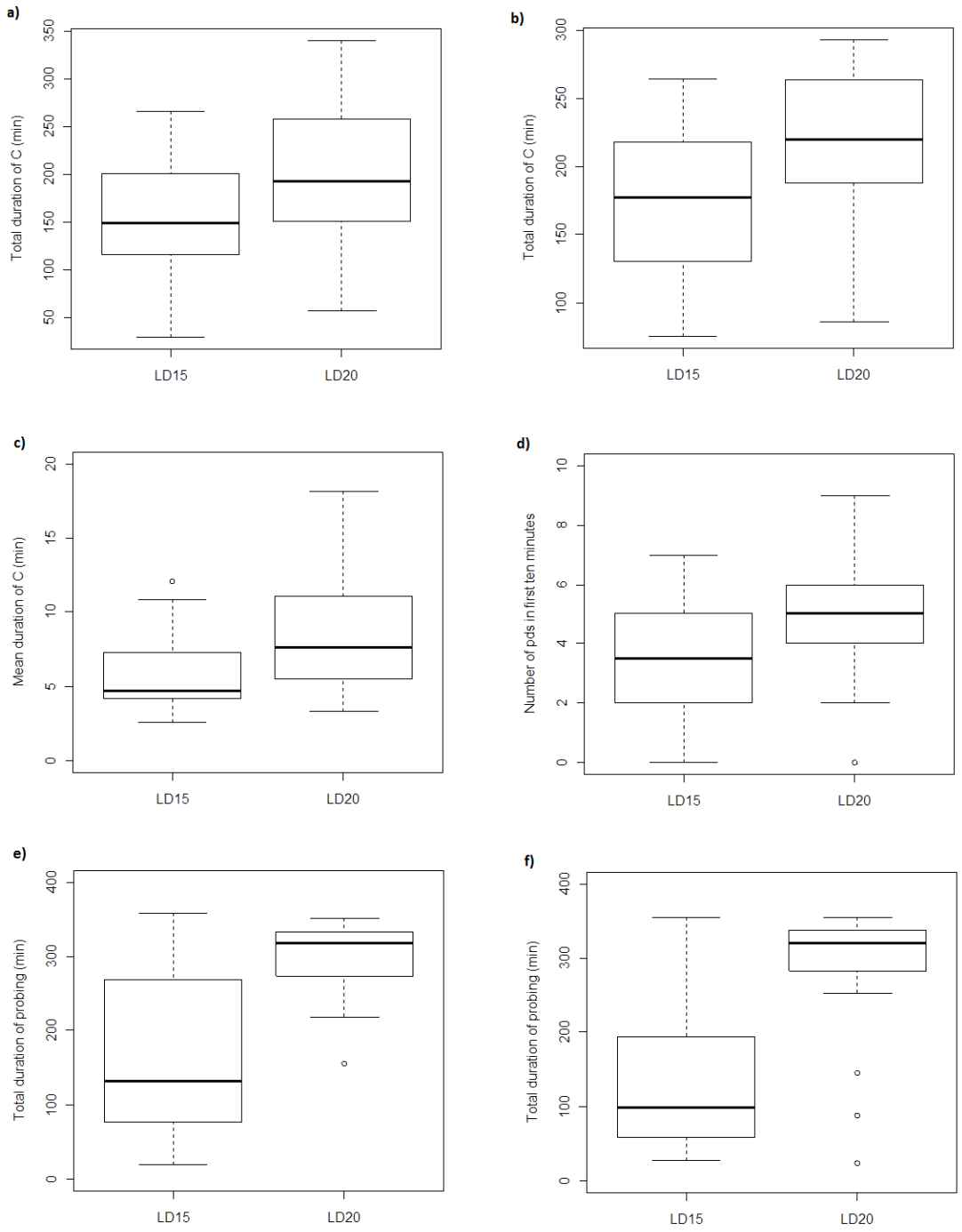


Figure 4.27 Behaviours shown to be significantly different between the LD_{15} and LD_{20} experiments when performed by the same aphid clone under the same treatment conditions. a) US1L on control treated plants b) FRC on control treated plants c) FRC on thiamethoxam (TMX) treated plants d) 5191A on control treated plants e) 5191A on TMX treated plants f) US1L on TMX treated plants.

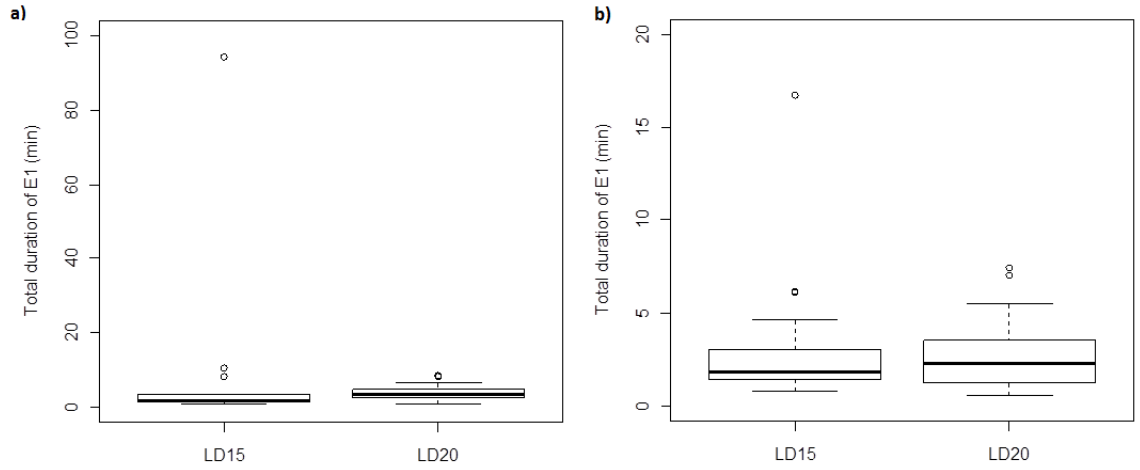


Figure 4.28 The total duration of E1 exhibited by the *Myzus persicae* clone 5191A as shown in the LD_{15} and LD_{20} experiments under the same conditions. A) 5191A on control treated plants b) 5191A on thiamethoxam treated plants

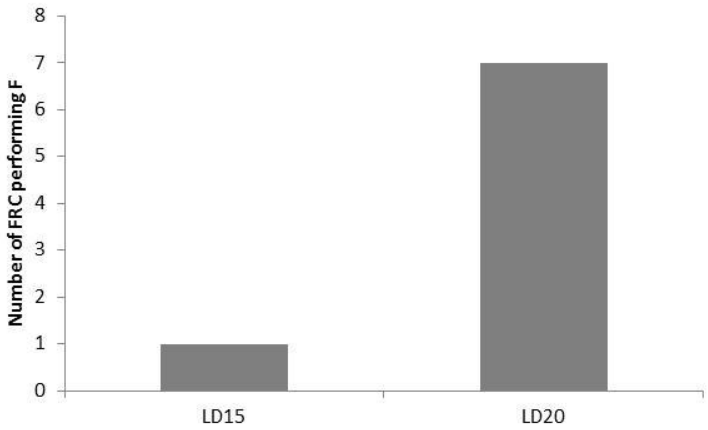


Figure 4.29 The number of *Myzus persicae* of the FRC clone performing F (disrupted stylet mechanics) when placed upon untreated plants in two separate experiments, one to see behaviour of aphids on plants treated with thiamethoxam at the LD_{15} concentration and one with plants treated at the LD_{20}. The aphids in the figure were placed upon plants forming the control for the separate experiments.

The FRC clone exhibited a greater mean duration of C on TMX treated plants during the LD_{20} experiment than the LD_{15} experiment. The total time spent in E1 and the percentage contribution of E1 to the phloem phase exhibited by this clone were greater, however, during the LD_{15} experiment than the LD_{20} experiment (Figure 4.30).

The behavioural parameters which differed between the LD_{15} and LD_{20} experiments (Table 4.6) did not correlate to those behaviours which were significantly different on TMX treated

and untreated plants in the LD_{15} experiment (Tables 4.2 & 4.3) but not the LD_{20} experiment (Tables 4.4 & 4.5), or the converse.

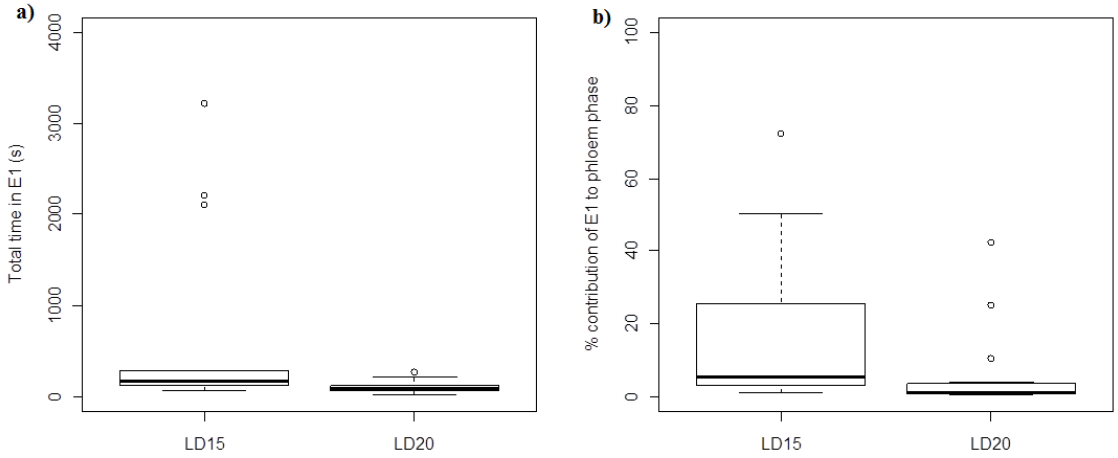


Figure 4.30 The a) total time spent in E1 and b) percentage contribution of E1 to the phloem phase exhibited by *Myzus persicae* of the FRC clone during the LD_{15} and LD_{20} experiments under the same conditions.

Please see Appendix 8.3 for the results of further statistical tests of interest not included in this chapter.

4.4 Discussion

4.4.1 The presence or absence of feeding behaviourally mediated neonicotinoid resistance in *Myzus persicae*

The behavioural parameters which significantly differed between the three *M. persicae* clones when offered TMX treated plants during the LD_{15} experiment were not consistent with those behaviours which significantly differed during the LD_{20} experiment. This may, however, not be due to the change in TMX concentration. The LD_{15} and LD_{20} experiments were not designed to investigate the effect of TMX concentration upon *M. persicae* feeding behaviour, but rather to examine the effect of the presence of TMX on feeding behaviour independently for the two concentrations. The purpose of repeating the original experiment at the LD_{15} using the LD_{20} dose instead was to see if several behaviours which showed a common pattern of being exhibited differently by the three clones but non significantly, would become further differentiated between clones when a greater TMX concentration was applied and so would become significantly different. This, however, did not occur. Equally, the behavioural parameters which varied significantly between the three *M. persicae* clones upon control treated plants during the LD_{15} and LD_{20} experiments were not consistent. This lack of consistency in terms of significantly differing behaviours in the LD_{15} and LD_{20} experiments makes it difficult to determine whether the three clones consistently significantly perform certain behavioural parameters differently, both in the presence and absence of TMX.

The majority of behaviours which were significantly different between clones differed between the two resistant clones, 5191A and FRC, rather than a resistant and susceptible clone. This makes these behavioural changes unlikely to contribute to neonicotinoid resistance. The only behavioural change which significantly differed between a resistant and susceptible clone was the total time spent in E1, with the FRC clone exhibiting significantly longer periods of phloem salivation than US1L during the LD_{15} experiment. It should be noted however that when offered control treated plants in the LD_{20} experiment almost the converse occurred, with the mean duration of E1 exhibited by the FRC significantly lower than that of US1L. It is therefore difficult to interpret this behaviour.

Although TMX is known to concentrate in the xylem (Maienfisch *et al.,* 2001a) it is well documented that it gives good control of piercing sucking pests that feed from the phloem (Maienfisch *et al.,* 2001b; Torres & Ruberson, 2004; Fuentes-Contreras *et al.*, 2007; Elbert *et al.,* 2008; Magalhaes *et al.,* 2009). The decreased duration of E1 exhibited by the FRC clone

in the LD_{20} experiment could potentially contribute to neonicotinoid resistance, as it reduces exposure to the phloem. For differences in feeding behaviour between clones to significantly contribute to their neonicotinoid resistance it would, however, be expected that a decrease in the duration and frequency of E2 would also occur, particularly as phloem feeding is often performed for longer than phloem salivation (Prado & Tjallingii, 1994). This was not seen for any clone at either TMX concentration.

In addition to this, any alterations in xylem feeding behaviour would also be expected to contribute to neonicotinoid resistance, given that TMX is highly concentrated within the xylem (Maienfisch *et al.,* 2001a). 5191A exhibited the G waveform significantly less than US1L upon TMX treated plants during the LD_{15} experiment, supporting this theory. However, the numbers of individuals of all three clones which performed waveform G were extremely low in all three experiments; hence this result is not very powerful statistically. Preliminary work using pea instead of Chinese cabbage (not shown) suggested that the lack of xylem feeding exhibited by the *M. persicae* may be a result of this particular combination of aphid and host plant species. Aphids of several species including *M. persicae* have been shown to vary their feeding behaviour when feeding from different plant species (Gabrys & Pawluk, 1999; Bournoville *et al.,* 2004; Davis & Radcliffe, 2008; Kordan *et al.,* 2008), and *M. persicae* appears to perform G infrequently upon Chinese cabbage compared to when feeding upon pea plants. Boquel *et al.* (2011) showed that apterous *M. persicae* did not exhibit xylem feeding upon *Solanum tuberosum* L., as opposed to alate individuals of the same clone. In order to determine whether 5191A shows a significantly reduced frequency of xylem feeding which contributes to the neonicotinoid resistance of this clone the experiment would have to be repeated in a situation in which much greater levels of waveform G were observed, for example by using a different host plant. In addition to this there was no significant difference in the frequency of xylem feeding observed between US1L and 5191A in the LD_{20} experiment.

When offered control treated plants during the LD_{15} experiment, the FRC clone probed more frequently but performed waveform C for shorter periods than 5191A. The FRC clone also exhibited enhanced phloem salivation activities upon TMX treated plants during the LD_{15} experiment, taking significantly longer to reach E1 and E2, performing more probes before entering E1 and performing E1 for longer than 5191A. Aphids have been shown to increase phloem salivation behaviour and probe more frequently but for shorter periods on non-host as opposed to host plants and resistant as opposed to non-resistant cultivars (Montllor &

Tjallingii, 1989; Cole, 1994; Paul *et al.,* 1996; Sauge *et al.,* 1998a; Pompon & Pelletier, 2012). For example, *Aphis craccivora* (Koch) exhibited an increased frequency of probing and a reduced duration of phloem feeding upon a resistant as opposed to a susceptible cowpea cultivar (Mesfin *et al.,* 1992) while *Brevicoryne brassicae* (L.) has been shown to exhibit an increased duration and frequency of E1 and a decreased duration and frequency of E2 upon non-host plant species (Gabrys & Pawluk, 1999). The increase in the performance of these behaviours by the FRC clone therefore suggests that this clone found plants treated with TMX at the LD_{15} less acceptable as host plants than 5191A. During the LD_{20} experiment however, the converse occurred with 5191A exhibiting a significantly greater mean duration of E1 than the FRC clone. The function of this discrepancy in behaviours between the two resistant clones is therefore not entirely clear.

Since it is not unknown for different clones of the same species to show differences in feeding behaviour, even upon the same host plant species, cultivar and variety (Wilkinson & Douglas, 1998; Bournoville *et al.,* 2004; Pallipparambil *et al.,* 2010), it is possible that the differences in behaviour between the *M. periscae* clones are linked to something other than neonicotinoid resistance, such as their host plant of origin. Although all three clones were reared on Chinese cabbage prior to the EPG tests, US1L originated on sugar beet in the UK, 5191A on tobacco in Greece (Puinean *et al.,* 2010), and the FRC clone on peach in France (Bass *et al.,* 2011). All three clones therefore originated on different plant species to Chinese cabbage and may find it suitable as a host plant to a greater or lesser extent. A clone which found Chinese cabbage less suitable as a host might be expected to perform behaviours such as increased phloem salivation and an increased number of short entries into pathway, given that such behaviours are commonly performed on host plants of lower suitability such as resistant cultivars (Montllor & Tjallingii, 1989; Cole, 1994; Paul *et al.,* 1996; Sauge *et al.,* 1998a; Pompon & Pelletier, 2012). Six different clones of the pea aphid *A. pisum* which originated upon different host species have been shown to significantly differ in frequency of probing and duration of E1 when feeding upon *V. faba* (Wilkinson & Douglas, 1998). These differences in feeding behaviour did not appear however, to correlate with the acceptability of *V. faba* to each of the clones.

The theory that the three *M. persicae* clones are exhibiting significant differences in feeding behaviour which are not linked to neonicotinoid resistance is supported by the comparison of the behaviour of the three clones in the absence of TMX. As with TMX treated plants, any behaviours which differed significantly between the three clones on untreated control plants

were not consistent across both the LD_{15} and LD_{20} experiments. In the LD_{15} experiment the clone 5191A exhibited a significantly greater total amount of time probing than the FRC clone and a significantly greater mean duration of C than both the FRC and US1L clones. In the LD_{20} experiment the 5191A clone exhibited a significantly greater amount of time in E1 than the US1L and FRC clones, but no significant difference in probing behaviours were observed between clones. Taken together with the findings on TMX treated plants in both the LD_{20} and LD_{15} experiments, it seems likely that 5191A does indeed find Chinese cabbage a more acceptable host plant than the FRC clone, and possibly the US1L clone. It is notable that a greater number of significant differences in behaviour were found between clones on TMX treated plants than untreated plants. It is possible that treating a plant with TMX increases the environmental stress presented to the aphids, enhancing any feeding behaviours commonly exhibited in the presence of a less optimal host plant. These behaviours possibly allow the aphid to cope with the greater environmental stress which occurs on an unsuitable host. This would increase the differences in behaviour exhibited by the 5191A and FRC clones, hence greater numbers of behavioural differences between these clones became significant under TMX treatment. These behaviours therefore do not indicate a behavioural mechanism to cope specifically with the presence of TMX, but rather indicate that the aphid is under greater environmental stress and less inclined to feed.

4.4.2 The effect of TMX on the feeding behaviour of *Myzus persicae*

Application of a sublethal dose of TMX to host plants appears to exert a slight antifeedant effect upon *M. persicae,* decreasing the mean duration of E2 for 5191A when applied at the LD_{20} concentration and increasing the frequency but decreasing the duration of probing in US1L on host plants during the artificial diet study. This supports many earlier studies which have shown neonicotinoids such as imidacloprid to exert an antifeedant effect upon treated aphids, even at low concentrations (Devine *et al.,* 1996; Daniels *et al.,* 2009; Cho *et al.,* 2011; Shi *et al.,* 2011).

4.4.3 Explanations for differences between LD_{20} and LD_{15} experiments

The discrepancies in the behavioural parameters which significantly differed between the three *M. persicae* clones during the LD_{15} as opposed to the LD_{20} experiment make it very difficult to accurately determine whether there are indeed inherent differences in feeding behaviour between the three clones.

It is possible that this lack of consistency between the LD_{15} and LD_{20} experiments is due to them being carried out at different times of year, the LD_{15} experiment over winter and the LD_{20} experiment over summer. Although care was taken to maintain the temperature, light and humidity levels between experiments no controlled lighting was available within the facilities available, and with the presence of windows in the experimental area it is possible that the aphids were able to detect the season via exposure to the natural photoperiod during the EPG trials and altered their feeding behaviour accordingly. This may have occurred despite all three clones being reared under the same controlled conditions prior to undergoing EPG. Equally, although the host plants were reared under environmentally controlled conditions they were within a glasshouse environment and it is possible that the plants were able to detect the change in season via the altered photoperiod between the two experiments. The contents of phloem sap are known to vary seasonally (Corbesier *et al.,* 1998; Gattolin *et al.,* 2008) and plants undergo other physiological changes with season. Some species of aphids appear capable of detecting these changes and altering their feeding behaviour in response (Pescod *et al.,* 2007; Dinant *et al.,* 2010), although to date such behaviour has not been shown in *M. persicae*. It is possible that this alteration in feeding behaviour maximises nutritional gain in terms of sucrose composition of phloem sap (Pescod *et al.,* 2007) but this is not proven. Aphids fed on plants sown during the summer may therefore exhibit different feeding behaviour to individuals of the same clone fed on plants sown during the winter.

The lack of consistency in the results of the LD_{15} and LD_{20} experiments highlights the complexity and plasticity of aphid feeding behaviour, and the need for a repetition of this experiment at a range of TMX concentrations over a range of seasons. Such an experiment could not be carried out during this study due to time constraints, but is suggested in section 4.4.5 below.

4.4.4 The effects of artificial diet and host plant based EPGs

When feeding on artificial diet the *M. persicae* produced different waveforms to those described by Nauen (1995). While this study examined the feeding behaviour of the US1L clone, Nauen (1995) used a clone denoted R3, which is described as being ‘highly resistant to insecticides with a conventional mode of action’ although the specific insecticides were not named. As mentioned earlier, separate clones of the same species may exhibit different in feeding behaviours (Wilkinson & Douglas, 1998; Bournoville *et al.,* 2004; Pallipparambil *et al.,* 2010). It is therefore not unfeasible that the feeding behaviour of the US1L and R3 clones

may differ, especially considering their differences in resistance and potential history of being cultured on different host plants for some time. US1L is completely susceptible to all insecticides and has been reared on Chinese cabbage at Imperial College London for at least 15 years, while the R3 strain was described as being reared on cabbage. It is also worth noting that Nauen (1995) used a sucrose solution while this study used a complex artificial diet containing many amino acids and ions. It is possible that the complex artificial diet used in this study gave a different level of electrical resistance than the sucrose solution used by Nauen (1995), hence the electrical signal would be affected and different waveforms would be produced. This may also explain the discrepancy between the two studies.

Since the waveforms between this study and that of Nauen (1995) did not correlate, no behaviours can be assigned to the waveforms produced on artificial diet other than the insertion of the stylets through the Parafilm membrane and into the solution, denoted 'probing'. Further work would be required to characterise the waveforms produced in this system and correlate them with other behaviours such as salivation. *Myzus persicae* will salivate in response to a decrease in sieve tube pressure (Will *et al.,* 2008). A sachet of artificial diet will unavoidably exert a much lower pressure upon the mouthparts of a feeding aphid than the highly pressurised phloem (Dinant *et al.,* 2010), hence it may be expected that US1L may have been exhibiting behavioural differences such as increased salivation when offered artificial diet as opposed to host plants. It is also reasonable to expect that further differences in feeding behaviour may exist between *M. persicae* feeding on artificial diet and individuals feeding on host plants beyond those shown in this study. The common brown leafhopper *Orosius orientalis* (Matsumura) also exhibits a range of different waveforms detectable by EPG when feeding on artificial diet as opposed to host plants (Trębicki *et al.,* 2012). The lack of ability to correlate the waveforms seen in the artificial diet EPGs of this study however prevent determining whether this were the case. This validates the choice made in this study to compare the feeding behaviours of the susceptible and resistant clones on host plants out of the two methods available.

