Title Resistance to conventional and novel insecticides in the glasshouse whitefly, *Trialeurodes vaporariorum*

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RESISTANCE TO CONVENTIONAL AND NOVEL INSECTICIDES IN THE GLASSHOUSE WHITEFLY, *TRIALEURODES VAPORARIORUM*

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A thesis submitted to the Faculty of Sciences, University of Luton, in partial fulfilment of the requirements for the degree of Doctor of Philosophy

January 2006

ABSTRACT

The incidence, influencing factors and mechanisms of resistance to insecticides from a range of chemical groups were examined in UK and European populations of the glasshouse whitefly, *Trialeurodes vaporariorum* (Westwood).

Toxicological assessments of populations from a range of plant production glasshouses and comparisons with the responses of a laboratory susceptible strain disclosed levels of resistance to pyrethroid, organophosphate, insect growth regulator (IGR) and neonicotinoid insecticides. Responses to conventional compounds indicated varying levels of resistance, potentially reflecting disparate usage between collection sites. All strains examined possessed resistance to the IGR, buprofezin; some populations were virtually immune to this commonly used control agent. Selection experiments demonstrated reciprocal cross-resistance between buprofezin and a further IGR, teflubenzuron, both of which are frequently incorporated into integrated pest management (IPM) programmes for this species. Results for the leading neonicotinoid, imidacloprid, revealed resistance in both UK and European strains, representing the first documented cases of neonicotinoid resistance in this species worldwide, and the first in any insect species within the UK.

The lethal effects of vapour emitted by applications of buprofezin and the anti-feedant effects of imidacloprid were demonstrated in *T. vaporariorum* for the first time. The potential consequences of these factors for both the control and selection of resistance were highlighted. Mechanistic studies using electrophoresis and kinetic spectrophotometer readings showed that neither non-specific esterases nor modified acetylcholinesterases were involved with resistance to either pyrethroid or specific organophosphate insecticides.

DEDICATION

The Road goes ever on and on,
Out from the door where it began.
Now far ahead the Road has gone,
Let others follow it who can!
Let them a journey new begin,
But I at last with weary feet
Will turn towards the lighted inn,
My evening-rest and sleep to meet.

For my brother John, to my daughter Lia

XXX

ACKNOWLEDGEMENTS

I would like to acknowledge and convey appreciation to my internal supervisors, Neville Punchard and Peter Coussons, for their expert guidance and advice; to my project mentor, Martin Williamson, for...for being there; to Graham Moores, whose expert biochemical tuition throughout both this project and my career has been invaluable; to Suzanne Clark for kindly contributing her profound statistical skills; to Bryan Lloyd and Kit Moores for their undying enthusiasm for editing; to my family, friends and colleagues who have helped maintain my insanity; to my wife Gabriela, who's support, encouragement and patience have been unwavering throughout; and lastly to my external supervisor, Ian Denholm, whose friendship, guidance and generosity over 17 years has meant so much.

DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Luton. It has not been submitted before for any degree or examination in any other University.

Kevin Gorman
5th day of January, 2006

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LIST OF ABBREVIATIONS

AChE = acetyl cholinesterase

cl = confidence limits

cv = cultivar

df = degrees of freedom

EC = emulsifiable concentrate

IGR = insect growth regulator

IPM = integrated pest management

IRM = insecticide resistance management

IUPAC = International Union of Pure and Applied Chemistry

kdr = knockdown resistance

MFO = mixed function oxidase

mOD = millioptical density

m/s = metres per second

OP = organophosphate

PAGE = polyacrylamide gel electrophoresis

ppm = parts per million

PSD = Pesticide Safety Directorate

SL = soluble liquid

T = treated with insecticide

UK = United Kingdom

UT = untreated with insecticide

V = volts

CHAPTER 1

GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

Insecticides have been in use for thousands of years. As early as 1000 BC, Homer reported the use of sulphur-based material to reduce pest damage and in around 200 BC fumigation with volatiles produced from heated bitumen was used to protect grape plantations (Fletcher, 1974). In the late 1800's dichlorodiphenyltrichloroethane (DDT) was discovered but without recognition of its insecticidal properties; placed in storage, it remained unexploited until the early 1940's. It's widespread use commenced with the suppression of mosquitoes, lice and fleas during World War II (Pedigo, 1999). DDT and related organochlorine molecules were subsequently adopted for controlling crop pests, signalling an agricultural revolution mediated by access to an increasing number of insecticides representing several chemical classes with distinct modes of action. Another compound with a long history of insecticidal applications is nicotine; still in use today as a fumigant and a foliar spray against a range of UK horticultural insect pests. By 1990 insecticide use had soared, in that year alone enough insecticide was bought to spray the entire earth's landmass twice (Wood Mackenzie, 2000).

Today, the insecticide market is a multi-billion dollar industry, dominated by large international agrochemical companies which have the scientific and financial resources required to meet mounting challenges with discovery, development and registration within increasingly strict environmental and safety constraints. This is further complicated by the need to maintain the efficacy and profitability of insecticides towards target pests, which under prolonged exposure to compounds or groups of compounds, often adapt to withstand their toxic effects.

1.2 EXTENT AND CAUSES OF INSECTICIDE RESISTANCE

Insecticide resistance is an evolutionary phenomenon that is now very widespread, affecting all known chemical classes and a wide range of species. By 1989, over 500 arthropod species had developed resistance to one or more compounds (Georghiou and Lagunes-Tejeda, 1991) and this figure has undoubtedly increased further since.

Insecticide resistance is a genetically inherited trait, enabling an organism to withstand higher doses of insecticide than susceptible counterparts. The resistant phenotype is therefore passed on to successive generations and in doing so, is subject to selection through exposure to insecticides and other environmental stimuli. When selection pressure is exerted on a population, individuals carrying beneficial gene(s) will possess a survival advantage. These 'resistant' individuals will begin to predominate as successive treatments kill a higher percentage of susceptible ones, resulting in a greater proportion of survivors carrying the resistant genotype through to subsequent generations (Plapp and Wang, 1979).

The development of resistance is thought to take place through the selection of naturally occurring genes, which confer resistance and have arisen via mutation within the susceptible 'wild-type' genome. With this in mind, it is not necessarily the appearance, but the frequency of these genes that we can attempt to manipulate in crop protection strategies. However, this is fraught with complications, as numerous ecological and genetic factors interact with the chemical itself and the operational tactics employed, to influence the evolution of resistance (Denholm, 1988). The rate of resistance development, relates amongst other things, to the intensity of the pressure exerted by the control agent. In general, increasing the degree of exposure (either through raising doses and/or application frequencies) will add to this pressure and result in a more rapid build-up of resistance. The 'pesticide treadmill syndrome', whereby

growers respond to a decrease in performance by reducing application intervals and increasing application rates, readily demonstrates this. This type of response, often due to over-reliance on a particular chemical, can render an insecticide ineffective in a very short time (Georghiou, 1979). Individual factors affecting resistance will vary in importance depending on the pest, control system and host plants under consideration (Riley and Tan, 2003).

1.3 WHITEFLIES AS CROP PESTS

Whiteflies are Hemipteran insects that inhabit tropical, sub tropical and temperate regions. They encompass 140 genera and more than 1500 species (Martin, 1987), 56 of which occur in Europe (Martin *et al.*, 2000). Although a wide variety of cropping systems are affected by whiteflies, the vast majority are not categorised as pests and even fewer are associated with annual cropping systems (Table 1.1). Only two are primary pests of global agricultural, namely the glasshouse whitefly, *Trialeurodes vaporariorum* (Westwood) and the cotton whitefly, *Bemisia tabaci* (Gennadius). Both of which have large host ranges that include many different plant genera (Byrne *et al.*, 1990). They have relatively short life cycles lasting 20-30 days (temperature dependent), are very fecund (females laying up to 30 eggs per day) and arrhenotokous.

Arrhenotoky is the use of a haplodiploid breeding system; females are diploid and contain the full chromosome complement while males contain half the genetic material and are therefore haploid. This system allows females to produce viable male (haploid) offspring asexually. Without successful mating, female whitefly will produce only male progeny. Sexual reproduction enables the development of diploid females, in addition to a proportion of haploid males. Male whiteflies cannot be heterozygous for genetically heritable traits and this has important implications for

resistance development and management. Heterozygotes are normally a valuable reserve of susceptible genes, and without such males, arrhenotoky facilitates the build-up of resistance within a population (Denholm *et al.*, 1998; Horowitz *et al.*, 1988). Whiteflies, along with many other agricultural insect pests, prefer the underside of leaves for feeding and breeding. Eggs, larvae and adults are sometimes protected from aerial sprays of insecticide, and in a dense canopy of leaves, even a hand-held lance can prove insufficient. These are just some of the combined characteristics that have enabled certain whitefly species to present such a serious and intractable threat to agriculture.

Table 1.1 Whiteflies associated with annual cropping systems (Byrne *et al.*, 1990).

Scientific name	Common name	Crop host examples
Aleurocybotus indicus	Cereal whitefly	Rice
Aleuroplatus malayanus		*Peuraria* spp.
Aleyrodes proletella	Cabbage whitefly	*Brassica* spp.
Aleyrodes lonicerae	Honeysuckle whitefly	Strawberry
Aleyrodes spiraeoides	Iris whitefly	Potato
Bemisia tabaci	Tobacco, sweetpotato or cotton whitefly	Various
Trialeurodes abutilonea	Bandedwinged whitefly	Cotton
Trialeurodes packardi	Strawberry whitefly	Strawberry
Trialeurodes vaporariorum	Glasshouse whitefly	Various

1.3.1 The glasshouse whitefly, *Trialeurodes vaporariorum*

Trialeurodes vaporariorum (Westwood), commonly known as the glasshouse or greenhouse whitefly inhabits the world's temperate regions. As the name implies, it is commonly found in glasshouses and other protected horticultural environments. It is a primary pest of many fruit, vegetable and ornamental crops. Suitable hosts in the UK are numerous and vary from tomatoes, cucurbits and peppers to fuchsias, gerberas and chrysanthemum. Adults are 1-2 mm in length, with yellowish bodies and four wax-coated wings held near parallel to the leaf surface (Figure 1.1a). Females are capable of mating less than 24 hours after emergence and begin to lay eggs within 48 hours. Eggs are pale yellow in colour, before turning grey prior to hatching. Newly hatched first instar larvae, often known as crawlers, are the only mobile immature life-stage; travelling just a short distance from the egg before inserting needle-like mouthparts into the phloem. During the first and second larval instars, the appearance is that of a pale yellow/translucent, flat scale which can be difficult to distinguish with the naked eye. During the fourth and final immature life-stage, the pupa, compound eyes and other body tissues become visible as the larvae thicken and rise from the leaf-surface (Figure 1.1b). This pupal stage usually lasts about one week (temperature dependent), prior to the predominantly morning emergence of adults, through a T-shaped, self-made slit (Martin *et al.*, 2000).

All life-stages apart from eggs and pupae cause crop damage through direct feeding, inserting their stylet into leaf veins and extracting nourishment from the phloem sap. As a by-product of feeding, honeydew is excreted and that alone can be a second, major source of damage; honeydew consists largely of sugars, rendering deposits very susceptible to infestations of moulds and fungi. When leaf surfaces become contaminated, there is a decline in photosynthesis and plant health (Byrne *et al.*, 1990). The third and potentially most damaging characteristic is the

ability of adults to transmit several 'clostero'-like plant viruses (Markham *et al.*, 1994; Jones, 2003). The crop hosts principally affected are vegetables such as cucurbits, potatoes and tomatoes, although a range of other crop and non-crop plants including weed species are susceptible, and can therefore harbour the infection.

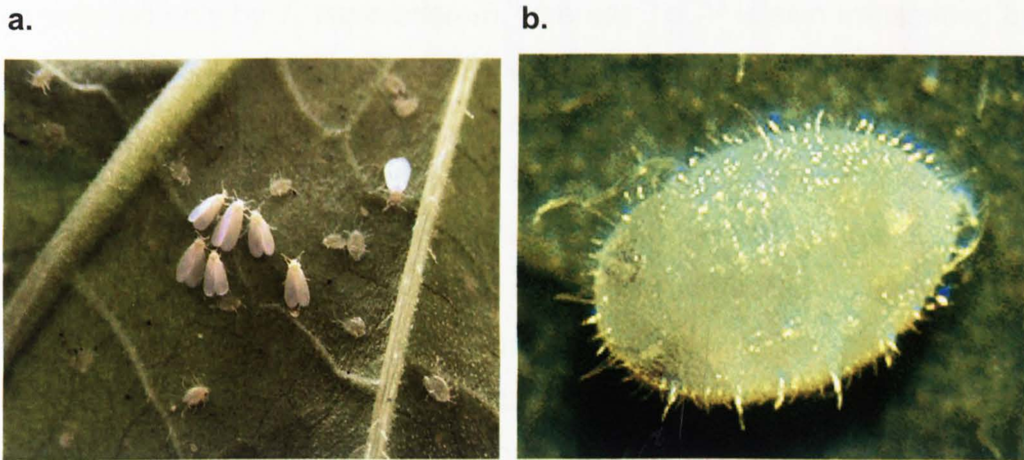


Figure 1.1 Life-stages of the glasshouse whitefly, *Trialeurodes vaporariorum*; **a.** adults and immatures **b.** pupa.

For over a decade, *T. vaporariorum* has been known as a primary vector of Melon yellows virus (MYV) (e.g. Jordagutierrez *et al.*, 1993), MYV has been responsible for severe crop failures of protected melons within Europe, particularly in south-eastern Spain and the Mediterranean Coast (Nuez *et al.*, 1999). Although known to be associated with *T. vaporariorum* for many years, it is not until relatively recently that outbreaks of potato yellow vein disease (PYVD) were directly attributed to the presence of potato yellow vein virus (PYVV), simultaneously confirming *T. vaporariorum* as a vector (Salazar *et al.*, 2000). Tomato infectious chlorosis virus (TICV) is another harmful, viral plant pathogen transmitted by this species. TICV has been shown to be accountable for losses of tomato produce of epidemic proportions in Italy since 2000 (Vaira *et al.*,

2002). There have been recent first reports for several countries and host plant species (Verhoeven *et al.*, 2003; Font *et al.*, 2004; Tsai *et al.*, 2004), demonstrating the capacity for establishment within new areas. Another whitefly-transmitted, phloem-limited, bipartite closterovirus that affects tomato is tomato chlorosis virus (ToCV). ToCV is distinct from tomato infectious chlorosis virus (TICV), based on a lack of serological and nucleic acid cross-reactions and differences in vector specificity. TICV is transmitted only by *T. vaporariorum*, whereas ToCV is also transmitted by the banded-wing whitefly (*T. abutilonea*) and *B. tabaci* (Wisler *et al.*, 1998). Cucumber yellows virus (CuYV) and Beet pseudo-yellows virus (BPYV) are also known to be transmitted by *T. vaporariorum* (Hartono *et al.*, 2003; Duffus, 1965), the latter causing symptoms identical to the *B. tabaci* transmitted Cucurbit yellow stunting disorder virus (CYSDV) (Livieratos *et al.*, 1998). In general, virus symptoms can include yellowing both of and between leaf veins, together with stunted and sometimes deformed new growth. Symptoms can be severe and persistent, leading to a reduction to both the quality and quantity of yields. Published virus transmission rates for *T. vaporariorum* are scarce; however, from data for *B. tabaci* it is apparent that rates can vary for different combinations of virus, host plant and whitefly. For some, a single feeding event is sufficient to acquire the virus and another for successful inoculation (Bedford *et al.*, 1994).

1.3.2 The cotton whitefly, *Bemisia tabaci*

As the subject of many references within this thesis, *Bemisia tabaci* Gennadius, commonly known as the cotton, tobacco or sweetpotato whitefly, warrants some description. It is an agricultural pest of more widespread economic importance and has commanded more detailed research than *T. vaporariorum*. Outdoors, *B. tabaci* inhabits tropical and sub-tropical regions, surviving on a wide range of favoured host plants including crops such as cotton, tobacco, tomatoes, cucurbits and ornamentals.

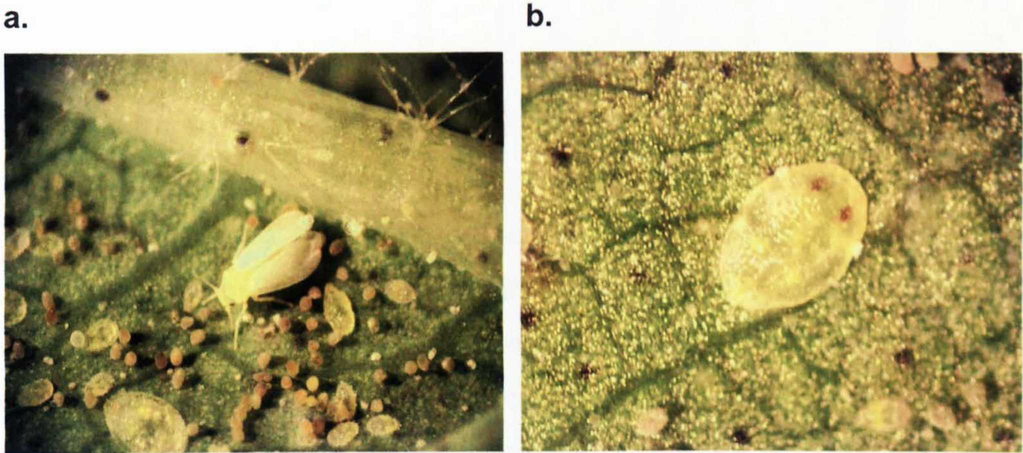


Figure 1.2 Life-stages of the cotton whitefly, *B. tabaci*; **a.** adult and immatures **b.** pupa.

B. tabaci is frequently found co-existing with *T. vaporariorum* and distinguishing features are few. *B. tabaci* adults are slightly smaller than *T. vaporariorum* and hold their wings closer to their bodies, at an angle of about 45° (Figure 1.2a). The other main visual difference is that pupae of *T. vaporariorum* possess numerous marginal setae while those of *B. tabaci* are relatively smooth in comparison (Figure 1.2b). The physiological host plant symptoms are essentially similar to those described for *T. vaporariorum* (section 1.3.1). However, *B. tabaci* transmits at least 111 different species of plant virus, the majority of which belong to the *Begomovirus* genus; the remaining 10% are members of the *Crinivirus*, *Closterovirus*, *Ipomovirus* or *Carlavirus* genera (Jones, 2003). They include some of the most severe and economically damaging plant viruses (Bedford *et al.*, 1994; Markham *et al.*, 1994) and as a consequence, even when present at low-levels this pest is capable of causing major crop failures (Bedford *et al.*, 1993).

1.4 CONTROL OF *T. VAPORARIORUM*

In temperate glasshouses, effective control has been provided for many years through the release of beneficial insects, principally the aphelinid parasitoid, *Encarsia formosa* Gahan (van Lenteren *et al.*, 1996; 1997). If required, integrated pest management (IPM) strategies can incorporate applications of selective chemical insecticides that complement natural enemies at times of high pest pressure or when environments are unsuitable to rely solely upon biological agents. For the majority of outdoor crops, employment of biological or cultural control strategies remains inadequate, and so chemicals are still the most widely used method. With advances in the development of transgenic technology and improvements in conventional breeding success, plant varieties with genetically enhanced protection are likely to become more widely accepted and available. However, as with insecticides the over-use of these control methods can result in the selection of insects capable of survival (Roush, 1997).

In the UK, the whitefly season begins around late February to early March, as glasshouse temperatures rise to around 15°C. This early season establishment can cause immediate problems for growers as the principal biocontrol agent for this species, *E. formosa,* only functions effectively at slightly higher temperatures (Qiu *et al.*, 2004). If unregulated, this time difference sometimes enables the pest to establish populations large enough to withstand maximum parasitoid inoculation rates from the outset. In addition to *E. formosa*, there are several other effective biocontrol agents available commercially including other parasitic Hymenoptera, predatory Hemiptera, predatory Coleoptera, and entomopathogenic fungi (Figure 1.3). Where simultaneous deployment of different beneficial organisms is desirable, knowledge of their associated interactions can be critical as both synergistic (Losey and Denno, 1998) and antagonistic (Lucas *et al.*, 2004) effects have been documented.

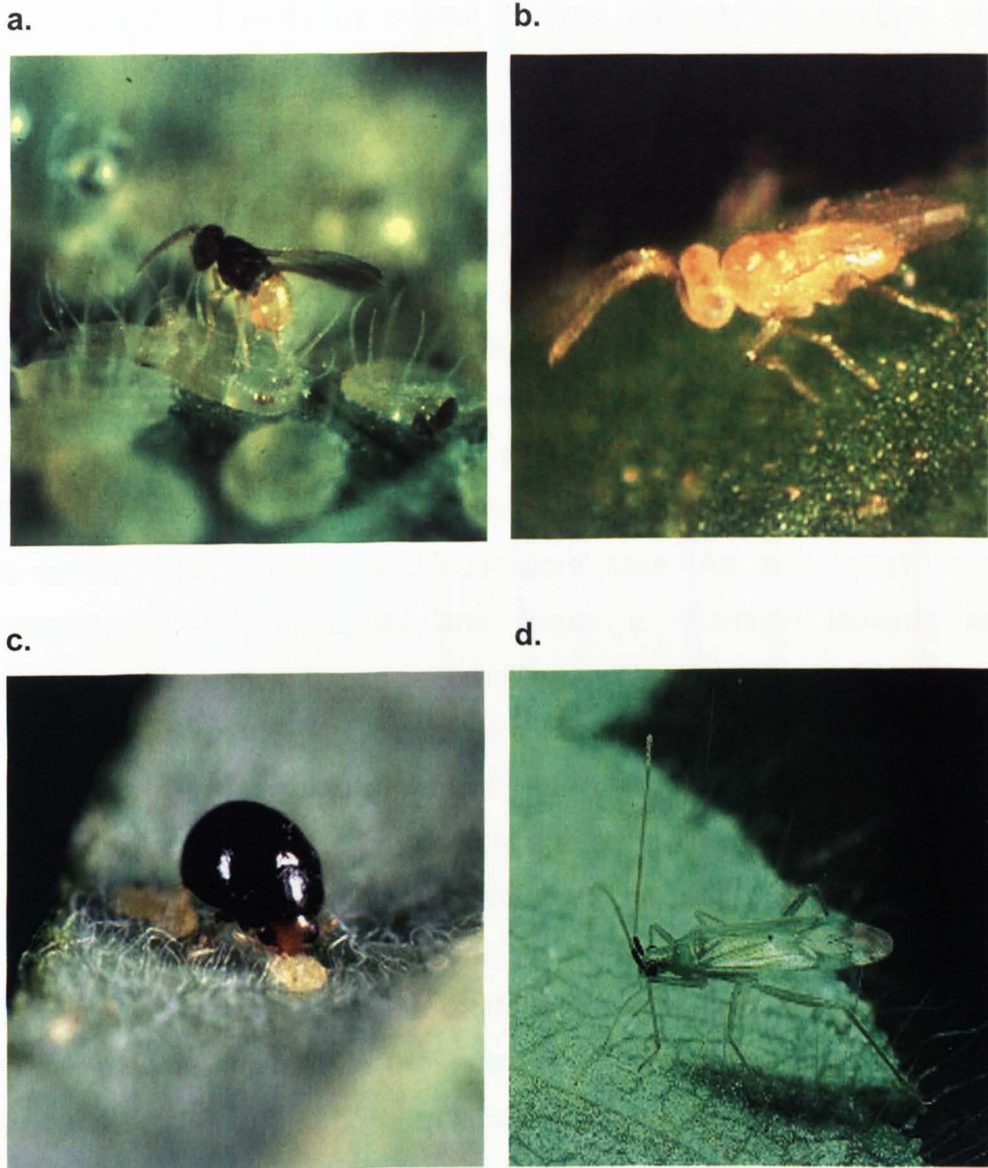


Figure 1.3 Commercially available beneficial insects used for the control of *T. vaporariorum* in UK horticulture; **a.** *Encarsia formosa* (Gahan) **b.** *Eretmocerus eremicus* (Rose and Zolnerowich) **c.** *Delphastus pusillus* (LeConte) **d.** *Macrolophus caliginosus* (Warner).

With a requirement for compatibility with beneficial insects, insecticidal control of *T. vaporariorum* within UK and European glasshouses has for some time centred on a limited number of IPM-compatible compounds, particularly insect growth regulators (IGR's) such as buprofezin and

teflubenzuron. Reports of control failures against this species have become increasingly common in recent years, both in the UK and mainland Europe. This may be as a consequence of increased reliance on a limited number of compounds, which has resulted in rising resistance levels that in some cases can confer survival beyond recommended application rates (Gorman *et al.*, 2002). As whitefly outbreaks have become increasingly problematical and with few, if any suitable alternatives, growers are sometimes forced to revert to more generic, conventional chemistries such as pyrethroids and organophosphates. These compounds have wide toxicity profiles and can be harmful to both bio-control agents and insect pollinators alike. As such, their usage compromises IPM strategies and forces a reversion towards less sustainable, chemical-based alternatives with a higher environmental impact. Consequently, selection pressures imposed by conventional chemistries may be rising as efficacy of these agents is also frequently reported as insufficient. In both edible and ornamental crops the situation appears to be worsening, and in many cases, poor control has led to harvest times being brought forward, an increase in production costs and significant losses of marketable produce (various growers, pers. comm.). For the UK, the insecticides most commonly targeted at whitefly over the last decade fall into one of four classes, representatives of each of these are included in work described in this thesis.

1.4.1 Pyrethroids

Initially discovered in the early 1970's at Rothamsted Experimental Station, the pyrethroids have proven to be one of the most successful and versatile chemical groups. Originally extracted from *Chrysanthemum* sp. (Elliot *et al.*, 1974), natural pyrethrum extract contains six insecticidal toxins on which the early, synthetic analogues were based. These compounds have in turn been modified to encompass a group of over 20 individual chemicals (Hassall, 1990).

Their intrinsic lack of stability in light and air compromised the application of the first synthetic pyrethroids, and replacement or modification of constituent functional groups and side-chains enabled synthesis chemists to resolve these issues. One of the major breakthroughs came with the substitution of the cyclopentenolone ring of the pyrethrin and allethrin alcohols, with an alternative unsaturated heterocyclic moiety (Elliot *et al.*, 1967). This resulted in the formation of resmethrin, the first synthetic pyrethroid to combine increased insecticidal potency with lower acute mammalian toxicity and photo-stability. The improved properties of resmethrin prompted further research that soon led to the development of permethrin, the first synthetic pyrethroid widely exploited by agriculture. As with all insecticides, performance, availability and cost denote success. The pyrethroids are no exception. Their relatively high intrinsic toxicity and low cost, combined with a range of affordable products that suit a variety of applications have made them a popular choice. By 1990, they accounted for 25% of the world insecticide market (Wood Mackenzie, 2000) and they retain a crucial role in many of today's chemical control programmes.

The pyrethroid investigated here, bifenthrin, is principally composed of two isomers (IUPAC: 2-methylbiphenyl-3-ylmethyl (*Z*)-(1*RS*,3*RS*)-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate or 2-methylbiphenyl-3-ylmethyl (*Z*)-(1*RS*)-*cis*-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate). The chemical structure of bifenthrin is shown in Figure 1.4. It is frequently used as an insecticide and/or acaricide against a range of UK horticultural pests including *T. vaporariorum*. It was first approved for UK use in 1988 and is sold commercially as an emulsifiable concentrate (EC) under the trade names 'Talstar' and 'Capture'. The active ingredients are comprised of up to eight stereo-isomers and at least 97% is known to be composed of the *cis*-isomers (Advisory Committee on Pesticides, 1989; Anon, 1991).