US1L took significantly longer to perform the first probe when offered untreated artificial diet than an untreated host plant. This is not surprising. Plants provide aphids with a wide range of stimuli which attract them to suitable hosts and induce them to feed. These include odours, colours and possibly textures (Prokopy & Owens, 1983; Hardie, 1989; Pettersson *et al.,* 1994, 2007; Powell *et al.,* 1995b; Park & Hardie, 2004). Even with a green plastic filter attached, artificial diet is unlikely to provide aphids with the same stimuli as a host plant;

hence they may be more reluctant to initially begin feeding. In addition, feeding by *M. persicae* has been shown to locally induce the synthesis of the glucosinolate 4-methoxyindol-3-ylmethylglucosinolate from its precursor indol-3-ylmethylglucosinolate in both *Arabadopsis thaliana* and *Brassica oleracea* (Kim & Jander, 2007). This compound, a known aphid feeding deterrant, may possibly have been present in the entire Chinese cabbage plants but would not have been synthesized within the artificial diet, potentially leading to changes in feeding behaviour between the two systems. It is not clear, however, why this difference in behaviour only occurred when the host plant and artificial diet were untreated and not when they were treated with TMX. Possibly application of TMX extended the time taken to initiate probing on a host plant, hence the time taken to reach the first probe was more comparable to that on a sachet of artificial diet. The time taken to reach the first probe was greater for TMX treated than untreated host plants even though the difference was not significant, supporting this theory. Application of TMX to a host plant may possibly change the stimuli that the *M. persicae* detected from the host plant, making it less appealing to feed from. It should be noted, however, that TMX is not very volatile (Maienfisch *et al.,* 2001a) so it is unlikely that aphids can detect plants treated with TMX by odour. Application of clothianidin, a metabolite of TMX, does appear to induce salicylate-associated plant defence responses in treated plants (Ford *et al.,* 2010). Although this pathway is not normally associated with defence against insect pests (Vlot *et al.,* 2009), it is possible that this may be linked to the increased time for the US1L to probe TMX treated host plants.

The total time spent probing was significantly greater when aphids were offered TMX treated artificial diet rather than TMX treated host plants. No significant difference in the duration of probing occurred between US1L feeding on untreated artificial diet and host plants, however, suggesting that this differentiation of behaviour was caused by the presence of TMX rather than environmental differences caused by the two EPG methods. This was accompanied by US1L exhibiting a significantly greater total duration of probing on untreated as opposed to TMX treated host plants, but there being no significant difference in this parameter between aphids offered TMX treated and untreated artificial diet. This suggests that the US1L may have been responding to a metabolite of TMX in the host plant, reducing the total amount of time spent probing. It is well documented that TMX is metabolised by host plants, producing metabolites which are themselves insecticidal such as clothianidin (Maeinfisch *et al.,* 2001a; Nauen *et al.,* 2003; Casida, 2011). Such metabolites would not be present in the artificial diet assay, hence the lack of changes in behaviour exhibited by US1L. This study therefore

suggests that US1L is responding to the presence of a metabolite of TMX by reducing the total duration of time spent probing. Such metabolites could be identified by processing treated plants using LCMS. Further application of EPGs using these metabolites in artificial diet assays would confirm their effects upon probing behaviour and exclude any effects of the parent compound TMX.

4.4.5 Further work, limitations and conclusions

This study clearly shows that when comparing behaviours between aphid clones a large number of factors must be taken into account with regards to experimental design. It appears that variables such as substituting a host plant for artificial diet, seasonal differences and even small changes in the concentration of insecticide applied may alter any differences in feeding behaviour exhibited between the three clones. It is recommended that in any future studies examining differences in feeding behaviour between aphid clones great care is taken to keep as many environmental factors conserved as possible. The effects of all clones and treatments should always be examined at the same time, as much as the limitations of the EPG technique will allow.

Since the LD_{15} and LD_{20} experiments gave different results but were not comparable, a further experiment incorporating a range of TMX concentrations could be carried out, ideally including the field rate. Although TMX is not generally applied to Chinese cabbage, the maximum recommended application rate of TMX on potato is 400 mg L^{-1}/ha (Syngenta Crop Protection, 2008), so a rate similar to this should be included in future work. In addition since the three clones potentially exhibited seasonal alterations in their feeding behaviour (Dinant *et al.*, 2010), further work comparing the feeding behaviour of the three clones during different seasons would be valuable and allow greater understanding of the differences in behaviour of the three clones to be gained.

Since *M. persicae* feeds differently on host as opposed to non host plants (Davis & Radcliffe, 2008), any differences in feeding behaviour which could potentially contribute to their neonicotinoid resistance may become more apparent on each clones host plant of origin. It would therefore be interesting to examine the feeding behaviour of the FRC clone as compared with a susceptible clone when feeding on peach, and 5191A likewise upon tobacco.

It is also worth noting that US1L, the susceptible clone used in this study, was collected from the field a long time ago. Many studies currently use the clone 4106A as an insecticide susceptible clone for comparison with clones exhibiting insecticide resistance (Foster *et al.*, 2008; Puinean *et al.*, 2010; Bass *et al.*, 2011). This clone was removed from the field much more recently. As the feeding behaviour of the two resistant clones 5191A and FRC varied, so it can be expected that the feeding behaviour of the two susceptible clones US1L and 4106A may vary. It may therefore be more relevant to compare the feeding behaviour of 5191A and FRC to a different susceptible clone such as 4106A. Such a comparison may highlight more consistent differences in feeding behaviour between the susceptible and resistant clones. This may potentially yet highlight a behavioural aspect to the neonicotinoid resistance of the FRC and 5191A clones in terms of altered feeding behaviour.

Only the behaviours of apterous aphids were examined during this study. The behaviours of apterous and alate morphs of two species however, *M. persicae* and *M. euphorbiae*, have been shown to significantly vary in terms of frequency of probing, frequency and duration of intercellular punctures, frequency of phloem salivation and ingestion, and duration of xylem feeding (Boquel *et al.*, 2011). Examining the feeding behaviour of alate individuals of the three *M. persicae* clones may yet reveal the presence of a feeding behaviourally-mediated component to the neonicotinoid resistance of 5191A and FRC.

It should be highlighted that the feeding behaviours exhibited by aphids during the EPG technique will not be identical to behaviour exhibited by aphids feeding freely on host plants in the field. The methodology of the EPG technique exerts some stress upon the aphid which has been shown to cause changes in feeding behaviour (Prado & Tjallingii, 1999; Caillaud, 1999). This is particularly true with regards to the attachment of the wire to the aphid (Tjallingii, 1986). The presence of this extra environmental stress may lead to earlier initiation of probing, an increased duration of the pathway phase, and decreased duration of phloem feeding (Prado & Tjallingii, 1999). EPG however, is frequently used to study the feeding behaviour of Hemiptera and remains a powerful technique for this purpose. The alternative, video recording of aphid feeding, is time consuming and does not allow the same depth of understanding into aphid feeding behaviour, such as phloem salivation and cell punctures, due to the lack of ability to determine the location of the aphid stylets within the plant tissue (Powell *et al.*, 1993; Nam & Hardie, 2012). At present, EPG appears to remain the preferred technique for studying the feeding behaviour of aphids in-depth.

The presence of altered feeding behaviours leading to neonicotinoid resistance, should they exist, could potentially aid the control of neonicotinoid resistant insects. Should the insects reduce feeding from the phloem for example, it may be possible to attempt control with an insecticide which is either highly concentrated in the phloem, or else sequested in plant tissues from which the resistant insects show increased levels of feeding. Knowledge of any differential feeding behaviour exhibited by the neonicotinoid resistant FRC clone would also aid in the development of new chemistries to control this pest, as chemistries which sequester in appropriate plant tissues could be specifically developed.

While the behaviours involved did not appear to contribute to neonicotinoid resistance, this study showed that the three *M. persicae* clones did exhibit differences in feeding behaviour under a wide range of conditions. Given the strong correlation between aphid feeding behaviour and plant virus transmission (Fereres & Moreno, 2009; Brault *et al.,* 2010) these differences in feeding behaviour may potentially lead to differences in vector ability between clones, both under TMX treated and untreated conditions. It is not unknown for aphid clones which differ in vector ability to also exhibit differential feeding behaviour (Pallipparambil *et al.,* 2010), including *M. persicae* (Khouaja *et al.,* 2011). Further work is required to determine whether this is also the case for the neonicotinoid resistant *M. persicae* clones, both for persistent and non-persistent plant viruses. Chapter five outlines a brief study made into this area.

Overall, this study suggests that the clones 5191A and FRC do not exhibit alterations in feeding behaviour which significantly contribute to their neonicotinoid resistance. It is clear however, that all three *M. perisicae* clones do exhibit differences in their feeding behaviour, even if these differences in feeding behaviour do not contribute to neonicotinoid resistance and were not consistent between the LD_{20} and LD_{15} treatments. Improved experimental design may yet potentially reveal a difference in the feeding behaviour of the 5191A and FRC clones that contributes to their neonicotinoid resistance, but the results of this study do not support such a theory. I therefore reject hypothesis three, that "the FRC clone will exhibit differences in feeding behaviour compared to the *M. persicae* clones US1L and 5191A when exposed to a sublethal dose of TMX. This altered feeding behaviour will contribute to the greater neonicotinoid resistance of the FRC clone" as the work shown here does not support such a hypothesis. It is recommended that this study be repeated, but with the revisions discussed taken into account.

Chapter 5: Viral transmission dynamics of neonicotinoid resistant and susceptible clones of *Myzus persicae*

5.1 Introduction

Earlier chapters have shown differences in both feeding and dispersal behaviour between the neonicotinoid resistant FRC and susceptible US1L *M. persicae* clones. Since these behaviours are closely linked to the transmission of plant viruses by aphids (Rice *et al.,* 1983; Ng & Falk, 2006; Fereres & Moreno, 2009; Brault *et al.,* 2010), it is possible that such behavioural differences could also lead to differences in the vectoring capability of these two clones. This chapter aims to investigate this possibility and determine whether the US1L and FRC clones exhibit differences as vectors.

As mentioned during the introductory chapter, aphids may transmit plant viruses by three different transmission methods, non-persistent, semi-persistent and persistent transmission (Ng & Falk, 2006; Hogenhout *et al.,* 2008). In brief, non-persistent viruses are inoculated and acquired by aphid vectors during the pathway phase of feeding, when the stylets enter the intracellular spaces and briefly penetrate epidermal cells (Martín *et al.,* 1997; Powell, 2005). As such, only a relatively short duration of feeding is required to transmit a non-persistent virus. Persistent viruses however must be inoculated into and acquired from the phloem tissue (Prado & Tjallingii, 1994; Limburg *et al.,* 1997). These viruses therefore require a greater duration of feeding of at least several hours in order for the aphid to reach the phloem and the virus to be inoculated or acquired (Katis *et al.,* 2007). Semi-persistent viruses appear to require an intermediate length of feeding time for acquisition and inoculation (Katis *et al.,* 2007), but are also mainly acquired from phloem tissues (Ng & Falk, 2006).

Although ideally the viral transmission ability of US1L and FRC would be compared using both a persistent and a non-persistent virus, time and space limitations made it necessary to limit this study to only a single virus. Given that FRC and US1L were shown to significantly differ in phloem salivation behaviour (See chapter 4), which is essential for the transmission of persistent but not non-persistent viruses, it was decided to use a persistent virus to investigate the vector behaviours of these two clones.

Certain viruses can only be vectored by specific aphid species (Conti *et al.,* 1979; Katis & Gibson, 1984, 1985; Katis *et al.,* 2007), a concept known as ‘vector specificity’, with non-persistent viruses exhibiting lower vector specificity than persistent and semi-persistent

viruses (Pirone & Harris, 1977). This is probably due to the briefer nature of the interaction of a non-persistent virus with the aphid vector. Viral strains may also differ in the efficiency with which they are transmitted by various aphid species (Du *et al.,* 2007; Katis *et al.,* 2007). There is evidence that for some viruses, vector specificity is mediated by the capsid protein which coats the virus, allowing it to bind to the aphid midgut, hindgut or stylets as appropriate (Brault *et al.,* 2005). Other viruses such as *Potyvirus* and *Caulimovirus,* appear to use an additional non-structural viral protein, known as a helper component (HC) to bridge the gap and bind the viral coat protein to vector receptor proteins (Perring *et al.,* 1999). These HC proteins have also been shown to sometimes play a role in vector specificity (Uzest *et al.,* 2007). Although the same virus was to be used to compare the vector ability of the two clones, it was essential that the virus chosen was capable of being efficiently vectored by *M. persicae.*

Since several aphid species have been shown to vary their feeding behaviour on different host plant species (Gabrys & Pawluk, 1999; Bournoville *et al.,* 2004; Davis & Radcliffe, 2008; Kordan *et al.,* 2008) it was not known whether the feeding and dispersal behavioural differences shown by the US1L and FRC clones would remain relevant if a different host plant was chosen. The virus therefore also had to be capable of infecting Chinese cabbage (*B. chinensis cv* Apex). Eventually *Turnip yellows virus* (TuYV) was identified as fitting all of the required criteria.

TuYV is closely related to *Beet western yellows virus* (BWYV), *Beet chlorosis virus* (BCV) and *Beet mild yellowing virus* (BMYV) (Graichen & Rabenstein, 1996) and should not be confused with *Turnip yellow mosaic virus* (TYMV), which is also capable of infecting *B. chinensis* (Dreher, 2004). TuYV, BWYV, BCV and BMYV have only been differentiated with some difficulty by extensive host range analysis (Graichen & Rabenstein, 1996) and phylogenetic studies of their protein sequences (Beuve *et al.,* 2008). *Myzus persicae* appears to be the principal vector of TuYV (Schliephake *et al.,* 2000) and sampling by Broom's Barn research centre has shown that up to 72% of alate *M. persicae* carry TuYV (Stevens *et al.,* 2008). Transmission only occurs through aphid vectors and mechanical and seed-based transmission do not appear possible (Stevens *et al.,* 2008). The main crops of interest which act as hosts include sugar beet and oil seed rape (Duffus & Russell, 1972) but TuYV has also been known to successfully infect *B. chinensis* (Graichen & Rabenstein, 1996).

Much work has also been done on the effects of applying insecticides, including neonicotinoids, on viral spread within crops (Rice *et al.,* 1983; Polston & Sherwood, 2003; Mowry, 2005). Such studies have shown the application of insecticides to be either beneficial in reducing the spread of viral infection or else have a neutral effect, with a reduction in viral infection more common for persistent than non-persistent viruses (Perring *et al.,* 1999). Given the considerable level of neonicotinoid resistance exhibited by the FRC clone it seemed sensible to examine the effect of the application of TMX upon viral transmission by FRC and whether this clone responded differently to the presence of TMX in terms of vector behaviour and efficiency than the susceptible clone US1L. It is well documented that different aphid species may vary in vector behaviour and ability to transmit the same viruses, particularly species specialised towards different host plants (Garzo *et al.,* 2004; Kanavaki *et al.,* 2006; Kozlowska-Makulska *et al.,* 2009; Mello *et al.,* 2011; Tian *et al.,* 2012; Wosula *et al.,* 2012). In some cases different clones of the same aphid species may also vary in vector ability. For example, six clones of the pea aphid *A. pisum* have been shown to exhibit varying ability to transmit *Bean yellow mosaic virus* (BYMV) (Jurík *et al.,* 1980), while several *R. padi* clones varied in their ability to transmit different BYDV isolates (Lucio-Zavaleta *et al.,* 2001). It is therefore possible that the US1L and FRC *M. persicae* clones may also exhibit different efficiencies as vectors of TuYV.

This study investigated the ability of the neonicotinoid resistant clone FRC and the susceptible clone US1L to transmit a persistent virus, TuYV, to TMX treated and untreated Chinese cabbage in a microcosm environment. It represents the first known investigation into the vector ability of the recently discovered neonicotinoid-resistant FRC clone.

5.2 Methods

5.2.1 Plants and aphid cultures

All Chinese cabbage plants (*B. chinensis cv* Apex) and *M. persicae* of the clones US1L and FRC were cultured as described in chapter 2, with the exception of viral plants described below. Two clones of *M. persicae*, US1L and FRC, were separately raised to a known age of 7 days in six well plates before use, as described earlier in chapter 2.2.1. The clone 5191A was excluded due to space limitations within the DEFRA-licenced facility and time.

5.2.2 Virus culture

The TuYV culture was initially obtained from Broom's Barn, Bury St Edmunds, Suffolk, in the form of a viral oil seed rape (*Brassica napus* L.) plant. This virus was initially transmitted to two-week old seedling stage Chinese cabbage plants by placing US1L onto the viral plant for 7 days, where they were allowed to feed and become infected. The aphids were then transferred to seedling stage Chinese cabbage plants, infecting them with TuYV.

TuYV was maintained in a culture consisting of nine virally infected seedling stage Chinese cabbage plants within a 30 x 30 x 15 cm netting cage, kept in a temperature control cabinet set at 22 ± 1 ºC, 16:8 h L:D photoperiod, 50 % r.h.. Plants were stood in groups of three in plastic saucers within the cage and watered as necessary. Three uninfected two-week old Chinese cabbage plants with two true leaves were added to the culture every seven days, and the three oldest virally infected plants removed.

Initially 100 apterous adult *M. persicae* of the US1L clone were added to the cage and then left to vector the virus between plants and reproduce freely. An identical set-up in a separate cage but within the same temperature control cabinet was also maintained using the FRC clone rather than US1L. When viral plants were required, 20 apterous adult individuals were removed from the culture as needed and placed within a 22 cm^3 clip cage on the underside of the leaf of a fresh two-week old seedling stage Chinese cabbage plant. All aphids were removed after 96 h using a soft paint brush. The viral plant was then left for a further 72 h before use in experiments, in order for sufficient levels of the virus to accumulate. Viral plants which were to be used in tests involving the US1L clone were initially infected using US1L, and plants to be used in tests involving the FRC clone were initially infected using FRC.

5.2.3 Thiamethoxam treatment of plants

Plants to be treated with formulated TMX (Actara) were stood on blue roll and left for 24 h at 22 ± 1 ºC, 16:8 h L:D photoperiod, 50 % r.h. to dry out the soil before treatment. 50 ml of TMX (Actara^{TM}) was then poured on to the soil of each plant pot, and the plants left for 24 h to take up the TMX from the soil before use in experiments. Plants were treated with the appropriate LD_{15} concentration for the aphid clone they were to be exposed to, 0.1 ppm or 42.4 ppm for US1L and FRC respectively. Control plants were treated with 50 ml of Milli-Q water instead of TMX.

5.2.4 Test set-up

Eight two-week old Chinese cabbage plants (two true leaf seedling stage) were arranged in a circle as in Figure 5.1. Each plant was 9 cm apart and 12 cm from the exact centre of the circle. The distance between the pot edges of the plants was 2.5 cm. Four plants were treated with TMX and four plants control treated. Treated and untreated plants alternated around the circle, but the same position in the cage consistently contained either a treated or untreated plant throughout all experimental repeats. A three week old viral Chinese cabbage plant at the two to three true leaf seedling stage was placed in the centre of the circle, with the rims of the pot 4 cm from those of the other plants. This viral plant could either be control treated or TMX treated. 200 apterous adult aphids of either the US1L or FRC clone were then very gently brushed on to the central leaves of the viral plant using a soft paint brush to ensure no harm was caused to the aphids.

The set-up was then left under conditions of 22 ± 1 ºC, 16:8 h L:D photoperiod, 50 % r.h. for a total of 72 h. The number of adult aphids on each of the nine plants, the number of aphids on the cage walls and the number of dead aphids were counted 24 h, 48 h and 72 h after the initial placement of aphids on to the plant. At 72 h the numbers of nymphs on each plant were also counted. Any aphids which could not be found were also recorded as dead.

All aphids were then brushed off the plants and the plants placed in a sealed netting cage. Temperature was maintained at 19 ºC ± 1 ºC. Light and humidity levels were not kept constant but the cage was placed next to a sealed window to allow light to reach the plants. This change in environmental conditions was not ideal but necessary due to the lack of space in the DEFRA facilities available. The plants were left under these conditions for a further 4 weeks, in order for the virus to build up to levels detectable by RT-PCR.

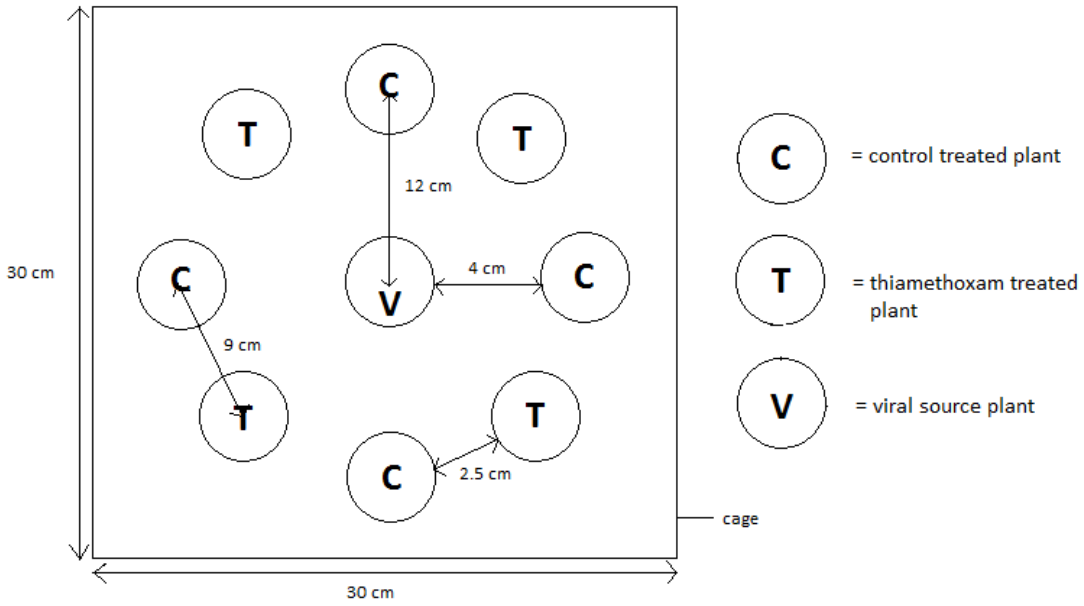


Figure 5.1 Cage set-up for measuring the viral transmission ability of *Myzus persicae.* Each circle represents a two week old two true leaf seedling stage Chinese cabbage plant (*Brassica chinensis* L. *cv* Apex).

At the four week mark, the plants were scored for the presence or absence of viral symptoms, including yellowing of the leaf tips and shrivelling and desiccating of the leaves (Figure 5.2). A small sample of plant tissue (approximately 170 µg) was taken from the tip of a true leaf of each plant, placed into an RNase-free 2 ml microcentrifuge tube, frozen with liquid nitrogen and crushed into powder within the tube using a pestle. The samples were stored at -80 ºC until RT-PCR could be performed.