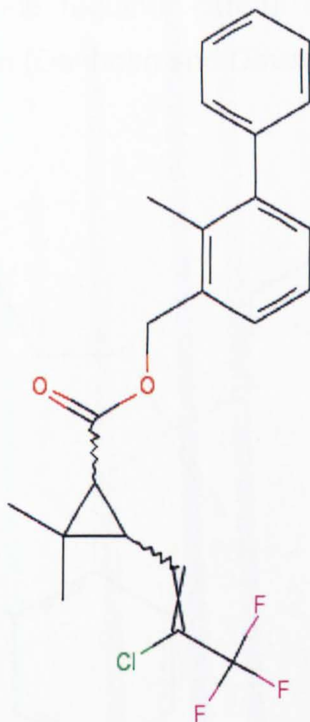


Figure 1.4 Chemical structure of bifenthrin.

1.4.2 Organophosphates

The most widely exploited class of chemical insecticides has been the organophosphates (OP's). Comprising over 40 individual compounds, these broad-spectrum insecticides have had a major impact since their introduction in the 1950's. Environmental concerns including long persistence times and high mammalian toxicity increased doubts about their safety and potential role in contemporary strategic use (Dutton, 2000). However, despite this they have retained a substantial share of the global insecticide market for the past 40 years and remain effective for a variety of applications. OP's retain a role, not only in agriculture but also in the public health and veterinary sectors (e.g. Grave, 1991). Their generic activity against a wide range of organisms means that there are numerous products available for whitefly control but as with all chemical classes,

intra-group cross-resistance requires careful consideration when using compounds in combination (Denholm and Devine, 2001).

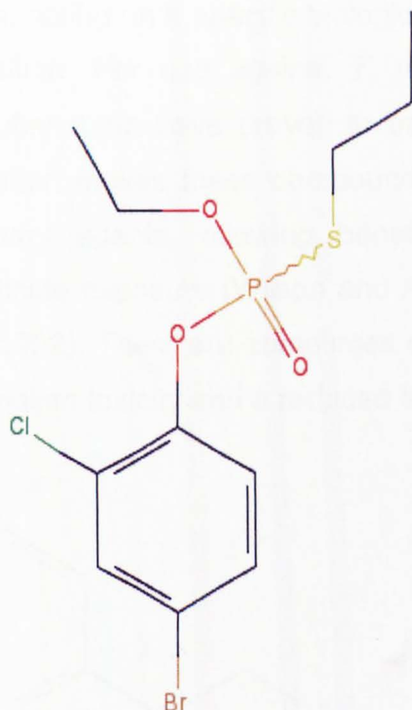


Figure 1.5 Chemical structure of profenofos.

The organophosphate investigated in this project is profenofos, whose structure is shown in Figure 1.5 (IUPAC: O-4-bromo-2-chlorophenyl O-ethyl S-propyl phosphorothioate). It is a non-systemic insecticide and acaricide first registered for use in 1982 by Ciba Geigy and marketed as an EC formulation under the trade name 'Curacron'. Although there is now little horticultural use within the UK, it is still used worldwide to control a range of pests on a variety of crops including rice, cotton, vegetables, citrus fruit and cereals, and serves as a valuable indicator of responses to the class as a whole.

1.4.3 Insect growth regulators

Insect growth regulators are compounds that affect the development of immature life-stages, acting on a specific biological process such as chitin synthesis or deposition. For use against *T. vaporariorum* in the UK, buprofezin and teflubenzuron have proven to be leading products. High species-specificity often makes these compounds ideal for incorporation with biological control agents, allowing beneficial insects to remain unharmed by insecticide exposure (Wilson and Anema, 1988; Ishaaya *et al.*, 1989; Ishaaya, 1992). There are sometimes other benefits, which can include lower mammalian toxicity and a reduced environmental risk.

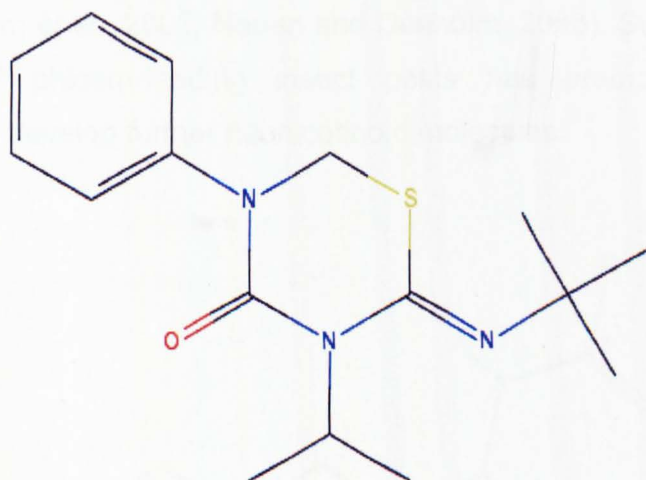


Figure 1.6 Chemical structure of buprofezin.

Buprofezin (IUPAC: -*tert*-butylimino-3-isopropyl-5-phenyl-1,3,5-thiadiazinan-4-one) is a thiadiazine (Figure 1.6), chitin synthesis inhibitor (Kanno *et al.*, 1981; DeCock and Degheele, 1993) marketed under the trade name 'Applaud'. When treated, susceptible individuals are unable to produce chitin and thereby form an exoskeleton, consequently dying during ecdysis. Due to this specific mode of action, buprofezin acts only on developing immature stages of certain species, which in addition to

whiteflies include other Hemiptera (mealybugs, leafhoppers, planthoppers and scale insects), Coleoptera and Acarina (Yarom *et al.*, 1988; Mendel *et al.*, 1991).

1.4.4 Neonicotinoids

Neonicotinoids, previously known as chloronicotinyls, are a novel class of insecticides that act on the nicotinic acetylcholine receptor of the insect nervous system, the same target-site as nicotine (Chao *et al.*, 1997). Since their introduction, resistance has developed gradually and is still only documented in a few insect pests (e.g. Grafius and Bishop, 1994; Devine *et al.*, 1995; Cahill *et al.*, 1996c; Prabhaker *et al.*, 1997; Elbert and Nauen, 2000; Denholm *et al.*, 2002; Nauen and Denholm, 2005). Success against a variety of phloem-feeding insect pests has prompted chemical companies to develop further neonicotinoid molecules.

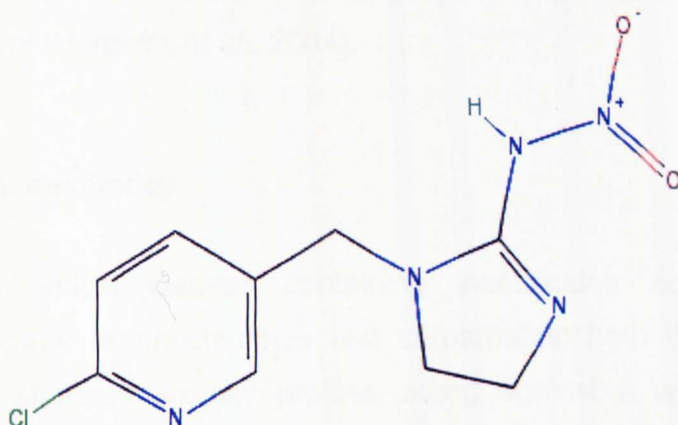


Figure 1.7 Chemical structure of imidacloprid.

The forerunner of the neonicotinoid class, imidacloprid (IUPAC: (*EZ*)-1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine), received its first UK registration for protected UK horticulture in 1997; its chemical structure is shown in Figure 1.7. Imidacloprid was initially available only for

a single use on containerised ornamentals; UK registration has since widened to incorporate bedding plants, herbaceous perennials and some edible crops. It is marketed as different formulations under the trade names 'Confidor', 'Gaucho', 'Intercept' and 'Chinook' and registered for UK use as seed treatments, formulated granules for integration with compost, or dissolved and applied as a root drench. Despite imidacloprid exhibiting good contact activity, foliar sprays are not currently permitted in the UK. Imidacloprid can act by contact and ingestion and against both adults and immature whitefly life-stages (van Lersel *et al.*, 2000). It possesses a high intrinsic toxicity against susceptible *T. vaporariorum* (Gorman *et al.*, 2002; Wang *et al.*, 2003) and *B. tabaci* (Cahill *et al.*, 1996c), and resistance develops relatively slowly (M. Cahill, unpublished data). Resistance may also be unstable in the absence of selection pressure (Prabhaker *et al.*, 1997). These characteristics have led to imidacloprid formulations becoming the world biggest-selling single insecticide and one of the most widely used against whiteflies. Thiamethoxam and acetamiprid are two of the other neonicotinoid compounds already making a global impact on whitefly control (Horowitz *et al.*, 2004).

1.4.5 Other insecticides

Additional chemical classes containing insecticides active against whiteflies include organochlorines and carbamates; both these classes have broad spectrum toxicity profiles acting against a wide range of organisms. As a result of environmental impact levels, loss of efficacy due to resistance and incomplete compatibility with beneficial insects, they are no longer used for whitefly control in the UK. Indeed, the majority of organochlorines and carbamates are not registered for use and are therefore of little relevance from a UK perspective. A number of other compounds targeting whitefly are available and include pymetrozine, a pyridine-azomethine marketed by Syngenta. Pymetrozine acts by arresting the feeding mechanism of susceptible insects, leading to incapacitation

and death due to starvation (Harrewijn and Kayser, 1997). Spiromesifen (Bretschneider *et al.*, 2003), spinosad (Salgado *et al.*, 1988), abamectin (Dybas, 1989) and pyriproxyfen (Ishaaya and Horowitz, 1995) are all thought to act uniquely and as such, may also prove to be important chemically acting compounds over the coming years.

There is an increasing number of physically acting insecticidal formulations that also demonstrate good efficacy against whitefly pests. They are often well-suited for incorporation into resistance management and integrated control strategies, helping to reduce the need for chemical products. Modes of action can vary and range from long-established soaps and detergents to more specific products, such as the spiracle-blocking Agri-50 (Murphy *et al.*, 2004).

1.5 INSECTICIDE RESISTANCE IN *T. VAPORARIORUM*

Due to the success of bio-control agents against *T. vaporariorum*, the majority of recent scientific literature has focused on relationships between whiteflies and their commercially available parasitoids and predators (van Lenteren *et al.*, 1997). Consequently, there is little published information regarding the status, mechanisms or impact of insecticide resistance in contemporary *T. vaporariorum* populations. This knowledge gap represents a real risk for protected horticulture as any fluctuation in resistance can not only proceed unnoticed, but also remain unchallenged by effective insecticide resistance management (IRM) strategies. Successful resistance management is heavily dependent upon early diagnosis of resistance and any delays are likely to reduce the potential of subsequent control attempts.

As a target of many different insecticides, *T. vaporariorum* is known to have developed resistance to several chemical classes. Pyrethroid and

organophosphate resistance was well documented in UK whiteflies during the 1970's and 1980's (Wardlow *et al.*, 1976; Wardlow, 1985) and still reduces efficacy of these conventional classes today. Resistance also affects performance of some insect growth regulators, including buprofezin (DeCock *et al.*, 1995; Workman and Martin, 1995). There are no documented cases of resistance to imidacloprid or other neonicotinoids. Despite the lack of up-to-date resistance monitoring data for this species, there have recently been a limited number of publications re-establishing baselines for a range of compounds that include some contemporary products (Bi *et al.*, 2002; Gorman *et al.*, 2002; Wang *et al.*, 2003).

1.6 CROSS-RESISTANCE

Cross-resistance occurs when resistance to one compound also provides protection against others. Although most common amongst members of the same chemical class or ones that are related structurally or functionally, cross-resistance can sometimes be unpredictable, affecting compounds considered as having different modes of action (e.g. Daborn *et al.*, 1995; Gorman *et al.*, 2002). The phenomenon of cross-resistance is particularly crucial to the success of new products, as those affected carry a greater risk of resistance development (Denholm and Devine, 2001).

For whiteflies, intra-group cross-resistance is known to affect three of the compounds under investigation in this thesis: bifenthrin (Cahill *et al.*, 1995; Wei *et al.*, 2001), profenofos (Cahill *et al.*, 1995; Miyo *et al.*, 2002) and imidacloprid (Nauen *et al.*, 2002; Gorman *et al.*, 2003; Rauch and Nauen, 2003). However, prior to this project buprofezin was considered to be unaffected by cross-resistance, including to other juvenile hormone mimics acting upon immature stages of the life cycle (Ishaaya and Horrowitz, 1995; DeCock *et al.*, 1995; Ishaaya *et al.*, 2003)

1.7 MECHANISMS OF RESISTANCE

Despite the publicised cases of insecticide resistance, to date, there are no documented mechanisms of resistance for *T. vaporariorum*. Mechanisms of resistance are the specific biological adaptations that confer the enhanced rate of survival, and these can for the most part be split into two types:

1. *Metabolic resistance* involves insecticides being broken down or converted into non-toxic metabolites within the insect.
2. *Target-site resistance* involves a modification of the target-site of the insecticide, either preventing or reducing the ability of the toxin to bind.

The mechanisms subject to biochemical investigation in this thesis are outlined in Table 1.2 and described in Chapter 5.

Table 1.2 The mechanisms of resistance under investigation.

Mechanism of resistance	Metabolic (M) or target-site (TS)	Documented to resist	Documented in *T. vaporariorum*
Elevated esterases	M	Pyrethroids, organophosphates	No
Modified acetylcholinesterase	TS	Organophosphates	No
Elevated mixed function oxidases	M	Pyrethroids, organophosphates, carbamates, neonicotinoids	No

1.8 AIMS AND OBJECTIVES

The overall aim of the project is to provide knowledge that furthers our understanding of the complexities associated with *T. vaporariorum* and the insecticide exposure it encounters, and in doing so, to provide contemporary data that enhance our ability to manage this insect pest safely and effectively within UK horticultural environments. To achieve this, a range of objectives tackled different research areas that were often refined by the resistance data disclosed from bioassays.

1. To examine the status of insecticide resistance in field-collected strains of *T. vaporariorum* (from the UK and mainland Europe) to four insecticides that represent the principal insecticidal groups recently used within the UK.

Responses to each of four compounds were assessed using a range of tailored, laboratory based bioassays. Levels of resistance were calculated through comparison with the response of an insecticide-susceptible laboratory strain (Chapter 2).

2. To disclose and investigate evidence of cross-resistance.

Bioassays provided the required information on the breadth and extent of resistance, giving an overall picture of the levels currently exhibited by UK whiteflies. In addition, resistance data were reviewed for correlations between compounds (i.e. across chemical classes) and reciprocal selection experiments used for verification (Chapter 2).

3. To establish the insecticidal activity of buprofezin vapour.

Consideration of any chemical characteristics that may influence the selection pressures imposed by chemical applications included the

insecticidal effects of vapour given off by the volatile compound buprofezin. Known to induce mortality in the cotton whitefly (De Cock *et al.*, 1990), this vapour action could have an impact on the selection pressures exerted by this chemical, particularly in glasshouses. Experiments were designed to examine the potency of any active vapours emanating from plants treated with foliar applications of buprofezin (Chapter 3).

4. To disclose any behavioural effects induced by imidacloprid applications.

One factor that can have a strong influence on insecticide dosage is insect behaviour. Imidacloprid has been shown to exert an anti-feedant response in other Hempitera (Nauen, 1995; Nauen and Elbert 1997), including *B. tabaci* (Nauen *et al.*, 1998, Isaacs *et al.*, 1999). This type of response could potentially stimulate migration from the treated area without acquisition of a lethal dose, consequently affecting pest and selection pressures on both treated and surrounding crops. To investigate the behavioural response of *T. vaporariorum* to this compound, choice tests were used to detect repellent properties (Chapter 4).

5. To investigate the presence of elevated esterases, as a potential mechanism of insecticide resistance.

Biochemical microplate assays and polyacrylamide gel electrophoresis were used to analyse and compare enzyme activities and types in both susceptible and resistant whitefly individuals. Data were reviewed for correlations between either enzyme activity or type, and resistance level (Chapter 5).

6. To investigate the presence of acetylcholinesterases, as a potential mechanism of insecticide resistance.

Biochemical microplate assays were designed and used to analyse the sensitivities of acetylcholinesterase (AChE) from susceptible and resistant individuals, both with and without the presence of insecticidal inhibitors. Data were reviewed for correlations between enzyme sensitivity and resistance level (Chapter 5).

7. To investigate the presence of mixed function oxidases (MFO's), as a potential mechanism of insecticide resistance.

Biochemical microplate assays were designed and used to analyse the activities of mixed function oxidases (MFO's) in susceptible and resistant insects. Correlations between enzyme activity and resistance level intended to elucidate any link between MFO's and resistance (Chapter 5).

CHAPTER 2

RESISTANCE PROFILES AND PATTERNS OF CROSS-RESISTANCE

2.1 INTRODUCTION

Quantification of the resistance that insect populations exhibit against specific chemical control agents is essential for monitoring both the performance of such products and the sustainability of resistance management strategies. This type of information often underpins research into the biology and behaviour of target species, the properties of chemicals, metabolic pathways and associated resistance mechanisms, and in doing so, the data presented in this Chapter helped to guide the direction of the other research described in this thesis.

In many cases, the most appropriate or only method for the evaluation of insecticide resistance is to measure the phenotypic response of insects to insecticides under controlled laboratory conditions. Usually termed bioassay, this type of assessment has previously provided consistent, repeatable results with many of the insecticides targeted at whiteflies (Cahill *et al.*, 1995; Prabhaker *et al.*, 1996; Elbert and Nauen, 2000; Ahmad *et al.*, 2002, El Kady and Devine, 2003). When the specific biochemical or molecular basis of insecticide resistance has been characterised, *in vitro* methods based on enzyme assays or DNA analysis may supplement bioassays and enable resistance to be diagnosed more rapidly and precisely in individual insects (Denholm, 1990). However, even when diagnostic biochemical or molecular markers are available, the relationship between genotype and phenotype needs to be fully understood. Additionally, the presence of multiple, or 'stacked' resistance mechanisms can complicate these relationships still further (Oakeshott *et al.*, 2003). For example, at least three independent resistance mechanisms are known to be present in some individuals of the peach-potato aphid, *Myzus persicae*. A detoxification system based on over-produced esterases and two target-site alterations collectively confer strong resistance to pyrethroids, organophosphates and carbamates (Devonshire *et al.*, 1998; Foster *et al.*, 2002). Even though these

mechanisms are detectable using a combination of biochemical and molecular techniques, predictions of phenotype have required detailed study of how the mechanisms interact and are vulnerable to the presence of additional, undetected mechanisms that also influence the phenotypic expression of resistance (Denholm *et al.*, 1990).

During bioassay, individuals of an insect species or strain are exposed to a prescribed amount of insecticide and the resulting mortality or sub-lethal effects recorded. The use of several concentrations or doses spanning the response-range enables median lethal doses or concentrations (LD_{50}'s or LC_{50}'s) to be calculated. Through comparison with the response of a known susceptible or 'wild-type' strain, resistance factors/ratios can then be estimated. Systematic use of such bioassays often underpins the design, validation and monitoring of pest control strategies (Jutsum *et al.*, 1998; Castle *et al.*, 2002), playing a pivotal role in the success of large-scale programmes in Arizona (Dennehy and Williams, 1997; Li *et al.*, 2003), Australia (Forrester *et al.*, 1993) and Israel (Horowitz *et al.*, 1993; Horowitz *et al.*, 1994).

Assessing the levels of resistance to a range of compounds yields a 'resistance profile' that aids the formulation of informed chemical control strategies. A comparison of such profiles can disclose consistent trends within or between functional chemical groups, thereby alerting to the potential threat of cross-resistance. However, regardless of consistency, correlations between the levels of resistance expressed to different compounds are little more than circumstantial evidence of cross-resistance, and the experimental confirmation is another area where bioassays can prove invaluable. Treatment (i.e. selection) with a particular compound in combination with pre- and post-selection bioassays can be used to detect shifts in responses to a chemical other than the selecting agent. Reciprocal cross-resistance occurs when this relationship holds in

both directions, i.e. selection with either product enhances levels of resistance to the other.

In order to relate bioassays to field performance, tests should mimic the field exposure as closely as possible (Sawicki, 1987). However, the choice of bioassay method is also driven by other criteria such as ease of operation, throughput, precision and repeatability (Denholm, 1990). Even when bioassays do at least approximate the mode of application and exposure in the field, a number of intervening factors can complicate these relationships (Welty *et al.*, 1989; Rowland *et al.*, 1991). Application technology, availability of alternative hosts, the weather and effects both of, and upon, natural enemies are just some of the variables that are usually excluded from laboratory-based bioassays. If discrepancies between bioassay data and field performance do exist, there is the potential for misguided predictions on both insecticide performance and the practical impact of resistance.

Disadvantages associated with bioassay relate to the significant time and resources required. For example, each larval bioassay used to assess resistance to IGR's during this project required 25 days, and the ability to culture age-structured populations under closely controlled environmental conditions (temperature, light and humidity) necessitated a well-equipped laboratory. Other workers have used similar methods to those employed here against *T. vaporariorum*, and the larval bioassay technique is analogous to that first described by French *et al.* (1973) and those used subsequently by Workman and Martin (1995) and DeCock *et al.* (1995). Adult leaf-dip and larval-dip assays have been used to determine glasshouse whitefly mortalities and/or document resistance against pyrethroid, organophosphate, organochlorines, carbamate and neonicotinoid insecticides (Wardlow *et al.*, 1976; 1985; Anis and Brennan, 1982; Collmann and All, 1982; Omer *et al.*, 1992; Bi *et al.*, 2002).

This Chapter describes bioassays designed to disclose contemporary levels of resistance and possible cross-resistance relationships, in populations of *T. vaporariorum* from the UK and two stains from mainland Europe.

2.2 MATERIALS AND METHODS

2.2.1 Insect strains

The strains of *T. vaporariorum* included a laboratory reference strain, originally established by L. S. Wardlow in the UK and subsequently reared for ten years in an untreated glasshouse at Cornell University, Ithaca, USA (J. P. Sanderson, pers. comm.), and 10 field-collected strains, whose geographical origins, host plants and collection dates are summarised in Table 2.1. The strain from Essex (UK-1) had been used by a bio-control company for c.15 years to rear *Encarsia formosa* commercially and had not knowingly been exposed to insecticides during that time. Other field strains came from commercial plant production glasshouses with varied treatment histories. All collection sites were known to have used pyrethroids, organophosphates and IGR's in their control programmes. Sites for UK-2, UK-4, UK-5, UK-6 and GER-1 were the only ones with known exposure to imidacloprid. All colonies were reared on French bean plants (cv 'Canadian Wonder') under a 16-hour photoperiod at 22°C, and maintained without exposure to insecticides.

2.2.2 Insecticides

Formulated insecticide products were used as follows: bifenthrin ('Capture', 25% emulsifiable concentrate (EC)); profenofos, ('Curacron', 50% EC); buprofezin ('Applaud', 25% EC); imidacloprid, ('Confidor', 25% SL); teflubenzuron ('Nemolt', 15% EC). To ensure even dispersal of active

ingredients, particularly at low concentrations, dilutions for residual assays (adult leaf-dip and larval-dip) were made by the addition of distilled water containing 0.01% of the non-ionic wetter Agral® (Zeneca Agrochemicals). For systemic assays, where the uptake of wetting agents such as Agral can induce phytotoxicity, dilutions were made by the addition of distilled water only.

Table 2.1 Strains of *T. vaporariorum*, including date of collection, *geographical origin and host plant information.*

Name	Origin	Host	Year collected
LAB-S	UK	French bean	1980
UK-1	Essex	Tobacco	1994
UK-2	Cambridgeshire	Hibiscus	1997
UK-3	Worcester	Tomato	1997
UK-4	Somerset	*Fuchsia* sp.	1997
UK-5	Jersey	Rose	1997
UK-6	Jersey	Rose	1997
UK-7	Surrey	Solanaceae	1997
UK-8	Surrey	Solanaceae	1997
NED-1	The Netherlands	Aubergine	1995
GER-1	Germany	Cucumber	1995

2.2.3 Bioassays

For the purposes of this project, it was necessary to utilise three different bioassay protocols relating to the different life-stages and chemical modes of action involved. The methods chosen were adaptations of protocols

published for use with the cotton whitefly, *B. tabaci* (Cahill *et al.*, 1995; 1996a; 1996b), modified to suit the requirements of *T. vaporariorum.*

Experimental design requires consideration of the environmental conditions, the life-stage under assessment and of the mode and speed of chemical action. Ideally, subjects should be representative of a fit, healthy population and of a consistent age, sex and size. For this reason, all whitefly cultures were reared in discrete generations and all adult insects used for bioassay were female and less than 10 days old. Females were chosen as they are diploid and therefore contain the full complement of genetic material, as opposed to haploid males. Appropriate endpoints, i.e. the time taken to reach maximum kill without significant (greater than natural response) control mortality, were initially adopted from assays on *B. tabaci.* 48 hours was found to be suitable for pyrethroids and organophosphates, however, for imidacloprid 72 hours was required to improve consistency. When compared to the corresponding *B. tabaci* assay, both treatment days and endpoints for the IGR assays were extended to accommodate the longer life-cycle of *T. vaporariorum.*

2.2.3.1 Adult leaf-dip

Resistance levels for pyrethroids and organophosphates were determined using a leaf-dip bioassay (Cahill *et al.*, 1995). French bean leaf-discs were dipped for 20 seconds into insecticide solutions, diluted to the required concentration with 0.01% Agral, or into the diluent alone for controls. Leaf discs were then laid on an agar bed held within a plastic Petri dish and after being air-dried, adult female insects were confined using a close-fitting ventilated lid (Figure 2.1a). For statistical purposes (see section 2.2.5), bioassays consisted of three replicates, each consisting of a group of 20-30 female insects for each concentration. All bioassays were maintained at 22°C with adult mortality scored after 48 hours.

2.2.3.2 Larval leaf-dip

Early instar whitefly nymphs were used to assess responses to the IGR's, buprofezin and teflubenzuron (following Cahill *et al.*, 1996b). Leaves on bean plants were trimmed into rectangles of approximately 40 mm x 50 mm. Adult females were confined to the trimmed leaves for 24 hours using clip cages (Figure 2.1b), thus providing an easily assessable cohort of eggs. Leaves were dipped 11 days later (when the majority of larvae had reached second instar), into insecticide solution diluted to the required concentration with 0.01% Agral, or for controls into the diluent only (Figure 2.1c). For statistical purposes (see section 2.2.5), bioassays consisted of three replicates per concentration, each consisting of a group of 50-500 unsexed larvae for each concentration (dependent upon fecundity rates). Bioassays were maintained at 22°C with larval mortality scored 22-25 days after eggs were laid, when surviving insects had reached late fourth instar.

2.2.3.3 Adult systemic

Responses to imidacloprid were determined using a systemic uptake assay, utilising a similar technique to the adult leaf-dip method (Cahill *et al.*, 1996c). French bean leaves were allowed 40 hours to take up either the required concentrations of imidacloprid diluted in distilled water, or water only for controls (Figure 2.1d). Leaf discs were cut and stored on an agar bed, held within a plastic Petri-dish. Adult female insects were added and confined using a close-fitting ventilated lid until endpoint. For statistical purposes (see section 2.2.5), bioassays consisted of three replicates, each consisting of a group of 20-30 adult females for each concentration. All bioassays were maintained at 22°C with adult mortality scored after 72 hours.

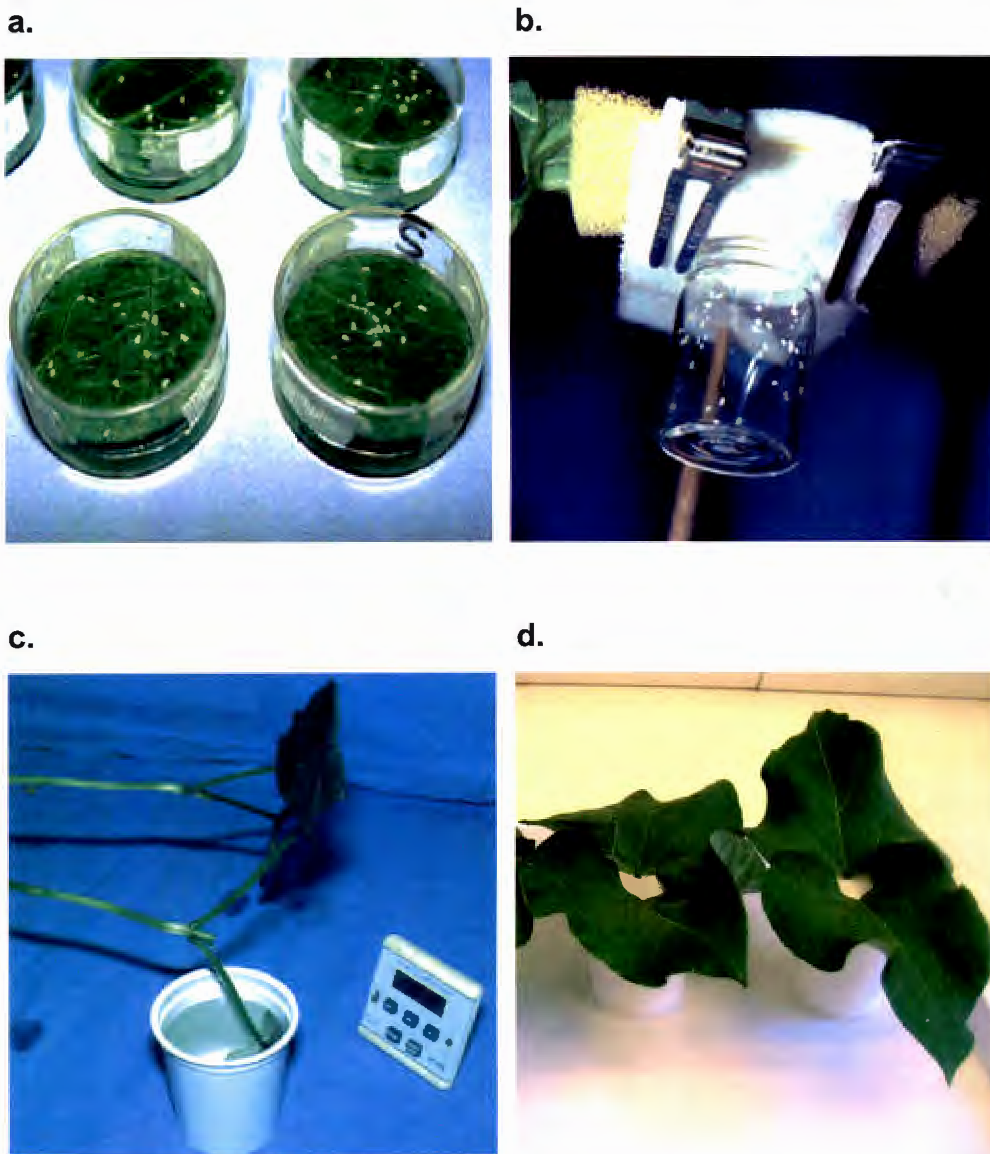


Figure 2.1 Experimental set-up of the different bioassay methods used; **a.** adult bioassay **b.** confinement of adults within clip-cages **c.** dipping larvae **d.** uptake of systemic insecticide.

2.2.4 Cross-resistance studies

Resistance assessments, both before and after the selection of a colony with the appropriate compound, were used to investigate suspected patterns of cross-resistance. All pre- and post-selection bioassays

contained a minimum of five replicates at the relevant field rate and followed the larval method outlined in section 2.2.3.2. Selections consisted of a single application of insecticide during a discrete whitefly generation. Buprofezin selections were performed at the field rate of 75 ppm, whilst for teflubenzuron two selecting concentrations were used, 75 ppm (field rate) and 1500 ppm.

2.2.5 Data analysis

Bioassays were designed in consultation with statisticians at Rothamsted Research. All adult bioassays consisted of a minimum of three replicates per concentration with an average number of 20 insects. Larval bioassays also used a minimum of three replicates and contained between 50-500 immature insects per replicate, dependent upon inoculation/fecundity rates. The cross-resistance experiments were repeated as in Table 2.4. When appropriate, dose-response data were subjected to probit analysis using the POLO computer programme (LeOra Software, Berkeley, California). This program firstly corrects for control mortality before calculating percent mortalities and relevant probit parameters. For resistant strains, mortality was sometimes too low or too heterogeneous for probit lines to be fitted. All LC_{50}'s given are listed with 95% confidence limits.

2.3 RESULTS

2.3.1 Bioassays

Results for bifenthrin demonstrated considerable variation between strains (Figure 2.2). The response range for LAB-S covered approximately three orders of magnitude (0.1-100 ppm) with 100% mortality being achieved at 128 ppm. Three of the field-collected strains (UK-1, NED-1 and GER-1)

gave a similar response to that of LAB-S and were considered as fully-susceptible. All other strains showed varying levels of resistance with mortalities at 128 ppm ranging from less than 5% (UK-4) to approximately 80 % (UK-8). The most resistant strains (UK-4, UK-5 and UK-6) were not completely controlled, even at concentrations high enough to induce phytotoxic effects.

Compared to the other compounds assessed, LAB-S gave a relatively steep probit line against profenofos (Figure 2.3) that covered a single order of magnitude (100-1000 ppm). UK-1, UK-2, UK-3, UK-7 and UK-8 gave responses that were not significantly different to that of LAB-S. The other field collected strains (UK-4, UK-5, UK-6, NED-1 and GER-1) were more than 10 fold resistant at LC_{50} and phytotoxic effects were apparent before complete control of these strains was achieved.

The whitefly strains also varied markedly in their response to buprofezin (Figure 2.4). Buprofezin has a high intrinsic toxicity against susceptible *T. vaporariorum* with LAB-S insects giving an LC_{50} of approximately 0.01 ppm, 100% kill was achieved at around 1-2 ppm. The response range for LAB-S spanned over four orders of magnitude. None of the field-collected strains gave a response comparable to LAB-S; all 10 strains contained a proportion of resistant individuals. UK-1 had a low LC_{50} (0.12 ppm) but exhibited a 'plateau' in its response from 1 ppm upwards, and even 10,000 ppm did not kill the most resistant individuals of this population. Other strains showed moderate to extreme levels of resistance and similar evidence of plateaux, over which increases in the concentration of buprofezin caused no corresponding increase in mortality. The most resistant strains (UK-3, UK-4, UK-5 and UK-6) were effectively immune to this chemical.

No resistance was found to imidacloprid (Figure 2.5); there was no significant difference between the responses of field collected strains and

that of LAB-S. The LC_{50}'s of all strains fell between 2 and 11 ppm. Some sites had a history of moderate imidacloprid exposure but no reflection of this was detected in the bioassay results. Indeed, LAB-S is at the upper end of the response range when compared to other strains and although insignificant at LC_{50}, a similar pattern of responses to imidacloprid was observed with a range of field collections of the currant-lettuce aphid, *Nasonovia ribisnigri* (Barber, 2002). Imidacloprid was the only compound for which responses were homogeneous enough to compute LC_{50}'s across all strains (Table 2.2).

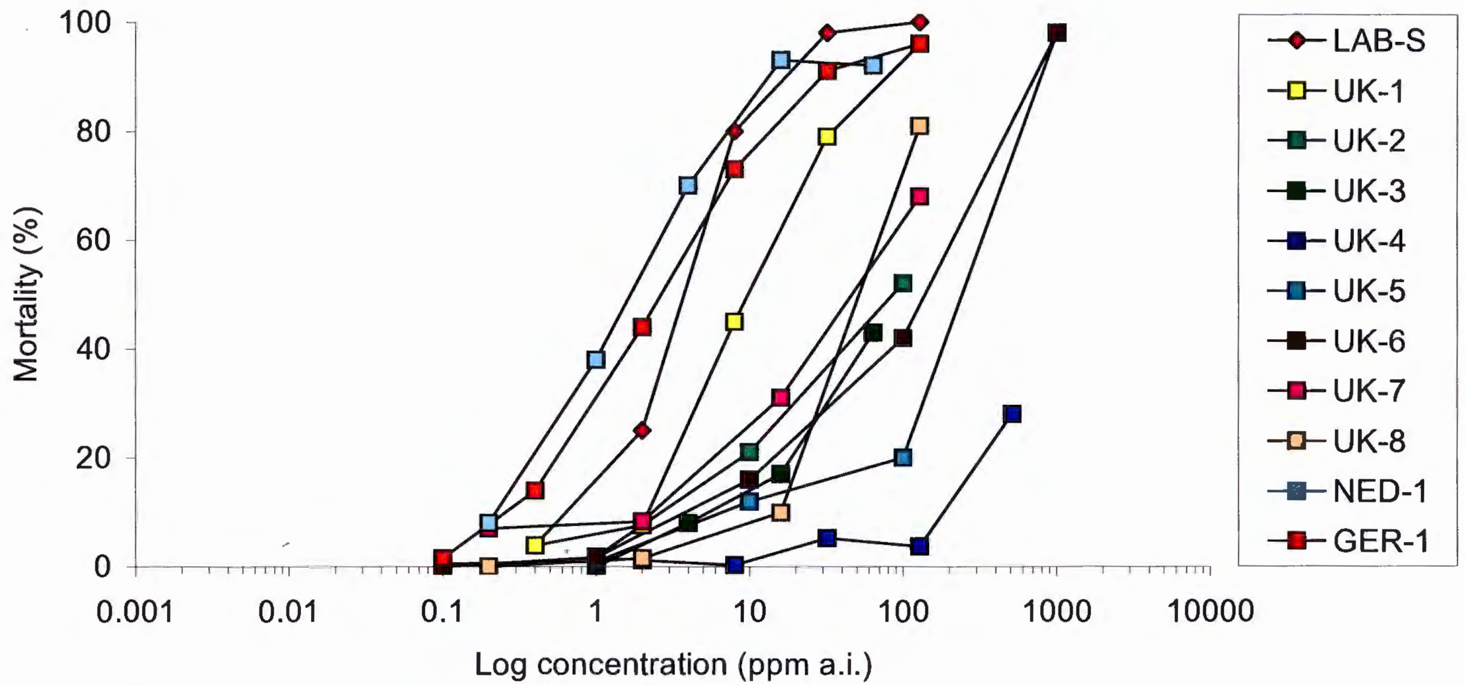


Figure 2.2 Responses of insect strains to the pyrethroid, bifenthrin.

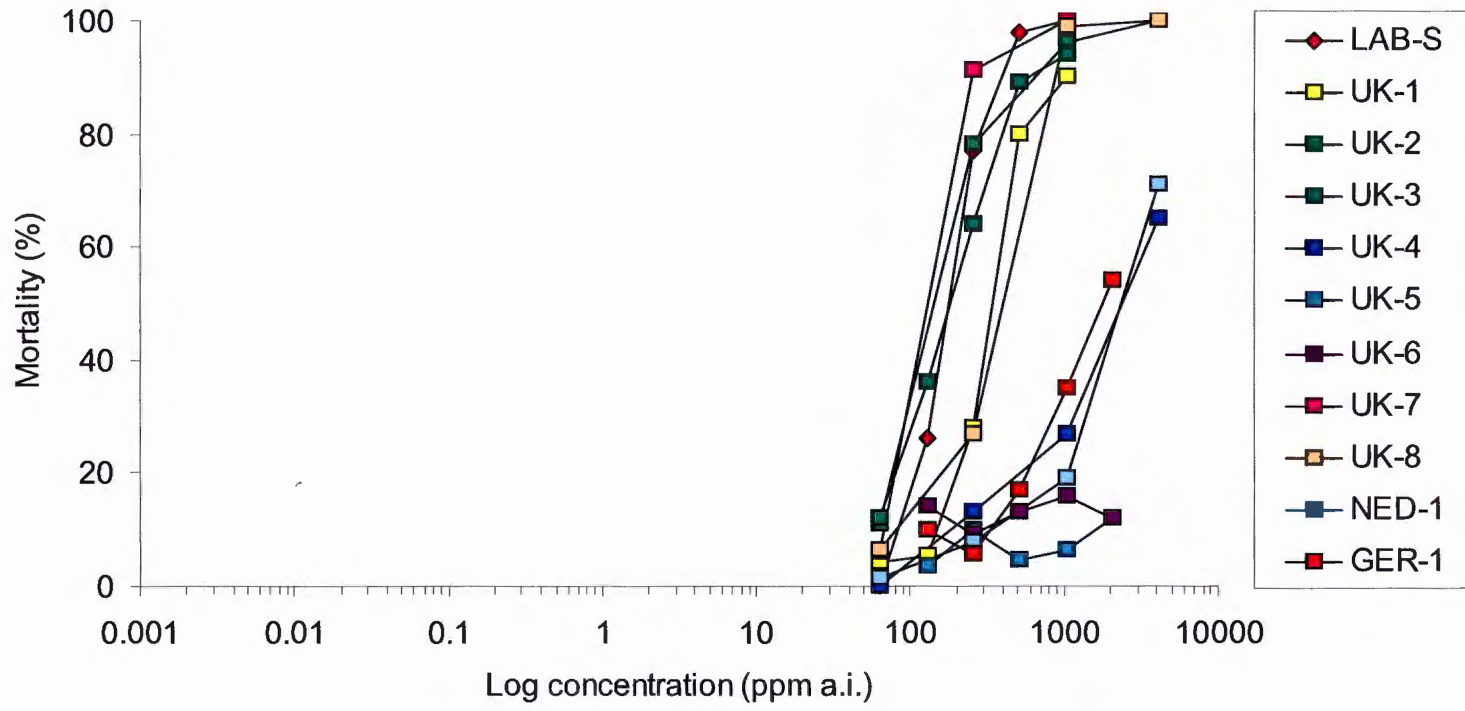


Figure 2.3 Responses of insect strains to the organophosphate, profenofos.

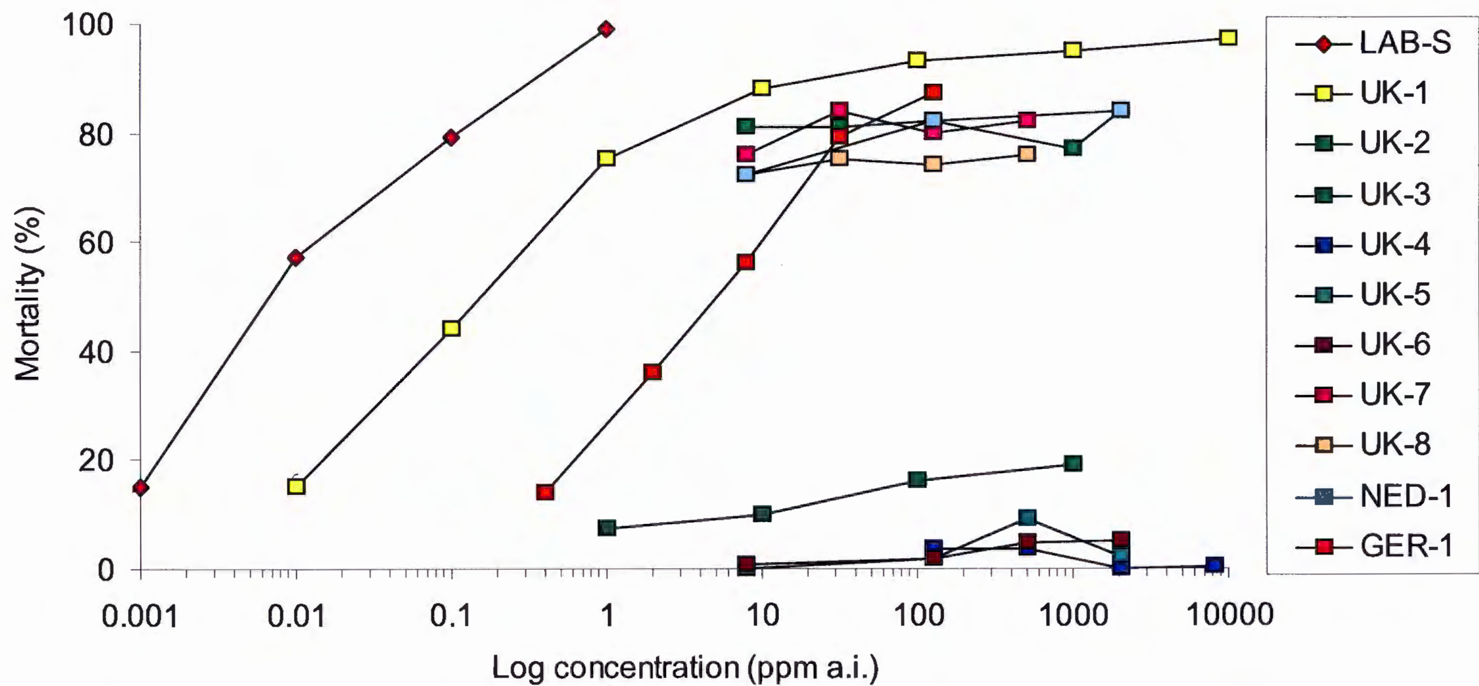


Figure 2.4 Responses of insect strains to the IGR, buprofezin.

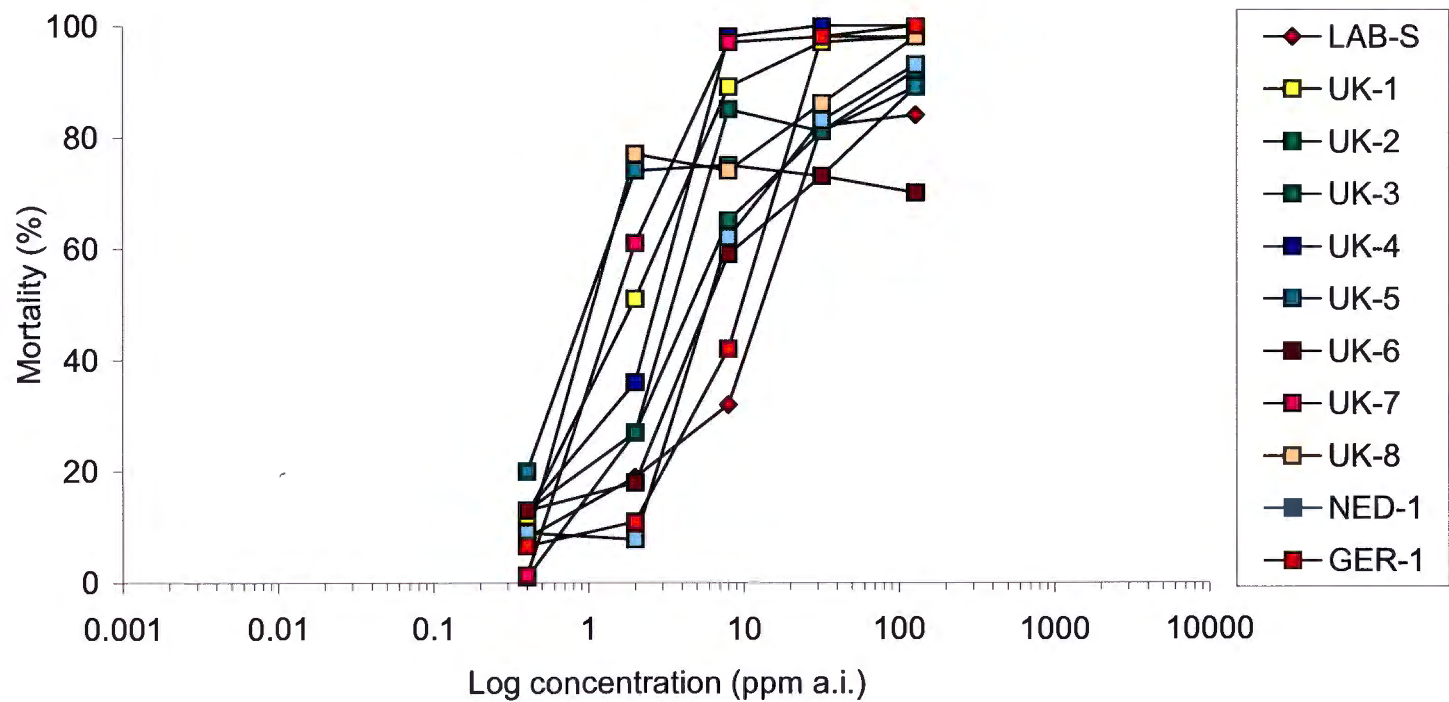


Figure 2.5 Responses of insect strains to the neonicotinoid, imidacloprid.

Table 2.2 LC_{50}'s with 95% confidence limits and probit line slopes for responses of *T. vaporariorum* strains to imidacloprid.

Strain	LC_{50} (ppm)	95% cl	Slope
LAB-S	11	4.2 - 22	1.1
UK-1	2.0	1.1 - 3.2	1.6
UK-2	7.0	3.9 - 11	1.2
UK-3	4.1	2.1 - 7.3	1.1
UK-4	2.6	2.1 - 3.2	4.2
UK-5	1.4	0.2 - 3.8	0.7
UK-6	11	4.8 - 25	0.8
UK-7	4.5	1.6 - 10	0.8
UK-8	1.9	0.7 - 4.0	1.1
NED-1	8.0	3.0 - 15	1.5
GER-1	9.3	7.0 - 11	3.7

2.3.2 Cross-resistance studies

The presence of buprofezin resistant individuals in UK-1 (section 2.3.1) was unexpected as this strain had been maintained in a closed glasshouse system from the 1980's until being sent to Rothamsted Research in 1994. It was isolated prior to the UK release of buprofezin in 1991 and as the colony had been maintained without exposure to insecticides, then cross-resistance to a compound used prior to that isolation may be responsible. To investigate the possibilities, bioassay data were reviewed for correlated patterns of resistance. The breadth of responses between strains was sufficient to demonstrate that the presence of resistance to one compound was not consistently associated with that of another (Table 2.3); suggesting that in *T. vaporariorum*,

cross-resistance does not predictably link any pair of these four insecticides.

Table 2.3 Responses of strains to each insecticide categorised as susceptible (S) and resistant (R).

Strain	Bifenthrin	Profenofos	Buprofezin	Imidacloprid
LAB-S	S	S	S	S
UK-1	S	S	**R**	S
UK-2	**R**	S	**R**	S
UK-3	**R**	S	**R**	S
UK-4	**R**	**R**	**R**	S
UK-5	**R**	**R**	**R**	S
UK-6	**R**	**R**	**R**	S
UK-7	**R**	S	**R**	S
UK-8	**R**	S	**R**	S
NED	S	**R**	**R**	S
GER	S	**R**	**R**	S

However, the presence of buprofezin resistance in UK-1 still required an explanation and so other compounds were considered. In whiteflies, buprofezin is known to act as a chitin synthesis inhibitor (Kanno *et al.*, 1981). Chitin is critical to the formation of a new exoskeleton after each larval moult and treated, susceptible larvae fail to successfully develop into the following instar. A further member of the IGR group is the benzoylurea, teflubenzuron. It also acts as an inhibitor of chitin synthesis in developing larvae but via an alternative mode of action (Ishaaya, 1992). Teflubenzuron's registration for use in the UK was approved in the early 1980's. UK-3 had no prior history of exposure to teflubenzuron and was

collected from a tomato glasshouse in which teflubenzuron is not registered for use. In addition to the extreme levels of buprofezin resistance, results disclosed that UK-3 and UK-4 showed similarly high levels of resistance to teflubenzuron (Figure 2.6). LAB-S proved to be the most susceptible with an LC_{50} value of 1 ppm. UK-1 responded similarly to LAB-S over most concentrations but showed some indication of greater survival at the highest concentration tested.

Further and unequivocal evidence for cross-resistance between buprofezin and teflubenzuron was obtained from reciprocal selection experiments. UK-1 was chosen for this work on the basis of apparently containing only low frequencies of resistant individuals and hence the greatest scope for marked changes in frequency upon selection. Reciprocal selection experiments accompanied by pre- and post-selection bioassays were initially done using the same concentration of buprofezin and teflubenzuron (75 ppm). Selection with buprofezin resulted in a large reduction in mortality by both compounds in the F_1 generation (Table 2.4). In contrast, selection with 75 ppm teflubenzuron did not enhance F_1 survival against either chemical. This could have been due to the significant proportion of susceptible insects that would have survived such a concentration (see Figure 2.5), subsequently contributing their genes to the following generation. In order to impose stronger selection with teflubenzuron, the experiment was repeated using 1500 ppm. This time strong selection for resistance to both compounds did take place. Replicate experiments showed good agreement and reinforced the finding of positive, reciprocal cross-resistance between buprofezin and teflubenzuron.

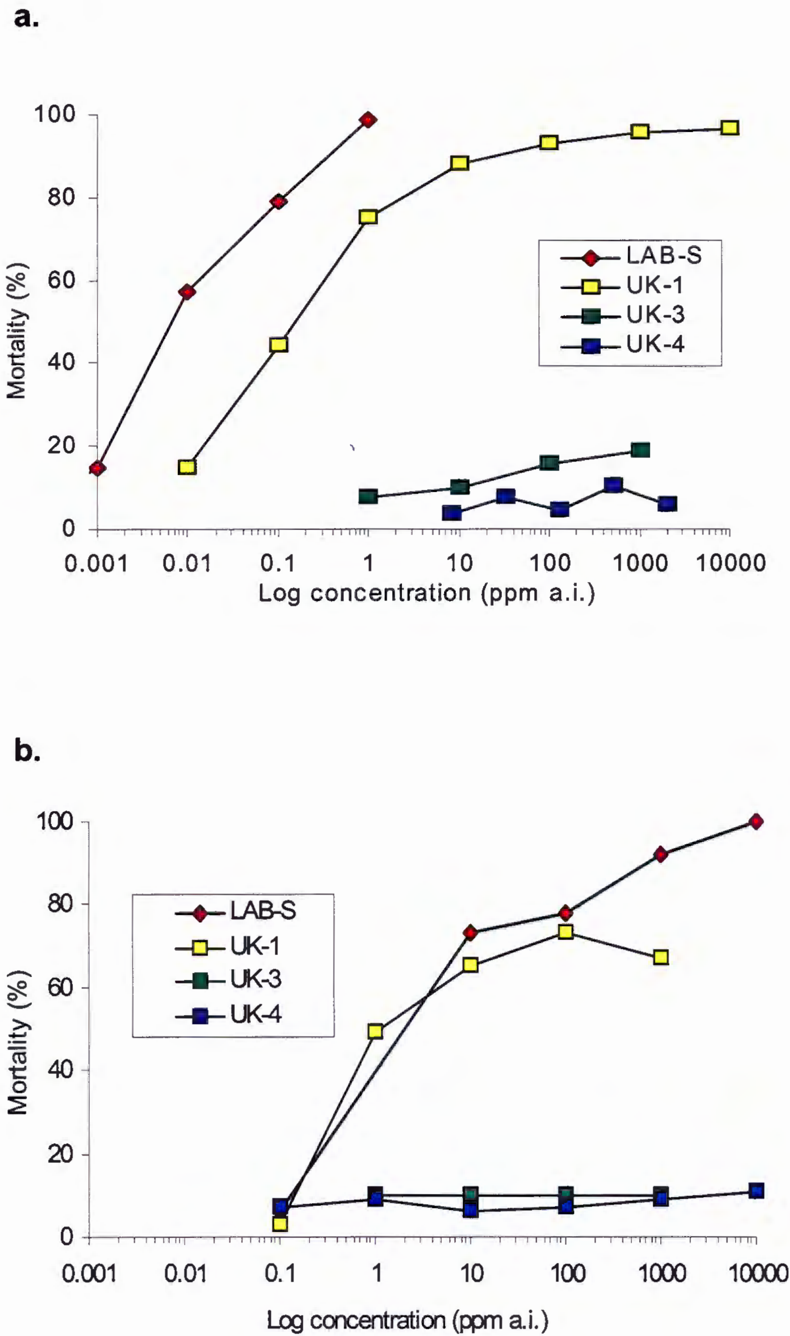


Figure 2.6 Responses of selected strains against **a.** buprofezin and **b.** teflubenzuron.