Since two treatments (TMX treated viral plant and control treated viral plant) were offered separately to two clones, four different treatment-clone combinations were tested. Each treatment-clone combination was repeated five times.

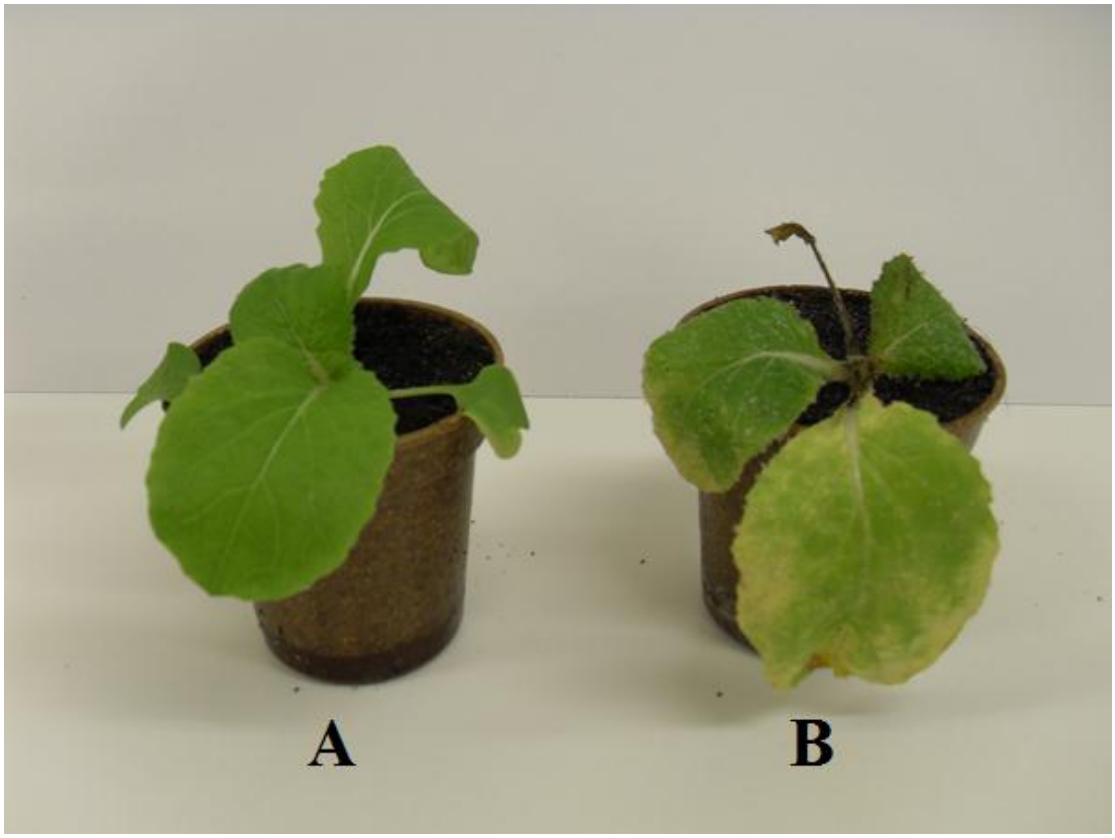


Figure 5.2 Chinese cabbage plants exposed to *Myzus persicae* infected with *Turnip Yellows virus* (TuYV) at two weeks of age, then allowed to grow and develop symptoms for a further two weeks. A) healthy control plant B) TuYV infected plant

5.2.5 RT-PCR of viral plants

Any plants which had not shown viral symptoms were tested for the presence of TuYV RNA by RT-PCR. In addition, 20 samples were tested from plants which had shown viral symptoms, to cross-check that these plants had been accurately scored as TuYV-infected. The methods followed for all molecular work were recommended by Mrs Felicita Vigano of Broom's Barn Research Centre, including the recommended primers.

RNA was purified from the frozen tissue samples using an RNeasy Plant Mini Kit from Qiagen. The protocol for purifying plant tissue provided with the kit was followed, including the option of the use of buffer RLT with the addition of 10 µl β-mercaptoethanol for each 1 ml of buffer RLT used. The quality of the RNA purified from each sample was checked using a NanoDrop^{TM} spectrophotometer.

The reverse transcription reaction was then performed on the RNA to produce viral cDNA using the viral coat protein primers (CP+ 5' ATGAATACGGTCGTGGGTAGGAG 3'; CP- 5' CCAGCTATCGATGAAGAACCATTG 3'). A master mix of reverse transcription stock

solution (Table 5.1) was made up as below and kept on ice. All reagents for the reverse transcription reaction were purchased from Promega.

Table 5.1 Amounts of reagents used to make up a reverse transcription stock solution for the conversion of viral RNA into cDNA.

Reagent	Amount
Nuclease free water	9 µl
5 x RT buffer	4 µl
DTT	2 µl
dNTPs (0.1 M stock)	1 µl
RT enzyme	0.5 µl
Rnasin	0.5 µl
Rev.primer CP- (20 µM stock)	1 µl

18 µl of the master mix reverse transcription stock solution was dispensed into an RNAse-free PCR tube, and 2 µl of RNA sample added. This was performed individually for all samples. The solutions were mixed by pipetting, vortexed gently and spun down in a centrifuge for ten seconds before being incubated in a 37 ºC water bath for 1 h. Once converted to cDNA all samples were stored at -20 ºC.

A PCR master mix (Table 5.2) was then made up in a microcentrifuge tube. 22.5 µl of PCR master mix was dispensed into 0.2 µl PCR tubes containing PCR beads, purchased from GE healthcare. 2.5 µl of cDNA was then added to each tube, which was gently vortexed and briefly spun down in a centrifuge. The PCR reaction was carried out using a PCR machine under the following cycling conditions:

94 ºC x 10 mins

30 x {94 ºC x 1 min, 55 ºC x 1 min, 72 µC x 2 mins}

72 ºC x 10 mins

PCR products were stored at -20 ºC until further needed.

Table 5.2 Amounts of reagents used to make up a PCR stock solution for amplifying viral cDNA.

Reagent	Amount
Nuclease-free water	21.5 µl
Coat protein primer +	0.5 µl
Coat protein primer -	0.5 µl

The PCR products were analysed on a 1% gel containing ethidium bromide with a 1 x TBE buffer. 10 µl of PCR product was mixed by pipetting with 2 µl of loading buffer in a PCR tube. 12 µl of each sample mixed with loading buffer was loaded on to the gel. 7 µl of a 1 kb DNA ladder was loaded alongside the samples. The gel was run at 110 mV. The size of the expected band to indicate the presence of TUYV in the sample was 600 bp.

5.2.6 Statistical analysis

Differences in mortality between clone and treatment were calculated using Analysis of variance tests (ANOVAs) separately for each time point. The percentage of surviving adults at each time period which had left the viral source plant was calculated using the formula below:

$$\%\ \text{of adults that have left the viral plant} = 100 * \frac{\text{Total number of live adults on all test plants and cage sides}}{\text{Total number of live adults in entire set-up}}$$

Any significant differences in the numbers of *M. persicae* leaving the viral source plant and the treatment of the viral source plant with TMX, aphid clone and any interaction between these parameters was then tested for using ANOVAs carried out separately for each time point. Percentage data were not arcsin transformed before analysis by ANOVA because no values were close to the 0% or 100% ends of the percentage scale. The assumptions of the ANOVA test were therefore not invalidated and it was thought that there was little advantage to be gained by transforming the data.

The percentages of adults that left the viral source plant and that were located on the TMX treated tests plants as opposed to the control treated plants were calculated using the formula overleaf:

$$\% \text{ of adults on TMX} = 100 * \frac{\text{Number of adults on TMX treated test plants}}{\text{Total number of adults that left the viral source plant}}$$

ANOVAs were then carried out separately at each time point to see if the proportion of adults on the TMX as opposed to control treated test plants significantly differed with TMX treatment of the viral source plant, aphid clone used and the interactions between these parameters. Again, percentage data were not arcsin transformed before analysis by ANOVA because no values were close to the 0% or 100% ends of the percentage scale.

Any preference of the *M. persicae* for the control treated test plants over the TMX treated test plants was determined separately for each clone and viral treatment by calculating the 95% confidence intervals around the treatment means. Confidence intervals excluding the 50% level indicated a preference of the *M. persicae* for TMX treated test plants over control treated plants or the converse.

In order to determine whether taking a count of the number of aphids on a plant every 24 h was truly representative of the number of individuals that had been on that plant over the 24 h period, the correlation coefficient between the log numbers of adults and nymphs per plant was calculated. This did not take into account plant treatment. All analyses were carried out using SAS version 9.2.

5.3 Results

5.3.1 Mortality rates

There was no significant difference in mortality between clones (Table 5.3). TMX treatment of the viral source plant significantly increased mortality of US1L, but not the FRC clone (Table 5.3). These findings occurred at all three time points (24, 48 and 72 h) (Figure 5.3).

Table 5.3 Percentage mortalities of two *Myzus persicae* clones, US1L and FRC, at three different time points, under two different treatments. In one treatment the central viral source plant was root drenched with thiamethoxam (TMX) at the LD_{15} dose for the appropriate clone, and in the other treatment the viral source plant was control treated with Milli-Q water. Differing superscript letters indicate a significant difference between treatments at the same time point. Error bars represent ± the standard error.

	24 h		48 h		72 h	
	Control	TMX	Control	TMX	Control	TMX
US1L	2.10 ± 0.51^{a}	18.00 ± 6.64^{b}	4.80 ± 1.23^{a}	21.60 ± 5.78^{b}	7.10 ± 1.02^{a}	25.20 ± 6.11^{b}
FRC	2.20 ± 0.66	8.50 ± 4.59	4.30 ± 0.64	13.40 ± 4.60	3.55 ± 0.51	19.80 ± 5.80

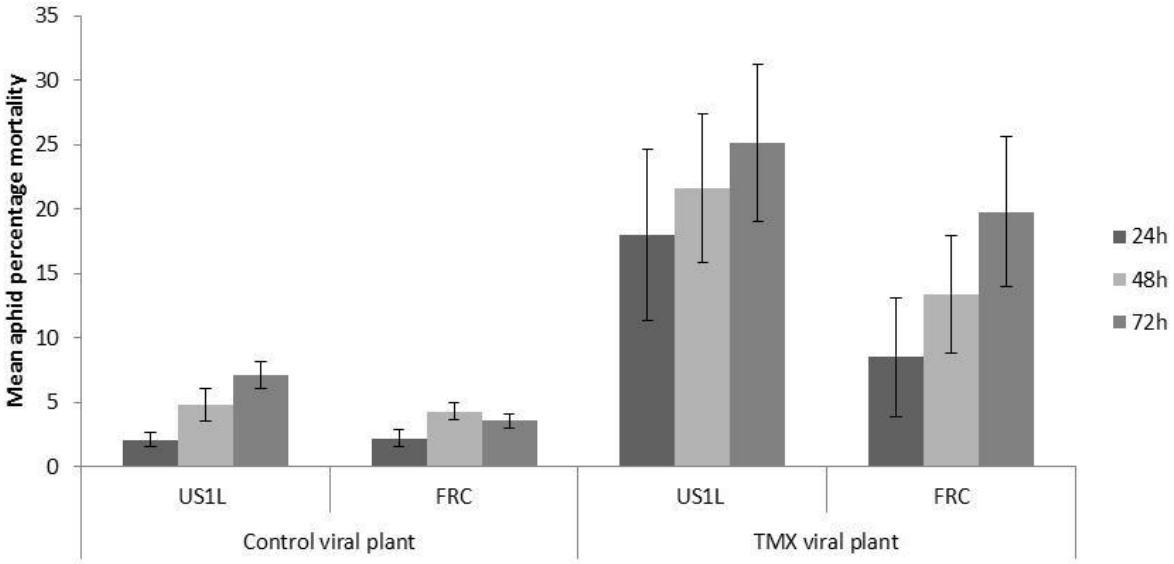


Figure 5.3 The percentage mortality of two *Myzus persicae* clones, US1L and FRC, under two different treatments, at three different time points. In one treatment the viral source plant was root drenched with thiamethoxam (TMX) and in the other treatment the viral source plant was control treated with Milli-Q water. Error bars represent ± standard error.

5.3.2 Dispersal of adults from the viral source plant

There was no significant difference in the percentages of adult *M. persicae* that left the viral source plant between either clones or viral plant treatments in the first 24 h (Table 5.4). After 48 h a significantly greater percentage of US1L had left the viral source plant when the source plants were TMX treated as opposed to control treated (Figure 5.4). This did not occur for the FRC clone and there was still no significant difference between the two clones at the 48 h mark (Table 5.4). At 72 h there was still a significant difference in the percentage of US1L that had left the viral source plants, with a greater percentage of US1L leaving the TMX treated source plants than the control treated plants (Figure 5.4). There was still no significant difference in the percentage of FRC that had left the TMX treated viral source plants as opposed to control treated source plants (Table 5.4). Most notably a significantly greater percentage of *M. persicae* of the US1L clone had left the viral source plant than the FRC clone when it was treated with TMX at this time point (Figure 5.4). No significant difference was seen between the clones on control treated source plants at any time point (Table 5.4).

Table 5.4 Mean percentages of *Myzus persicae* of two different clones, US1L and FRC, leaving a viral source plant over a 72 h time period. The viral source plant could be either treated with thiamethoxam (TMX) or else control treated with Milli-Q water. Note that deceased aphids were not included in these calculations. Differing superscript letters indicate a significant difference in the number of *M. persicae* leaving the viral source plant at the time point between viral source plant treatments or clones. Error bars represent ± the standard error.

	Control		**TMX**	
	US1L	**FRC**	**US1L**	**FRC**
24 h	13.0 ± 6.80	17.1 ± 4.75	31.7 ± 9.90	20.8 ± 8.02
48 h	18.3^{a} ± 7.23	28.7^{ab} ± 8.29	57.1^{b} ± 8.34	38.9^{ab} ± 8.84
72 h	44.1^{ab} ± 9.64	53.5^{ab} ± 7.42	77.4^{a} ± 8.86	63.8^{b} ± 8.23

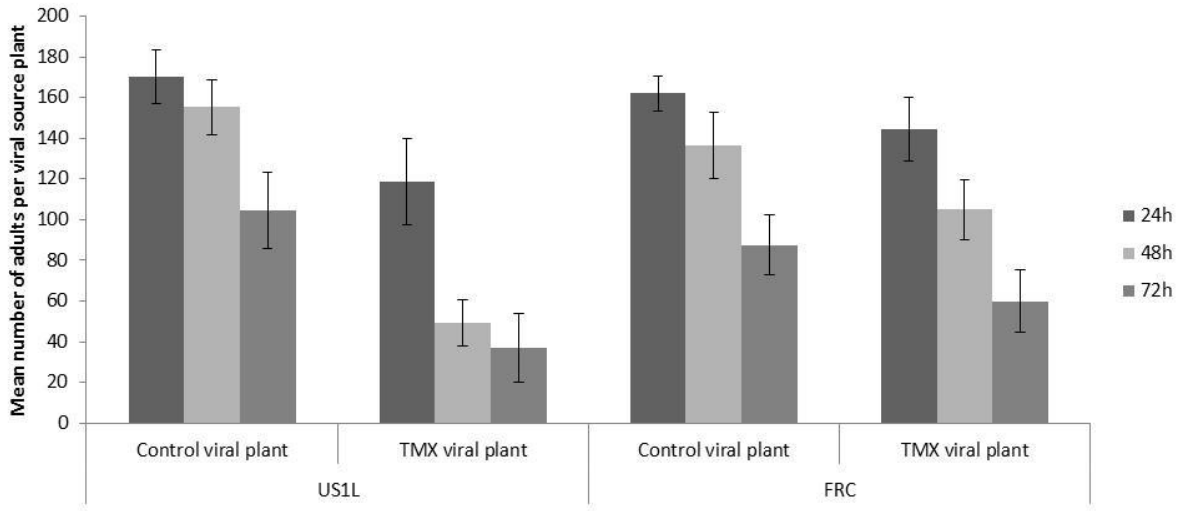


Figure 5.4 The mean number of adult apterous *Myzus persicae* of two different clones, US1L and FRC, remaining on a viral source plant at three different time points. The viral source plant could either be root drenched with a sublethal dose of thiamethoxam (TMX) equivalent to the LD_{15} or control treated with Milli-Q water. Error bars represent ± the standard error.

5.3.3 Preference of *M. persicae* for TMX treated and untreated test plants

Significantly greater percentages of both US1L and FRC were located on control treated test plants as opposed to TMX treated test plants at the 24 h period, when the viral source plant was control treated (Table 5.5 & Figure 5.5). There was no other significant difference between the percentages of either of these two clones on the two types of treated test plants at any other time point during the control treated viral source plant experiment (Table 5.5). There was no significant difference between the percentages of either US1L or FRC on the two types of treated test plants at any time point during the TMX treated viral source plant experiment (Table 5.5 & Figure 5.6).

Table 5.5 Confidence intervals calculated around the mean percentages of *Myzus persicae* of two clones, US1L and FRC, located upon thiamethoxam (TMX) treated as opposed to control treated test plants root drenched with Milli-Q water, at three different time points. Two treatments were investigated, one in which the viral source plant was treated with TMX, and one in which it was control treated. Values marked in bold indicate that there was a 95% confidence that there was a preference of aphids for one type of test plant over another in that treatment.

	Control treated source plant				TMX treated source plant			
	US1L		FRC		US1L		FRC	
	Lower CI	Upper CI	Lower CI	Upper CI	Lower CI	Upper CI	Lower CI	Upper CI
24 h	**4.4**	**49.2**	**0.0**	**43.8**	11.1	55.9	10.3	55.1
48 h	9.1	55.5	15.9	62.4	20.3	66.8	23.1	69.5
72 h	21.1	54.4	18.3	51.6	32.7	66.0	26.6	59.9

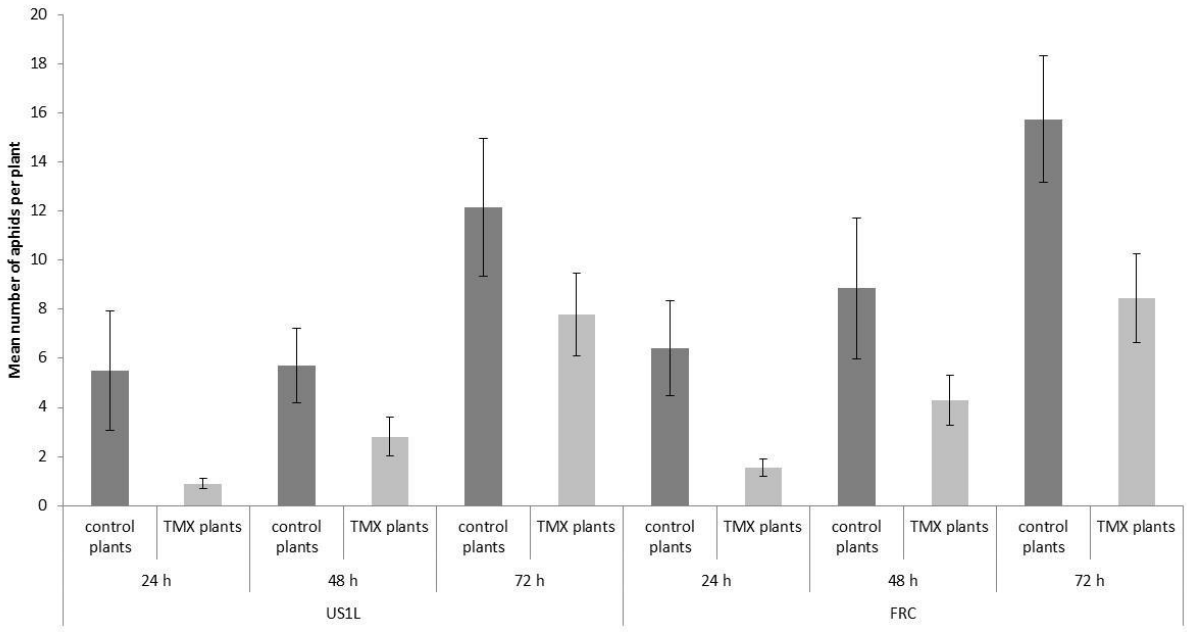


Figure 5.5 The mean numbers of adult apterous *Myzus persicae* of two different clones, US1L and FRC, located on test plants either root drenched with thiamethoxam (TMX) or else control treated with Milli-Q water, at three different time points over a 72 h period. In this set-up aphids were originally placed on a viral source plant control treated with water, before dispersal to the test plants. Error bars represent ± the standard error.

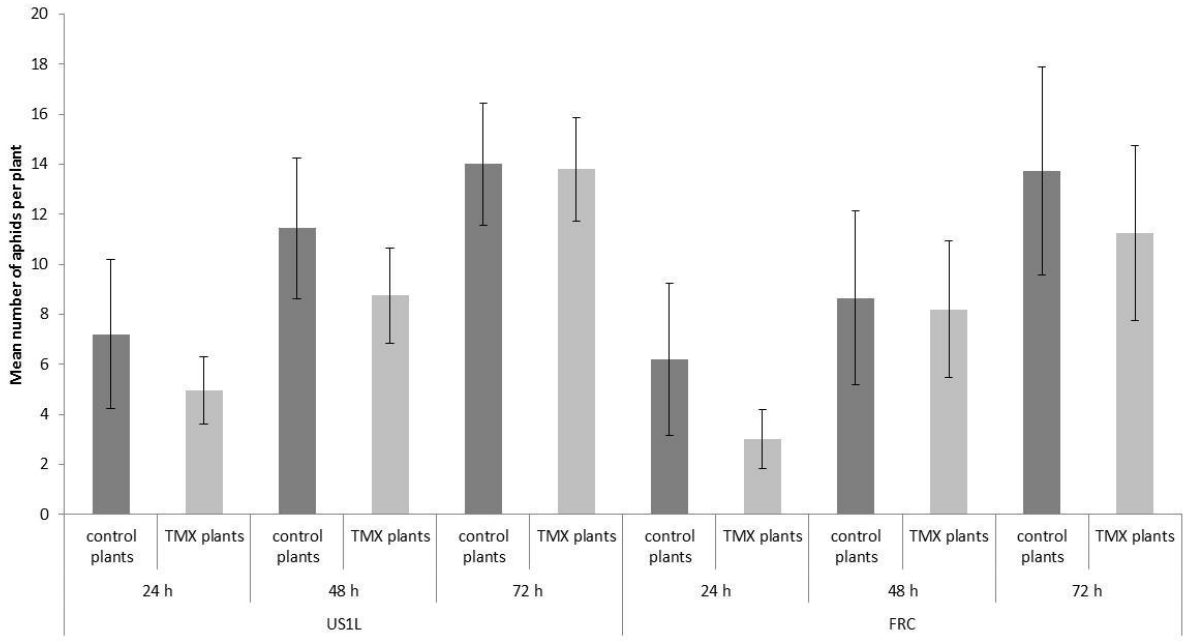


Figure 5.6 The mean numbers of adult apterous *Myzus persicae* of two different clones, US1L and FRC, located on test plants either root drenched with thiamethoxam (TMX) or else control treated with Milli-Q water, at three different time points over a 72 h period. In this set-up aphids were originally placed on a TMX treated viral source plant, before dispersal to the test plants. Error bars represent ± the standard error.