Table 2.4 Cross-resistance bioassay results after selection with either compound for UK-1 against buprofezin and teflubenzuron.

Colony details		**% Mortality of F_1 generation**	
Name	**Selection**	**Bup-75 ppm**	**Tef-75 ppm**
UK-1	Control-no selection	71	68
UK-1	Bup-75 ppm	18	27
UK-1[1]	Bup-75 ppm	14	24
UK-1	Tef-75 ppm	79	71
UK-1	Tef-1500 ppm	33	24
UK-1[1]	Tef-1500 ppm	29	23

[1] *Replicate experiment*

2.4 DISCUSSION

The present work represents the first comprehensive investigation of insecticide resistance in *T. vaporariorum* from the UK since the work of Wardlow in the 70's and 80's, and provides important insights into the status of contemporary populations and the persistence of resistance mechanisms.

Use of synthetic pyrethroids and organophosphates against whiteflies has declined over the past decade or more, although they are still in use against spider mites, thrips and a host of other co-existing pest species (various growers, pers. comm.). After a protracted period of exposure in the past, the presence of susceptible wild-type populations is evidence that, in the absence of selection, resistance to these compounds may decline. This could be due either of two reasons; immigration of susceptible insects into the population from outside or negative effects

associated with the possession/expression of the resistant gene(s). Such negative effects, termed 'fitness costs', are an expanding research area and one of the best documented cases refers to populations of the peach-potato aphid, *Myzus persicae*, that contain elevated carboxyl esterase levels (Foster *et al.*, 1996). Detoxifying enzymes known as E4 and FE4 carboxylesterases protect resistant *M. persicae* primarily against organophosphates and to a lesser extent pyrethroids and carbamates (Devonshire and Moores, 1982). In this example, there was a correlation between levels of esterases and a lower winter survival during cold, wet conditions. Further to those findings, Foster *et al.*, (1999) found that another mechanism of pyrethroid resistance, kdr, also seemed to be associated with a fitness cost. Peach-potato aphids with the kdr mutation showed a reduced response to alarm pheromone. This could potentially increase the rate at which these individuals suffer from predation and parasitism, thus conferring the fitness disadvantage. If either of these mechanisms is a predominant defence against pyrethroids and/or organophosphates in *T. vaporariorum*, there could be a comparable relationship that has contributed to the restoration of insecticide susceptibility as usage has declined.

Pyrethroids and organophosphates possess generic toxicity profiles and are potentially harmful to beneficial insects, including biological control agents and pollinators. Primarily due to concerns over safety and a high environmental impact, there has been a move away from these conventional chemistries. Combined with the near industry-wide uptake of biological control for *T. vaporariorum*, targeted use of such compounds against this species has declined since the early 1990's. More recently, as alternative compounds have failed, usage may again be increasing in some areas and this fluctuating history may be reflected in the variable results observed. Although incompatibility with beneficial insects precludes the use of broad-spectrum compounds in the majority of control situations,

if appropriate, use of carefully selected conventional products could provide adequate control of hotspots and outbreaks at some localities.
The bioassay data for bifenthrin and profenofos showed no common trends or patterns. UK-1 was the only strain whose response was similar to that of LAB-S against both compounds, in keeping with its isolated and pesticide-free history. UK-4, UK-5 and UK-6 were strongly resistant to these insecticides, showing survival even at concentrations at which phytotoxic effects become apparent. However, UK-2, UK-3, UK-7 and UK-8, which showed appreciable resistance to bifenthrin, were comparatively susceptible to the organophosphate. The continental strains NED-1 and GER-1 showed the opposite trend.

For the IGR's assessed, there are additional factors that have contributed to the current situation. Buprofezin data have shown some startling developments and demonstrated the threat posed when unrestricted applications are combined with a lack of awareness. Increased application frequencies as a response to lower efficacy, combined with the unwitting concurrent use of a cross-resisted product (teflubenzuron), have conspired to render both these valuable chemicals wholly ineffective at some sites (Gorman *et al.*, 2002). Insects capable of surviving the field rate were shown to be unaffected by concentrations of over 100 times that rate in bioassays, and could be considered virtually immune. Such products, which are ideally suited for IPM and have enabled a significant reduction in total insecticide usage, are now severely compromised as a result.

The consequences of a decline in the efficacy of buprofezin and teflubenzuron are only now being realised but with few, if any suitable replacements, successful biological control will become more problematic and require a greater expenditure to maintain existing levels of success. In addition to financial considerations, a reversion towards more generic insecticides must be feared, which would additionally impinge upon environmental and sociological issues. It should also be noted, that such a

response may conspire to elevate selection pressures against broader spectrum compounds, exacerbating the situation and giving little more than a temporary respite. At this stage it is unclear if and when susceptibility to buprofezin may return to affected localities (see Chapter 3). On-site monitoring of UK-3 for 7 successive years without use, revealed no significant reduction in the level of resistance to buprofezin. Additionally, resistance levels in strains with a range of responses were shown to be stable for more than 3 years when reared under laboratory conditions.

The neonicotinoid class forerunner, imidacloprid, received its first UK registration in 1997. Foreseeing widespread and potentially intense usage, initial registrations issued by the Department for Food and Rural Affairs (Defra) were limited in an attempt to minimise selection for resistance. As no resistance was detected in any of the populations tested, the success of this strategy may be reflected in the results observed. Originally registered only for a single application on containerised ornamentals or as stem paint on hops, imidacloprid now has a variety of 'off-label' approvals including protected lettuce and an assortment of brassicas. The results reveal that even at sites of known exposure, resistance has not yet reached detectable levels. However, as other compounds fail and registrations widen, the pressure on imidacloprid will rise. Many UK growers of edible crops are calling for further registrations as they are presently disadvantaged; imidacloprid is registered for use on a much wider range of edibles in competing European countries, whose produce is also sold on the UK market. With other neonicotinoids receiving approval, many factors are conspiring to increase the selection pressure exerted by neonicotinoids on UK whitefly populations. With imidacloprid resistance in *B. tabaci* now causing severe problems in many areas (Nauen and Denholm, 2005) and as exemplified by *T. vaporariorum* and buprofezin, well-informed choices regarding regulation and management will continue

to be essential if these compounds are to remain effective for the near future.

The lack of any consistent association between responses to the four compounds was not unexpected, although there are examples of detoxification mechanisms which confer cross-resistance to members of pyrethroid and organophosphate classes (Rodriguez *et al.*, 2002). In contrast, toxicity of the organophosphate, diazinon, was found to be enhanced in pyrethroid resistant populations of the horn fly, *Haematobia iritans* (L.), demonstrating negative cross-resistance between these two classes (Cilek *et al.*, 1995). Some strains (e.g. NED-1 and GER-1) showing strong resistance to profenofos retained full susceptibility to bifenthrin, precluding the presence of metabolic cross-resistance between these compounds. This is discussed further in Chapter 6.

Although there are some reports of cross-resistance affecting different classes of IGR's (Ishaaya, 1992), this is the first report of cross-resistance to involve buprofezin. Both buprofezin and teflubenzuron are considered IPM compatible products and considered as having different modes of action. Buprofezin inhibits incorporation of N-acetyl-D (1-H) glucosamine into chitin, in a similar manner to benzoylphenylureas such as teflubenzuron (Ishaaya *et al.*, 1989); however, their exact target-sites remain undisclosed. If target-site resistance were involved, then it would appear that despite differences in their chemical structures, at some point along the chitin synthesis/deposition pathway the activities of these compounds are combated by a common mechanism of defence.

Despite the presence of resistant individuals in all field strains examined, it should be remembered that whitefly collections were taken from areas suffering control failures and were not intended to represent all UK sites. Use of buprofezin and teflubenzuron in rotation should be avoided and if either is used, a regular appraisal of resistance is advised. There is no

evidence to date that this cross-resistance extends to any other compounds, or indeed to other species or geographical areas. Two other insecticides, pyriproxyfen and diafenthiuron, were shown to be unaffected by cross-resistance to buprofezin in *T. vaporariorum* (Ishaaya and Horowitz, 1995). Interestingly, there is evidence of cross-resistance between pyriproxyfen and buprofezin in *B. tabaci* populations from Australia, which appears to be based on detoxification by esterases (Gunning, pers. comm. 2004).

CHAPTER 3

FACTORS INFLUENCING RESISTANCE TO BUPROFEZIN

3.1 INTRODUCTION

The data obtained from buprofezin bioassays in Chapter 2 indicated widespread, and in some cases highly potent, resistance in the UK and mainland Europe. This is of concern, particularly as buprofezin has assumed a key role within integrated *T. vaporariorum* control strategies over the past decade. The range of responses observed for buprofezin may reflect the differing selection pressures to which these populations had previously been exposed. This exposure is attributable either directly to buprofezin or to compounds linked by cross-resistance; teflubenzuron is now the first to be implicated (section 2.3.2). It is well established that the potency and developmental rate of resistance depends upon numerous ecological and operational factors that may differ for individual compounds or geographical areas. Understanding these variables is a prerequisite for making correct predictions regarding likely selection pressures (Denholm and Rowland, 1992; Dennehy, 1995).

All tactics for reducing insecticide resistance have a common approach; to reduce overall exposure to the selecting agent(s). This strategic use of insecticides, termed insecticide resistance management (IRM), although often primarily imposed to alleviate resistance in a single pest, often requires consideration of a pest complex and each of their associated selection pressures (Denholm, 1988). Strategies can employ the use of untreated areas (refugia), insecticide rotations, synergistic compounds, limited treatment times and frequencies and may necessitate the discontinued use of specific products. There are various examples of IRM strategies that have improved or sustained efficacy of products through the restoration or maintenance of insecticide susceptibility (*e.g.* Dennehy and Williams, 1997). Frequently, reducing the use of a particular product necessitates the availability of alternative insecticides with independent modes of action. Unfortunately there are few suitable alternatives registered in the UK for use against pests of edible produce that also

possess the required compatibility with IPM practices. Over the last 10-15 years this has often led to a reliance on buprofezin and/or teflubenzuron in particular.

The rate at which resistance responds to management attempts is discussed further in Chapter 6; however, although buprofezin resistance appeared stable within unselected, isolated laboratory conditions during the period of this study, this does not necessarily reflect populations within a horticultural environment. The potential influences of migration, fitness costs and glasshouse management practices are unpredictable. Resistance monitoring of either phenotypes or genotypes provides the only true indication of the influence that a change in usage has had on the resistance dynamics associated with any given situation (Denholm, 1990).

As a contact insecticide, buprofezin has a high intrinsic toxicity against susceptible species (Kanno *et al.*, 1981). It is additionally known to possess significant vapour activity, shown to be capable of inducing mortality of susceptible *B. tabaci* over a distance of several inches (DeCock *et al.*, 1990). Although insecticidal fumigants are commonly volatile compounds (Hammond *et. al.*, 2000; Park *et. al.*, 2004) such activity is not usual for foliar applied insecticides although there are several exceptions (Siebers *et. al.*, 2003). Due to the fact that *T. vaporariorum* is primarily a pest of enclosed environments, the implications of buprofezin vapour action could be of importance, and a better awareness of its complications could improve the chances of more effective employment.

3.2 MATERIALS AND METHODS

3.2.1 Insect strains

The strains used for this work demonstrated a range of responses to buprofezin in addition to repeat collections from a single site. These strains are detailed in Table 3.1.

Table 3.1 Strains of *T. vaporariorum* including dates of collection, geographical origins and host plant information.

Name	Origin	Host	Year collected
LAB-S	UK	F. bean	1980
UK-1	Essex	Tobacco	1994
UK-4	Somerset	*Fuchsia* sp.	1997
UK-3*	Worcester	tomato	1997
UK-19*	Worcester	tomato	1998
UK-20*	Worcester	tomato	1999
UK-22*	Worcester	tomato	2000
UK-24*	Worcester	tomato	2004

** came from same site*

3.2.2 Insecticides

Formulated buprofezin ('Applaud', 25% EC) was used throughout. Dilutions required for systemic bioassays used a diluent of distilled water; all others were performed by the addition of distilled water containing 0.01% of the non-ionic wetter Agral® (Zeneca Agrochemicals).

3.2.3 Field simulator experiments

Experiments to determine the phenotypic expression of buprofezin resistance under semi-field conditions and compare that to the responses observed in bioassays utilised 'field simulator' technology, designed at Rothamsted Research for the study of large-scale populations of up to 20,000 individuals and first described by Rowland *et al.* (1990). Cahill *et al.* (1996a) used the same technology to examine the relationships between bioassay data and field performance of insecticides against *B. tabaci*.

Eight French bean plants were housed in each field simulator cage and onto these 400 adult females (50 insects per plant) were released. After 12 days, insecticide was applied until 'run-off' to both the upper and lower leaf surfaces using a hand-held lance sprayer. The rate used was 75 ppm, as this equates to the recommended field-rate at this particular spray volume. Each experiment was run for a single generation (maximum of 35 days) with numbers of survivors being periodically counted using a rigid endoscope, attached to a light box via a cable of optic-fibres. The endoscope gave magnified views of the undersides of leaves from outside the chamber, without disturbing the leaves (Figure 3.1). For each assessment, all adults on all leaves in a particular simulator were counted one-by-one, until the total population exceeded 3000. At that point, a randomly selected half of every leaf was counted, and the figure doubled on a leaf by leaf basis. Although time-consuming, these counts enabled accurate monitoring of changes in population size.

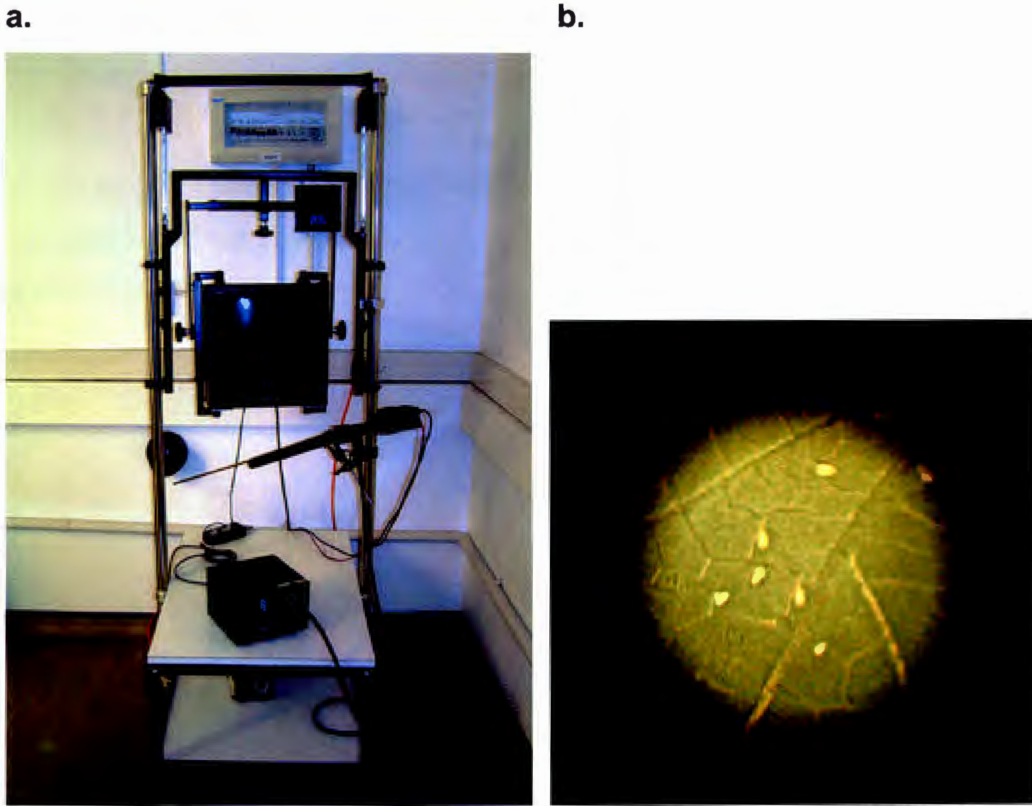


Figure 3.1 To assess fluctuations in population size, adult whiteflies were counted individually using endoscope technology; **a.** endoscope, light source and monitoring equipment **b.** observed view using endoscope.

3.2.4 Volatility studies

Experiments were designed to examine the effect of vapour produced by spray applications of buprofezin, as shown in Figure 3.2. A group of four French bean plants was placed at each end (plant sets 1 and 3) and at the centre (set 2) of a field simulator cage (1.7 x 1.2 x 1.0 m high). The distance between each set was 0.5 m. A simulated wind, produced via a rotary blade extractor fan at one end of the cage, was set at 5 m/s to ensure any released vapours were drawn in one direction at a constant velocity, thereby producing a concentration gradient effect. 50 adult UK-1 females were simultaneously released onto each plant and allowed to

oviposit for 24 hours before removal. After a further 11 days the set of plants and resulting early instar larvae furthest from the fan (set 1) were sprayed with the field rate (75 ppm) of buprofezin. Immediately prior to emergence of survivors, larval mortality (pre-selection) in all plant sets was recorded and each was isolated to collect adults for subsequent bioassay assessment (post-selection). Due to numbers of surviving immatures (F_1) being low, all bioassays were done using F_2 adults; as a single untreated generation was required to provide sufficient individuals.

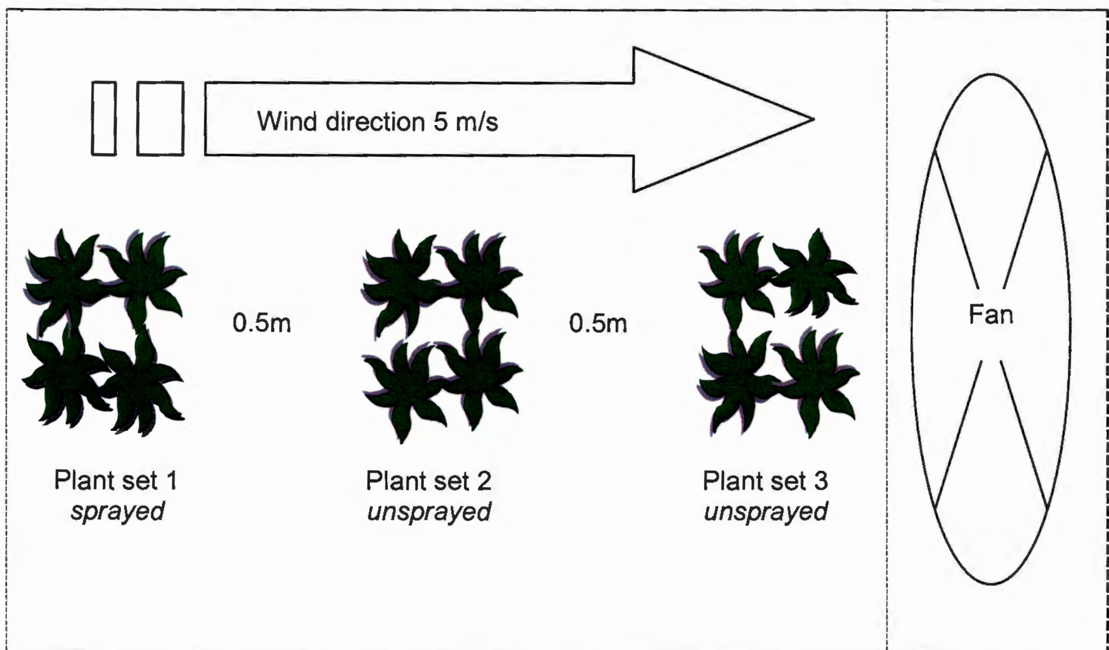


Figure 3.2 Design of volatility studies showing aerial view of field-simulator.

3.2.5 Monitoring of buprofezin resistance

Repeat whitefly collections (5 in total) were taken from the same commercial glasshouse over a 7 year period. After an initial discovery of high resistance to buprofezin (UK-3 in 1997), use of insect growth regulators ceased and during the following seven years no buprofezin or teflubenzuron were used at this site. The whitefly samples were tested at a single discriminating concentration of formulated buprofezin using the bioassay method described in Chapter 2. Due to the near immunity of resistant individuals and the resulting flat response lines observed against buprofezin resistant insects (Chapter 2), it was possible for a diagnostic dose to be chosen (512 ppm) well above the concentration at which susceptible insects can survive.

3.2.6 Data analysis

The bioassay methodologies were generated through consultation with statisticians at Rothamsted Research. All larval bioassays used a minimum of three replicates and contained between 50-300 immature insects dependent upon fecundity rates. Due to their long duration, field simulator experiments (buprofezin performance trial and volatility investigations) were done once using large numbers of insects. When appropriate, dose-response data were subjected to probit analysis using the POLO computer programme (LeOra Software, Berkeley, California). This software is a statistical programme that firstly corrects for control mortality before calculating percent mortalities and relevant probit values.

3.3 RESULTS

3.3.1 Field simulator experiments

Results obtained from these experiments mirrored those from bioassays. Through comparison of untreated and treated responses for individual strains it can be seen from Figure 3.3 that LAB-S (buprofezin susceptible) was completely controlled (i.e. no F_1 emergence) by a single application of buprofezin at 75 ppm (equating to the recommended field-rate). UK-1 showed substantial mortality when compared to its untreated control; however, there was some emergence of F_1 individuals. In contrast, the UK-4 colony showed no buprofezin-induced mortality and the population growth was unaffected by this application rate.

3.3.2 Volatility Studies

The data obtained from these investigations clearly showed that the vapours produced by spraying plant set 1, gave substantial mortality in the unsprayed plant sets (Table 3.2). In addition to the direct mortality, selection for resistant insects by vapour alone was as efficient as the direct spray; post-selection bioassay results show this to be the case even at the maximum distance tested (1 metre).

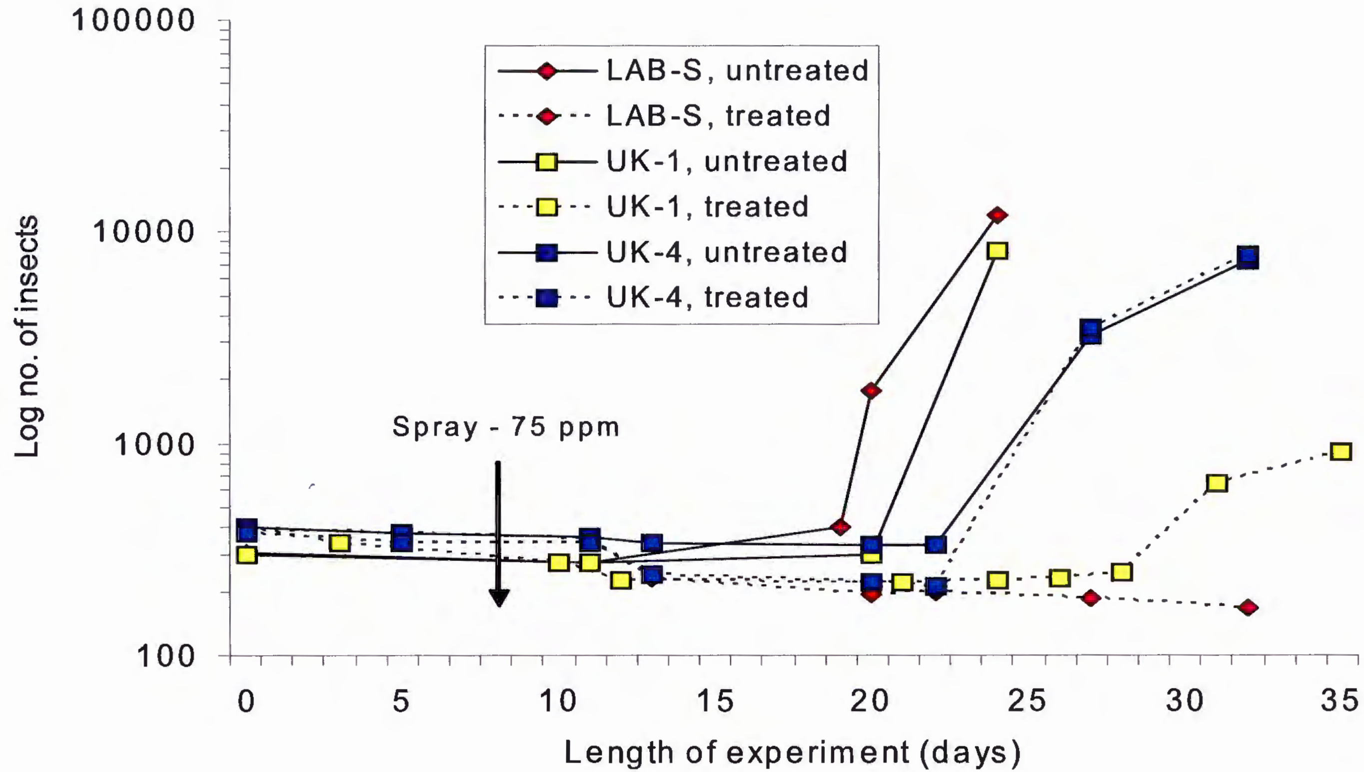


Figure 3.3 Population sizes observed during field simulator experiments.

Table 3.2 Pre- and post-selection mortalities (%) of UK-1 progenies from plant sets 1-3, against 75 ppm buprofezin.

Plant set	Pre-selection (F_1)	Post-selection (F_3)
1 (sprayed - 75 ppm)	82	46
2 (unsprayed – distance 0.5 m)	70	48
3 (unsprayed – distance 1.0 m)	65	46

These results demonstrate that vapour produced by buprofezin's high volatility has insecticidal action. Inevitably, this will increase selection pressures in treated areas and speed-up the rate of resistance development. In addition, it could affect untreated areas that are adjacent to treated plots.

3.3.3 Monitoring of buprofezin resistance

The diagnostic dose assays aimed to document any change in resistance levels due to the discontinued use of buprofezin at a single site. The concentration used was above that giving 100% mortality of fully susceptible whiteflies. Table 3.3 shows that resistant individuals remained at a relatively consistent level throughout the entire 7 year monitoring period, close to that of the 1997 starting point. There was no evidence for a systematic decline in resistance level despite such a sustained period without selection at this locality.

Table 3.3 Mortality of repeat samples against a discriminating concentration of 512 ppm buprofezin.