There were no significant effects of either clone or treatment of viral source plant on the percentages of adult *M. persicae* which were located on TMX treated test plants, out of all the aphids which left the viral source plant, at any time point (Table 5.6).

Table 5.6 Mean percentages of *Myzus persicae* of two clones, US1L and FRC, located on thiamethoxam (TMX) treated as opposed to control treated test plants root drenched with Milli-Q water, at three different time points. Two treatments were investigated, one in which the viral source plant was treated with TMX, and one in which it was control treated. Differing superscript letters indicate a significant difference in the percentage of *M. persicae* upon the TMX treated test plants between viral source plant treatments or clones. Error bars represent ± the standard error.

	Control treated source plant		**TMX treated source plant**	
	US1L	**FRC**	**US1L**	**FRC**
24 h	26.8 ± 8.79	21.4 ± 7.21	33.5 ± 10.20	32.7 ± 8.45
48 h	32.3 ± 3.56	39.1 ± 12.71	43.5 ± 3.19	46.3 ± 12.9
72 h	37.8 ± 8.36	34.9 ± 8.69	49.3 ± 3.06	43.2± 11.96

5.3.4 Correlations between numbers of adults and nymphs on plants

There was a significant correlation between the numbers of adults and the numbers of nymphs on a plant, ignoring the effects of plant treatment or clone ($r = 0.86$, $p < 0.0001$), with the number of nymphs per plant increasing with increasing numbers of adults (Figure 5.7). When the correlation between the numbers of adults and nymphs on a plant was investigated separately for each clone and treatment it was always significant, both on control and TMX treated test plants (Figures 5.8, 5.9 & Table 5.7). This indicated that the number of nymphs on a plant and the number of adults on a plant were representative of the same level of aphid dispersal.

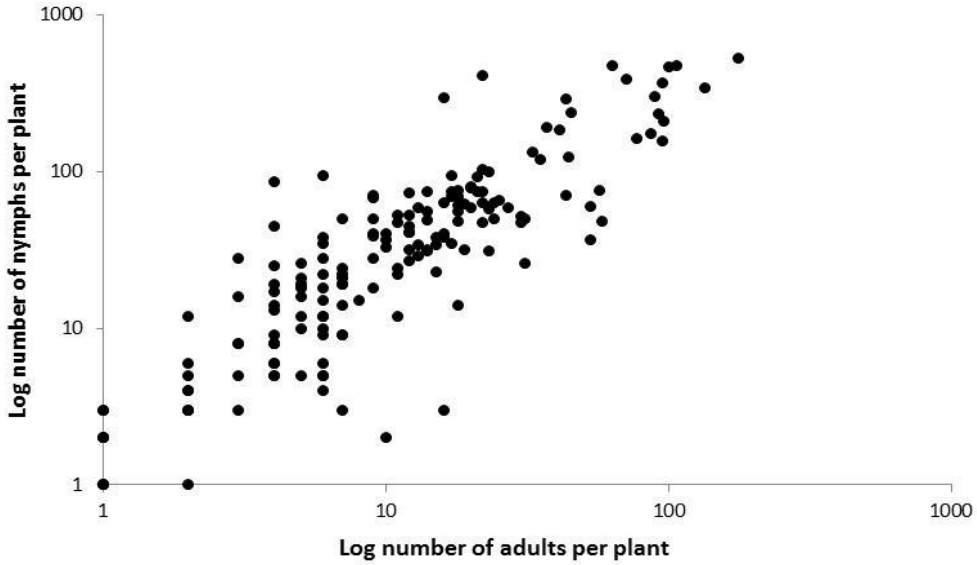


Figure 5.7 The numbers of *Myzus persicae* adults and nymphs per plant, ignoring any effects of clone and viral plant treatment ($r = 0.86$, $p < 0.0001$).

Table 5.7 Correlation coefficients of numbers of adults and numbers of nymphs of two *Myzus persicae* clones, US1L and FRC, on plants under different treatment conditions. Plants could either be treated with thiamethoxam (TMX) or control treated with Milli-Q water.

	Control treated viral plant		TMX treated viral plant	
	US1L	FRC	US1L	FRC
Control treated test plants	0.857	0.903	0.949	0.925
TMX treated test plants	0.877	0.473	0.680	0.845

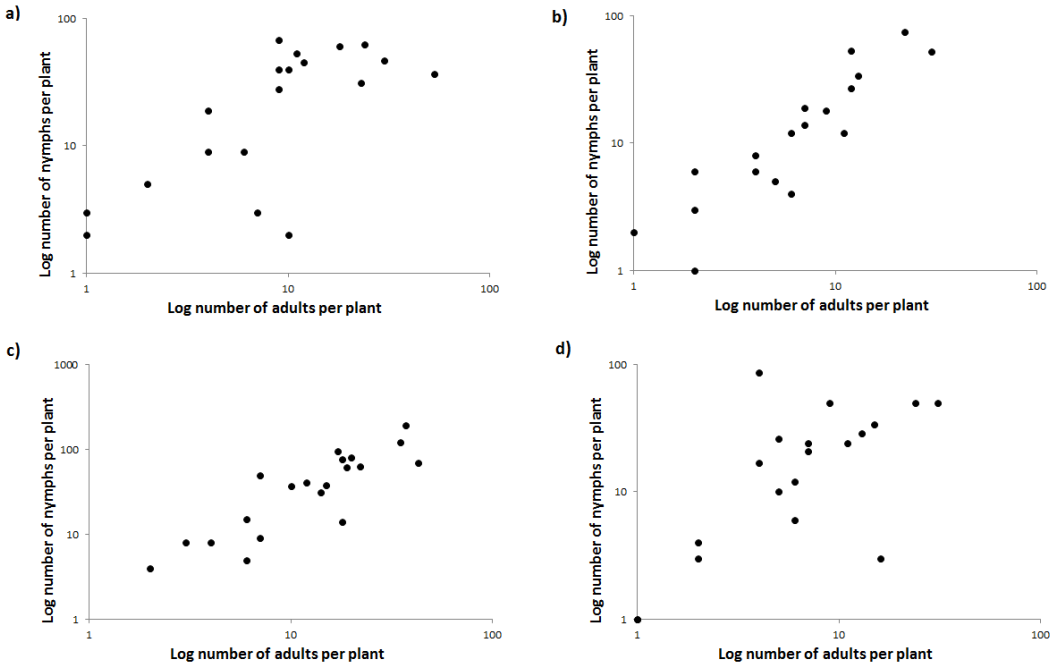


Figure 5.8 The numbers of *Myzus persicae* adults and nymphs per plant, of the clones US1L and FRC, on two different types of test plants, when the viral source plant was control treated with Milli-Q water. Aphids could be on test plants either root drenched with a sublethal dose of thiamethoxam (TMX) or else control treated. a) US1L on control treated plants b) US1L on TMX treated plants c) FRC on control treated plants d) FRC on TMX treated plants.

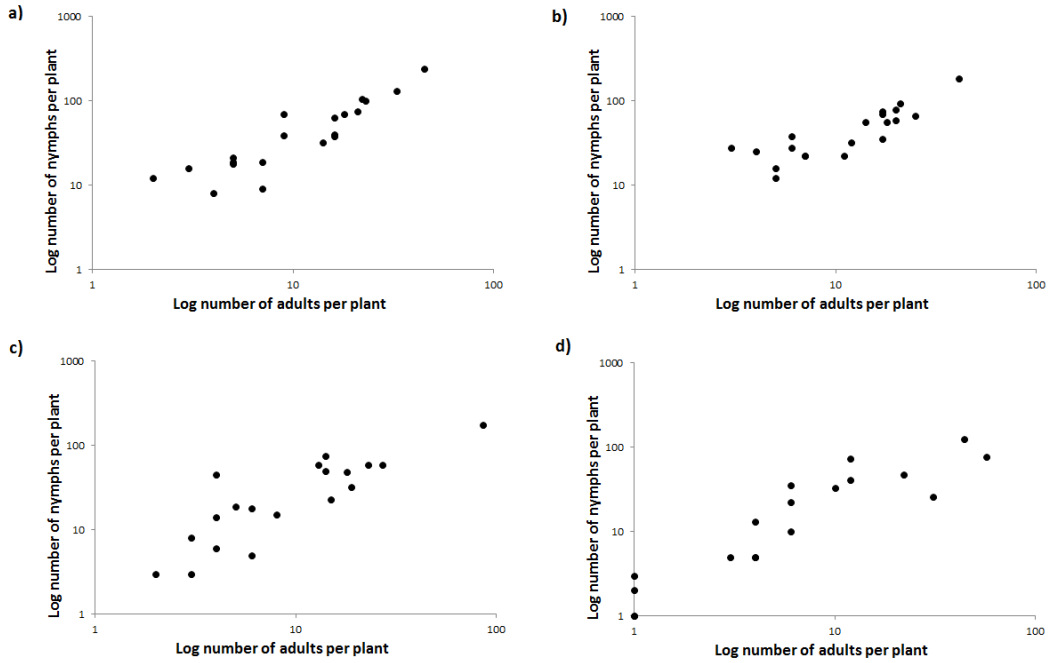


Figure 5.9 The numbers of *Myzus persicae* adults and nymphs per plant, of the clones US1L and FRC, on two different types of test plants, when the viral source plant was root drenched with thiamethoxam (TMX). Aphids could be on test plants either treated with TMX or else control treated with Milli-Q water. a) US1L on control treated plants b) US1L on TMX treated plants c) FRC on control treated plants d) FRC on TMX treated plants.

5.3.5 Viral infection of plants

Since there was not the time or resources to test all 160 test plants for the presence of TUYV using RT-PCR, twenty plants which exhibited viral symptoms were confirmed as infected with TUYV by RT-PCR (Figure 5.10 & Table 5.8). It was assumed that all plants exhibiting viral symptoms had been successfully infected with TUYV, based on this sample of twenty confirmed infected symptomatic plants. All viral source plants were confirmed to be infected with TUYV using RT-PCR, except for two which showed viral symptoms but died before a sample of leaf tissue could be collected for testing.

The majority of test plants in each of the treatment-clone combinations became infected with TUYV, as confirmed by both observation of symptoms and RT-PCR of all asymptomatic plants (Tables 5.9, 5.10 & Figure 5.11). Since so few plants were uninfected it was not possible to perform any statistical tests on these data. Visually, there did not appear to be any obviously significant difference in infection rates between the US1L and FRC clones, or between treatments. A slightly greater percentage of plants exhibited symptoms in

experiments where the source plant was untreated than when it was treated for both clones (Table 5.8).

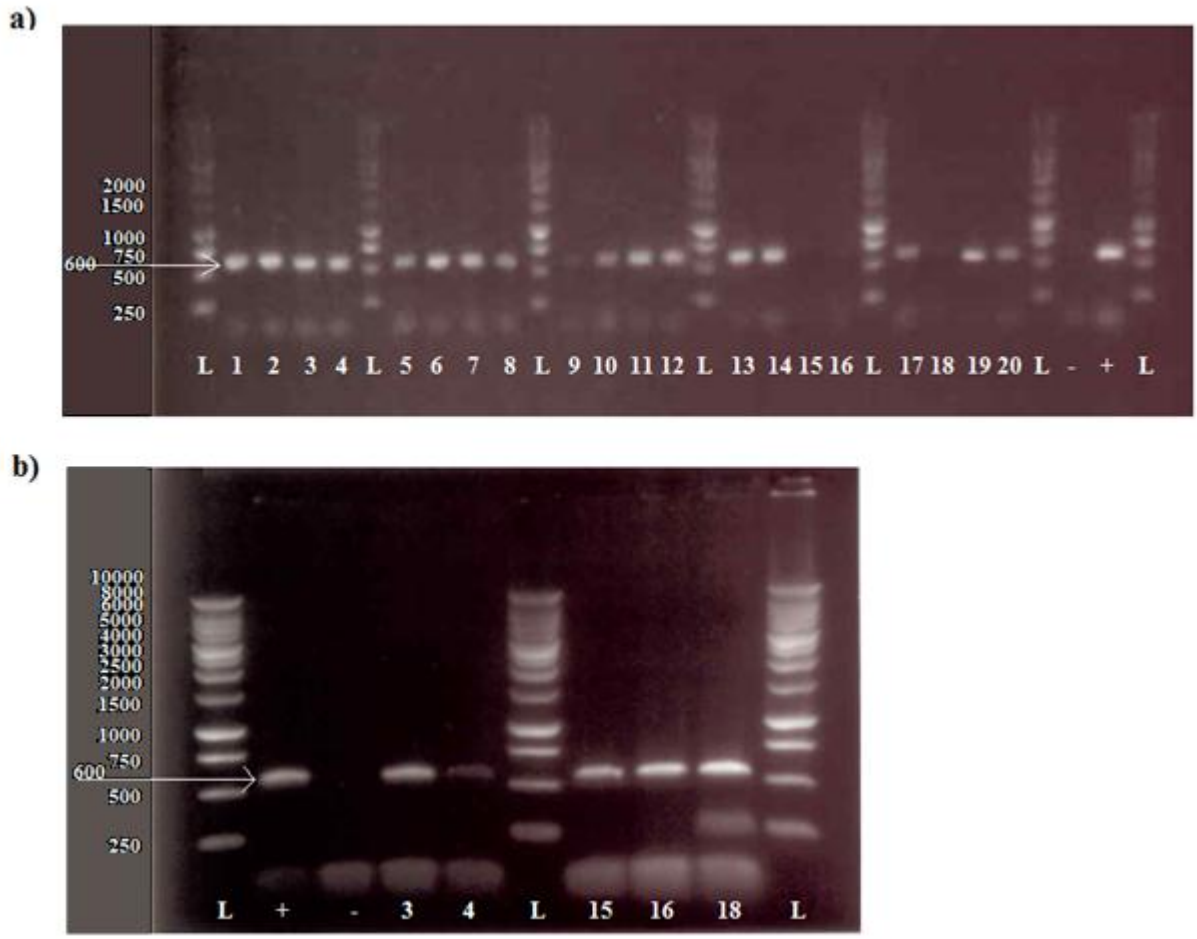


Figure 5.10 Confirmation of the presence or absence of *Turnip yellows virus* (TuYV) within symptomatic Chinese cabbage plants as inoculated by *Myzus persicae*. Ladder 1 kb, gel 1% agarose with ethidium bromide. The presence of a 600 bp band (highlighted using arrow) indicates the presence of the virus in the plant sample while the absence of the band indicates the plant tissue contained no TuYV RNA. 'L' indicates 1 kb ladder, '+' positive control, '-' negative control. a) Original test, containing individual samples from 20 symptomatic plants. Since the RNA quality in samples 15, 16 and 18 was poor, fresh samples from the same plants were re-tested in b).

Table 5.8 Confirmation of the presence of a 600 bp band after RT-PCR and gel electrophoresis of Chinese cabbage plant leaf tissue samples showing symptoms of *Turnip yellows virus* (TuYV) after exposure to *Myzus persicae* carrying TuYV. The presence of the 600 bp band indicates successful infection by TuYV.

Sample	600 bp band present?	Sample	600 bp band present?
1	yes	11	✔
2	yes	12	✔
3	yes	13	✔
4	yes	14	✔
5	yes	15	✔
6	yes	16	✔
7	yes	17	✔
8	yes	18	✔
9	yes	19	✔
10	yes	20	✔

Table 5.9 Presence or absence of 600 bp band after RT-PCR and gel electrophoresis of Chinese cabbage plant leaf tissue samples exposed to *Myzus persicae* infected with *Turnip yellows virus* (TuYV) but not showing viral symptoms. The presence of the 600 bp band indicates successful infection by TuYV.

Sample	600 bp band present?	Sample	600 bp band present?
1	no	24	**X**
2	yes	25	✓
3	yes	26	✓
4	yes	27	**X**
5	no	28	**X**
6	no	29	✓
7	no	30	**X**
8	no	31	**X**
9	no	32	**X**
10	no	33	**X**
11	yes	34	✓
12	yes	35	✓
13	yes	36	**X**
14	no	37	**X**
15	yes	38	**X**
16	yes	39	✓
17	no	40	✓
18	no	41	✓
19	yes	42	**X**
20	yes	43	✓
21	yes	44	✓
22	yes	45	**X**
23	yes	46	✓

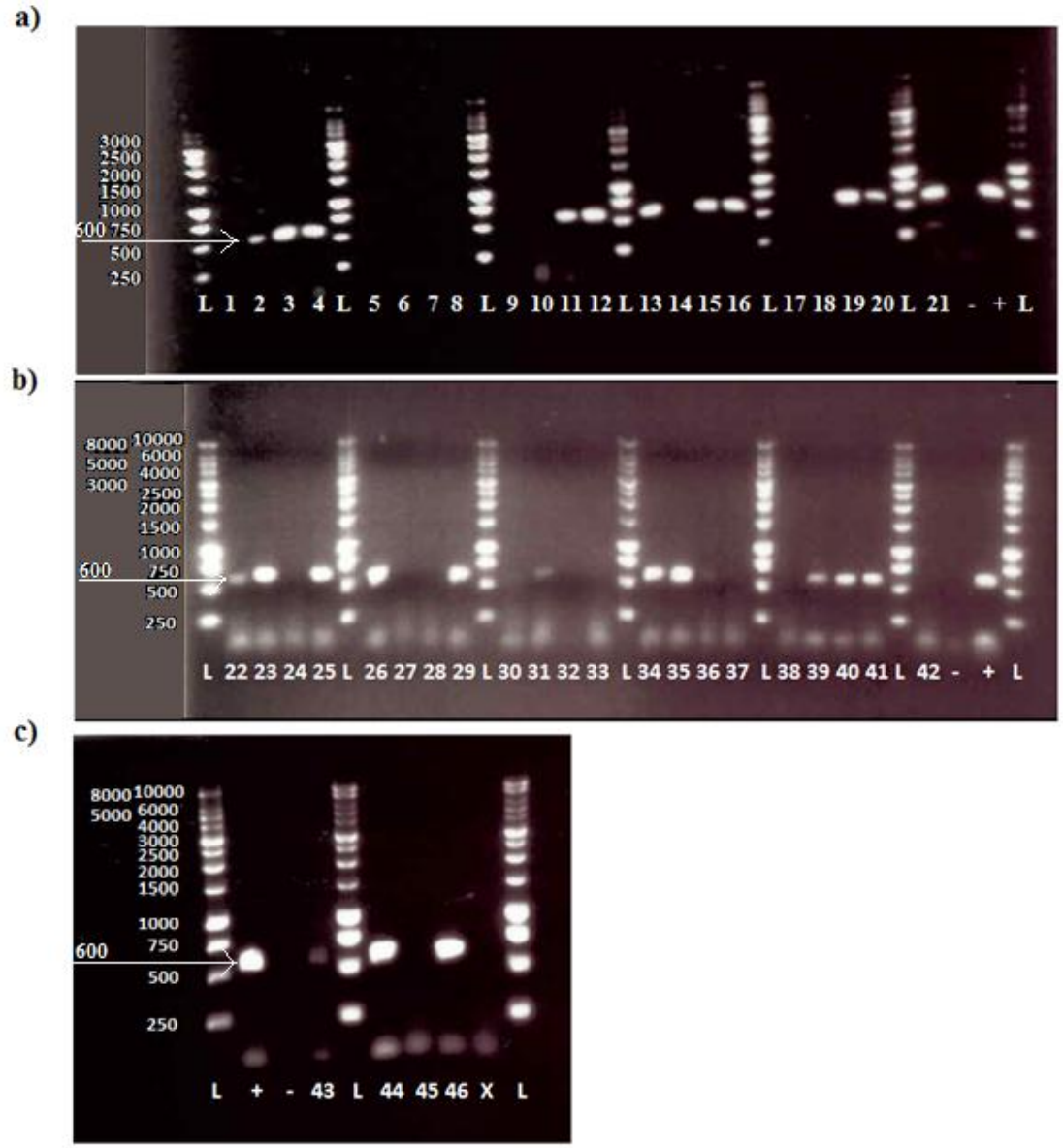


Figure 5.11 Confirmation of the presence or absence of *Turnip yellows virus* (TUYV) within 46 asymptomatic Chinese cabbage plants as inoculated by *Myzus persicae.* All ladders shown are 1 kb. Gel 1 % agarose with ethidium bromide. The presence of a 600 bp band indicates the presence of the virus in the plant sample while the absence of the band indicates the plant tissue contained no TUYV RNA. In the interests of time the plant samples underwent RT-PCR on three different days a) b) and c). 'L' indicates a 1 kb ladder, '+' positive control, '-' negative control, 'X' that the well in the gel was damaged and not used.

Table 5.10 Percentage viral infection rates of Chinese cabbage with *Turnip yellows virus* by two *Myzus persicae* clones, US1L and FRC.

Treatment of viral source plant and clone	Control		TMX	
	US1L	FRC	US1L	FRC
Mean percentage of plants that showed viral symptoms	85.0	85.0	67.5	65.0
Mean percentage of plants that did not show viral symptoms but were viral, out of plants that showed symptoms	50.0	50.0	63.3	52.5
Mean percentage of virally infected plants	90.0	92.5	87.5	80.0
Percentage of total plants virally infected	90.0	92.5	90.0	82.5

5.4 Discussion

This study revealed no significant difference in the ability of the two *M. persicae* clones, US1L and FRC, to transmit TuYV. Despite exhibiting some limited differences in dispersal, there was no significant difference in the numbers of infected plants produced by the two clones. In addition, treatment of the test plants with TMX did not protect them from viral infection by either clone. While it would have been ideal to confirm the presence or absence of TuYV in all test plants by molecular means, this was not possible due to time constraints. In future it is recommended that this be done via an ELISA test that is also available (Smith *et al.,* 1996) so larger amounts of samples can be processed. Viral symptoms, however, were so obvious in the majority of cases that it was highly unlikely that such plants did not contain TuYV, and it is probable that the results accurately reflect the high prevalence of TuYV within the test plants.

It has been shown that *M. persicae* will disperse up to 1.8 m to reach new host plants (Boiteau, 1997), but due to space limitations in the DEFRA facilities available the boundaries of the arenas utilised in this study were much smaller. Many studies investigating the spread of plant viruses by free aphids use much larger test areas, such as glasshouse bays or field studies (Perring *et al.,* 1999; Groves *et al.,* 2001; Polston & Sherwood, 2003; Coutts *et al.,* 2010; Tian *et al.,* 2012). It is possible that the arena used in this study was not large enough for the differences in dispersal behaviour of the two clones to exert an effect upon viral spread. In such a small area, the majority of plants were visited by *M. persicae* and inoculated with TuYV. From the data it appears even a small number of aphids infected with TuYV may cause infection of a plant, as has been shown for many other viruses including PVY (Margaritopoulos *et al.,* 2010), CMV (Martín *et al.,* 1997) and *Beet yellows closterovirus* (BYCV) (Limburg *et al.,* 1997). Utilisation of a larger test area may reveal differences in vector ability between the *M. persicae* clones not shown by this study.