Strain	Date collected	Mortality (%)
UK-3	1997	17
UK-19	1998	20
UK-20	1999	25
UK-22	2000	22
UK-24	2004	28

3.4 DISCUSSION

The performance of buprofezin applications in field simulators correlated with the responses observed under bioassay conditions and mimicked the lack of control some growers are experiencing in the field. Although there may be other influences, the rapid rate at which buprofezin resistance can be selected (see Chapter 2) warranted further investigation. The high volatility of buprofezin is known to produce vapour that can induce mortality of susceptible *B. tabaci* larvae several inches from the source (De Cock *et al.*, 1990), however, this had not previously been studied with *T. vaporariorum*. Buprofezin exhibits significantly higher intrinsic contact toxicity towards susceptible *T. vaporariorum* larvae compared to those of *B. tabaci*. Despite their larger size, the LC_{50} for *T. vaporariorum* is approximately 0.01 ppm, 50 times less than for *B. tabaci* (Gorman *et al.*, 2002; Cahill *et al.*, 1996b). The data produced here confirmed that *T. vaporariorum* are similarly susceptible to the activity of buprofezin vapour. It was shown that the vapour produced by sprayed, foliar applications was not only active over the maximum distance tested (1 m), but that the potency (in terms of its level of selection exerted) was

undiminished and close to that of a direct spray. The effects of such vapour activity in a confined glasshouse could be significant. It is rare for insecticides to possess such activity and it may complicate the incorporation of buprofezin into indoor, refugia-based management strategies against *T. vaporariorum*. These strategies utilise untreated areas to provide a reserve of susceptible genes and/or beneficial insects within the population, thereby extending the longevity of control (Verkerk *et al.*, 1998). However, with such active vapours produced it would be difficult to confine treatment to the desired location, thereby compromising the efficacy of the strategy. Not only does this high level of vapour action affect untreated areas but also within treated plots, there will be little escape for the target species. Consequently, the selection pressure exerted will be above that associated with spraying non-volatile compounds and this may be a contributing factor towards the acceleration of buprofezin resistance development.

During the single site monitoring study an attempt was made to track the effect that ceasing use of insect growth regulators had on the resistance levels within an established glasshouse population. Prior to 1997, whiteflies at this site (UK-3) had been exposed to buprofezin and teflubenzuron annually and around 90% of individuals were shown to be resistant (Chapter 2). Use of both buprofezin and teflubenzuron ceased in the summer of 1997 and monitoring continued using the same bioassay method. After seven years the resistance to buprofezin had not changed significantly, as was observed with laboratory based populations. This level of stability suggests that there is little or no fitness cost associated with the possession or expression of the resistance mechanisms. It also implies that any immigration of genotypes over the seven year monitoring period was either negligible or entailed introgression with populations of similar resistance genotype ratios. The locality of the site is such that several other horticultural sites are within close proximity; some integration between populations is considered likely.

CHAPTER 4

FACTORS INFLUENCING RESISTANCE TO IMIDACLOPRID

4.1 INTRODUCTION

As the world's current top-selling insecticide (Wood Mackenzie, 2004), and the commercial forerunner of a new insecticidal group (the neonicotinoids), the importance of imidacloprid to UK agriculture cannot be over-estimated. With efficacy against a range of often co-existing pest species (Mullins, 1993), assessing and managing levels of exposure to minimise resistance can be challenging even within a background of strict regulation. In the UK, the Pesticides Safety Directorate (PSD) is responsible for the award and monitoring of insecticide approvals, label recommendations and the enforcement of restrictions. Imidacloprid first gained registration in the UK for use as a sugar beet seed treatment in 1994. With continued successful control of both soil and foliar pests, including the primary threat of aphids as vectors of virus yellows, usage has risen to over 70% of sugar beet currently grown in the UK (A. Dewar, pers. comm.). Subsequently, imidacloprid has received further approvals permitting seed treatments for oilseed rape, cereals, lettuce and a number of brassica crops, and systemic applications to hops. The principal target of these registrations are aphids and it was not until 1997 that imidacloprid was approved for use against glasshouse whitefly. PSD granted an initial approval for *T. vaporariorum* of a single systemic application per year on containerised ornamentals only, applied either as a soil drench or as granules integrated into compost. Imidacloprid registrations within protected environments still impose a significant restriction in usage and resulting selection pressures. Current legislation for glasshouses still permits only systemic application of a single treatment per crop against aphids, whiteflies and leaf-miners that are affecting ornamentals or protected lettuce. Crops for which it is not permitted therefore include some common whitefly hosts such as tomatoes, cucurbits, beans, peppers and a range of herbs. In some countries, including others within the European Union, imidacloprid has received relatively open registrations permitting more frequent and widespread usage, often together with foliar applications. UK registrations

for another neonicotinoid, thiacloprid, are also increasing. In 2005, UK legislation permits applications of thiacloprid against pests such as cotton whiteflies, western flower thrips and palm thrips in a range of protected vegetables (aubergines, courgettes, cucumbers, peppers and tomatoes). It is conceivable that co-existing glasshouse whiteflies could be exposed to thiacloprid treatments in such circumstances.

Reports of resistance to imidacloprid have now been published for a range of agricultural pests including Colorado potato beetles *(Leptinotarsa decemlineata*), (Grafius and Bishop, 1996; Zhau *et al.,* 2000); cotton whiteflies (*Bemisia tabaci*), (Cahill *et al.,* 1996c; Nauen *et al.,* 2002); peach-potato aphids (*Myzus persicae*), (Devine *et al.,* 1996; Nauen and Elbert, 1997; Foster *et al.,* 2003) and more recently brown plant-hoppers (*Nilaparvata lugens*), (Liu *et al.,* 2003). Non-agricultural pests to have demonstrated neonicotinoid resistance include the German cockroach (*Blattella germanica*) and the housefly (*Musca domestica*), (Wen and Scott, 1996).

Resistance in *B. tabaci* was first documented by Cahill *et al.* (1996c) in Spanish populations sampled in 1994 from the intensive horticultural region close to Almeria, where imidacloprid was being used continuously as both systemic and foliar applications. A range of collections from protected tomato crops (grown inside plastic screen houses) were found to exhibit up to 25-fold resistance using a systemic bioassay technique. Since then, subsequent collections from the same region in 1996, 1999 and 2000 have been shown to contain individuals with increasing levels of resistance; those collected in 2000 having resistance factors in excess of 100-fold (Gorman *et al.*, 2003; Nauen *et al.*, 2002). These data have provided a means of tracking resistance development in the Almeria region, highlighting the time-scale in which high-level resistance can build up in some species. Comparable levels of resistance have now been observed in other areas; for example, Q-biotype *B. tabaci* collected from

Cyprus also demonstrated resistance levels at LC_{50} of over 100 times that of a fully-susceptible strain, precluding any control at recommended rates (M. Hadjistylii, unpublished data).

In response to an escalation of imidacloprid resistance in *B. tabaci*, some countries have implemented IRM strategies advocating limited usage. One current and successful example of imidacloprid resistance management being that practised in the cotton and vegetable growing regions of Arizona in North America. A whitefly resistance management programme (Dennehy *et al.*, 1996) was introduced in 1996 after levels of resistance to conventional compounds had reached crisis point. Imidacloprid, buprofezin and pyriproxyfen were introduced to the region with usage guidelines limiting growers to a single application of each per crop. Buprofezin and pyriproxyfen received emergency clearance for use only on cotton, whereas imidacloprid was restricted to spring and fall melon crops. By minimising application frequencies through a rotational strategy, imidacloprid has provided successful season-long whitefly control in Arizona vegetables since 1993 (Palumbo *et al.*, 2003) and resistance levels continue to remain at a low level (Dennehy *et al.*, 2004). This contrasts markedly with the indiscriminate use in Almeria.

Q-biotype populations of *B. tabaci* from Almeria have provided the clearest evidence for a metabolic mechanism of resistance to imidacloprid (Nauen *et al.*, 2002; Stumpf *et al.* 2002). Synergism with a monooxygenase inhibitor increased activity of imidacloprid against a resistant strain, suggesting that resistance to this compound was mediated by detoxifying cytochrome P-450 monooxygenases. Ligand competition studies showed no direct correlation between the phenotype of resistant Q-type *B. tabaci* with the binding level of tritium-labelled [^{3}H] imidacloprid at the nicotinic acetylcholine receptor (Nauen *et al.*, 2002); thus discounting the possibility of target site insensitivity in these strains. Recent work on imidacloprid resistance in *N. lugens* (Liu *et al.*, 2003) has yielded contrasting results,

and represents the first case of target-site resistance to imidacloprid (Liu *et al.*, 2005).

Encouragingly, published work also suggests that in some cases resistance to imidacloprid has been relatively slow to develop when compared to other compounds, and additionally seems to respond well to management attempts (Denholm *et al.*, 2002; Nauen and Denholm, 2005).

Numerous generic factors including application rate and frequency influence the selection pressures imposed by any compound (Denholm and Rowland, 1992) but sometimes specific chemical and/or biological characteristics induce responses that can also be influential. For example, imidacloprid applications can provoke a behavioural avoidance by acting as an anti-feedant against some species. Devine *et al.* (1996) showed that imidacloprid elicited anti-feedant effects against *M. persicae* that were subsequently implicated with resistance (Nauen and Elbert, 1997). Isaacs *et al.* (1997) demonstrated anti-feedant properties of imidacloprid against *B. tabaci* on leaves systemically treated with a sub-lethal concentration of imidacloprid. Categorisation of individual behaviours enabled measurements of a range of responses, which included a reduced feeding rate. Additional studies of the same species by Nauen *et al.* (1998) showed comparable behavioural responses with systemic applications using choice tests between treated and untreated leaf-surfaces. Interestingly, no effect was observed using foliar applications.

After uptake through the root system, a systemically applied insecticide is transported around the plant via the phloem and associated translocation vessels. As there are no surface residues present, contact is only possible by feeding on plant tissues, or as in the case of whiteflies, direct phloem feeding. As such, any reduction in feeding rate will lead to a corresponding reduction in exposure, and consequently selection pressures may also be

altered. Experiments were designed to investigate the presence of imidacloprid-induced behavioural effects in *T. vaporariorum*.

Despite the apparent absence of resistance to imidacloprid observed in bioassays, either in populations from the UK or abroad, a primary consideration is the potential for resistance to develop in the near future. As with other techniques, it is possible that the bioassay method used was not sensitive enough to detect extremely low frequencies of individuals possessing an imidacloprid-resistant genotype. Subjecting populations to intense selection in the laboratory can sometimes bring such individuals to the forefront, enabling pre-emptive studies on the nature of mechanisms that may arise through field exposure (e.g. Prabhaker *et al.*, 1997). Additionally, to maintain vigilance for the presence of imidacloprid resistance in *T. vaporariorum*, collections from suspect sites continued to be assessed up until project completion. These strains are additional to ones reported in Chapter 2 and contribute to a more comprehensive, contemporary survey of the status of imidacloprid resistance in *T. vaporariorum*.

4.2 MATERIALS AND METHODS

4.2.1 Insect strains

The insect strains used are listed in Table 4.1. The strain MIXED, was generated by combining samples of over 250 insects from each of UK-2, UK-4, UK-5, UK-6 and GER-1 (section 2.2.1) into a single strain. Strains other than LAB-S and MIXED were collected and analysed specifically to provide further information on the response of *T. vaporariorum* populations to imidacloprid.

Table 4.1 List of *T. vaporariorum* strains used in this Chapter, including year of collection, geographical origin and host plant information.

Strain	Year	Origin	Host plant
LAB-S	1980	UK	French bean
UK-9	1998	Hertfordshire, UK	*Fuchsia* sp.
UK-10	1999	Lancashire, UK	Mixed ornamentals
UK-11	1999	Hertfordshire, UK	Solanaceae
UK-12	1999	Cambridgeshire, UK	*Fuchsia* sp.
UK-13	1999	Worcestershire, UK	Poinsettia
UK-14	1998	Cambridgeshire, UK	Mixed ornamentals
UK-15	1998	Cambridgeshire, UK	Mixed ornamentals
UK-16	1999	Essex, UK	Cucumber
UK-17	1999	Essex, UK	Cucumber
UK-18	1999	Essex, UK	Cucumber
UK-19	1998	Worcestershire, UK	Tomato
UK-20	1999	Worcestershire, UK	Tomato
UK-21	1999	Hampshire, UK	*Chrysanthemum* sp.
UK-22	2000	Worcestershire, UK	Tomato
UK-23	2004	Suffolk, UK	Mixed ornamentals
UK-24	2004	Worcestershire, UK	Tomato
NED-2	2004	The Netherlands	Gerbera
SPAIN-1	1994	Spain	Tomato
SPAIN-2	1998	Spain	Tomato
CHINA	2004	China	Cucumber
MIXED	Various	Various	Various

4.2.2 Insecticides

Formulated imidacloprid ('Confidor', 20% SL) was used throughout, diluted to the required concentration in distilled water.

4.2.3 Laboratory selections with imidacloprid

To enhance detection of neonicotinoid resistance, successive and intensive imidacloprid treatments were used in an attempt to select for resistance in the strain 'MIXED'. The pooling of strains to form 'MIXED' was done to maximise the gene pool and thereby enhance the chances of successful selection. After a single untreated generation to allow the strains to introgress, the population was subjected to a systemic application of 128 ppm imidacloprid. Seven repeat selections (eight in total) were made during the following 15 generations. Due to the numbers of survivors often being low, up to two untreated generations were sometimes required before repeat selections were possible.

4.2.4 Behavioural studies

Behavioural studies used two sizes of experimental arena. Firstly, closed Petri-dish experiments in which insects were confined to leaf-discs (37 mm in diameter). The Petri-dish height was 10 mm giving a total volume for the experimental arena of 10.74 cm^3. The second experimental scale employed field simulators containing two whole plants at one end, onto which insects were released from the centre. The field simulator chambers were 2 m x 1 m x 1 m (l x w x h), giving a total volume of 2 m^3.

4.2.4.1 Petri-dish experiments

The petioles of excised French bean leaves were immersed in solutions of either imidacloprid diluted to the required concentration in distilled water, or water only. After an uptake period of 40 hours, leaf-discs were cut and halved. Untreated and treated (with 37.5 ppm or 75 ppm imidacloprid) halves were placed side-by-side on an agar bed within a plastic Petri-dish (37 mm diameter). LAB-S whiteflies aged between 1 and 8 days old were taken from rearing colonies and lightly anaesthetised using CO_2. A single healthy adult was selected and placed at the centre of each Petri-dish and contained using a close-fitting ventilated lid (Figure 4.1). Once insects had recovered, dishes were inverted so that the abaxial leaf-surface faced downwards. Experiments were maintained beneath a large light bank (to minimise any bias due to positive phototropism) under a 16-hour photoperiod at 22°C. After the required time interval, individuals were scored as being either on the untreated half, the treated half, the plastic dish, or dead. Experiments for males and females were done separately and for each sex/dose combination there were 20 replicates. Each experiment was repeated four times.

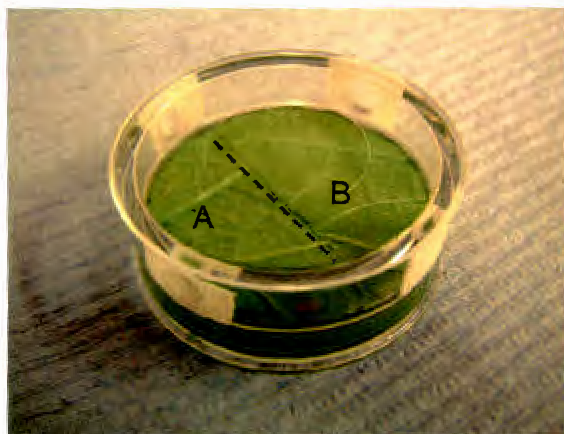


Figure 4.1 Experimental design of Petri-dish experiments (A = imidacloprid treated, B = untreated).

4.2.4.2 Field simulator experiments

Samples of LAB-S aged between 1 and 8 days old were taken from rearing colonies and lightly anaesthetised using CO_2. 200 healthy adult females were transferred to a glass vial which was then placed at the centre of a field simulator. Two plants had been placed towards the fan (Chapter 3, Figure 3.2) and at an equal distance from the centre of the chamber (Figure 4.2), one plant was untreated and one had been systemically treated 5 days earlier with 100 ml of 37.5 ppm imidacloprid. Once insects had recovered, individuals were free to disperse in any direction. After 24 hours, the number of individuals on each plant was counted. Initial assessments were at three rates of 0.2 ppm, 37.5 ppm (approximately 1/2 recommended field-rate) and 75 ppm (approximately full field-rate) imidacloprid. The half field-rate dose (37.5 ppm) was chosen for a further seven replicate experiments as this was the lowest, potentially discriminating concentration; any insecticide induced mortality needed to be minimised as this may have distorted results.

4.2.4.3 Insect retrieval from field simulators

Although Petri-dish experiments showed 37.5 ppm to be largely sub-lethal over a 24 hour period, it was necessary to confirm that mortality was not a significant factor under field simulator conditions. During experiments 4 and 5, dead insects were retrieved from the floor, on and around the plants, the glass introduction vial and the wire mesh that guards the ventilation fan. To aid detection of such individuals, the chamber floor and plant pot were covered with black cloth (Figure 4.2).

4.2.5 Additional strains

In addition to field strains tested with a range of products that included imidacloprid and reported on in Chapter 2, a number of other strains of *T. vaporariorum* were obtained and established in laboratory culture.

These were used to provide further information on the possible occurrence of imidacloprid resistance and came primarily from a range of hosts in the UK (Table 4.1). Insects from these strains were tested as described previously (section 2.2.3.3) using a single discriminating concentration of 128 ppm, which equates approximately to the LC_{95} of LAB-S.

4.2.6 Data analysis

The bioassay methodologies were generated through consultation with statisticians at Rothamsted Research. All adult bioassays consisted of a minimum of three replicates per concentration with an average number of 20 insects. When appropriate, dose-response data were corrected for control mortality and subjected to probit analysis using the POLO computer programme (LeOra Software, Berkeley, California). In some cases mortality was too low or too heterogeneous for probit lines to be fitted. All LC_{50}'s given are listed with 95% confidence limits. Behavioural data statistics were analysed using Genstat, a computer-based statistical programme designed at Rothamsted Research. Error margins are shown where appropriate, specific details are given within section 4.3.2.

Figure 4.2 Experimental design of a field simulator during behavioural studies; including host plants, glass insect vial, fan, mesh and black floor cloth to aid retrieval of dead insects.

4.3 RESULTS

4.3.1 Laboratory selections with imidacloprid

Periodic assessments showed that sensitivity to imidacloprid in the MIXED strain did not decrease as a result of repeated selection with this compound (Table 4.2). The lack of response to selection for resistance accords with the apparent absence of resistance disclosed during bioassays of the individual cultures used to generate this composite strain (Chapter 2), and failed to disclose the presence of even a low frequency of genes conferring resistance to imidacloprid.

Table 4.2 Mean mortalities (%) for LAB-S (unselected) and MIXED (selected with successive exposure to 128 ppm imidacloprid) against a discriminating dose of 128 ppm imidacloprid.

Generation	LAB-S	MIXED	No. of selections
1	93	94	0
6	90	79	3
15	95	96	8

4.3.2 Behavioural studies

4.3.2.1 Petri-dish experiments

There was no consistent significant difference found across either the four time intervals or the two imidacloprid concentrations (37.5 and 75 ppm),

between the number of survivors, the number settling on the leaf or the preference of those settled for either untreated or treated surfaces (Tables 4.4 and 4.5). Despite overall proportions of adults on 75 ppm treated leaf segments sometimes being higher than those on untreated, this relationship was not significant across all experiments. There was no discernable movement over time, of individuals towards or away from either treated surfaces or the plastic surfaces of the Petri-dish, and the majority of categorised responses were relatively consistent throughout. Overall average mortality rates for experiments using 37.5 ppm were 5% for ♂♂ and 2.5% for ♀♀ and the maximum mortality in any individual experiment was 10% (2/20). For those using 75 ppm overall mortality averages were 10% and 3.75% for ♂♂ and ♀♀ respectively, the maximum within any individual experiment was 20% (4/20).

Table 4.4a Numbers of individuals (i.e. replicates) recorded at 4 time intervals as either on untreated leaf sections (UT), 37.5 ppm treated leaf sections (T) or plastic (P) surfaces, or dead (D).

		30 min				**1 hour**				**2 hours**				**24 hours**			
Experiment	**Sex**	**UT**	**T**	**P**	**D**	**UT**	**T**	**P**	**D**	**UT**	**T**	**P**	**D**	**UT**	**T**	**P**	**D**
1	♂	7	10	3	0	6	12	2	0	7	9	4	0	10	8	1	1
2	♂	9	11	0	0	10	10	0	0	10	8	2	0	6	10	3	1
3	♂	7	11	2	0	8	10	2	0	8	8	4	0	7	7	5	1
4	♂	9	7	4	0	10	8	2	0	10	7	3	0	10	8	1	1
Proportion		**0.4**	**0.49**	**0.11**	**0**	**0.43**	**0.5**	**0.07**	**0**	**0.44**	**0.4**	**0.16**	**0**	**0.41**	**0.41**	**0.13**	**0.05**
1	♀	7	10	3	0	7	12	1	0	8	12	0	0	8	4	6	2
2	♀	9	10	1	0	11	9	0	0	9	9	2	0	9	11	0	0
3	♀	13	5	2	0	12	8	0	0	11	9	0	0	11	8	1	0
4	♀	4	15	1	0	4	15	1	0	7	11	2	0	13	3	4	0
Proportion		**0.41**	**0.5**	**0.09**	**0**	**0.43**	**0.55**	**0.02**	**0**	**0.44**	**0.51**	**0.05**	**0**	**0.51**	**0.33**	**0.14**	**0.02**

Table 4.4b Statistical analyses for Petri-dish experiments at 37.5 ppm.

	30 min			1 hr			2 hrs			24 hrs		
	A*	**B**	**C**	**A***	**B**	**C**	**A***	**B**	**C**	**A**	**B**	**C**
£GLM:												
rmd	-	1.104	2.948	-	0.4569	1.927	-	2.207	0.3785	1.296	5.116	0.3446
Test* **	-	chi	F	-	chi	chi	-	chi	chi	chi	F	chi
Expt.	-	NS	NS	-	NS	NS	-	NS	NS	NS	NS	NS
Treat	-	NS	NS	-	NS	NS	-	p=0.018	NS	NS	NS	NS
Male	-	0.89	0.55	-	0.92	0.54	-	0.84	0.48	0.95	0.87	0.51
(se)		(0.035)	(0.100)		(0.029)	(0.057)		(0.041)	(0.061)	(0.024)	(0.087)	(0.060)
Female	-	0.91	0.55	-	0.98	0.56	-	0.95	0.54	0.98	0.86	0.38
(se)		(0.031)	(0.099)		(0.017)	(0.055)		(0.024)	(0.057)	(0.017)	(0.089)	(0.058)
Overall @	-	0.90	0.55	-	0.95	0.55	-	0.89	0.51	0.96	0.86	0.44
95% CI @	-	(0.80, 0.95)	(0.38, 0.71)	-	(0.86, 0.98)	(0.42, 0.67)	-	(0.79, 0.95)	(0.38, 0.64)	(0.87, 0.99)	(0.66, 0.95)	(0.31, 0.58)
$Contingency table:												
Chi-sq (p)	-	**0.28 (0.598)**	**0.00 (0.987)**	**-**	**p=0.276^^**	**0.09 (0.770)**	**-**	**5.33 (0.021)**	**0.55 (0.460)**	**0.03 (0.864)**	**p=0.681^^**	**1.69 (0.194)**

Table 4.5a Numbers of individuals (i.e. replicates) recorded at 4 time intervals as either on untreated leaf sections (UT), 75 ppm treated leaf sections (T) or plastic (P) surfaces, or dead (D).

		30 min				1 hour				2 hours				24 hours			
Experiment	**Sex**	**UT**	**T**	**P**	**D**	**UT**	**T**	**P**	**D**	**UT**	**T**	**P**	**D**	**UT**	**T**	**P**	**D**
1	♂	8	8	4	0	10	6	4	0	8	9	3	0	4	14	1	1
2	♂	6	10	4	0	8	12	0	0	7	12	1	0	4	7	5	4
3	♂	1	7	12	0	6	9	5	0	4	10	6	0	5	8	6	1
4	♂	9	8	3	0	10	8	2	0	10	7	2	1	7	8	3	2
Proportion		**0.3**	**0.41**	**0.29**	**0**	**0.42**	**0.44**	**0.14**	**0**	**0.36**	**0.48**	**0.15**	**0.01**	**0.25**	**0.46**	**0.19**	**0.1**
1	♀	8	9	3	0	8	11	1	0	8	12	0	0	7	11	2	0
2	♀	4	10	6	0	6	12	2	0	5	12	3	0	5	9	4	2
3	♀	7	12	1	0	5	14	1	0	6	13	1	0	10	9	1	0
4	♀	7	3	10	0	9	7	3	1	11	7	1	1	6	10	3	1
Proportion		**0.33**	**0.42**	**0.25**	**0**	**0.35**	**0.55**	**0.09**	**0.01**	**0.38**	**0.55**	**0.06**	**0.01**	**0.35**	**0.49**	**0.12**	**0.04**

Table 4.5b Statistical analyses for Petri-dish experiments at 75 ppm.

	30 min			1 hr			2 hrs			24 hrs		
	A*	**B**	**C**	**A****	**B**	**C**	**A****	**B**	**C**	**A**	**B**	**C**
[£]GLM:												
rmd	-	7.272	0.827	-	2.593	0.295	-	2.426	0.1231	0.4521	1.326	0.5356
Test***	-	F	chi	-	chi	chi	-	chi	chi	chi	chi	chi
Expt.	-	NS	NS	-	NS	NS	-	NS	NS(5%)	NS	NS	NS
Treat	-	NS	NS	-	NS	NS	-	NS(6%)	NS	NS	NS	NS
Male (se)	-	0.71 (0.135)	0.60 (0.063)	-	0.86 (0.038)	0.51 (0.059)	-	0.85 (0.040)	0.57 (0.059)	0.90 (0.032)	0.79 (0.047)	0.64 (0.063)
Female (se)	-	0.75 (0.129)	0.54 (0.063)	-	0.91 (0.032)	0.61 (0.057)	-	0.94 (0.027)	0.59 (0.056)	0.96 (0.021)	0.87 (0.038)	0.59 (0.060)
Overall [@]	-	0.73	0.57	-	0.89	0.56	-	0.89	0.58	0.93	0.83	0.61
95% CI [@]	-	(0.48, 0.89)	(0.43, 0.71)	-	(0.78, 0.95)	(0.43, 0.69)	-	(0.79, 0.95)	(0.45, 0.71)	(0.83, 0.97)	(0.71, 0.91)	(0.47, 0.74)
[$]Contingency table:												
Chi-sq (p)	**-**	**0.29 (0.593)**	**0.02 (0.893)**	**-**	**0.95 (0.331)**	**1.54 (0.214)**	**-**	**3.23 (0.072)**	**0.11 (0.742)**	**1.64 (0.200)**	**2.44 (0.118)**	**0.58 (0.445)**

Tests = comparisons between males and females in terms of:
A = proportion alive (= [UT + T + P] / 20)
B = proportion on base of dish [made a choice] of those alive (= [UT + T] / [UT + T + P])
C = proportion of those on base that chose T (= T / [UT + T])
£Analyses = logistic regressions (GLM with binomial error and logit link) allowing for overall differences between experiments before testing the difference between treatments (male/female), and for over-dispersion where appropriate
GLM = generalized linear model
rmd = residual mean deviance (3 df)
*Tests*** = either chi-square tests (no evidence of over-dispersion present) or F-tests (when over-dispersion present)*
Expt. = significance of test for overall differences between the four experiments (3 df)
Treat = significance of test for differences between the two sexes (1 df)
NS = not significant at 5% level (P>0.05
@ = estimates based on null model excluding experiment and sex effects; back transformed from logit scale. Proportions for each sex given in two rows above are obtained from full model with both sex and experiment effects.
** None dead*
*** Too few dead for analyses (1 and 2, for 1 hr and 2 hrs, respectively)*
$Also given are results of Chi-squared tests (1 df) on 2 x 2 contingency tables of the form Male/Female x class1/class2 (as appropriate for the required test)
^^ = analysed with Fisher's Exact Test as at least one expected value is less than 5 invalidating the Chi-square test.