It is also possible that while there are no differences between the vector ability of the US1L and FRC clones, there may be differences between either of these clones and the clone 5191A, which exhibits a low level of neonicotinoid resistance (Puinean *et al.,* 2010). The vector ability of 5191A was not investigated during this study due to time and space constraints. The FRC clone was chosen instead, due to its greater neonicotinoid resistance (Bass *et al.,* 2011). Given this high level of resistance, any differences in the vector ability of the FRC clone could potentially be of greater impact and relevance within the field. 5191A

exhibited differences in a greater number of feeding behavioural parameters than both US1L and FRC during the EPG experiments described in chapter four, however, and given these feeding differences and the greater overall rate of dispersal shown in chapter three it is possible that 5191A may be more likely to exhibit a difference in vector ability than the FRC clone when compared to US1L. While several studies have shown differences in vector ability between clones of the same species (Rice *et al.,* 1982; Lucio-Zavaleta *et al.,* 2001; Mowry & Ophus, 2002; Kozlowska-Makulska *et al.,* 2009) other studies have shown the converse (Garzo *et al.,* 2004). It therefore follows that certain clones of certain species may differ in vector ability, but not all clones of all aphid species; hence it is possible that 5191A may differ in vector ability compared to other *M. persicae* clones even though US1L and FRC do not.

While the US1L and FRC clones did not exhibit differences in transmission efficiency when vectoring the persistently transmitted virus TuYV, it is possible they may exhibit a difference in ability to vector a non-persistent virus, such as PVY (Kostiw & Trojanowska, 2011). Non-persistent viruses are capable of being transmitted during brief probes and cell punctures and do not require sustained phloem feeding for either inoculation or acquisition (Martín *et al.,* 1997; Moreno *et al.,* 2005). Given however, that no significant differences were exhibited in either the duration or frequency of pathway phase and cell punctures between the US1L and FRC clones during the experiments of chapter four, there is little evidence to suggest a difference in vector ability with regards to non-persistently transmitted viruses occurs between these two clones. Despite this, further work should certainly be carried out upon the ability of the neonicotinoid resistant and susceptible *M. persicae* clones to transmit a non-persistent virus, as until this is completed the full extent of any differences in their vector behaviour and ability remains unknown. Since only a brief probe is required for transmission of a non-persistent virus (Perring *et al.,* 1999), differences in dispersal behaviour may possibly exert a greater effect upon the spread of a non-persistent rather than a persistent virus, as even brief probes by transient unsettled aphids would contribute to viral spread. The vector ability of the 5191A clone should be included in any comparisons, as this clone did exhibit differences in the duration and frequency of probing and pathway phase, though generally such differences were significantly different from the FRC clone rather than US1L.

Although this study showed no significant differences in vector ability between the US1L and FRC clones, there were some differences in dispersal behaviour. In contrast, however, to the differences in dispersal behaviour between US1L and FRC described in chapter three in

which significant differences were sustained throughout the first 24 h, the only significant difference in dispersal behaviour between the two clones in this study was in the number of individuals which had left the viral source plant at the 72 h time point, with a greater percentage of US1L having left the viral source plant than FRC. In addition this only occurred when the viral source plant was treated with TMX and not when it was control treated. This is not a large amount of evidence to support the existence of a significant difference in dispersal behaviour between the two clones in a microcosm environment. It does suggest that any such differences in dispersal within a large area only begin to occur after 72 h and only after exposure to TMX has occurred. A further study repeated over a greater time period of up to 1 week is recommended, in order to observe whether this difference in dispersal behaviour between the US1L and FRC clones continues to be consistently observed over a longer time period. Such a difference may be of relevance within the field where a growing season may last several months, particularly if the FRC clone remains in a field for a substantial amount of time, given the difficulty of control of this clone (Bass *et al.,* 2011).

Dispersal of US1L away from TMX treated source plants as opposed to untreated plants may be interpreted as a repellency effect of TMX. Neonicotinoids and many other insecticides have been shown to exert repellency effects upon target insects (Rodtakis *et al.,* 2000; Rose *et al.,* 2005; 2006; Vernon *et al.,* 2007; Smitii *et al.,* 2008) with such effects often considered a beneficial attribute. It is possible that the FRC clone was not repelled by TMX in the same way that US1L was, due to the upregulated metabolism and target site mutations that confer this clone neonicotinoid resistance (Bass *et al.,* 2011). These resistance mechanisms may have reduced any repellent effect of TMX upon this clone. It should be noted however, that both clones received an equivalent dose of TMX at their respective LD_{15} concentrations. In addition only a sublethal dose of TMX was applied in this study. Neonicotinoids act quickly and even an application greater than the LD_{15} concentrations utilised in this study, but still below that of the field rate, can cause significant mortality within 72 h (Devine *et al.,* 1996; Nauen *et al.,* 1998c; Magalhaes *et al.,* 2008). It is therefore not expected that this difference in dispersal between the two clones would be observed in the field, simply due to the high levels of mortality of susceptible aphid clones at field rates. To achieve a greater understanding of the more complex transmission dynamics which may occur in the field situation this study should be repeated at the field rate.

In terms of preference between TMX treated and untreated test plants, significantly greater numbers of adults of both the US1L and FRC clones were found upon control treated rather than TMX treated test plants for the first 24 h, when the viral source plant was also control treated. Other than this, there was no significant difference in the numbers of aphids of either clone found on the TMX and control treated test plants. This is unusual, given that it is well documented that treatment of plants with neonicotinoids may exert an antifeedant effect (Nauen, 1995; Nauen & Elbert, 1997; Nauen *et al.,* 1998a,c; Gomes *et al.,* 2008) as well as exert repellency effects in general (Vernon *et al.,* 2007; Smitii *et al.,* 2008) hence the *M. persicae* would be expected to seek out untreated plants. This finding is also at odds with the results of chapter three, where the FRC clone showed a significant preference for control treated over TMX treated plant tissue. Although it is possible that this difference is due to the increased distance between the plants in the microcosm experiment, as mentioned earlier, is more probable that the differences between the results of the two experiments are due to the use of whole plants as opposed to excised leaves or leaf discs. Treated plants will metabolise and break down many insecticides, including TMX (Maeinfisch *et al.,* 2001; Nauen *et al.,* 2003; Casida, 2011), into other metabolites. Some of these metabolites, such as clothianidin, still exert insecticidal action whereas others do not (Nauen *et al.,* 2003; Casida, 2011, XueXiang *et al.,* 2011). Bearing in mind that the test plants were treated with a sublethal dose of TMX equivalent to the LD_{15} of each clone, it is possible that over the 72 h period the test plants metabolised the TMX into a range of metabolites. If the altered dispersal behaviour of the FRC clone is triggered in response to the presence of TMX rather than a metabolite of this compound, it is possible that the breakdown of TMX into its metabolites reduced the behavioural response of the FRC clone. This would explain why no significant difference was seen in the numbers of aphids on TMX treated and untreated plants, except in the initial 24 h before sufficient metabolic breakdown of the TMX had occurred. It is also reasonable to suppose that the excised leaves in the dispersal experiment possibly exhibited a reduced rate of TMX metabolism compared to the whole plants in the viral experiment. Even though the excised tissue was alive and set in agar containing sugar and water, it did not have a root system and was under environmental stress from its excision. This could have potentially reduced the rate of TMX metabolism, as it is well documented that many metabolism pathways alter in response to plant stress (Obata & Fernie, 2012; Rasmussen *et al.,* 2012). If greater concentrations of TMX were present in the excised leaves than in treated whole plants this may have been enough to cause the FRC to exhibit an altered dispersal behaviour of seeking out untreated areas of leaf tissue. In order to determine whether this was the case, the

dispersal experiment described in chapter three should be repeated with whole plants rather than excised leaves, to see if the FRC clone still exhibits differential dispersal behaviour from US1L. In addition the levels of both TMX and TMX metabolites in excised leaves and whole plants could be compared using LCMS.

Some plant viruses may influence the behaviour of their aphid vectors (Alvarez *el al.,* 2007; Stafford *et al.,* 2011; Boquel *et al.,* 2012; Ingwell *et al.,* 2012; McMenemy *et al.,* 2012). It has been shown that *M. persicae* is capable of discriminating between the headspace volatiles of potato (*S. tuberosum*) plants infected with PLRV and uninfected plants (Alvarez *et al.,* 2007). *Myzus persicae* also performed different penetration behaviours on infected as opposed to uninfected plants. All of these behaviours were dependant on the age of the leaf, however, with leaves that showed extensive viral symptoms eliciting the greatest behaviour changes. Boquel *et al.,* (2012) also showed *M. persicae* to alter its behaviour in response to *S. tuberosum* plants infected with a different virus, PVY, exhibiting an increased duration of phloem phase activities on infected plants. *S. avenae* showed a similar behavioural change within the same study, while conversely *Aphis fabae* Scop. exhibited a decreased duration of phloem phase behaviour and increased xylem feeding on infected plants. The thrip *Frankliniella occidentalis* (Pergande) has also been shown to alter its feeding behaviour after infection with *Tomato spotted wilt virus* (TSWV), with infected individuals increasing the frequency of probes in which they salivate but do not ingest (Stafford *et al.,* 2011). Other studies have shown aphids to discriminate between virally infected and non-infected plants in terms of their dispersal and plant choice. In a study by Hodge & Powell (2008), *A. pisum* was preferentially attracted to plants infected with PEMV, BYMV and *Broad bean mottle virus* (BBMV) over uninfected plants, although attraction to viral plants was not linked to whether the virus was aphid-transmitted. *Rhopalosiphum padi* will feed preferentially from wheat plants infected with BYDV when non-viruliferous, but once they have acquired BYDV show an active preference for feeding upon uninfected wheat plants (Ingwell *et al.,* 2012). McMenemy *et al.* (2012) showed that the large raspberry aphid *Amphorophora idaei* Börner was initially attracted to plants infected with *Black raspberry necrosis virus* (BRNV) and *Raspberry leaf mottle virus* (RLMV) but within 12 h had distributed equally between viral and non-viral plants.

Since all *M. persicae* were initially placed upon the viral source plant it can be assumed that the majority of them became infected with TuYV, especially as large percentages of individuals remained upon the source plant for the first 24 h in all treatment and clone

combinations. Since a persistent virus requires phloem feeding for a period of several hours to be acquired by the vector (Hogenhout *et al.*, 2008), this is more than adequate for the aphids to become viruliferous. If the majority of the *M. persicae* were infected with TuYV then any effects of TuYV infection upon aphid behaviour would have influenced the dispersal and or feeding of the majority of individuals. This may explain the differences in dispersal behaviour exhibited by the *M. persicae* clones in this study as opposed to the results described in chapter three. If acquisition of TuYV increased the probability of dispersal of aphids away from the viral source plant, or caused them to probe the test plants more frequently then any behavioural effects of infection with TuYV may have disrupted any dispersal behaviours usually exhibited by non-viruliferous aphids in response to the presence or absence of TMX, such as those described in chapter three. Future work should involve a second microcosm identical to the setup described in this study, run in parallel but with no virus present. In this manner the dispersal behaviour of the US1L and FRC clones in a microcosm environment could be compared in the absence of any behavioural changes caused by the presence of a plant virus.

In general, a relatively small percentage of aphids of either clone actually left the viral source plant within the 72 h, regardless of treatment with TMX. This is similar to behaviour observed by Tian *et al.* (2012), where few *M. persicae* left tobacco viral source plants infected with CMV compared to *A. gossypii*, *Lipaphis erysimi* (Kaltenbach), *A. craccivora* and *Megoura viciae* Buckton within a ten day trial. This reluctance of *M. persicae* to leave the viral source plant may also explain the lack of a significant preference between the TMX treated and untreated test plants. If few aphids left the viral source plant, then relatively few aphids would have come into contact with tests plants of both types of treatments. Any preference shown by the aphids for either of the two test plants would therefore have been difficult to detect at a significant level.

The time points at which the locations of the aphids were observed were relatively far apart and much aphid movement may have occurred in the intervening 24 h between each observation. Previous studies on the dispersal of aphids in microcosms have suggested that additional unobserved movement by adults may be shown by the deposition of nymphs upon plants in the absence of settled feeding adults (Harrington & Taylor, 1990; Hodgson, 1991). The number of nymphs per plant however, correlated strongly with the number of adults for all treatments and time points in this study. It is possible that adult dispersal between test plants was extremely brief in nature and dispersing adults did not remain upon test plants for

long enough to produce nymphs. Extremely few adults, however, were observed upon the cage floor or walls at any time (data not shown), making this unlikely. It is also possible that the nymphs themselves were dispersing between test plants. Since nymphs as are also capable of transmitting TuYV (Stevens *et al.,* 2008) the dispersal of nymphs is also of interest as it could have potentially exerted an effect upon viral spread. Although significant dispersal of first instar *M. persicae* nymphs from plants has been recorded (Boiteau, 1997; Sauge *et al.,* 1998b), personal observations have shown that first instar *M. persicae* nymphs appear to move very little in the environmental conditions applied in all four of the studies described in this thesis; hence the positions of nymphs should have correlated with the plants they were produced upon during the 72 h, making a high level of nymph dispersal unlikely. Basing a comparison of the dispersal of the three *M. persicae* clones on the distribution of adult individuals at the three 24 h time points during this study therefore appears appropriate.

One of the great limitations of this study is that it was impossible to study the feeding behaviour of the *M. persicae* within the microcosm environment and see whether it differed from behaviour exhibited within the EPG set-up described in chapter four. Since the two *M. persicae* clones US1L and FRC exhibited different feeding behaviours on plants during the EPG experiments, had the two clones exhibited any differences in viral transmission ability we would have been unable to say whether such differences were due to differences in feeding, dispersal, or differences in a combination of both behaviours between the clones. It is recommended that any future work into the viral transmission behaviour of the neonicotinoid resistant and susceptible *M. persicae* clones involves the EPG technique in order to incorporate an understanding of any differences in the feeding behaviour of the clones when free within a microcosm environment. It is possible to attach a short gold wire electrode to the back of an aphid with silver conductive glue, and then leave it to roam freely upon a plant (Tjallingii, 1986). Periodically the gold wire may be touched to the input electrode of an EPG setup, allowing the feeding behaviour of the aphid to be examined by EPG. Were all of the plants in the arena connected to different channels of an EPG machine by separate copper electrodes, it would be possible to study the feeding behaviour of free individual *M. persicae* within the microcosm environment and compare it to the EPG experiments described in chapter four. This would increase the understanding of how any differences in feeding behaviour contribute to any potential differences in the vector ability between the clones.

The environmental conditions present within this experiment are not representative of those in the field and field based experiments are required in order to truly understand the consequences of the emergence of the neonicotinoid resistant FRC clone on viral spread throughout crops. In the field aphids would be able to disperse over a far greater distance, although the spacing of individual crop plants is unlikely to be much greater than that used within this experiment. Aphids within the field would also be exposed to the effects of fluctuating temperature, light, weather and predation, all of which have been found to significantly affect aphid dispersal (Bailey *et al.*, 1995; Mann *et al.*, 1995; Narayandas & Alyokhin, 2006) and are therefore likely to affect viral spread. Although *M. persicae* is polyphagous and can feed on over 400 plant species (Blackman and Eastop, 2007), different crops will be affected by different viruses and even fed upon by different *M. persicae* clones (Weber, 1985; Fenton *et al.*, 2010). The FRC clone has only been collected within the field from peaches and nectarines at present (Bass *et al.*, 2011; Porter, 2012); hence the viral transmission dynamics in Chinese cabbage may not be appropriate to apply to the field situation. Even when a crop is treated with an insecticide, alates may migrate into the crop from alate populations outside of the treated area. Such migrants may or may not be viral. Indeed, alate aphids are often considered more important than apterous individuals in viral transmission (Blua & Perring, 1992; Boiteau, 1997; Katis *et al.*, 2007) and further work is required on the vector abilities of alate individuals of both the FRC and US1L clones in order to fully understand any potential differences in vector ability between these two clones. Viral transmission dynamics have also been found to be affected by the different combinations of aphid vectors available within the field (Tian *et al.*, 2012), and it has been shown that *M. persicae* may transmit at least two viral strains of PVY at the same time (Srinivasan *et al.*, 2012). Since multiple plant viruses, viral strains, vector species and even aphid clones of the same species may all exist within the same field and crop, viral transmission dynamics are extremely complicated and very difficult to predict merely from lab based experiments. This study represents an initial investigation into the vector behaviour of the FRC and US1L clones, but much further work is required before such information can be applied to a field situation.

Aphid-vectored plant viruses cause substantial economic damage in the agricultural industry, with transmission of plant viruses being the major cause of crop damage by aphids (Dedryver *et al.*, 2010). Should a difference in vector ability and efficiency be found to exist between neonicotinoid-resistant and susceptible *M. persicae* clones, this would be a major cause for

concern, especially given the significant difficulty of control of the FRC clone at present. Knowledge of any differences in vector ability would at least provide guidance in the suitability of the control measures to be taken in a situation where both the FRC clone and a plant virus were present within the same crop. Since this study found no evidence of a significant difference in vector ability between the FRC and US1L clones it offers no predictive power for field scale outcomes. Further investigation is required into the relative vector efficiencies of neonicotinoid resistant and susceptible *M. persicae* clones with different plant viruses and under conditions more representative of the field in order to increase understanding and determine whether there is truly no difference in vector ability and efficiency between neonicotinoid-resistant and susceptible *M. persicae* clones.

Overall I reject hypothsis four that "the differential feeding and dispersal behaviour of the FRC clone will lead to this clone exhibiting an increased efficiency as a vector of a persistent virus, compared to the *M. persicae* clones US1L and 5191A, when exposed to a sublethal dose of TMX" as the results of the study described here do not support such a hypothesis. Much further investigation is required, however, to establish a fuller understanding of the dynamics of the vector behaviour of neonicotinoid resistant *M. persicae* clones.

Chapter 6: Overall summary

6.1 Introduction

Significant neonicotinoid resistance has only recently emerged in *M. persicae.* Prior to the discovery of the FRC clone from peach orchards in 2009 (Bass *et al.,* 2011), neonicotinoid resistance was only detectable within the laboratory (Devine *et al.,* 1996; Nauen *et al.,* 1996; Foster *et al.,* 2002; Cox *et al.,* 2004; Puinean *et al.,* 2010). Neonicotinoids, however, are widely used on a variety of crops and make up a large percentage of the agrochemical market (Jeschke *et al.,* 2011). Neonicotinoid resistance has also been reported in other insect species, including the whiteflies *B. tabaci* and *Trialeurodes vaporariorum* (Westwood) (Cahill *et al.,* 1996; Gorman *et al.,* 2007), the brown planthopper *N. lugens* (Nauen & Denholm, 2005), the Colorado potato beetle *L. decleminita* (Alyokhin *et al.,* 2008) and a few other species (Elbert *et al.,* 2008). The emergence of neonicotinoid resistance in aphids was therefore predicted to occur much earlier than happened in reality.

The emergence of the neonicotinoid-resistant FRC clone presents a serious agricultural concern. It is likely that this clone will eventually disperse further throughout Europe. The only insecticides known to be effective against this pest at present are antifeedant compounds such as pymetrozine and the novel insecticide spirotetramat. Spirotetramat is a fully systemic broad-spectrum insecticide which shows no cross-resistance with neonicotinoids and is effective against sap-feeding pests (Nauen *et al.,* 2008). The mode of action appears to involve the inhibition of ACCase (Biche *et al.,* 2011). Pymetrozine is an antifeedant (Harrewijn & Kayser, 1997; Kaufmann *et al.,* 2004) and so aphids survive for a longer period after application than when acutely neurotoxic neonicotinoids are applied. This is not ideal for reducing the spread of plant viruses, as the longer duration of survival of moribund aphids allows them to continue to disperse and spread plant viruses for several days after treatment, although it should be noted that this would occur at a reduced rate and there is much evidence that application of the chronic toxic pymetrozine has a beneficial effect in reducing the spread of viral disease (Bedford *et al.,* 1998; Margaritopoulos *et al.,* 2010). Nevertheless a shorter time to mortality, as found with the acutely toxic neonicotinoids, is preferable, both for the rapid impact in reducing viral disease and direct damage to the crop, but also to inspire grower confidence. There is therefore a serious need for new chemistries and agricultural practices to effectively control the FRC clone and any further neonicotinoid resistant clones that emerge within the future. In addition, since the market for neonicotinoids is extremely

large (Jeschke *et al.*, 2011) the resistant FRC clone may potentially cause serious economic damage in future to the agricultural industry; hence much further research is likely to be invested into further chemistries to control this pest.

While understanding the behaviour of the FRC clone does not directly provide a means for its control, it adds to the understanding of the mechanisms that cause the high levels of neonicotinoid resistance it exhibits. While the metabolic and target site based resistance mechanisms are already well characterised (Bass *et al.*, 2011), there has been no investigation into potential behavioural based mechanisms of resistance of this clone until the present study. Defining such behavioural resistance may aid in the design of alternate control strategies, such as IPM strategies where the behaviour of pest insects is often taken into account (Verkerk *et al.*, 1998). This may be of particular relevance in light of the reduced number of chemistries capable of controlling the FRC clone at present.

In addition, behavioural aspects of insecticide resistance in insects do not always appear as thoroughly studied as more conventional mechanisms of resistance, such as target site mutation, increased metabolism, increased excretion and reduced penetration (ffrench-Constant, 1999; Scott, 1999; Despres *et al.*, 2007; Alyokhin *et al.*, 2008; Hardstone & Scott, 2008; Boyer *et al.*, 2012). This may be because behavioural resistance mechanisms are often more difficult to study and define. A behaviour which appears to increase insecticide resistance in the laboratory may not actually occur within the field, and studying complex insect behaviour within the field is difficult. This study into the feeding and dispersal behaviours of three *M. persicae* clones which differ in neonicotinoid resistance is therefore of great interest in the field of behavioural based insecticide resistance.

6.2 Sublethal doses

The LD_{15} of the FRC clone was greater than that of both 5191A and US1L when host plants were root drenched with TMX, supporting previous work which shows the neonicotinoid resistance of the FRC clone to be greater than 5191A (Puinean *et al.*, 2010; Bass *et al.*, 2011). When host plants were treated with the antifeedant pymetrozine, however, although the LD_{15} dose of the FRC clone was still greater than that of US1L, the LD_{15} value of 5191A was greater than that of the FRC clone. This may illustrate the different selection pressures applied in the different geographic regions where these clones originated (Philippou *et al.*, 2010; Bass *et al.*, 2011). Since tobacco is commonly treated with pymetrozine (Margaritopoulos *et al.*, 2010), but not peach (Syngenta Crop Protection, 2011) these clones have probably been exposed to different levels of pymetrozine and TMX previous to their collection from the field.

A lower concentration of TMX was required to achieve the LD_{15} of the susceptible US1L clone when applied in artificial diet as opposed to a root drenched host plant. This suggests that not all of the TMX applied by root drench reached the target pest. It is known that TMX is xylem mobile (Maienfisch *et al.*, 2001) and the EPG work in chapter four showed that *M. persicae* appears to feed very little from the xylem of Chinese cabbage. It is therefore possible that greater concentrations of TMX are required to achieve the same levels of mortality when aphids are offered treated host plants as opposed to when they are offered treated artificial diet. Since TMX is xylem-mobile and *M. persicae* ingest primarily from the phloem of Chinese cabbage (chapter four), the aphids probably receive a lower dose than the concentration applied to the host plant. Alternately, in artificial diet the TMX is spread evenly throughout the sachet and so the *M. persicae* receive the full concentration of TMX applied. It is also possible that some TMX remained in the soil and was not taken up by the host plant, exacerbating this effect. Future work comparing the phloem and xylem concentrations of TMX in treated Chinese cabbage with the TMX concentration present in the artificial diet would allow validation of this theory. This could be achieved using an analytical approach such as LCMS. In addition, this study highlights the danger of using laboratory-based assays to predict LD_{15} values within the field, as even though this study did not include a field trial significantly different LD_{15} values were produced by different treatment methods even when involving the same compound and *M. persicae* clone.

When offered in treated artificial diet, the neonicotinoids imidacloprid and TMX required a lower concentration to achieve the LD_{15} than the antifeedant compounds pymetrozine and flonicamid. This is probably linked to the modes of action of these compounds; Neonicotinoids are agonists which bind with high affinity to the nicotinic acetylcholine receptors, producing prolonged nervous signals and causing death by toxicity (Matsuda *et al.,* 2005, 2009; Brown *et al.,* 2006). Pymetrozine and flonicamid are antifeedants which cause target aphids to starve to death (Harrewijn & Kayser, 1997; Morita *et al.,* 2007; He *et al.,* 2011). It takes longer for the same number of *M. persicae* to die by pymetrozine and flonicamid than by neonicotinoid compounds if the same time point is used to asses mortality (Chapter two); hence greater concentrations of antifeedants are required.

Further work would be required into the effect of sulfoxaflor upon the mortality of the FRC clone to determine whether this compound is a suitable candidate for control of this pest. Given however that resistance to one neonicotinoid compound often confers resistance to all other neonicotinoid compounds (Elbert & Nauen, 2000; Foster *et al.,* 2003, 2008), and the mode of action of sulphoxflor resembling that of other neonicotinoids, it is unlikely that this compound is a good alternative chemistry for control of the FRC clone. This study suggests that pymetrozine and potentially flonicamid would be better candidates, supporting commonly held views at present. Although this study did not include work on spirotetramat, it should be noted that this compound also has a different mode of action to that of the neonicotinoids (Biche *et al.,* 2011) and may be another promising alternative choice for control of the FRC clone.

6.3 Dispersal behaviour of neonicotinoid-resistant and susceptible *Myzus persicae* clones

The 5191A clone exhibited a significantly increased rate of movement compared to the clones FRC and US1L, even in the complete absence of TMX. This suggests that different clones of the same aphid species may disperse at different rates. Different aphid clones may therefore spread viral disease at different rates throughout a crop field. It should be noted however, that the situation in the field is more complex than the laboratory, with mixtures of different clones, viruses and host plants present within at same location. Viral transmission dynamics may therefore not directly relate to the flat rate of dispersal of the aphid clones shown by this study. In addition, in the field aphids are exposed to the effects of weather and predation, both of which affect their dispersal behaviour (Bailey *et al.*, 1995; Mann *et al.*, 1995; Narayandas & Alyokhin, 2006).

This information may also be useful in the context of aphid ecology. If the apterous individuals of different *M. persicae* clones disperse at different rates then it is possible that their populations may also potentially migrate at different rates. Different clones may also distribute themselves differently upon their host plants. Further work is required to see whether the dispersal differences between the *M. persicae* clones US1L, 5191A and FRC also occur within a field environment however before this study can be applied to *M. persicae* ecology.

It is interesting that the dispersal behaviour of the clones 5191A and FRC differed from the behaviour of the susceptible clone US1L in different manners (chapter three). Such deviations suggest that, should either of these dispersal behaviours contribute to resistance, they do so by different mechanisms. Alternately it is possible that the FRC clone exhibits more complex, developed dispersal behaviour than the 5191A clone. While 5191A moved at a faster rate and was often found upon the agar and dish sides compared to the other clones, the FRC clone relocated specifically to control treated plant tissue when in an environment of mixed TMX and control treatment. This suggests that 5191A may be dispersing generally away from stressed plant tissue, whether from TMX treatment or from the physical stress of excising the leaves and leaf discs. 5191A appears to disperse away from TMX but does not show any specificity in the new location chosen, while the FRC clone is able to relocate specifically to untreated plant tissue. This potentially makes the FRC clone a more serious crop pest as it may be able to continue feeding and reproducing for a longer time after TMX treatment compared to the 5191A clone, due to this enhanced ability to relocate to untreated

plant tissue. It may not even need to leave the host plant if parts of the host plant are not protected, such as when plants are sprayed with TMX rather than root drenched, leaving unsprayed areas. This behaviour is all the more powerful when taking into account the substantial neonicotinoid resistance the FRC clone exhibits from both target site mutation and enhanced P450 metabolism (Bass *et al.*, 2011), as FRC individuals will be more difficult to control at field rates than other clones, with surviving individuals able to perform relocation behaviours after sprays.

It should be noted that the altered dispersal behaviour of the FRC clone has only been demonstrated in an artificial laboratory setting, and the dispersal behaviour of this clone within the field remains theoretical until appropriate field studies are undertaken. Due to the DEFRA status of this clone within the UK such studies may however be limited to larger microcosms more consistent with a small field plot.

The difficulty of predicting the behaviour of the FRC in the field from an experiment conducted under artificial conditions is highlighted by the differences in dispersal behaviour exhibited by the FRC clone in chapter three as opposed to chapter five. In chapter three the FRC clone demonstrated an ability to relocated to untreated plant tissue in an environment consisting of a mixture of TMX treated and untreated plant tissue, a behaviour not shown by either 5191A or US1L. In chapter five however, the FRC clone exhibited no preference for untreated control plants over TMX treated plants. Since in chapter five the plants were root drenched with TMX it can be assumed that the TMX treated plants did not contain any untreated areas of tissue, as the insecticide should have spread evenly throughout all leaves and stems of the plant, although it would have been expected to concentrate in the xylem (Maienfisch *et al.*, 2001). This difference in behaviour may be due to the experiment in chapter three utilising excised leaves set in agar, while chapter five used whole host plants. It would be expected that excised leaves would be under greater environmental stress than whole plants, and potentially may have had greater levels of defensive chemicals associated with plant stress than the whole plants. The presence of these defensive chemicals may have affected the behaviour of the FRC clone. It would be useful were these experiments repeated in future to sample the phloem contents of the excised leaves and compare the levels of stress associated signalling molecules to those in the phloem contents of whole Chinese cabbage plants. Alternatively, it is possible that lower levels of metabolism of TMX occurred in the stressed excised leaves than in the whole host plants and that the FRC were responding to the greater levels of TMX AI present in the excised leaves. Analysis of the TMX concentration in

the phloem contents of both excised leaves and whole host plants would possibly allow validation of this theory, and could be achieved using an analytical method such as LCMS.

There is however, another possibility. Acquisition of several plant viruses has been shown to affect the behaviour of aphid vectors, in terms of dispersal, feeding behaviour and host plant choice (Alvarez *el al.,* 2007; Boquel *et al.,* 2011; Ingwell *et al.,* 2012; McMenemy *et al.,* 2012). Since large percentages of both the US1L and FRC clones remained upon the viral source plants for over 24 h (chapter five) it can be assumed that the majority of the aphid population acquired TuYV. This could be confirmed in future work in a manner similar to the confirmation of viral infection of host plants described in this study, using either RT-PCR or ELISA (Smith *et al.,* 1996; Chomič *et al.,* 2011). If TuYV also manipulates the behaviour of its vectors in some way, or infection of plants with TuYV alters the attractiveness of the host plant to *M. persicae,* then these changes in behaviour in response to the presence of TuYV may have overridden and masked any unique behaviours usually exhibited by the FRC clone in response to the presence of TMX treated plants. This would explain the differences between the results of chapters three and five. In order to confirm whether this is the case, the experiment described should be repeated, but with a second setup identical save for the complete absence of plant viruses. This would allow any effects of TuYV on the behaviour of the *M. persicae* clones to be distinguished from the behaviour of the clones in the presence and absence of TMX in a microcosm environment.

6.4 Feeding behaviour of neonicotinoid-resistant and susceptible *Myzus persicae* clones

There were many significant differences in the feeding behaviour of the three clones (chapter four). The majority of these differences however were between the resistant clones 5191A and FRC rather than a resistant clone and the susceptible clone US1L. The only significant difference in feeding behaviour between the FRC clone and US1L was the total time spent performing phloem salivation, but the dynamics of this behaviour were not clear. The FRC clone exhibited significantly greater periods of phloem salivation than US1L during the LD_{15} experiment but during the LD_{20} experiment US1L exhibited significantly greater periods of phloem salivation than the FRC clone, although on control treated plants. Overall it does not appear clear whether there is a consistent difference in feeding behaviour between the FRC and US1L clones and it seems unlikely that differences in feeding behaviour contribute to the neonicotinoid resistance of the FRC clone. Equally, since few behaviours were significantly different between the US1L and 5191A clones and these were not consistent between experiments it is unlikely that differences in feeding behaviour contribute to the neonicotinoid resistance of the 5191A clone.

There was a large number of feeding behavioural differences between the FRC and 5191A clones. Although none of these behavioural parameters were consistently different between the LD_{15} and LD_{20} experiments, the presence of so many differences between these two clones suggests that their feeding behaviour is generally significantly different, both in terms of phloem phase and pathway phase activities. Many of the behavioural parameters that were different involved E1, phloem salivation. It is possible that these differences in feeding behaviour therefore relate to the differences in host plants of origin rather than neonicotinoid resistance. Phloem salivation unblocks the forisomes and overcomes plant defences, allowing aphids to feed (Knoblauch & Peters, 2004). Aphids will perform phloem salivation more frequently and for greater periods when on resistant cultivars and non-host plant species (Montllor & Tjallingii, 1989; Sauge *et al.*, 1998a; Pompon & Pelletier, 2012). While the FRC clone took longer to reach phloem salivation and performed more probes before entering E1, 5191A exhibited a greater amount of time performing this same behaviour despite reaching it earlier (chapter four). It therefore seems feasible that Chinese cabbage may have been less acceptable as a host plant for one clone than another. It should be noted however, that both clones were raised on Chinese cabbage for many generations prior to the experiments described in chapter three, although the FRC clone has been removed from the field more recently than 5191A. Comparing the feeding behaviour of these two clones on a different

plant species may aid further understanding of the potential purpose of the differences in their feeding behaviour. Feeding behavioural differences between aphid clones of the same species are not unknown (Wilkinson & Douglas, 1998; Bournoville *et al.,* 2004; Pallipparambil *et al.,* 2010), although this study appears to represent the first work comparing the feeding behavioural differences of neonicotinoid resistant and susceptible aphid clones.

Given that the 5191A clone originated on tobacco plants, it is possible that it is the tobacco-adapted form of *M. persicae* known as *M. persicae* ssp. *nicotianae*. Although this form has many morphological distinctions from other populations of *M. persicae* (Blackman, 1987) molecular methods have shown that it is not distinct at a species level (Field *et al.,* 1994; Margaritopoulos *et al.,* 1998) and so *M. nicotianae* is considered another form of *M. persicae* rather than another species. This potential difference in form is another possible explanation for the large number of differences in feeding behaviour between the 5191A and FRC clones. *Myzus nicotianae* may have developed alternate, specialised feeding behaviour in order to avoid the insecticidal metabolites of tobacco, alkaloids. These compounds would not be present in peach, from which the FRC clone originated. *M. persicae* populations collected from peach and tobacco in Serbia have been shown to be morphologically different (Vucetic *et al.,* 2010) hence there is potential for them also to exhibit differences in feeding behaviour. The exact form of 5191A could be determined using DNA sequencing.

The feeding behaviours which were significantly different between clones were not consistent between the LD_{15} and LD_{20} experiments, both on control and TMX treated plants. These two experiments were not designed to examine the effect of TMX concentration on the feeding behaviour of *M. persicae,* however there is clearly potential for an investigation into this in future. Such an experiment would require the application of different concentrations of TMX to aphids simultaneously, to minimise the effects of other environmental variables on the aphid's feeding behaviour. Since the LD_{15} experiment was undertaken in winter and the LD_{20} experiment in summer, it is possible that the discrepancies between the results of the two experiments are due to the effect of season upon the aphids rather than TMX concentration. Even though it was attempted to maintain consistent environmental conditions during the two experiments including light, humidity and temperature, the DEFRA facilities did contain windows and it is possible that even short exposure to the outside photoperiod altered the feeding behaviours of the *M. persicae*. Equally, the host plants used during the experiments were grown in glasshouse bays with controlled temperature and humidity, and a degree of controlled artificial lighting, but the plants would still have been exposed to the

seasonal photoperiod effects to some degree through the glass roof. This could have caused physiological changes in the phloem contents of the host plants (Corbesier *et al.*, 1998; Gattolin *et al.,* 2008) to which the aphids may have responded by altering their feeding behaviour.

Application of sublethal doses of TMX appeared to exert a slight antifeedant effect on *M. persicae,* in line with many other reported studies which have shown neonicotinoids to be antifeedants (Devine *et al.,* 1996; Daniels *et al.,* 2009; Cho *et al.,* 2011; Shi *et al.,* 2011). The majority of feeding behaviours of most clones were unaffected by the application of TMX, with the exception of a decrease in the mean duration of phloem feeding of 5191A, and an increased frequency but decreased duration of probing of US1L upon plants, though the latter only occurred in the artificial diet study. It is probable that the low levels of response to the presence of TMX is due to sublethal doses being used, rather than greater doses more equivalent to the field rate. The application of such doses would have made comparing the behaviour of resistant and susceptible clones difficult however, as the majority of neonicotinoid-susceptible individuals would have died during the experiments. Nevertheless, the feeding behaviour of the three *M. persicae* clones on plants treated with TMX at the field rate should still be investigated. The FRC clone will be capable of surviving this treatment but may further alter its feeding behaviour in response to the presence of such high concentrations of TMX. Since there is already evidence that an investigation into the effect of TMX concentration upon *M. persicae* feeding behaviour is required in the future, given the differences in feeding behaviours observed between the LD_{15} and LD_{20} experiments, the field application rate should also be included in any future investigations. In addition to clarifying the differences in feeding behaviour between neonicotinoid resistant and susceptible *M. persicae* clones, this would also make the findings of this study more relevant to the field.

The comparison of the feeding behaviour of US1L upon TMX treated artificial diet and host plants suggested that US1L may potentially respond to a metabolite of TMX by reducing the total time spent probing, as it exhibited a greater probing time upon artificial diet than host plants. This cannot be confirmed however unless the concentrations of TMX metabolites such as clothianidin (Casida, 2011) in the phloem contents of treated Chinese cabbage are analysed and the feeding behaviour of US1L on artificial diet treated with TMX is compared with that on artificial diet treated with different TMX metabolites. If US1L responded to the presence of a TMX metabolite by reducing probing time this would be evidence that the metabolite was exerting an antifeedant effect. In order to compare more complex feeding

behaviours of the three clones using artificial diet, characterisation of the different waveforms produced by the three *M. persicae* clones when feeding on artificial diet would first be required, as they did not match those described by Nauen (1995).

6.5 Vector ability of neonicotinoid-resistant and susceptible *Myzus persicae* clones

Despite the dispersal and feeding behavioural differences described in chapters three and four, there was no evidence of a significant difference in the ability of the US1L and FRC clones to vector the persistently transmitted *Turnip yellows virus* (TuYV) in a microcosm environment. The work described in chapter five represents a very preliminary initial study into the vector abilities of these two clones, and much further work should be undertaken before it can be confidently stated that these two clones do not significantly differ in their ability to vector plant viruses. In particular, work should be carried out upon the transmission of a non-persistent virus such as PYV. The only significant difference in feeding behaviour which differed between the FRC and US1L clones described in chapter four was in the total time spent in E1. This behaviour would be expected to affect the transmission of persistent viruses, which require phloem feeding for inoculation and acquisition (Prado & Tjallingii, 1994; Hogenhout *et al.,* 2008), rather than non-persistent viruses which do not (Martín *et al.,* 1997; Powell, 2005). It is therefore possible that these two clones will also not differ in ability to vector a non-persistent virus.

The vectoring ability of the 5191A clone was not investigated due to space and time constraints. This clone significantly differed from the other clones in a large number of feeding behaviours, although the majority of these behaviours differed from those of the resistant FRC rather than the susceptible US1L clone (chapter four). In addition 5191A exhibited a significantly greater rate of movement than the other *M. persicae* clones during the dispersal experiments of chapter three, regardless of the presence or absence of insecticide treatment. This suggests that it is possible that 5191A may exhibit a significant difference in vector ability compared to the FRC and US1L clones and this should be investigated in future. Other studies have shown clones of other aphid species to differ in vector ability (Rice *et al.,* 1983; Lucio-Zavaleta *et al.,* 2001; Mowry & Ophus, 2002; Kozlowska-Makulska *et al.,* 2009) making this potentially feasible, although these studies have not included differentially resistant clones.

Treatment of plants with sublethal doses of TMX caused no significant difference in the frequency of viral infection for either the FRC or US1L clones. This is unusual given that many insecticides including TMX are beneficial for the treatment of plant viruses, causing a reduction in their spread (Nauen, 1995; Nauen & Elbert, 1997; Nauen *et al.,* 1998a,c; Gomes *et al.,* 2008). It is possible that application of a greater concentration of TMX similar to the

field rate rather than a sublethal dose would protect plants from viral infection. Alternatively it is possible that the arena utilised for these experiments was too small and not representative of the field environment, where aphids may disperse over far greater areas (Boiteau, 1997). This work should be repeated at the field rate, and ideally in a much larger arena more similar to field conditions, since the DEFRA status of the FRC clone makes field trials difficult.

6.6. Further work

Many of the investigations carried out in this study require repeating under consistent conditions for greater understanding to be obtained. In particular the effects of a greater number of chemistries on the feeding and dispersal behaviour of the three clones should be investigated. This could involve some compounds which were discarded after chapter two due to time constraints, such as pymetrozine and sulfoxaflor, but also the compound spirotetramat which although not included in this study is currently being suggested as a suitable means of control of the FRC clone. Such work if completed would be valuable information useful towards the development of new control measures for this newly emerged resistant clone.

The investigations into the dispersal behaviour (chapter three), feeding behaviour (chapter four) and vector ability (chapter five) of the three clones were all undertaken using sublethal doses of TMX. While this was required to compare the behaviour of the three clones, given that the susceptible clone US1L would not have been able to survive high TMX concentrations, repeating all of these experiments at the field rate is required to increase the relevance of this study to the field situation. In addition, it is difficult to compare the behaviour of aphids in an artificial laboratory environment with their behaviour in the field. Ideally further work is required to ascertain whether the differences in behaviour between the *M. persicae* clones shown by this study also occur in the field, particularly the dispersal and relocation behaviour of the FRC clone described in chapter three which may potentially contribute to its high level of neonicotinoid resistance. The DEFRA status of both the FRC and 5191A clones within the UK however makes this intangible. Larger DEFRA facilities in which to conduct such experiments may be a suitable compromise.

As mentioned in section 6.5 above, chapter five represented a very brief investigation into the vector ability of the FRC and US1L clones, and much further work is required before a judgement can be made on whether these two clones differ in vector ability. In particular, the study should be repeated in a larger set up more comparable to the field, and with a non-persistent as well as a persistent virus. Given the large number of differences observed in the dispersal (chapter three) and feeding (chapter four) behaviours of 5191A the vector abilities of this clone should also be investigated. Since the presence of some aphid vectored plant viruses has been shown to alter the behaviour of aphids (Alvarez *et al.,* 2007; Ingwell *et al.,* 2012; McMenemy *et al.,* 2012) identical set-ups should be run alongside future experiments

in which plant viruses are completely absent. This will require larger DEFRA facilities than were available at the time of this study, but will allow determination of whether the presence of plant viruses alters the behaviour of the three clones differentially. It may also be useful to investigate a plant virus of greater economic importance than TuYV is at present, such as PYV (Mowry & Ophus, 2002; Palukaitis, 2012); although it should be noted that this would require switching to a different host plant species which may itself alter the feeding and dispersal behaviours of the three clones, and TuYV still causes much economic damage annually (Stevens *et al.,* 2008).

Neonicotinoids have also been shown to affect the reproduction of target aphid species including *M. persicae* (Devine *et al.,* 1996; Magalhaes *et al.,* 2008; Shi *et al.,* 2011; Cho *et al.,* 2011). Some preliminary work was performed during this study to investigate whether the reproductive behaviour of the three clones was altered differentially by the presence of TMX but this work was unsuccessful and discontinued due to time constraints. Such an investigation however would be beneficial, as a clone which could reproduce at higher rates under neonicotinoid treated conditions would be more likely to survive. Alternatively many insecticide resistant lines, clones and species have fitness trade-offs, such as decreased reproduction or a decreased response to alarm pheromone (Boivin *et al.,* 2003; Higginson *et al.,* 2005; Foster *et al.,* 2011; Rivero *et al.,* 2011; Kliot & Ghanim, 2012), the latter of which occurs for the 5191A clone (Foster, 2009, personal comms.). It does not appear that a study has been conducted into this for the FRC clone. Additionally, the preliminary work attempted to determine whether the ratio of alate to apterous offspring of the FRC clone was altered by the presence of TMX, and whether the alate:apterous ratio of offspring differed between clones. Since alates are more capable of dispersal to distantly located new host plants than aptarae, an aphid which produces more alate offspring when under environmental stress such as when treated with TMX may be more likely to produce more surviving offspring than an aphid which produces greater numbers of apterous offspring. This has potential implications for the spread of both the FRC clone and plant viruses in the field. Since the high level of neonicotinoid resistance exhibited by the FRC clone allows survival at field rates (Bass *et al.,* 2011) alate offspring may potentially be capable of developing to adulthood upon treated plants. Considering both this and the unique dispersal and relocation behaviour exhibited by the FRC clone in chapter three such an investigation is highly recommended.

All of these studies have compared the behaviour of the neonicotinoid resistant clones 5191A and FRC with that of the susceptible clone US1L. This clone was however obtained from the

field in the 1980s. High levels of exposure to various classes of insecticides which came into more frequent use subsequent to the collection of this clone (Devonshire *et al.*, 1998), along with the natural migration and genetic recombination of aphid populations, have led to susceptible clones in the field today which may be very different in terms of genetics, physiology, insecticide resistance and even behaviour than US1L. Current studies often use the clone 4106A as a susceptible baseline to which to compare resistant clones (Foster *et al.*, 2008; Puinean *et al.*, 2010; Bass *et al.*, 2011). This study utilised US1L because it was available at Imperial College at the beginning of the PhD, before this study had been fully planned. At this initial stage US1L was appropriate for the purposes of the proposed study. Future investigations however may benefit from comparing the behaviour of the FRC and 5191A clones to 4106A rather than US1L. It is possible that behaviours which are significantly different between US1L and FRC will not be significantly different between 4106A and FRC. Alternatively new behaviours which are significantly different between the 4106A and FRC clones may emerge, some of which may contribute to the neonicotinoid resistance of the FRC clone.