4.3.2.2 Field simulator experiments

The first three 'range finders' indicated that there was no discriminatory effect at 0.2 ppm but that at the two higher concentrations (37.5 and 75 ppm), the majority of adults were settling on untreated plants (Table 4.6). In order to minimise the influence of mortality, 37.5 ppm was chosen for the remaining experiments (numbers 4-10). All 8 experiments at 37.5 ppm demonstrated a preferential settling on untreated plants with an average ratio of approximately 4 untreated: 1 treated (Figure 4.3).

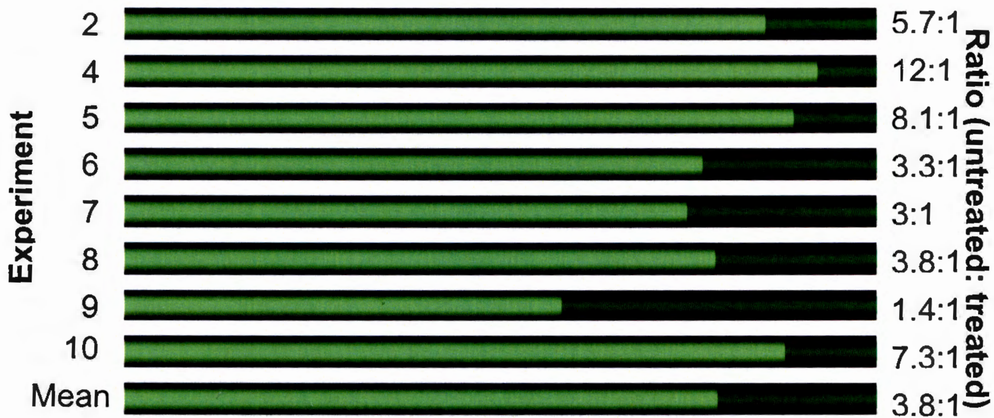


Figure 4.3 Proportions of adults remaining on untreated (light green) and 37.5 ppm imidacloprid treated (dark green) plants after 24 hours. Experiments using concentrations other than 37.5 ppm (numbers 1 and 3) are excluded.

A logistic regression of the number of adults on either plant shows that of the total number released in each experiment (200), an average of 42% of whiteflies were found on the plants (95% confidence interval = 0.30 - 0.55). This regression analysis makes allowance for any over-dispersion present in the 8 individual experiments. The number of whiteflies that were on the treated plant as a proportion of those on either plant, again using logistic regression allowing for over-dispersion, shows that 21% of the whiteflies on plants were on treated plants (95% confidence interval = 0.13 - 0.32). This therefore shows departure from the null hypothesis that 50% would be on untreated and 50% on treated plants, towards the majority (79%) being on untreated plants.

Table 4.6 Distributions of adults and eggs in field simulator experiments.

Experiment number	Dose (ppm)	Adults: untreated	Adults: treated	Eggs: untreated	Eggs: treated	Adults: proportion on untreated
1	0.2	68	126	651	1136	0.35
2	37.5	93	16	53	9	0.85
3	75	86	40	169	35	0.68
4	37.5	60	5	49	2	0.92
5	37.5	33	4	31	0	0.89
6	37.5	57	17	23	5	0.77
7	37.5	57	19	28	9	0.75
8	37.5	52	14	26	3	0.79
9	37.5	70	50	54	29	0.58
10	37.5	108	15	70	13	0.88
Mean (37.5ppm)	37.5	530	140	334	70	0.79

The plot of eggs against adults for each treatment/plant using a parallel regression model shows that a single line fits both sets of data adequately, i.e. the underlying relationship appears to be the same for each treatment with treated plants forming the lower part and untreated plants the upper part of the line (Figure 4.4). Computed correlations (both Pearson's parametric and Spearman's non-parametric were used for comparison) between eggs and adults, for both treatments combined (n=16) and for each treatment separately (n=8) were calculated. As Figure 4.4 suggests these all exhibit significant

correlation; for Pearson's correlations the 5% tabulated value with n - 2 = 6 (df = 0.707), and with n - 2 = 14 (df = 0.497).

Numbers of eggs per adult present (number of eggs/number of adults) for each treatment were computed and a Wilcoxon non-parametric test used to analyze the differences between the untreated and treated plant values. Because the sample size is relatively small the result is quite strongly influenced by one experiment which had T > UT and yields a non-significant value for p of 0.078. Using the total number of eggs as the variable does result in a strongly significant difference with p = 0.008 (as all experiments have T < UT in terms of number of eggs alone).

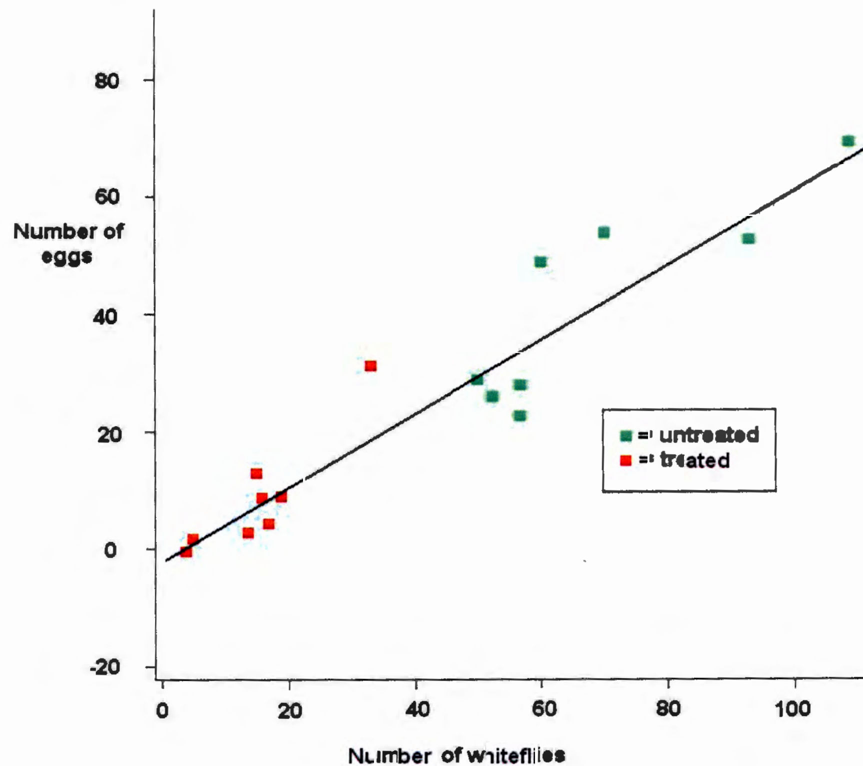


Figure 4.4 Parallel model regression plot showing relationships between numbers of adults and eggs on all 16 individual plants.

correlation; for Pearson's correlations the 5% tabulated value with n - 2 = 6 (df = 0.707), and with n - 2 = 14 (df = 0.497).

Numbers of eggs per adult present (number of eggs/number of adults) for each treatment were computed and a Wilcoxon non-parametric test used to analyze the differences between the untreated and treated plant values. Because the sample size is relatively small the result is quite strongly influenced by one experiment which had T > UT and yields a non-significant value for p of 0.078. Using the total number of eggs as the variable does result in a strongly significant difference with p = 0.008 (as all experiments have T < UT in terms of number of eggs alone).

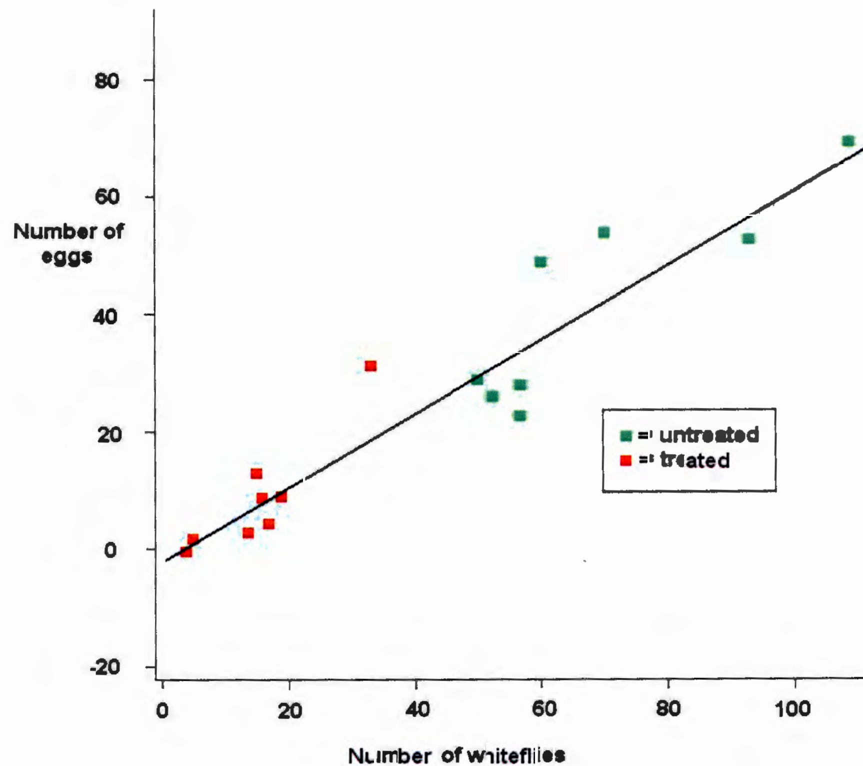


Figure 4.4 Parallel model regression plot showing relationships between numbers of adults and eggs on all 16 individual plants.

4.3.2.3 Insect retrieval

The number and distribution of dead insects within field simulators indicated that mortality due to insecticidal exposure was low. The vast majority of dead insects either remained in the inoculation vial or had been drawn against the fan mesh by the 5 m/s air current (Table 4.7). These mortality factors were not associated with the presence or absence of imidacloprid residues on plants within the simulators. Average mortality (excluding insects in vial and on mesh) in all simulators averaged 14%. Within treatments, there was no significant difference between mortalities around clean or untreated plants.

Table 4.7 Insect retrieval data from field simulators, mortality percentages given exclude those recovered from the vial and mesh.

Exp. no.	Live adults	Dead on untreated	Dead on treated	Dead (floor)	Dead (vial)	Dead (mesh)	% mort
4	65	4	7	4	25	28	18.8
5	37	1	1	2	48	17	9.8

4.3.3 Responses of additional strains to imidacloprid

For the majority of the additional strains, although there was minor variation between their responses, results with the diagnostic concentration of 128 ppm imidacloprid were consistent with those strains tested previously (Chapter 2) and judged to be susceptible to this compound. Several of these had previously been exposed to successive imidacloprid treatments but this did not lead to a

detectable reduction in susceptibility. Mortality at 128 ppm ranged from 84% (UK-14) to 100% (UK-9, 11, 13, 20, 21 and 22, SPAIN-1, SPAIN-2 and CHINA). However, two strains collected in 2004 (UK-23 and NED-2) demonstrated considerably increased levels of survival that resulted in 68% and 19% mortality in discriminating dose assays (Figure 4.5). Although not significantly resistant at LC_{50} (Table 4.8) dose-response assays spanning a range of concentrations (Figure 4.6) showed that a proportion of individuals from these two field-collected strains survived the highest concentration tested (1024 ppm). An important feature of the results is that mortality did not increase with an increase in imidacloprid concentration from 128 to 1024 ppm. Such a 'plateau' is a hallmark of resistance and identifies a proportion of individuals effectively immune to the highest concentration that can be effectively applied in a systemic leaf-based bioassay.

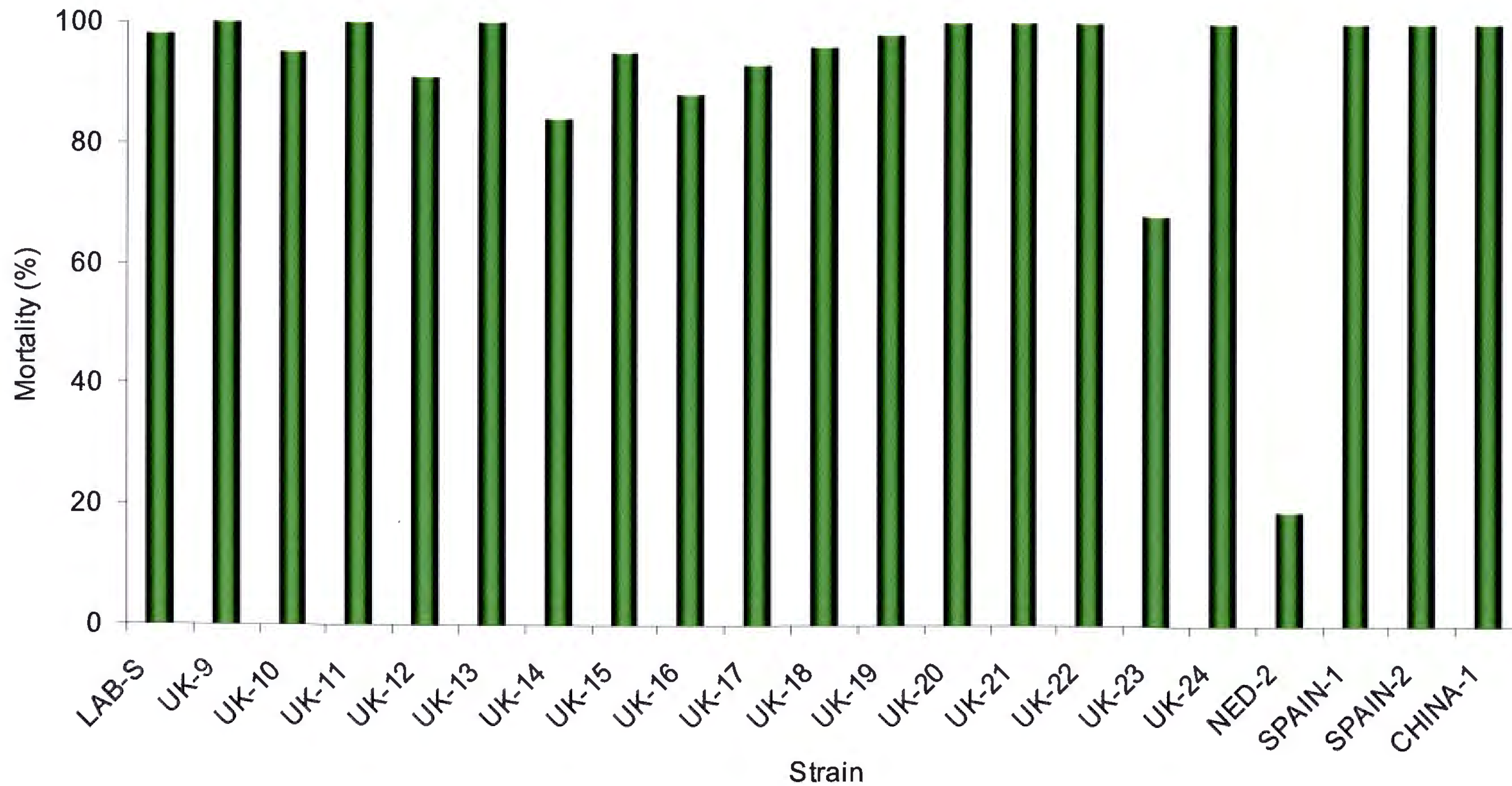
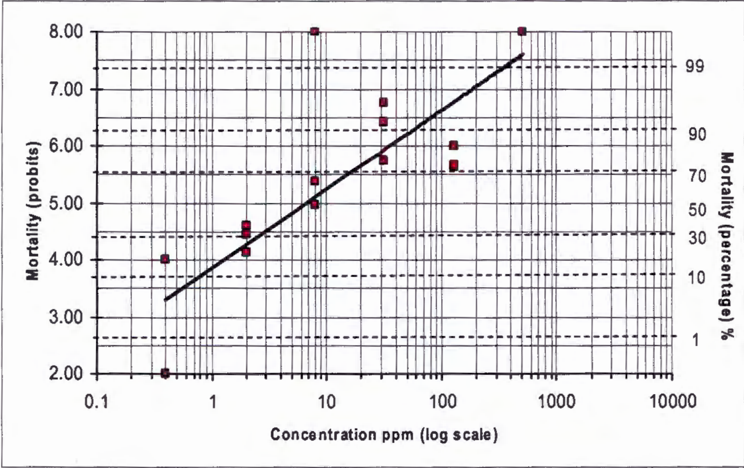
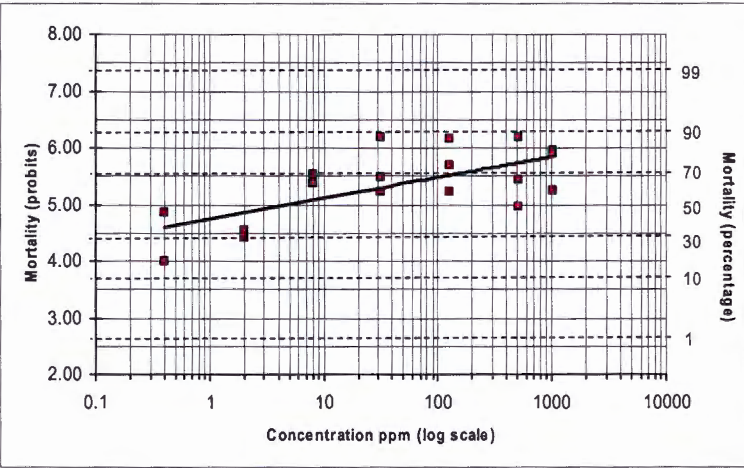


Figure 4.5 Responses of additional strains to a diagnostic dose of 128 ppm imidacloprid.

a.



b.



c.

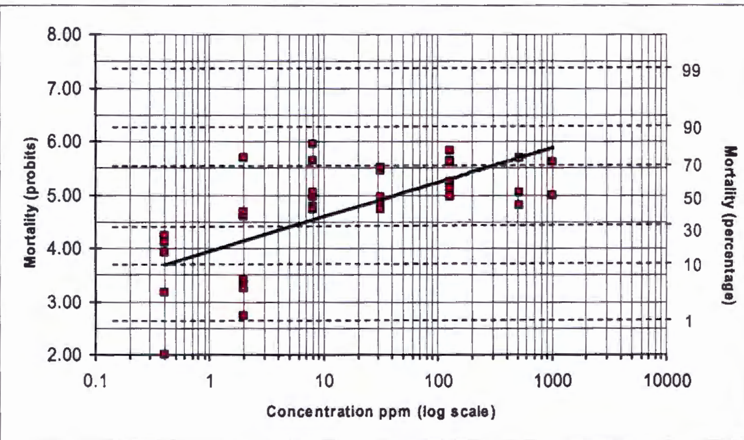


Figure 4.6 Dose-response relationships against imidacloprid for a. LAB-S, b. UK-23 and c. NED-2.

Table 4.8 LC_{50} and LC_{90} values obtained during dose response assays for LAB-S, UK-23 and NED-2 against imidacloprid.

Strain	LC_{50}	95% cl	LC_{90}	95% cl
LAB-S	6.3	2.8 - 13	100	40 to 590
UK-23	4.0	0.02 - 29	>2000	NC
NED-2	31	8.8 - 110	10600	1500 -670000

NC = not calculable

4.4 DISCUSSION

The lack of any detectable shift in response during successive selections with imidacloprid demonstrated that none of the cultures that were combined to form the MIXED strain, contained individuals carrying alleles capable of conferring strong resistance to this compound. This supports conclusions drawn from bioassay data in Chapter 2, that despite established resistance to other compounds, genes encoding for imidacloprid resistance were not present in the parental populations.

In contrast, UK-23 and NED-2 (collected from sites of reported control failures), were shown to contain resistant individuals and represent the first documented case of imidacloprid resistance in the UK, and the first in this species worldwide. These findings are of grave concern in view of the reliance being placed on imidacloprid for control of *T. vaporariorum*. Movement of insects within the extensive plant trade industry has previously been implicated with the establishment of novel resistance traits in new areas (e.g. Kirk and Terry, 2003) and it appears that many other regions may soon be threatened. This scenario resembles the early findings of resistance in *B. tabaci*, which in addition to extending to other

neonicotinoids, has since increased in potency and geographical distribution (Nauen and Denholm, 2005).

Field simulator experiments showed that the anti-feedant effects of imidacloprid documented for *B. tabaci* (Nauen *et al*, 1998; Isaacs *et al.*, 1999) and aphids (Devine *et al.*, 1996) are also manifest in *T. vaporariorum*. As a systemic application was used, it appears that this is a true anti-feedant response (as feeding was the only possible route of exposure). It should be noted that imidacloprid can be applied either systemically or as a foliar spray and the application method may influence the extent of behavioural responses to this compound. Ingestion may not be a prerequisite for eliciting such responses and this will to some extent, dictate whether systemic or foliar applications are the most effective inducers of this repellent effect. This highlights an interesting area for future research. Another factor that influences the impact of anti-feedant responses, particularly in a closed glasshouse system, is the accessibility of untreated host plants. This could be in the form of areas of poor application within the target crop, or surrounding untreated and possibly less-favoured hosts including weeds. The existence of such untreated refuges favoured by whiteflies may have an indirect influence over the selection for resistance, since it reduces the proportion of an overall population exposed to a selecting agent (Denholm and Rowland, 1992). In environments lacking such refuges, the implications of behavioural responses is likely to be far less.

The anti-feedant response observed during field simulator studies was not replicated in Petri dish experiments. The reasons for this remain unclear; however, it is possible that the small arena volume (10.74 cm^3) and leaf area (10.74 cm^2), or the close proximity of treated and untreated surfaces may have influenced or impaired the ability of whiteflies to discriminate during Petri-dish experiments.

Several collections included in this study were taken as adults from sites that had recently used imidacloprid, in some cases with unsatisfactory results. Despite this, bioassays showed the majority of these populations to be fully-susceptible; it may be that in their glasshouse environments, some individuals were able to avoid lethal dosages and survive for protracted periods, thereby giving the impression of reduced efficacy. Further studies could aid our understanding of the mechanism(s) that governs this anti-feedant response and its intrinsic potency; nonetheless, there are potential consequences of anti-feedant effects regarding insecticide efficacy and selection pressures:

1. In environments containing both treated and untreated plant hosts, some individuals may be able to avoid lethal doses.
2. As a consequence of reduced dosage, selection pressure for resistance may alter.
3. The effects of inadequate or spatially selective imidacloprid applications (refugia/alternative untreated hosts/poor application) upon efficacy and selection may be more complex than with insecticides not exerting behavioural effects.
4. In some situations, pest pressures on surrounding untreated hosts may increase.

The behavioural response of *T. vaporariorum* to imidacloprid has additional interest from a pest management perspective; further research is required to investigate the potential of imidacloprid treatments to complement and improve the function of trap crops. Trap crops are areas adjacent to agricultural produce, planted with more favoured host species and holding no commercial value (Hokkanen, 1986; 1991). They are designed to lure pests away from crops of importance and can if desired, be treated with high volumes of insecticides (e.g. Todd and Schumann, 1988). Although mixed results have been reported for trap crops used against whiteflies (Smith and McSorley, 2000; Stansly *et al.*, 1998), those

surrounding an imidacloprid treated area may have an enhanced performance due to the combination of their attractant and imidacloprid's repellent effects. If established, this type of combination strategy may provide improved control whilst only exposing the breeding pest population to a minimal number of imidacloprid applications, thereby maintaining a low selection pressure and improving sustainability. Commonly referred to as a 'push-pull' strategy, other examples have been explored in more detail; Khan *et al.* (2000) demonstrated the improved function of perimeter trap crops when combined with an intercropped repellent plant. Despite a reduction in potential insecticidal efficacy under certain circumstances there may be a number of behavioural side-effects associated with imidacloprid treatments that if understood and utilised effectively, could expand the suitability of this important compound for integration into contemporary pest management practices.

CHAPTER 5

BIOCHEMISTRY OF RESISTANCE MECHANISMS

5.1 INTRODUCTION

The resolution and detection of specific insecticide resistance mechanisms can aid our understanding of their development, inheritance and potency. The development of diagnostic assays based on qualitative or quantitative changes in detoxifying enzymes or target proteins, provides the ability to correlate mechanistic data with resistance profiles and can also enhance investigations into insecticidal modes of action (Horowitz and Denholm, 2001). Such assays also offer a convenient and effective way of monitoring genotype and/or phenotype frequencies, which is particularly advantageous for evaluating the effectiveness of insecticide resistance management (IRM) tactics. By definition, IRM is likely to influence levels of resistance and therefore, in order to maximise efficiency and minimise selection pressures it is essential to review performance periodically and to react accordingly, emphasising the need for a responsive, dynamic approach (Denholm, 1990; Sawicki, 1986; Dennehy, 1995). Consequently, improvements to monitoring capabilities that reduce this 'reaction time' are likely to have a positive impact on the sustainability of the strategy concerned. Information on resistance mechanisms can also contribute to alternative research areas such as pesticide development and application technology that have important implications for combating resistance (Horowitz and Denholm, 2001).

As demonstrated for *B. tabaci* (Elbert and Nauen, 2000) and *M. persicae* (Devonshire and Moores, 1982), resistance to a single compound may involve the presence of more than one mechanism of defence and equally, a single mechanism of defence may be responsible for resistance to more than one compound. As such, the *in vivo* phenotypic expression of resistance observed during bioassay does not identify the mechanism(s) responsible. Such diagnoses require *in vitro* molecular and/or biochemical techniques that have the sensitivity required to detect either the genetic mutations or phenotypes associated with specific mechanisms. When the

relationships between genotype and phenotype are understood, high throughput assays can sometimes be developed for use as reliable resistance monitoring tools (e.g.; Foster *et al.*, 2002; Nauen and Stumpf, 2002; Anstead *et al.*, 2004).

Mechanisms of insecticide resistance fall largely into one of two types: Metabolic resistance is normally associated with an over-production or increase in activity of detoxifying enzymes, which enhance the abilities of resistant insects to breakdown insecticidal molecules into non-toxic components or sequester them away from the target site (Devonshire and Moores, 1982). Target-site resistance results in a reduction in the binding capabilities of an insecticide at its site of action, and can be due to alterations in the shape or size of the target molecule, affecting either the pathway to, or the binding site itself (Moores *et al.*, 1994; Williamson *et al.*, 1993).

Rare exceptions include physical mechanisms such as reduced cuticular penetration that structurally interfere with the transport of an insecticide to its target-site (*e.g.* Vandebaan and Croft, 1991; Anspaugh *et al.*, 1994; Sugiyama *et* al., 2001), and the evolution of behavioural responses that limit contact between insects and insecticides (Sarfraz *et al.*, 2005).