In addition all of the work described in this study has been conducted using apterous aphids. Alates however occur frequently within aphid populations, and exhibit both different feeding and dispersal behaviours to apterous individuals (Boiteau & Parry, 1985; Boiteau, 1997; Boquel *et al.*, 2011). Alate individuals also exert different effects upon viral transmission dynamics to apterous aphids (Blua & Perring, 1992; Boiteau, 1997; Katis *et al.*, 2007). In order to fully understand the differences in behaviour between the *M. persicae* clones US1L, 5191A and FRC and how these differences may contribute to neonicotinoid resistance all of the experiments described in this study should be repeated for alate individuals of the three clones, with modifications where required to take into account their flying ability. This would increase the relevance of the findings of this study to the field, where alates are prevalent.

Finally, it is notable that the three clones compared in this study also vary in terms of the presence and extent of the esterase phenotype and MACE and kdr genotypes, all of which confer resistance to different insecticide classes. It is possible that is it one of these resistance mechanisms which is associated with the differences in behaviour exhibited between the three clones, rather than neonicotinoid resistance. Given, however, that the altered dispersal behaviour of the FRC clone is exhibited in response to the presence of TMX, a neonicotinoid, it seems more likely that these behaviours are associated with neonicotinoid resistance rather than other insecticidal resistance mechanisms. Further work into the behaviours exhibited by

further *M. persicae* clones in which these mechanisms are either present or absent, but which do not exhibit neonicotinoid resistance, would confirm or disprove any link between the alterations in behaviour and these other resistance mechanisms.

6.7 Conclusions

There is significant evidence of a behavioural component to the neonicotinoid resistance of the FRC *M. persicae* clone in terms of its enhanced dispersal behaviour. This component exists along with the already documented target site mutation and enhanced metabolic mechanisms (Bass *et al.,* 2011), although it may not contribute as equally to the neonicotinoid resistance as these other mechanisms. Further work is required in conditions more representative of the field in order to determine whether this altered behaviour significantly contributes to the neonicotinoid resistance of this clone.

The *M. persicae* clone 5191A exhibits differences in behaviour compared to the neonicotinoid susceptible US1L clone, but these behaviours also differ from those of the FRC clone and do not appear to contribute directly to its neonicotinoid resistance. Many feeding behaviours differ between the 5191A and FRC clones. It is possible that these behavioural differences occur because of differences in the host plant of origin of the two clones (Philippou *et al.,* 2010; Bass *et al.,* 2011). Such differences in feeding behaviour may benefit each clone by achieving greater nutrition or avoiding defensive chemistry or other defence mechanisms on their host plants of origin.

There is no evidence that any of the differences in the feeding behaviour of the FRC clone compared to other clones significantly contribute to its high level of neonicotinoid resistance. Again, such differences in behaviour may be due to differences in host plant of origin between the US1L and FRC clones. The results of this study make it seem more probable that the altered dispersal behaviour exhibited by the FRC clone is more likely to contribute to its increased neonicotinoid resistance than any differences in feeding behaviour.

There is no evidence that any of the differences in either dispersal or feeding behaviour exhibited by the FRC clone contribute to a difference in its ability to act as a vector of the persistently transmitted TuYV virus. Although the neonicotinoid resistance of this clone is still of concern for plant viral transmission in terms of the increased survival of this clone at high neonicotinoid rates (Bass *et al.,* 2011), it does not appear that the behavioural differences of this clone affect its ability as a vector of persistent viruses in either a positive or negative manner.

This study has revealed the presence of a behavioural aspect to the neonicotinoid resistance of *M. persicae* for the first time. Much further work is required to ascertain the contribution

of this behavioural mechanism to the neonicotinoid resistance of the FRC clone, and whether such behavioural changes can be exploited to improve control of this agricultural pest.

7.0 References

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Appendix 8.1

Commonly used abbreviations

AC Alternating current

DC Direct current

DEFRA Department for environment, food and rural affairs

ELISA Enzyme-linked immunosorbent assay

EPG Electrical penetration graph (technique)

FLN Flonicamid

IMD Imidacloprid

LBD Ligand binding domain

LD Lethal Dose

nAChR Nicotinic acetylcholine receptor

PYM Pymetrozine

RT-PCR Reverse Transcription Polymerase Chain Reaction

SFX Sulfoxaflor

TMX Thiamethoxam

Appendix 8.2

Virus abbreviations

BBMV *Broad bean mottle virus*

BCV *Beet chlorosis virus*

BMYV *Beet mild yellowing virus*

BRNV *Black raspberry necrosis virus*

BWYV *Beet western yellows virus*

BYCV *Beet yellows closterovirus*

BYDV *Barley yellow dwarf virus*

BYMV *Bean yellow mosaic virus*

CaMV *Cauliflower mosaic virus*

CMV *Cucumber mosaic virus*

CTV *Citrus tristeca virus*

CYDV *Cereal yellow dwarf virus*

PEMV *Pea enation mosaic virus*

PLRV *Potato leafroll virus*

PVY *Potato virus Y*

RLMV *Raspberry leaf mottle virus*

TSWV *Tomato spotted wilt virus*

TuYV *Turnip yellows virus*

TYMV *Turnip yellow mosaic virus*

TYLCV *Tomato yellow leaf curl virus*

ZYMV *Zucchini yellow mosaic virus*

Appendix 8.3 Further feeding behaviour statisitcs from chapter four

Table 8.1 p values indicating the significance of differences in feeding behaviour of three *Myzus persicae* clones when placed on a host plant treated with TMX at the LD_{15} concentration for each respective clone, or a control plant treated with Milli-Q water. Bolded values represent a significant difference between clones for that behavioural parameter.

	US1L	5191A	FRC
Total time probing (min)	0.55	0.45	0.28
Number of probes	0.79	0.28	0.70
Total time in C (min)	0.24	0.23	0.93
Number of C	0.83	0.21	0.69
Mean duration of C (min)	0.46	0.70	0.36
Number of E1	0.34	0.40	0.47
Number of E2	0.53	0.47	0.67
Number of sustained E2 (> 10 min)	0.32	0.35	1.00
Time to first probe (min)	0.35	0.42	0.72
Time taken to reach E1 from first probe (min)	0.99	0.97	0.47
Time taken to reach E2 from first probe (min)	0.94	0.95	0.76

Table 8.2 p values indicating the significance of differences in feeding behaviour of three *Myzus persicae* clones when placed on a host plant treated with TMX at the LD_{15} concentration for each respective clone, or a control plant treated with Milli-Q water. Bolded values represent a significant difference between clones for that behavioural parameter.

	US1L	5191A	FRC
Total Time in E1 (min)	0.25	0.99	0.12
Mean duration of E1 (min)	0.83	0.05	0.10
Number of probes to the first E1	0.68	0.41	0.11
Total time in E2 (min)	0.52	0.55	0.24
Mean duration of E2 (min)	0.85	0.92	0.40
% contribution of E1 to the phloem phase	0.81	0.59	0.18
Total number of pd	0.78	0.19	0.47
Total number of pds in first 10 min	0.31	0.72	0.87
Total duration of G	0.93	n.a.	0.77
Number of G	n.a.	n.a.	n.a.
Total duration of F	0.21	0.82	n.a.
Number of F	n.a.	n.a.	n.a.

Table 8.3 p values indicating the significance of differences in feeding behaviour of three *Myzus persicae* clones when placed on a host plant treated with TMX at the LD_{20} concentration for each respective clone, or a control plant treated with Milli-Q water. Bolded values represent a significant difference between clones for that behavioural parameter.

	US1L	5191A	FRC
Total time probing (min)	0.55	0.90	0.41
Number of probes	0.24	0.59	0.54
Total time in C (min)	0.32	0.59	0.48
Number of C	0.21	0.33	0.47
Mean duration of C (min)	0.12	0.99	0.80
Number of E1	0.18	0.06	0.99
Number of E2	0.10	0.06	0.99
Number of sustained E2 (> 10 min)	0.14	0.10	0.65
Time to first probe (min)	0.82	0.92	0.48
Time taken to reach E1 from first probe (min)	0.46	0.74	0.35
Time taken to reach E2 from first probe (min)	0.40	0.64	0.47

Table 8.4 p values indicating the significance of differences in feeding behaviour of three *Myzus persicae* clones when placed on a host plant treated with TMX at the LD_{20} concentration for each respective clone, or a control plant treated with Milli-Q water. Bolded values represent a significant difference between clones for that behavioural parameter. n.a. indicates there was not enough data available to statistically test that parameter.

	US1L	5191A	FRC
Total Time in E1 (min)	0.71	0.09	0.94
Mean duration of E1 (min)	0.11	0.92	0.43
Number of probes to the first E1	0.70	0.83	0.66
Total time in E2 (min)	0.33	0.42	0.58
Mean duration of E2 (min)	0.80	**0.02**	0.36
% contribution of E1 to the phloem phase	0.67	0.17	0.53
Total number of pd	0.81	0.16	0.56
Total number of pds in first 10 min	0.49	0.22	0.50
Total duration of G	0.11	n.a	n.a
Number of G	n.a	1.00	n.a
Total duration of F	0.23	n.a	0.42
Number of F	n.a	n.a	n.a

Table 8.5 p values indicating the significance of differences in the feeding behaviours of clones upon either plants treated with TMX at a dose equivalent to the LD_{15}, or else plants control treated with Milli-Q water. Bolded values represent a significant difference between clones for that behavioural parameter. n.a. indicates there was not enough data available to statistically test that parameter.

	Control	**TMX**
Total time probing (min)	**0.01**	**0.03**
Number of probes	0.06	**0.001**
Total time in C (min)	0.07	0.86
Number of C	0.06	**0.001**
Mean duration of C (min)	**0.003**	**0.001**
Number of E1	0.39	0.65
Number of E2	0.38	0.63
Number of sustained E2 (> 10 min)	0.24	0.24
Time to first probe (min)	0.18	0.69
Time taken to reach E1 from first probe (min)	0.60	**0.04**
Time taken to reach E2 from first probe (min)	0.19	**0.01**
Total Time in E1 (min)	0.84	**0.04**
Mean duration of E1 (min)	0.20	0.60
Number of probes to the first E1	0.59	**0.02**
Total time in E2 (min)	0.61	0.41
Mean duration of E2 (min)	0.61	0.20
% contribution of E1 to the phloem phase	0.59	0.17
Total number of pd	0.35	0.79
Total number of pds in first 10 min	0.91	0.30
Total duration of G	n.a.	n.a.
Number of G	0.32	**0.04**
Total duration of F	n.a.	n.a.
Number of F	0.17	0.26

Table 8.6 p values indicating the significance of differences in the feeding behaviours of clones upon either plants treated with TMX at a dose equivalent to the LD_{20}, or else plants control treated with Milli-Q water. Bolded values represent a significant difference between clones for that behavioural parameter. n.a. indicates there was not enough data available to statistically test that parameter.

	Control	**TMX**
Total time probing (min)	0.61	0.84
Number of probes	0.08	0.21
Total time in C (min)	0.78	0.50
Number of C	0.09	0.20
Mean duration of C (min)	0.17	0.06
Number of E1	0.31	0.84
Number of E2	0.40	0.64
Number of sustained E2 (> 10 min)	0.44	0.19
Time to first probe (min)	0.72	0.56
Time taken to reach E1 from first probe (min)	0.16	1.00
Time taken to reach E2 from first probe (min)	0.67	0.40
Total Time in E1 (min)	**0.008**	0.43
Mean duration of E1 (min)	**0.001**	**0.03**
Number of probes to the first E1	0.48	0.36
Total time in E2 (min)	0.83	0.15
Mean duration of E2 (min)	0.64	0.40
% contribution of E1 to the phloem phase	0.13	0.12
Total number of pd	0.21	0.45
Total number of pds in first 10 min	0.10	0.99
Total duration of G	n.a.	n.a.
Number of G	0.76	0.57
Total duration of F	n.a.	n.a.
Number of F	0.17	0.08

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Behavioural avoidance and enhanced dispersal in neonicotinoid-resistant *Myzus persicae* (Sulzer)

Lucy M. Fray[1], Simon R. Leather[2], Glen Powell[1], Russell Slater[3], Eddie McIndoe[4] & Robert J. Lind[4].

[1]Imperial College London, South Kensington Campus, London, SW7 2AZ.

[2]Imperial College London, Silwood Park Campus, Buckhurst Road, Ascot, Berkshire, SL5 7PY, UK.

[3]Syngenta Crop Protection, Werk Stein, Schaffhauserstrasse, Stein, CH4332, Switzerland.

[4]Syngenta, Jealott's Hill International Research Station, Bracknell, Berkshire, RG42 6EY, UK.

Correspondence to: Lucy Fray, Syngenta, Jealott's Hill International Research Station, Bracknell, Berkshire, RG42 6EY, UK. Phone: +44 01344 41 4648 E-mail: lucy.fray@syngenta.com

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Abstract

BACKGROUND: The peach potato aphid *Myzus persicae* is a major agricultural pest capable of transmitting over 100 plant viruses to a wide range of crops. Control relies largely upon treatment with neonicotinoid insecticides such as thiamethoxam (TMX). In 2009, a strain denoted FRC, was discovered in France which exhibits between 255-1679 fold resistance to current neonicotinoids previously linked to metabolic and target site resistance. Dispersal behaviour may potentially further enhance the resistance of this strain. This study investigated this possibility and is the first to compare the dispersal behaviour of aphid clones of the same species with differing levels of neonicotinoid resistance.

RESULTS: Comparing the dispersal behaviour of the FRC strain with that of a clone of lower neonicotinoid resistance (5191A), and a susceptible clone (US1L) highlighted several differences. Most importantly, the FRC strain exhibited an increased ability to locate untreated areas when presented with an environment consisting of both TMX-treated and untreated plant tissue.

CONCLUSION: The altered dispersal behaviour of the FRC may partially account for the high level of neonicotinoid-resistance exhibited by this strain in the field. Since the dispersal of aphid vectors is key to the transmission of viruses across crop fields this has implications for current crop protection practise.

Key words

Myzus persicae, dispersal, behaviour, resistance, neonicotinoid, thiamethoxam

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1. Introduction

The recently discovered peach potato aphid *Myzus persicae* (Sulzer) FRC population exhibits a resistance factor (RF) of 1679 to the neonicotinoid imidacloprid and 225 to thiamethoxam in topical bioassays. [1] Resistance in this population appears to be due to a combination of two separate mechanisms, a target-site mutation within the nicotinic acetylcholine receptor (nAChR) and enhanced detoxification of the insecticide by cytochrome P450 monooxygenases. While a mixed population and not a clone, all FRC individuals carry the same mutation within the nAChR and exhibit the same levels of neonicotinoid resistance, hence for the purposes of this study they will be treated as a clone. Neonicotinoid resistance is also achieved by the upregulation of P450 genes in the Greek *M. persicae* clone 5191A. [2] 5191A however exhibits a 60-fold RF to imidacloprid in topical bioassays [3] hence this clone's resistance, unlike that of FRC, is only detectable within the laboratory. *Myzus persicae* is already resistant to three other classes of insecticide by four other distinct mutations. [4] On the basis that resistance to one neonicotinoid chemical generally confers resistance across a whole insecticide class [5] and that the major method of control of this pest is by the application of insecticides the discovery of the FRC resistant strain presents a serious agricultural threat.

Resistance to neonicotinoids in insect pests is commonly achieved by upregulation of metabolic pathways. [6] It has been shown that, compared with susceptible strains, neonicotinoid resistant strains of *Bemisia tabaci* (Genn.) produce larger amounts of the primary metabolite 5-hydroxy-imidacloprid, which has a lower binding affinity for the nAChR than imidacloprid and would be subjected to secondary metabolism through inactivating conjugation reactions. This upregulated metabolism appears to be due to increased

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expression of P450s, with monoxygenase activity correlating strongly with neonicotinoid resistance in this species. Other species which show upregulation of P450s correlated with resistance to neonicotinoids include *Nilaparvarta Lugens* (Stal), [7] *Drosophila melanogaster* (Meigen) [8] and *Musca domestica* (L.). [9] In terms of neonicotinoid resistance, metabolic changes occur more frequently than target site mutations. Alteration of the nAChR target site has occurred in *N. lugens* [10] but in this case it arose in an artificially selected laboratory strain. It has also been suggested that target site resistance may occur in the Colorado potato beetle *Leptinotarsa decemlineata* (Say), but the mutation remains unproven. [11] Other than the *M. persicae* FRC population there is therefore little evidence of target site based neonicotinoid resistance arising in the field.

Although less commonly cited, behavioural changes may also contribute to insecticide resistance. For example, an imidacloprid resistant strain of *Myzus nicotianae* Blackman only exhibited such tolerance when the insecticide was applied in an oral ingestion assay rather than topically. This increased tolerance appeared to be linked to an alteration in feeding behaviour shown by the resistant strain compared with a susceptible strain. [12] A resistant strain of the German cockroach *Blattella germanica* (L.) showed increased aversion to agar containing fructose, glucose, maltose and sucrose, all ingredients commonly contained within commercial gel baits, than a susceptible strain. [13] Topical application of abamectin or fipronil reduced the difference in mortality between resistant and susceptible strains, indicating that this change in feeding behaviour contributed significantly to the resistance.

In theory, dispersal behaviour might also contribute to resistance. An individual which shows an increased tendency to quickly disperse from an insecticide-treated to an untreated plant may ingest a lower dose of insecticide and so be more likely to survive. In

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this way a resistant strain could potentially arise. This has happened in the Colorado potato beetle *Leptinotarsa decemlineata* (Say), where a Bt resistant strain shows increased flight activity compared with a susceptible strain upon ingestion of Bt toxin. [14] Larvae from Bt resistant strains have also been shown to be more behaviourally responsive to the presence of Bt than susceptible larvae, moving away from foliage treated with the toxin (Hoy and Head, 1995). [15] While previous work has linked enhanced dispersal behaviour to increased levels of pyrethroid resistance in two resistant *M. persicae* clones, [16] this is the first study to investigate the possibility of a behavioural component to neonicotinoid resistance in aphids by comparing the dispersal behaviours of neonicotinoid resistant and susceptible *M. persicae* clones. This study reports the novel finding of a behavioural component to the high levels of neonicotinoid-resistance exhibited by the *M. persicae* FRC population.

2. Methods

2.1 Insects and plant material

The susceptible *M. persicae* clone US1L was obtained from Imperial College London, while 5191A and FRC were obtained from Syngenta, with the permission of Rothamsted Research, UK. All three clones were reared on Chinese cabbage (*Brassica chinensis* L. *cv* Apex) in separate environmentally controlled chambers (22 ± 1 ºC, 16:8 h L:D photoperiod, 50 % relative humidity). Chinese cabbage plants for the FRC culture were sprayed to run off with 10 mg L^{-1} thiamethoxam [17] (TMX) (Actara®, provided by Syngenta Crop Protection UK) diluted with Milli-Q water. Such pressuring was required to retain the resistance of the population. To obtain adults of known age, apterous aphids were removed from the main culture and reared on leaf discs. Three and a half cm discs were cut from the tissue either side of the central vein of two-week old true Chinese cabbage leaves, inverted, and set into

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2% agar within the wells of a six-well plate. Five adult aphids were placed on the disc and allowed to reproduce for 24 h before removal. The remaining nymphs were raised to the first day of adulthood before being used in experiments. Aphids were starved in a Petri dish for 1.5 h prior to use.

Chinese cabbage plants were grown individually in 3 inch pots filled with compost (Levington F2 + sand) in a growth room (20 ± 1 ºC, 16:8 h L:D photoperiod and 50% relative humidity). Plants for all experiments were used at the seedling stage, when two to three true leaves had formed.

2.2 Sublethal dose work

Dose-response experiments were carried out prior to the dispersal experiment, in order to calculate a sublethal dose potentially capable of altering the behaviour of *M. persicae* without causing a high rate of mortality. Literature often cites the LD_{15}, the dose which causes the mortality of 15% of the population, as a suitable concentration for this purpose.[18,19,20] Due to their differing levels of resistance, the three clones required different concentrations of TMX to reach this LD_{15} dose.

Chinese cabbage plants as described earlier, were not watered for 24 h to allow the compost to dry. They were then root drenched with 50 ml of TMX and left for a further 24 h to allow uptake of the insecticide. Ten adult aphids of the same clone were then placed on each plant, which was covered with a 1240 cm^3 cylindrical plastic ventilated cage and placed into an environmentally controlled chamber under conditions of 22 ± 1 ºC, 16:8 h L:D photoperiod, 50 % relative humidity. After a further 96 h mortality of the aphids was assessed.

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Plants were treated with a range of at least five different TMX concentrations, diluted with Milli-Q water. Control mortality was assessed from aphids on plants root drenched with pure Milli-Q water. The dose response tests were repeated three times and the results plotted and fitted in Microsoft Excel to produce dose-response curves for each clone, in order to calculate LD_{15} doses. [21] This provided a suitable sublethal concentration at which to assess the dispersal behaviour of the three clones. For a more comprehensive comparison of the resistance factors of the 5191A and FRC strains see Bass *et al.*. [1]

2.3 Dispersal test

Chinese cabbage plants at the seedling stage with two to three true leaves were left to dry for 24 h and then root drenched with 50 ml TMX. Plants were drenched with a TMX concentration within 0.1 ppm of the LD_{15} value of the appropriate clone (Table 1). Control plants were root drenched with 50 ml Milli-Q water. All plants were left to stand for 24 h at conditions of 22 ± 1 ºC, 16:8 h L:D photoperiod, 50 % relative humidity to take up the TMX or Milli-Q water.

A pipette was used to transfer 20 ml of 2% agar into a 5 cm diameter Petri dish. A 3.5 cm diameter hole was then punched into the middle of a leaf, freshly removed from the plant. The leaf was placed abaxial surface uppermost within the agar before it fully set, ensuring that the outer edges protruded underneath the agar to prevent any aphids from accessing the lower surface. A 3.5 cm diameter disc punched from the same location on a different leaf was also placed into the partially set agar, positioned within the vacant hole left in the original leaf (Fig.1). The agar was then left for 1 h to set. Ten adult apterous *M. persicae* of the same clone were placed onto the centre of the disc. A second Petri dish in which holes

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had been punched and covered by netting was inverted and placed over the first Petri dish to form a ventilated lid, and the edges sealed together using parafilm 'M'.