Although the modes of action of some insecticides targeted at *T. vaporariorum* are known, there are currently no published reports of specific mechanisms of resistance either to conventional or novel chemical agents for this species. In comparison, *B. tabaci* has been the subject of considerable mechanistic research and some individuals have been found to possess a range of different resistance mechanisms, either singly or in combination, that confer broad-spectrum protection from agrochemicals (Denholm *et al.*, 1996; 1998; Morin *et al.*, 2002).

One mechanism known to protect *B. tabaci* and a number of other insect species against pyrethroids is termed knockdown resistance (kdr) and is sometimes also present in an enhanced form (super-kdr) (Martinez-Torres *et. al.*, 1999; Soderlund and Knipple, 1999; Lee *et. al.*, 2000; Chandre *et. al.*, 2000; Morin *et al.*, 2002; Williamson *et al.*, 1993; Williamson *et al.*, 1996). Single amino acid substitutions in the para-type sodium channel protein are known to be responsible for both kdr and super-kdr (Dong *et. al.*, 2000; Williamson *et al.*, 1996), and positive verification of their presence requires molecular techniques based on the development of specific primers tailored to suit the species in question (e.g. Anstead *et al.*, 2004). Investigations of kdr mutations in *T. vaporariorum* were not included in the current project but deserve priority given the apparent importance of the mechanism in *B. tabaci* (Morin *et al.*, 2002; M. S. Williamson, unpublished data).

Three other mechanisms that can be biochemically diagnosed and are known in *B. tabaci* are studied in this Chapter (Table 5.1). The work presented is intended as a preliminary examination of these potential mechanisms, aiming to establish either their presence or absence through the use of techniques used for their detection in *B. tabaci* and other pests.

Table 5.1 The mechanisms of resistance under investigation.

Mechanism	Type	Potential protection against
Elevated carboxylesterases	M	Pyrethroids, organophosphates, carbamates
Modified acetylcholinesterases	TS	Organophosphates, carbamates
Mixed function oxidases	M	Carbamates, pyrethroids, organophosphates, neonicotinoids

TS = target site; M = metabolic

5.1.1 Non-specific esterases

Elevated levels or additional types of non-specific esterases in some insects can contribute to detoxifying pyrethroid, organophosphate and carbamates insecticides (Devonshire and Moores, 1982; Devonshire and Field, 1991; Byrne *et. al.*, 1994). In the majority of cases, this is due to esterases sequestering toxic insecticidal components or hydrolysing structural ester bonds that are present in some insecticidal molecules (Devonshire and Moores, 1989). By combining esterase specific substrates with colour indicators in the presence of insect homogenates, spectrophotometer readings can be used to quantify the total level of esterases present. Determination of the different esterase types and their relevant quantities enables comparisons between insects of differing resistance status, indicating additional or elevated esterase variants that may be implicated with resistance. Polyacrylamide gel electrophoresis (PAGE) can be used to separate, stain and visualise esterase isozymes in the form of horizontal dark bands whose intensities are proportional to their titres (Brown *et al.*, 1995; Byrne *et al.*, 2000).

5.1.2 Modified acetylcholinesterases

Acetylcholinesterases (AChE) are crucial to the correct firing of neurones within the nervous system. Also present in vertebrates, their function is to terminate neurotransmissions through the breakdown of acetylcholine, thereby allowing restoration of a latent response. Inhibition of their activity through the application of organophosphate or carbamate insecticides can result in continual, repetitive firing and quickly leads to incapacitation and death (Fournier *et. al.*, 1996). Modified forms of AChE have altered target-sites that whilst retaining their affinity for acetylcholine, reduce the rate at which insecticides bind, leading to a lower insecticidal toxicity. By quantifying the levels of AChE activity in the absence and presence of

insecticidal AChE inhibitors, the sensitivities of susceptible and resistant individuals can be compared (Moores *et al.*, 1988; Byrne *et* al., 1994).

5.1.3 Mixed function oxidases

Mixed function oxidases (MFO's), sometimes termed cytochrome P450-dependent mono-oxygenases, are generic metabolic enzymes, capable of detoxifying insecticidal compounds from several chemical classes as well as a range of other xenobiotics. In addition to recent evidence linking MFO activity with imidacloprid resistance in *B. tabaci* (Nauen *et al.*, 2002), they have also been implicated with resistance to organophosphates, pyrethroids and carbamates in several other pest species such as the tobacco budworm, *Helicoverpa virescens* (Rose *et. al.*, 1995; Zhao *et al.*, 1996*)* and the cotton bollworm, *Helicoverpa armigera* (Yang *et al.*, 2004). Detection of MFO activity utilises spectrophotometer or fluorometer readings to detect the level of oxidase binding to a range of artificial substrates, indicated by a corresponding colour change. This biochemical approach to quantifying MFO activity has often been sensitive enough for measurements from individual insects, however, prior to this project there was no report of this technique being successfully applied to *T. vaporariorum*.

5.2 MATERIALS AND METHODS

5.2.1 Insect strains

The strains chosen for investigation had demonstrated a range of responses against bifenthrin, profenofos and buprofezin during bioassay (Chapter 2). Comparisons between susceptible and resistant strains aimed to highlight any consistent correlations between biochemical markers and

resistance phenotypes. Two strains of *B. tabaci* (one susceptible and one resistant) were also included in some experiments (Table 5.2).

Table 5.2 Whitefly strains including species, geographical origin, original host plant and responses to three insecticides (S = susceptible, LR = low resistance and HR = high resistance).

Name	Origin	Host	Bifenthrin	Profenofos	Buprofezin
T.vaporariorum					
LAB-S	UK	Bean	S	S	S
UK-1	Essex	Tobacco	LR	S	LR
UK-4	Somerset	*Fuchsia* sp.	HR	HR	HR
UK-5	Jersey	Rose	HR	HR	HR
UK-6	Jersey	Rose	HR	HR	HR
UK-7	Surrey	Solanaceae	HR	S	LR
UK-8	Surrey	Solanaceae	HR	S	LR
B. tabaci					
SUD-S	Sudan	Cotton	S	S	S
ISR-R	Israel	Cotton	HR	HR	S

5.2.2 Esterases

Different analytical methods aimed to compare total esterase activities and variation of esterase isozymes in a range of *T. vaporariorum* strains with differing levels of pyrethroid and organophosphate resistance. In addition, a comparison of esterase activities with a susceptible (SUD-S) and a pyrethroid and organophosphate resistant (ISR-R) *B. tabaci* strain were included. Electrophoresis protocols and a 96-well microplate assay sensitive enough for analysis of esterase activities in individual insects were adapted from protocols for *B. tabaci* (Byrne *et al.*, 2000). All insect

homogenates were prepared using 3 repetitions of 20 clockwise and 20 anti-clockwise turns of a multi-homogeniser (Burkard Scientific).

5.2.2.1 Total esterase activities in *T. vaporariorum*

Adult female *T. vaporariorum* were homogenised in individual microplate wells containing 5µl of 1.6% Triton X-100 in distilled H_2O and diluted to 200µl with phosphate buffer (pH 6.0). These diluted homogenates were then separated into two 100µl aliquots providing replicate plates for total esterase and total protein assays.

In order to account for the different sizes of individual insects when measuring total esterase activities, readings were adjusted to compensate for individual total protein contents. 200µl of Bradford reagent (ready-made combination of substrates and colour indicators) was added to 100µl aliquots of diluted insect homogenates and the plate stored for an incubation period of 10 minutes to allow colours to stabilise. Endpoint readings were then taken at room temperature, at a wavelength of 620nm using a V_{max} microplate reader. A reaction curve for experimental standards was obtained through the use of simultaneous readings of 200µl of Bradford reagent with increasing concentrations (2 - 14µg) of bovine serum albumin (BSA) in 100µl of phosphate buffer (pH 6.0).

For measurements of total esterase activities, 200µl of phosphate buffer (pH 6.0) containing 0.06% fast blue RR salt and 0.15mM of either α-naphthyl acetate or α-naphthyl butyrate was added to 100µl aliquots of diluted insect homogenates. Microplates were then read kinetically at 450nm and intervals of 10 seconds for 20 minutes at room temperature, using a V_{max} kinetic microplate reader (Molecular Devices) that is capable of simultaneously analysing the optical densities of all 96 reactions. Two model esterase specific substrates (α-naphthyl acetate and α-naphthyl butyrate) were chosen as between them they preferentially bind to serine

hydrolases with differing-sized acyl pockets (G. D. Moores, pers. comm., 2001).

5.2.2.2 Comparisons between *T. vaporariorum* and *B. tabaci*

Adult female *B. tabaci* and *T. vaporariorum* were homogenised in individual microplate wells containing 5µl of 1.6% Triton X-100 in distilled H_2O and diluted to 200µl with phosphate buffer (pH 6.0) containing 0.06% fast blue RR salt and 0.15mM of either α–naphthyl acetate or α–naphthyl butyrate. Microplates were then read kinetically at 450nm and intervals of 10 seconds for 20 minutes at room temperature, using a V_{max} kinetic microplate reader (Molecular Devices).

5.2.2.3 Polyacrylamide gel electrophoresis

For single insect analyses, adult female whiteflies were homogenised separately in individual microplate wells containing 5µl of 1.6% Triton X-100 in distilled H_2O. For mass homogenates, 50 female whiteflies for each strain were collectively homogenised in 200µl of 1.6% Triton X-100 in distilled H_2O, containing 10% sucrose and 0.01% bromocresol purple. 15µl (equivalent to 3.75 whole insects) of this homogenate was added to each well of a 7.5% polyacrylamide gel.

Gels were run at 250V for $1^1/_2$ hours in a 0.55% barbitone buffer (pH 6.0). After removal, each gel was bathed in 50ml of phosphate buffer (pH 6.0) containing 0.2% fast blue RR salt and 0.15mM of either substrate (α–naphthyl acetate or α–naphthyl butyrate) for a further 45-90 minutes. Gels were then rinsed in distilled water before bathing in 7% acetic acid for approximately 72 hours, or until excess stain had cleared.

5.2.3 Acetylcholinesterases

Single whiteflies were homogenised in individual microplate wells containing 5µl of 1.6% Triton X-100 in distilled H_2O. All insect homogenates were prepared using 3 repetitions of 20 clockwise and 20 anti-clockwise turns of a multi-homogeniser (Burkard Scientific). The volume was adjusted to 250µl with phosphate buffer (pH 7.5) containing 0.1% Triton TX-100, and then split into three 75µl aliquots. To each aliquot a further 25µl of phosphate buffer was added and then 200µl of a substrate solution. Substrate solutions consisted of phosphate buffer (pH 7.5) containing 0.1% Triton TX-100, 0.75mM acetylthiocholine iodide (ATChI) and 0.075mM 5, 5-dithiobis (2-nitrobenzoic acid), (DTNB), either with or without the addition of insecticidal inhibitors. Inhibitor concentrations were determined from preliminary experiments and in the final reactions were 100µM, 30µM and 10µM for both pirimicarb and Demeton-S-methyl. Microplates were read kinetically at 405 nm and intervals of 30 seconds for 60 minutes at 25°C using a V_{max} kinetic microplate reader.

5.2.4 Mixed function oxidases

Determination of mixed function oxidase (MFO) activities was attempted using a protocol used by Rose *et al.*, (1995) to successfully measure MFO activity in a range of organophosphate-, pyrethroid- and carbamate-resistant collections of *H. virescens*. Initial experiments used 3rd instar larvae of *H. armigera* to validate a working protocol. Once confirmed, this protocol was adapted and used with the LAB-S strain in an attempt to measure total MFO activity in *T. vaporariorum*.

Individual adult female whiteflies were homogenised in 5µl of 1.6% Triton X-100 and then diluted to 80µl in phosphate buffer (pH 7.5) containing 0.1% Triton X-100. For bollworm, 10 larvae were collectively homogenised

in 80µl of 1.6% Triton X-100, and then diluted to 80µl of the required homogenate concentration in phosphate buffer (pH 7.5) containing 0.1% Triton X-100. All insect homogenates were prepared using 3 repetitions of 20 clockwise and 20 anti-clockwise turns of a multi-homogeniser (Burkard Scientific). To this was added 10µl of a NADPH regenerating solution (0.25mM NADP+, 2.5mM glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase) and 100µl of 3mM *p*-nitroanisole (PNA). Microplates were read kinetically at 405 nm and 30°C for 15 minutes using a V_{max} kinetic microplate reader.

5.3 RESULTS

5.3.1 Esterases

Results obtained using α-naphthyl acetate and α-naphthyl butyrate were generally similar. However, banding patterns using PAGE were clearer when using α-naphthyl acetate as the substrate.

5.3.1.1 Total esterase activities for *T. vaporariorum*

Measurements of total protein contents using Bradford reagent with a range of bovine serum albumin quantities gave an appropriate standard curve (Figure 5.1). Simultaneous readings of protein content within individual adult females from LAB-S, UK-1 and UK-4 were relatively consistent both within and between strains (Table 5.3). Protein adjusted kinetic esterase activities did not differ significantly between strains and there was no consistent correlation between esterase activity and resistance level with either substrate (Figures 5.2 and 5.3). During analyses with α-naphthyl butyrate, two individuals belonging to the LAB-S strain did demonstrate a higher level of esterase activity. Although this was not correlated with insecticide resistance, it does suggest that either

mutated esterase alleles with enhanced binding characteristics, or additional/elevated esterase isozymes were present.

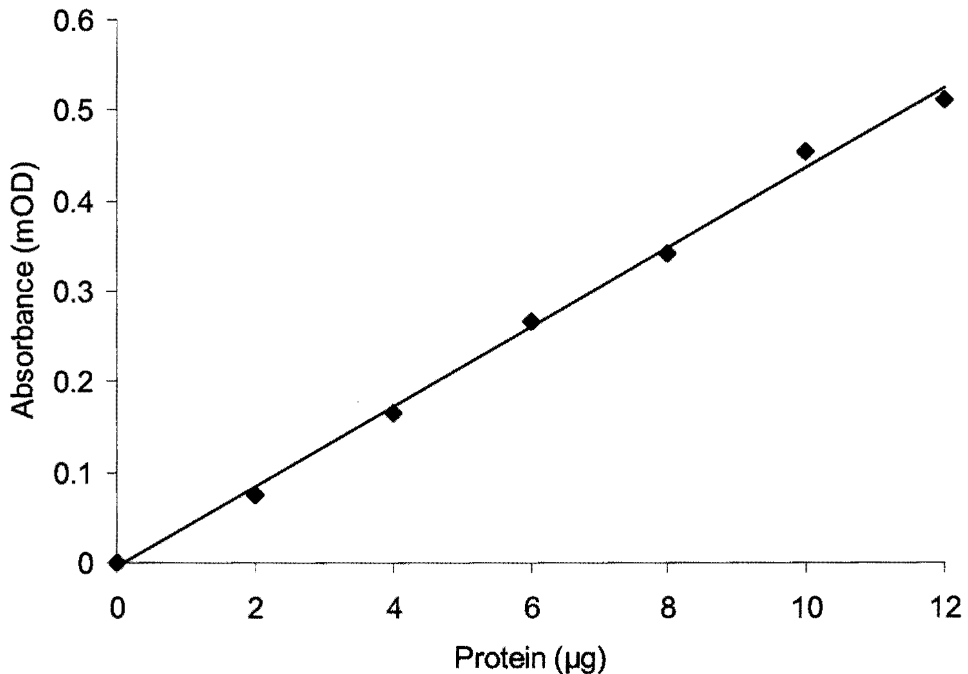


Figure 5.1 Standard curve obtained using Bradford reagent with increasing quantities of bovine serum albumin (BSA).

5.3.1.2 Comparisons between *T. vaporariorum* and *B. tabaci*

The esterase activities for individual *T. vaporariorum* females were similar regardless of resistance status (Figure 5.4). All *T. vaporariorum* strains spanned activity categories up to 16-20mOD, comparable to that of the susceptible *B. tabaci* strain (up to 20-24mOD). In contrast, the pyrethroid resistant *B. tabaci* strain contained individuals with a broad range of esterase activities, ranging from 24-28mOD to 72-76mOD, reflecting the 22 fold resistance level observed in this strain (Byrne *et al.*, 2000).

When amalgamated results for susceptible and resistant strains of both species were compared, complete separation of susceptible and resistant *B. tabaci* strains is evident (Figure 5.5). For *T. vaporariorum* there was no distinction in the esterase activities of susceptible and resistant strains.

Table 5.3 Standard curve and total protein contents for LAB-S (pyrethroid and organophosphate susceptible), UK-1 (pyrethroid resistant, organophosphate susceptible) and UK-4 (highly resistant to pyrethroids and organophosphates) individuals.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0	0.075	0.166	0.267	0.343	0.455	0.511	0.506	blank	blank	blank	blank
B	1.181	1.146	1.188	1.247	1.206	1.211	1.236	1.19	1.227	1.23	1.212	1.192
C	1.249	1.216	1.201	1.205	1.237	1.23	1.24	1.2	1.222	1.218	1.24	1.232
D	1.219	1.184	1.211	1.234	1.248	1.233	1.221	1.216	1.239	1.24	1.238	1.218
E	1.138	1.162	1.21	1.201	1.2	1.245	1.208	1.207	1.241	1.19	1.171	1.207
F	1.213	1.19	1.205	1.213	1.2	1.216	1.222	1.223	1.209	1.243	1.217	1.246
G	1.216	1.195	1.216	1.213	1.232	1.235	1.215	1.203	1.214	1.214	1.234	1.228
H	1.166	1.11	1.206	1.224	1.13	1.16	1.209	1.108	1.164	1.138	1.145	1.091

Row A, columns 1-8 = total protein standard curve (0, 2, 4, 6, 8, 10, 12 and 14μg BSA)

Row A, columns 9-12 = no reaction

Rows B-H, columns 1, 2, 7 and 8 = LAB-S

Rows B-H, columns 3, 4, 9 and 10 = UK-1

Rows B-H, columns 5, 6, 11 and 12 = UK-4

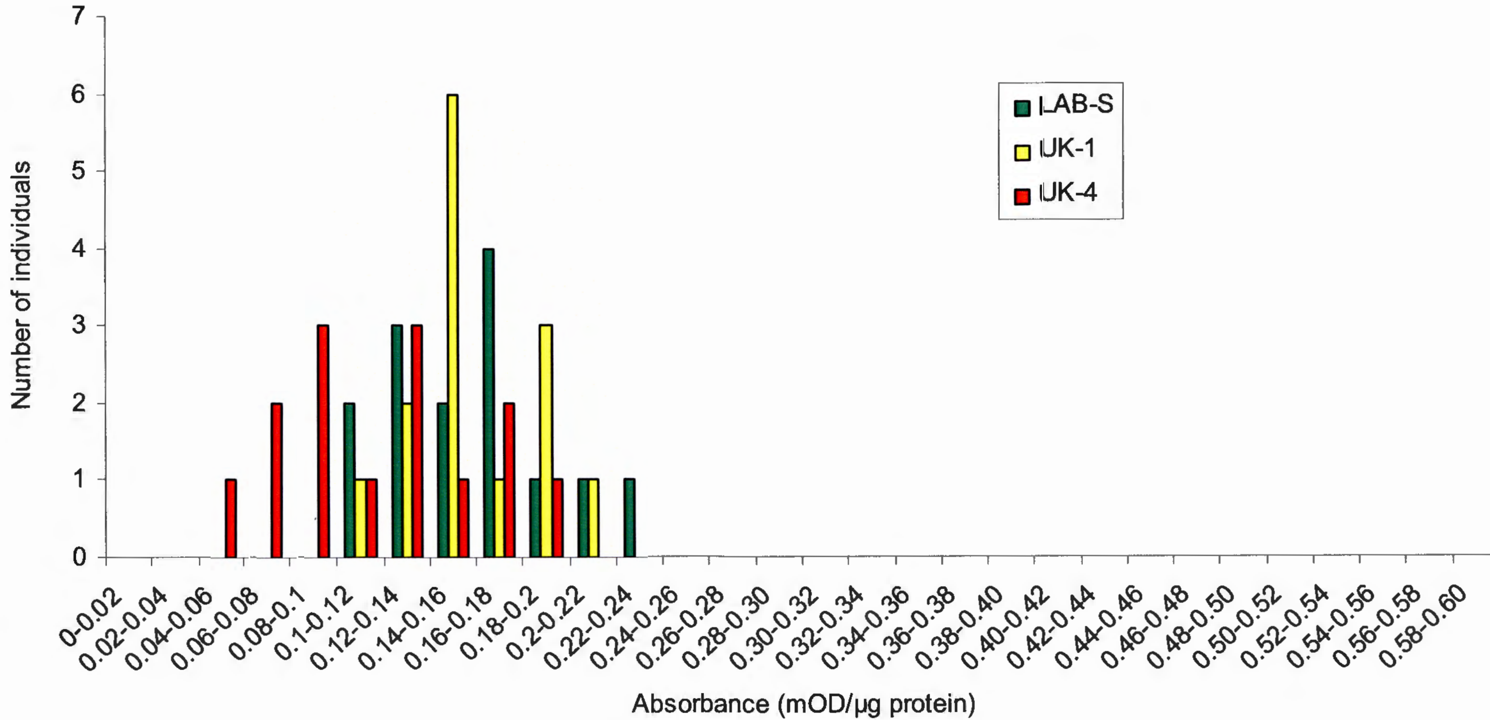


Figure 5.2 Inter- and intra-strain variations in esterase activities/μg protein, between individual *T. vaporariorum* (LAB-S, UK-1 and UK-4) females using α-naphthyl acetate as the substrate.

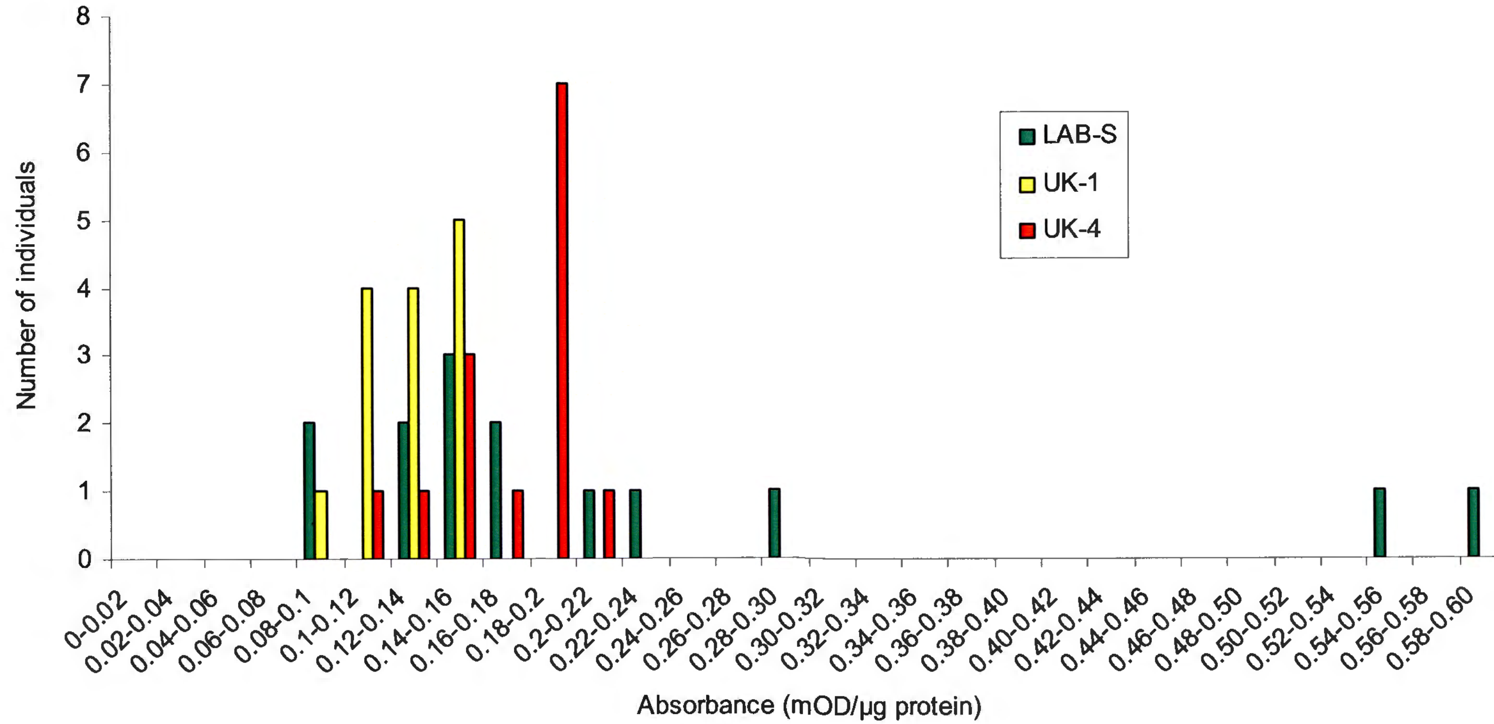


Figure 5.3 Inter- and intra-strain variations in esterase activities/µg protein, between individual *T. vaporariorum* (LAB-S, UK-1 and UK-4) females using α-naphthyl butyrate as the substrate.