The setup was then placed on a light box underneath the lens of a time lapse camera. A total of 12 microcosms could be recorded under the camera at the same time, each representing a different treatment/clone combination. Four different combinations of disc and leaf TMX treatments were offered separately to each aphid clone. These were both the leaf and disc coming from separate control plants treated with Milli-Q water, both the leaf and the disc coming from plants treated with TMX, the disc treated with TMX and the leaf from a control plant and finally the disc from a control plant and the leaf treated with TMX. In each case, when an arena contained either a leaf or a leaf disc treated with TMX, the plant had been treated with a dose of TMX equivalent to the LD_{15} value for the corresponding aphid clone contained within the arena. The camera was set up to record an image of all twelve dishes every five minutes for a total of 24 h. The position of the treatments under the camera was randomised for each of the ten repeats.

2.4 Statistical analysis

Image analysis was carried out using a macro written in the program ImageJ (Rasband, http://imagej.nih.gov/ij/) which was capable of calculating the change in pixels between two successive images. Since leaf shrinkage over a 5 minute period was minimal, change in pixels was considered equivalent to aphid movement between frames. The macro was capable of detecting the change in pixels within two different regions of interest in the dish, with the central disc forming one region and the leaf/agar/dish sides forming the other. The number of aphids located on the leaf, disc and agar were manually recorded for each treatment at hourly intervals from the still images. Dividing the change in number of pixels in a region of

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the dish by the number of aphids located there gave the rate of aphid movement. The rate of movement across the entire dish area for treatments where all plant tissue was either control or TMX treated were determined. The proportion of aphids on the disc out of the number found on both the leaf and disc in each treatment and the proportion of aphids on the agar out of the total number of aphids in the dish were also calculated.

Data were analysed by a series of t-tests on pre-planned treatment comparisons at each hourly time point following an initial F-test for an overall treatment effect. Differences in the proportion of aphids located on the disc and the proportion of aphids located on the agar were compared between dishes containing only control treated plant tissue and the other three possible treatments. Differences in the rate of movement were compared between dishes containing completely control treated and completely TMX treated plant tissue. Proportions of aphids upon the disc and agar were arcsine transformed and the rates of movement log transformed prior to analysis. All analyses were carried out using SAS software, version 9.2.

3. Results

3.1 Rate of movement

When under completely control treated conditions, the resistant clone 5191A exhibited significantly higher rates of movement over the entire arena than the susceptible clone US1L at 18 out of the 24 hourly time points (Fig.2). There was no evidence of any consistent significant pattern of difference in rate of movement between the 5191A and FRC strains in a completely control treated environment, or a completely TMX treated environment. There

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was a slight decrease in the rate of movement of US1L in the completely thiamethoxam treated environment towards the end of the experiment (Fig.2).

3.2 Aphid location

There were no consistent patterns of significant differences in the proportions of US1L on the disc between any treatments (Fig.3a). The proportions of 5191A upon the disc were significantly greater in the control disc TMX leaf treatment than the control leaf and disc treatment for 11 time points (Fig.3b), and in the control disc TMX leaf treatment rather than the TMX leaf and disc treatment for 19 time points, with greater proportions of 5191A upon the disc in the control disc TMX leaf treatment than all other possible treatments for the last 3 h of the experiment. There were significantly greater proportions of FRC upon the disc in the control disk TMX leaf treatment than all three other treatments for a total of 18 time points, more than any other clone (Fig.3c). This significant difference between treatments began 4 h into the experiment and lasted until the end.

The proportions of 5191A on the agar were significantly greater than those of both FRC and US1L for 22 time points in the control disc and leaf treatment (Fig.4a) and the last seven time points of the TMX leaf control disc treatment (Fig.4b). There were no other consistent patterns of significant difference in the proportions of aphids on the agar between clones for any other treatments (Figs 4c & 4d).

3.3 Aphid mortality and reproduction

No mortality of aphids occurred during the experiment, although there was a low level of reproduction. Since newborn nymphs move very little initially this was not judged to significantly affect the change in pixels between images as detected by the software.

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4. Discussion

Studies into the behavioural aspects of insecticide resistance in hemiptera have typically focused on differences in feeding behaviour [14,18,22,23] rather than dispersal. In terms of differences in dispersal between resistant and susceptible clones of the same species no studies have yet been published using neonicotinoids. Differences in dispersal behaviour have, however, been shown to exist between *M. persicae* clones resistant to other insecticides. Clones carrying carboxylesterase resistance exhibit a reduced tendency to disperse from wilting leaves as compared with susceptible clones (Foster *et al.,* 1997). [24] Differences in dispersal behaviour in response to parasitoid attack have also been shown between susceptible *M. persicae* clones and clones carrying either carboxylesterase or *kdr* resistance, with resistant clones less likely to disperse from leaves after exposure to parasitoids and subsequently more likely to be parasitized (Foster *et al.,* 2007; 2011). [25,26] Finally, clones carrying *kdr* and carboxylesterase resistance have been shown to exhibit a reduced dispersal response to aphid alarm pheromone compared with susceptible clones (Foster *et al.,* 1999; 2003a; 2005). [27,28,29] MACE resistant *M. persicae* clones, however, appear more responsive to aphid alarm pheromone than non-MACE forms and suffer lower levels of parasitism (Foster *et al.,* 2003b). [30] MACE resistant and sensitive forms also distribute differently upon the host plant, with larger numbers of MACE forms being found on the growing points of pepper plants. Pyrethroid-resistant *M. persicae* have been shown to walk off of deltamethrin-treated excised potato leaves in greater numbers than a susceptible clone [16] but aphids were only observed for ten minutes and were never offered a mix of treated and untreated leaf tissue. Najar-Rodríguez *et al.* [31] showed differences in landing and settling behaviour of two separate lineages of *Aphis gossypii* Glover, but the

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clones differed in preferred host plant species rather than insecticide resistance. Studies showing differences in dispersal between aphid species are more common, but do not link such behaviours with insecticide resistance. [32,33,34]

Two different dispersal behaviours were noted in 5191A and FRC, the two resistant clones or strains within this study. While 5191A showed an increased tendency to disperse away from all plant tissue regardless of insecticide treatment, the FRC population exhibited an increased ability to locate itself to areas of untreated plant tissue in an environment consisting of mixed insecticide treatment. While these changes in dispersal behaviour should increase the fitness of both clones under thiamethoxam treated conditions and so contribute significantly to their neonicotinoid resistance, the enhanced ability of the FRC population to locate untreated plant tissue and therefore target its dispersal may be more relevant given the high level of resistance already exhibited by this strain. [1]

The differential dispersal behaviour exhibited by the neonicotinoid resistant FRC population in this study should not be confused with the general repellency effect of some insecticides, which may also cause changes in feeding and dispersal. These effects are often seen and considered beneficial in insect control. [35,36,37,38] For example, the large pine weevil *Hylobius abietis* (L.) will avoid feeding on Scots pine twigs treated with lambda-cyhalothrin but will feed from untreated areas on the same twig [39,40] while exposure to imidacloprid-infused leaves has been shown to increase the general motility of *M. persicae*. [41] The study reported here, however, reports the discovery of a dispersal behaviour performed in response to TMX which differs between a resistant and susceptible clone of the same species, thus potentially linking this behaviour to the presence of neonicotinoid resistance in *M. persicae*.

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The dispersal behaviour of the FRC population has significant implications for agricultural practises, particularly when spraying infested crops with neonicotinoids, as it is likely that the FRC, if present, will relocate to any untreated areas within a field. This finding therefore emphasises the importance of ensuring that an insecticide is applied evenly throughout a crop. The modified dispersal behaviour exhibited by the FRC population may also possibly be of benefit in the case of an unsprayed trap crop [42] as there is potential for the aphid to move into this untreated region for destruction by other means.

Alterations in dispersal behaviour could lead to changes in the transmission of aphid-vectored plant viruses. Transmission of plant viruses is the major cause of yield loss and economic damage to crop systems by aphids. [43] *Myzus persicae* is a known vector of at least 100 plant viruses. [44] Aphids acquire plant viruses during feeding (for a review see [45]). Virions enter and adhere to the aphid stylets or midgut [46] during either short probes of the mouthparts into the plant tissue in the case of non-persistent viruses, [47] or longer sustained phloem feeding in the case of persistent viruses. [48,49] Transmission occurs when a viruliferous aphid disperses from a viral host plant and relocates to another, healthy, host plant. The inoculation of non-persistent viruses is associated with salivation during brief cell punctures by the stylets [50,51] while that of persistent viruses is thought to occur during phloem salivation. [47] Previous studies have often shown the application of insecticides to be beneficial in reducing the spread of plant viruses. [20,52,53,54,55] From this study it appears that *M. persicae* disperses at the same rate regardless of the presence or absence of neonicotinoid treatment, hence in theory, the application of neonicotinoids should exert either a beneficial (due to increased vector mortality) or else no effect on the rates at which plant viruses spread within a crop, supporting such findings.

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Of more relevance from a control perspective, the FRC may be more difficult to remove from the field than other *M. persicae* clones due to this combination of behavioural, metabolic and target site resistance. Treating plants with neonicotinoids would probably cause the FRC to relocate to any untreated or partially sprayed plants. In the case of a persistent virus where feeding must occur for several hours in order for the virus to successfully infect a new host plant, [56] this could limit or slow the spread of plant viruses to untreated plants, with treated plants gaining fuller protection from plant viruses. A plant virus however, may be more difficult to remove from a crop when resistant aphids are present due to the persistence of the vector.

Different aphid species have been shown to disperse at different rates [33,34] as do alate individuals of the same clone that differ in ovariole number. [57,58] This is the first study however to show differences in dispersal rates between different aphid clones of the same species with regard to apterous individuals. The greater rate of movement of 5191A compared with the other clones or strains, even under completely control-treated conditions, suggests that different clones may disperse across a field at different rates. It is therefore also possible that the rate of viral spread within a field may vary according to the different aphid clones present. In particular, the presence of 5191A and potentially other resistant clones may lead to the spread of crop disease at a greater rate, although the lack of an enhanced dispersal rate exhibited by the resistant FRC population should be noted. The effect of the enhanced dispersal of resistant *M. persicae* on the spread of plant viruses however remains theoretical and further work is urgently required to both determine whether this enhanced dispersal occurs under field conditions, and to link such changes in behaviour to any possible effects on vector ability.

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It is difficult to elucidate the mechanisms behind the unique dispersal behaviour shown by the FRC in this study, and further work would be required to do so. Potentially it is possible that the FRC population responds to either olfactory cues from TMX treated host plants, or else chemical signalling molecules ingested in the phloem of TMX treated plants, in a manner different to that of the neonicotinoid susceptible US1L clone. TMX has been shown to induce upregulation of salicylic acid-associated plant defence and stress responses in treated plants. [59] *Myzus persicae* appears to use phloem constituents in host discrimination [60] so it is possible that when feeding on treated plant tissue FRC and 5191A detect such stress chemical markers, leading to the induction of the alternative dispersal behaviours shown in this study. US1L may be either incapable of detecting such chemical markers, or else may not respond to them behaviourally. Alternatively the resistant aphids may be responding to olfactory signals produced by the host plant or insecticide. Aphids are capable of detecting plant volatiles and appear to use them when locating host plants. [61] It is the blend of different host volatiles, rather than individual volatiles, which appears attractive to aphids [62] and that certain plant volatiles offered individually may even be repellent. It is therefore possible that treatment of plants with TMX masks or changes such mixes of host plant volatiles and makes them less attractive to resistant *M. persicae*, while the susceptible clone US1L makes no such distinction. Different lines of the cotton aphid *A. gossypii* show different behavioural responses to the volatiles of the same host plants; [31] hence it is possible for different clones of *M. persicae* to also show different behavioural responses to the same volatiles. Further olfactometry work would be needed to determine whether this is truly the case.

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Since resistance to one neonicotinoid compound usually confers resistance to all other compounds within that class [63,64,65] it is possible that the FRC and 5191A *M. persicae* clones will exhibit similar behavioural differences in response to all neonicotinoid compounds. Although the nature of the relationship between behavioural and physiological insecticide resistance is in debate [66,67,68,69,70,71,72] it is still potentially possible that the behavioural resistance exhibited by the FRC clone would occur in the presence of other neonicotinoid compounds, although this would require further work to confirm. Indeed, the resistance factor for the FRC population is substantially higher for imidacloprid than TMX. It should be noted that in this study the FRC aphids were treated with a dose greater than the conventional field dose. TMX is not generally applied to Chinese cabbage, but the maximum recommended application rate on potato is 400 mg L^{-1}/ha. [73] Further study is therefore needed to determine whether the FRC would behave in a similar way at conventional field rates. US1L and 5191A would be unable to survive such rates, and so would present no problem under current control regimes. In addition, the dispersal behaviour of the FRC population should also be investigated at 0.1 mg L^{-1} and 0.9 mg L^{-1}, to verify that any changes in behaviour are due to the resistance of the strain and not due to the increased concentration of TMX applied.

It is difficult to accurately predict the behaviour of insects in the field from laboratory results. Aphids in the field would be exposed to a range of temperature and light conditions, both of which have been shown to affect host-finding ability of the potato aphid *Macrosiphum euphorbiae* (Thomas) [74] as well as the effects of weather, predation and physical barriers to movement between host plants such as soil. [75,76] While it has been

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shown that *M. euphorbiae* can travel up to 1.8 m over bare soil to successfully locate host plants [26] this experiment is not comparable to such conditions.

Aphids have been shown to disperse upwards from the older leaves at the bottom of the plant to the younger, newer leaves at the top. [77] It is suggested that this is a mechanism for finding suitable feeding spots upon a host as the population rapidly grows. While the leaf tissue in each treatment of this study always originated from two separate plants, it is possible that the dispersal behaviour exhibited by these clones is representative of intra-plant movement on the same host rather than inter-plant dispersal throughout a field. Understanding the differences in intra and inter-plant dispersal between these clones would require further work in suitable field conditions.

Much dispersal of aphid populations is mediated by winged individuals, known as alates. [78] There is however, evidence that dispersal of unwinged apterae can also cause substantially large infestations of plants. [32] While revealing several interesting implications, further work on alates is needed before a full understanding of the dispersal differences between these clones can be obtained. In addition, this study only compared dispersal behaviour between single examples of a susceptible, resistant and highly resistant clone or strain. Comparisons between 5191A and FRC and a different susceptible clone, such as 4106A, or further highly neonicotinoid resistant clones which may arise in the future, could reveal differences in behaviour not exhibited by the three clones included in this study. Nevertheless this study reveals significant differences in the dispersal behaviour of *M. persicae* clones of different neonicotinoid resistance levels, and it would not be unreasonable to suppose that similar behavioural differences might also occur between these clones under field conditions. The lack of literature on the behaviour of neonicotinoid resistant aphid clones makes this an

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especially interesting study. This may be the first step towards discovering the presence of such behavioural differences in the field between neonicotinoid resistant *M. persicae*.

In summary, this is the first characterisation of a behavioural resistance in aphids that actively disperse away from neonicotinoid treated leaves to untreated plant material. Together with the intrinsic biochemical and target site resistance it represents new challenges in the field for aphid control and the viruses they spread.

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Table 1 The LD_{15} concentrations of thiamethoxam for three *Myzus persicae* clones with differing levels of neonicotinoid resistance, as calculated from dose-response tests.

Clone	Rep	LD15 ± standard error	LD50 ± standard error	Slope ± standard error	P-value	Mean LD15 (mg L^{-1})	Drench concentration (mg L^{-1})
US1L	1	0.02 ± 0.01	0.08 ± 0.01	1.16 ± 0.16	0.997	0.1	0.1
	2	0.18 ± 0.06	2.20 ± 0.63	0.69 ± 0.14	0.875		
	3	0.11 ± 0.05	3.20 ± 1.24	0.51 ± 0.10	0.991		
5191A	1	0.92 ± 0.08	3.65 ± 0.17	1.26 ± 0.07	0.861	0.8	0.9
	2	0.66 ± 0.06	2.17 ± 0.10	1.45 ± 0.08	0.901		
	3	0.81 ± 0.04	1.90 ± 0.06	2.04 ± 0.10	0.605		
FRC	1	41.20 ± 3.60	166 ± 7.80	1.24 ± 0.07	0.839	42.4	42.5
	2	41.60 ± 2.00	105.30 ± 3.20	1.87 ± 0.08	0.178		
	3	44.40 ± 3.70	125.90 ± 6.20	1.66 ± 0.11	0.682		

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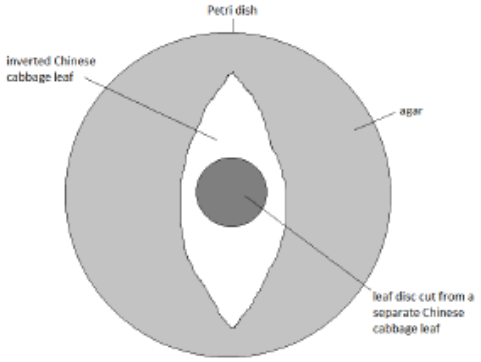


Fig.1 The setup of the arenas used to test aphid dispersal as viewed from above. Both the leaf and the leaf disc were inverted before being set into agar. Aphids were therefore exposed to the undersides of the leaves.

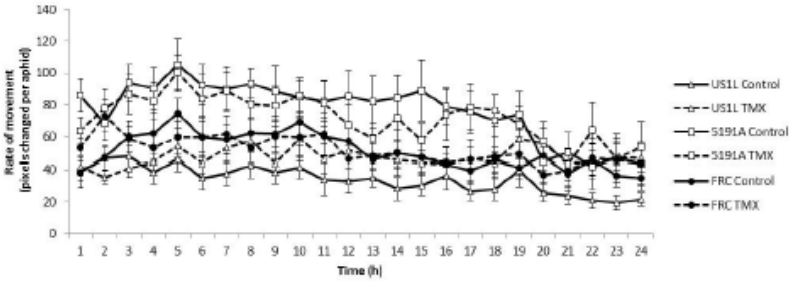


Fig.2 The rate of movement of the three *Myzus persicae* clones US1L, 5191A and FRC within arenas containing either completely control treated or completely thiamethoxam treated plant material.

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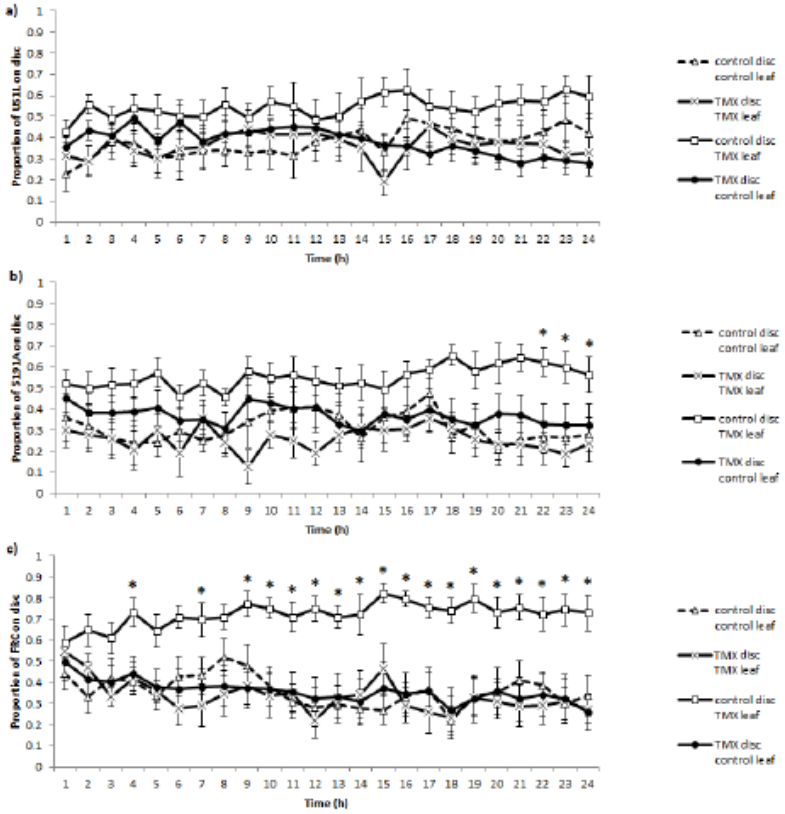


Fig.3 The proportion of a) US1L b) 5191A c) FRC on the disc out of the total number of *Myzus persicae* on leaf tissue in Petri dish arenas containing four different possible thiamethoxam (TMX) treatments, control treated leaf and disc, TMX treated leaf and disc, TMX treated leaf and control treated disc and control treated leaf and TMX treated disc. Asterisks indicate that the proportion of aphids on the disc in the control disc TMX leaf treatment was significantly greater than that for all other treatments at that time point. Error bars represent ± the standard error.

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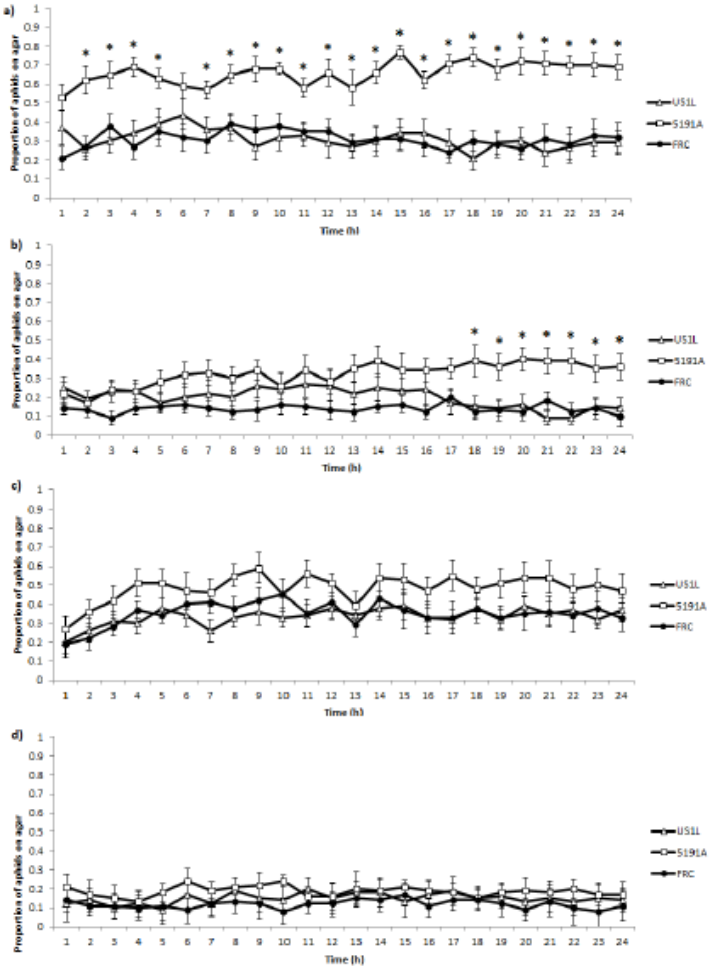


Fig.4 The proportion of three *Myzus persicae* clones on the agar in a Petri dish arena where a) all plant tissue is control treated with Milli-Q water b) all plant tissue is treated with thiamethoxam (TMX) c) the disc is control treated and leaf TMX treated d) the disc is TMX treated and leaf control treated. Asterisks indicate that the proportion of 5191A on the agar was significantly greater than all other clones at that time point. Error bars represent ± the standard error.