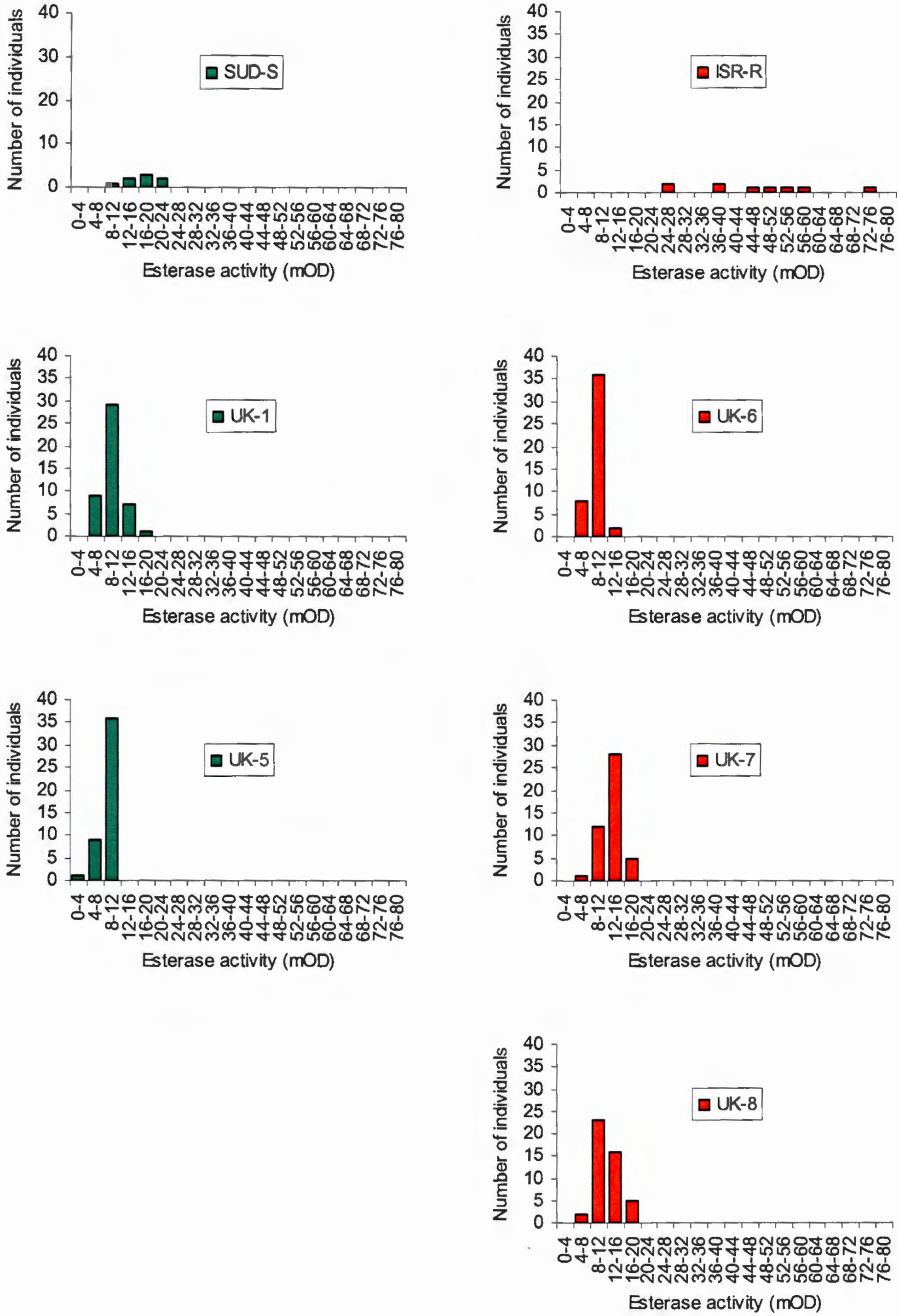
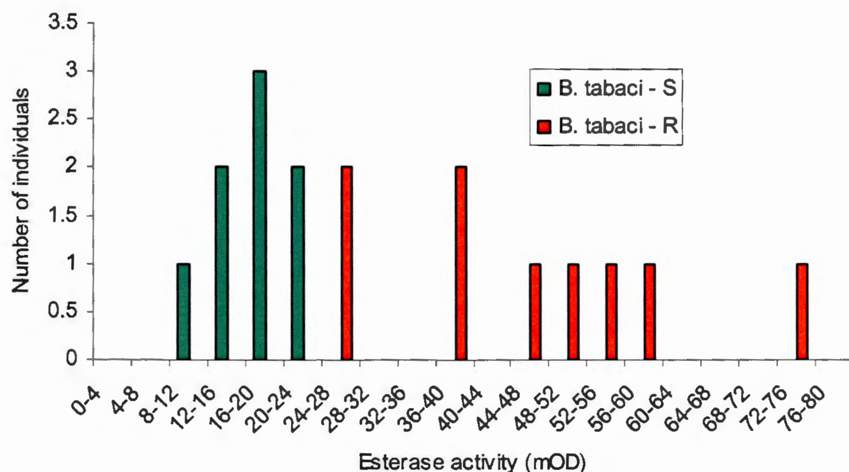


Figure 5.4 Inter- and intra-strain variation in esterase activity of *B. tabaci* (SUD-S and ISR-R) and *T. vaporariorum* strains (UK-1, 5, 6, 7 & 8); green = pyrethroid susceptible, red = pyrethroid resistant.



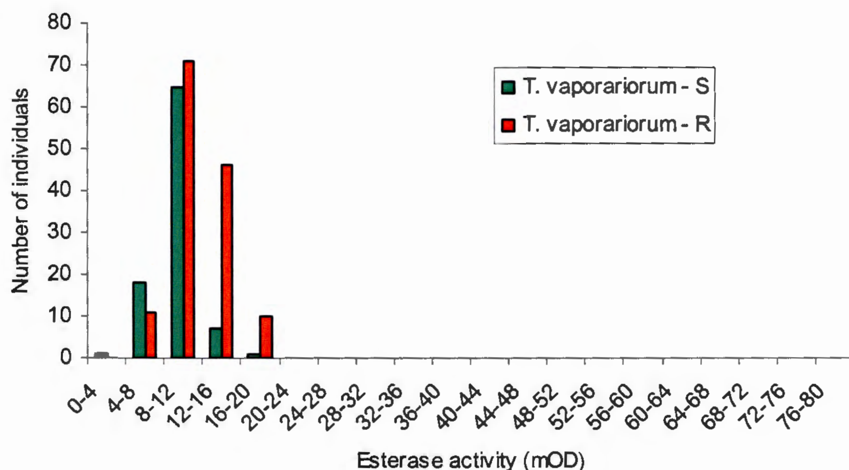


Figure 5.5 Variation in esterase activities between susceptible and pyrethroid resistant individuals of *B. tabaci* and *T. vaporariorum*; green = pyrethroid susceptible, red = pyrethroid resistant.

5.3.1.3 Polyacrylamide gel electrophoresis

Banding patterns disclosed during individual and mass homogenate analyses demonstrated similar esterase profiles for all four strains (Figure 5.6). All individuals possessed a combination of up to four different

esterase types, all of which were common to at least some members of each strain. Staining intensities reflected the relatively low esterase quantities present, there was no correlation between intensity and resistance level for any particular band.

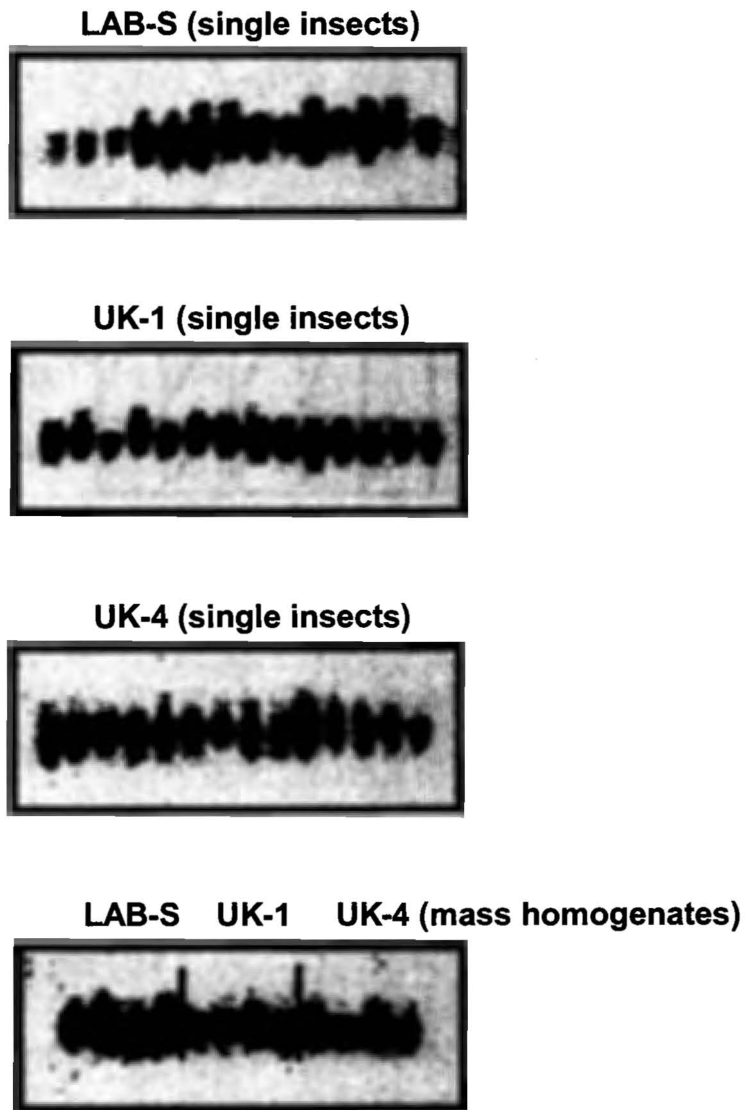


Figure 5.6 Esterase banding patterns for different strains of *T. vaporariorum*, obtained using PAGE and α-naphthyl acetate as substrate.

5.3.2 Acetylcholinesterases

Three strains with varying levels of resistance to organophosphate insecticides were analysed for AChE activities using spectrophotometer microplate assays (Figure 5.7). Decreasing concentrations of two insecticidal inhibitors, pirimicarb and demeton-s-methyl (DSM), revealed no significant difference between the strains.

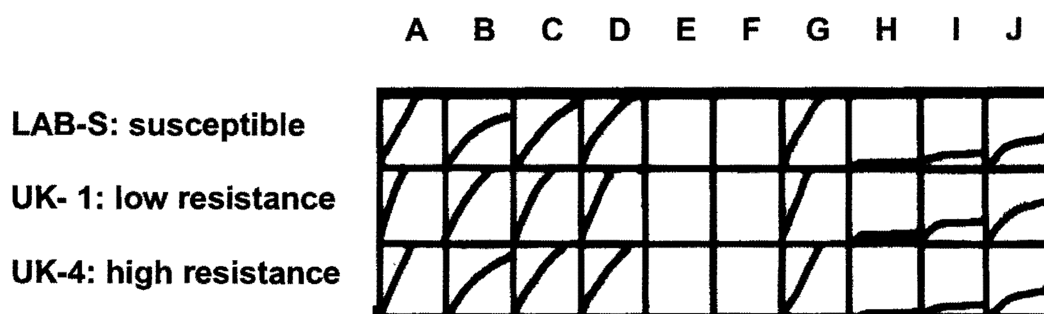


Figure 5.7 AChE activities over time for three *T. vaporariorum* strains (column A = uninhibited, B = 100µM pirimicarb, C = 30µM pirimicarb, D = 10µM pirimicarb, E = blank, F = blank, G = uninhibited, H = 100µM DSM, I = 30µM DSM, J = 10µM DSM).

5.3.3 Mixed function oxidases

Initial studies were done using the cotton bollworm, *H. armigera*. Activities obtained correlated with homogenate concentrations (Figure 5.8), demonstrating that a working protocol was in place. However, subsequent assays with *T. vaporariorum* failed to provide repeatable values; results were inconsistent with the homogenate amounts used and despite further attempts with adjusted substrate quantities, failure to obtain similar results in repeat experiments rendered the method unusable for the determination of MFO activities in this species.

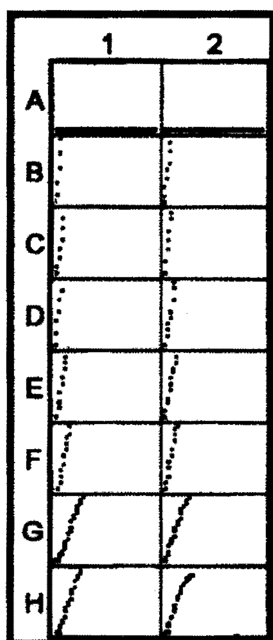


Figure 5.8 Kinetic spectrophotometer plots of *H. armigera* MFO activities over time. Row A = blank, row B = 2 larvae, rows C & D = 1 larvae, rows E & F = ½ larvae, rows G & H = ¼ larvae. Column two is a replicate of column one.

5.4 DISCUSSION

Individuals from all strains were shown to possess relatively low total esterase activities compared to those of *B. tabaci*, despite high overall protein content. Banding patterns present in mass homogenates showed that all observed bands were common to at least some individuals of each strain. As there were no bands found in the resistant UK-1 and UK-4 populations that were not present in LAB-S, it was concluded that the resistant strains did not possess any additional or altered carboxylesterases detectable with the substrates used. If discovered, further investigation would have been required in order to clarify any involvement with resistance. Intensities of bands are also similar between susceptible and resistant strains, indicating that the over-production of

specific esterases is also unlikely to be a contributing factor (this was potentially undetectable during kinetic microplate assays). The similarity of the results with both substrates reinforces the finding of a relatively narrow esterase spectrum.

As the strains used possessed differing pyrethroid and organophosphate resistance levels that did not correlate with esterase activities, in these strains it is unlikely that esterases confer a significant degree of protection against either of these chemical classes. This is in contrast to several pest species including *B. tabaci*, where elevated esterases are known to be associated with pyrethroid resistance (Byrne *et. al.*, 2000) and *Myzus persicae,* which utilises esterase-based resistance to counter both these groups of compounds (Devonshire and Moores, 1982; Field and Foster, 2002).

Results from AChE assays showed all strains to respond in a similar manner; requiring a similar concentration of either inhibitor to reduce AChE activity regardless of resistance status. This suggests that in the strains tested, modified AChE is not conferring resistance against these inhibitors and that the activity levels observed are indicative of a susceptible, wild-type response. It is possible that the use of alternative insecticidal inhibitors may differentiate between AChE activities in this species, representing an area for future mechanistic research.

When comparing the LC_{50}'s from profenofos bioassays with the recommended field rate (<200 ppm using low volume sprays) and comparative data for susceptible and resistant *B. tabaci* generated with the same method (Table 5.4), the relatively high values obtained for the LAB-S strain indicate that complete control in the field would likely be compromised. This may be due either to an inherently less sensitive response to organophosphate compounds in *T. vaporariorum* than *B. tabaci*, or as LAB-S was initially collected and isolated in 1980 (many

years after organophosphate insecticides had been introduced), a baseline level of resistance in all the strains examined.

Table 5.4 Comparison of profenofos bioassay data between *T. vaporariorum* and *B .tabaci.*

	T. vaporariorum		***B. tabaci****	
OP resistance status	S	R	S	R
Strain name	LAB-S	UK-4	SUD-S	ISR-R
Profenofos LC_{50} (ppm)	≈200	≈2000	≈20	≈200

* *Data obtained from Cahill et al., 1995*

The failure to produce acceptable readings for MFO activity using the techniques described was disappointing; however, in retrospect it may not be surprising. Both *B. tabaci* and *T. vaporariorum* are common agricultural pests, the former being of primary pest status. As such, they have a long history of insecticidal exposure and despite the breadth of associated research within this area there was no documented methodology available for either species. It is conceivable that this assay, as a convenient and obvious preliminary experimental choice, may have been tried without success before; if so, it would have been likely to evade publication in peer reviewed journals. Subsequently to this work being undertaken, a fluorometric technique was published for measurements of MFO activity in *B. tabaci* (Rauch and Nauen, 2003). Unfortunately, the technology required was at that time unavailable for inclusion in this project. Although still not yet proven with *T. vaporariorum*, this system may provide a viable option for subsequent studies, and be especially valuable when analysing any cases of neonicotinoid resistance that develop in this species.

CHAPTER 6

GENERAL DISCUSSION

6.1 GENERAL SUMMARY

Although the complexities of pest management are often related to environmental, operational and political influences that may be specific to a given situation, there are common threads and principles that are sometimes more widely applicable. Indeed, the overlapping nature of pest management practices are exemplified within this project, as numerous insect pests including those of agriculture, domestic environments and human health, either have been or are, target-species of at least one of the four insecticidal groups represented. This project has centred on the chemical control of a particular indigenous insect pest of UK horticulture. However, it should be remembered that the majority of insects co-exist within a species-complex (Janssen *et al.*, 1998), which in the case of *T. vaporariorum*, can include a wide-range of both pest and beneficial counterparts. For the UK horticultural industry, the data presented represents a contemporary characterisation of resistance that sheds some light on problems of the past, discloses current concerns and provides some warning of potential problems for the foreseeable future. Nevertheless, it does not necessarily reflect the situation in other areas, where both environments and whitefly biology may differ.

This study aimed to investigate resistance in *T. vaporariorum* to a range of insecticides, and as such required strains from areas of control failure that were likely to possess detectable levels of resistance conferring genes. In addition, the presence of whiteflies in today's competitive and highly demanding glasshouse crops industry has come to exemplify a severe resistance risk due to their proven capacity to adapt to man-made environments and to withstand control measures. For these reasons, whitefly samples were primarily collected from sites of moderate to high insecticide exposure, where control failures were either suspected or evident. Monitoring of samples from organic growers may provide valuable insight into the seasonal and longer-term stability of certain resistance

traits within pesticide-free environments. The distribution map of collection sites (Figure 6.1) shows their geographical spread across the UK.

6.2 RESULTS

For the pyrethroid, bifenthrin, the variable levels of resistance observed in bioassays of individual strains are to some extent, likely to reflect the levels of local pyrethroid usage. The selecting agent(s) could have been bifenthrin, and/or any cross-resisted product, which principally includes the other pyrethroid molecules. The susceptible-type responses of the strains from mainland Europe, NED-1 and GER-1, provided evidence that resistance to these compounds could be managed given appropriate circumstances, and may reflect a low level of exposure to pyrethroids as a consequence of sustained use of IPM in these technically advanced horticultural systems. The potential involvement of fitness costs associated with the possession of resistance genes has already been discussed in Chapter 2. If confirmed, IRM strategies could be designed to exploit any benefits through, for example, strategies involving the rotation of different modes of action (Denholm, 1988; Denholm and Rowland, 1992).

In the case of compounds resisted by enhanced metabolic systems and particularly those that are MFO or esterase mediated, the use of appropriate synergistic enzyme inhibitors can lead to enhanced activity. For example, pyrethroids resisted by *B. tabaci* have been restored to near full-efficacy through pre-treatment with the oxidase and esterase inhibitor, piperonyl butoxide (PBO) (Devine *et al.*, 1998). Although elevated carboxylesterase levels were not evident in the *T. vaporariorum* strains examined as part of this study and measurements of MFO activities were unattainable, synergism studies could lead to an improved pyrethroid performance and/or provide further information regarding the mechanisms likely or unlikely to be involved.

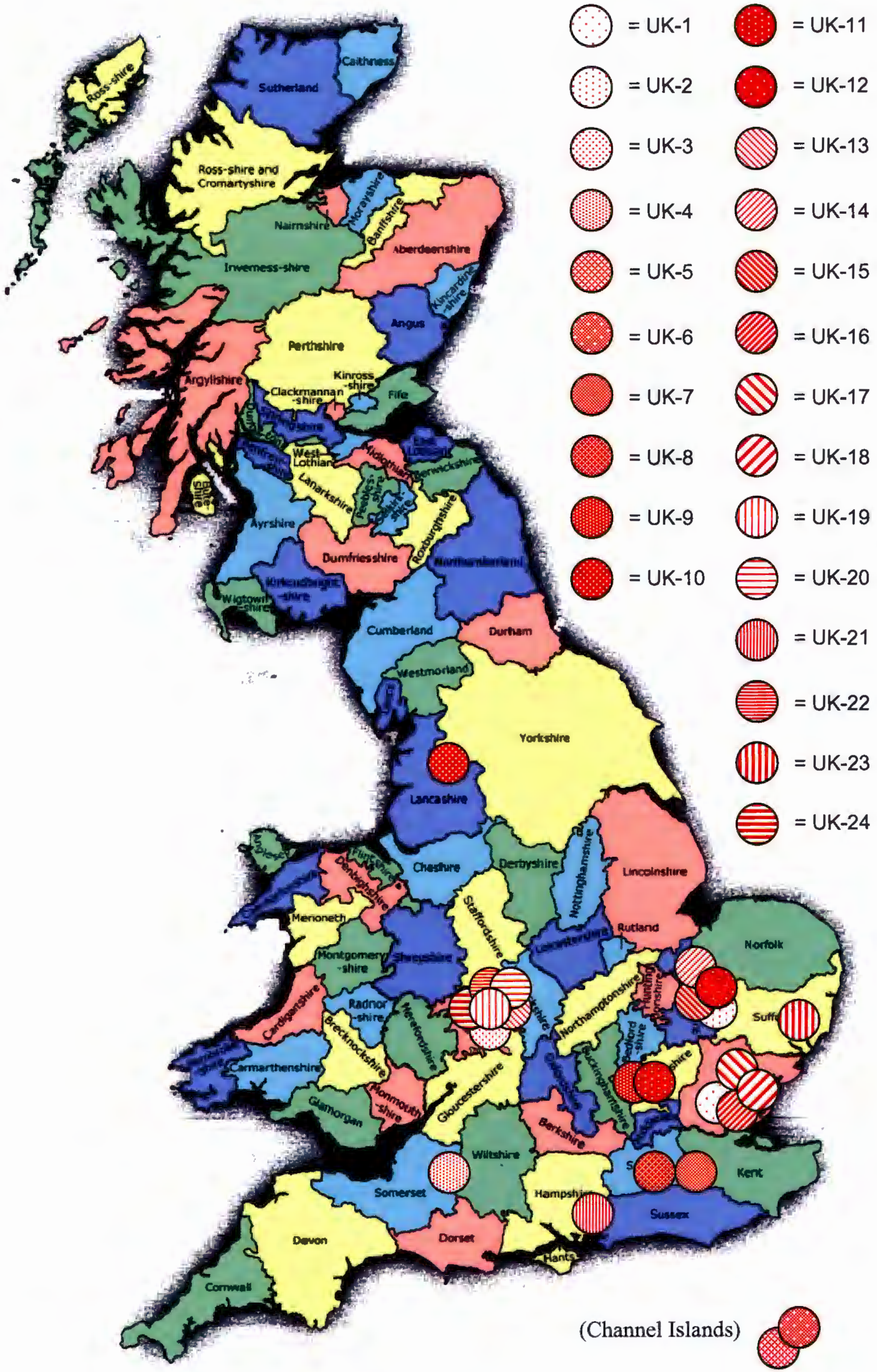


Figure 6.1 Distribution of UK *T. vaporariorum* collection sites.

On a worldwide scale, the immediate future of pyrethroids as key agricultural insecticides is relatively secure. Although some registrations will likely be removed and the overall market share may reduce, they have attained a central role in many pest management practices; their affordability and availability are likely to ensure continued use even though more suitable alternatives are becoming accessible. For control of glasshouse based pests such as *T. vaporariorum*, their role is less assured. The successes of IPM against this species have generated a welcome move away from conventional, broad-spectrum pesticides that is likely to inhibit any prolonged involvement for pyrethroids (van Lenteren *et al.*, 1996). However, the fact remains that members of this class retain efficacy at some sites and as such, could prove valuable if preferred alternatives fail.

Although variable, the responses of strains to the organophosphate insecticide, profenofos, appeared to fall into two groups. The intrinsic activity of profenofos against the more susceptible grouping, which included LAB-S, was lower than expected. Indeed, recommended application rates would be insufficient to achieve sufficient control of these strains, leading to the conclusion that all strains assessed exhibited some level of organophosphate resistance. It should be remembered that organophosphate insecticides had been in use for several decades prior to the isolation of the LAB-S strain.

The lack of discrimination within AChE assays indicates that all strains either do or do not possess a relevant target-site alteration. Given the bioassay, AChE and metabolic data, it appears likely that all strains did possess some form of modified AChE that is responsible for at least part of the resistant phenotype. It is conceivable that the less susceptible group of strains (NED-1, GER-1, UK-4, UK-5 and UK-6) possess either further enhanced or additional mechanism(s) of resistance, although the relevant contribution of different metabolic enzymes or target-site alterations

remains unclear. The non-corresponding pyrethroid and organophosphate resistance profiles of individual strains suggest that there are no consistent cross-resisting mechanisms within the whiteflies analysed.

If resistance to organophosphates is indeed widespread, their future role in IPM of *T. vaporariorum* is compromised beyond the political pressures that are already prompting their withdrawal from European markets. They are largely incompatible with beneficial insects, including predators, parasitoids and pollinators but also have generic toxicities that render them hazardous to vertebrate and invertebrate organisms (Dutton, 2000). Their broad-spectrum toxicity can be volatile, and necessitates significant and strict harvest intervals. Without appreciable efficacy, there can be no valid reason for *T. vaporariorum* remaining as a target-species of this class of compounds. The stability of organophosphate resistance is not documented as dependent upon fitness costs and although immigration of susceptible genes could play a role in restoring susceptibility, the lack of any fully susceptible populations suggests an established genetic trait that is likely to persist in the long-term.

IPM compatibility and environmental risk are not issues with which IGR's generally conflict. Due to their species-specificity, these compounds are ideal for use alongside biological control agents and have so far provided an important line of defence within multi-disciplinary approaches. Previously thought to be largely unaffected by cross-resistance between chemical groups, it is now evident that the two principal IGR's targeted at *T. vaporariorum* within the UK, buprofezin and teflubenzuron, do select for the same mechanism of resistance. Remedial IRM may help this situation; however, monitoring studies at a single site of high resistance revealed no significant increase in susceptibility throughout 7 years of discontinued use. In addition, resistance to buprofezin was stable in laboratory culture (without exposure to insecticides) over a similar time period. Although this may not be indicative of the outcome of large-scale IRM practices, or

indeed IRM at other individual sites, discontinued use of buprofezin and/or teflubenzuron cannot guarantee the restoration of susceptibility.

The common mechanism of defence that protects *T. vaporariorum* from the toxicity of buprofezin and teflubenzuron remains unknown. Although the different modes of action that these compounds utilise may have suggested a common metabolic resistance mechanism as opposed to a target-site modification, biochemical data have revealed that there is no correlation between the levels of buprofezin resistance and carboxylesterase enzyme activity. More detailed characterisations or successful determination of MFO activities in this species may provide additional information.

It appears that within the UK, resistance to buprofezin and teflubenzuron is relatively widespread although levels do vary. The continued use of already resisted products can only exacerbate resistance problems and as such, should be minimised whenever possible. Within glasshouse situations, the selection pressures imposed by applications of buprofezin are likely to be enhanced due to its high volatility and active vapours, and this further supports a strategy of restricted use (Ishaaya, 1992).

Avoiding indiscriminate applications of insecticides in exchange for timely, efficiently delivered treatments is a common-sense approach that maximises efficacy and minimises usage. Limiting selection pressures by avoiding inefficient use of insecticides is also likely to be crucial to the longevity of the neonicotinoid class of insecticides (Nauen and Denholm, 2005). In contrast to the variable levels of resistance found to the other insecticidal classes, characterisation of resistance in over 30 populations spanning a 7 year collection period has indicated that resistance to neonicotinoids in *T. vaporariorum* is still at an early stage. Considering that these early confirmations of resistance include at least one UK population,

it must be assumed that resistance to this compound is now of immediate concern to UK horticulture.

As discussed in Chapter 4, since the publication of low-level neonicotinoid resistance in Spanish populations of *B. tabaci* in 1996, there are complementary works detailing the effects that the subsequent, unabated neonicotinoid use in that region had on resistance levels. The situation quickly worsened and resulted in some of the most resistant populations currently documented (Nauen *et al.*, 2002; Gorman *et al.*, 2003). Contrastingly, the Arizona state-wide IRM strategy for cotton pests managed and monitored resistance in an analogous, intensively farmed region with developing, low-level neonicotinoid resistance (Dennehy, 1995). In 1995, imidacloprid use was restricted to a single application per season on Arizona cotton, to be used within a specified calendar period or 'application window', and in rotation with other insecticidal classes. The strategy successfully maintained imidacloprid resistance at near susceptible levels in *B. tabaci* for the following 9 years and continues to do so today. Imidacloprid remains a component compound of the strategy with the single-use tactic still in place. However, the strategy is presently threatened by a number of developments including approvals of neonicotinoids for other crops and the recent discovery of a highly imidacloprid-resistant Q-biotype strain of *B. tabaci* in a glasshouse in Arizona (T. J. Dennehy, pers. comm., 2005)

As imidacloprid resistant populations of *T. vaporariorum* have only become available recently, the biochemical studies of this project had already been completed. Consequently, this Chapter has not produced any information relating to the likely mechanism(s) of neonicotinoid resistance in *T. vaporariorum*. With evidence of both metabolic and target-site mechanisms in other species (Nauen *et al.*, 2002; Liu *et al.*, 2003) this now represents an important and exciting area for further research.

6.3 FURTHER RESEARCH

There are several areas of research that either remain outstanding, or would provide particularly pertinent data. These include:

1. Monitoring of the spread and development of imidacloprid resistance in *T. vaporariorum*. Now known to be detectable, resistance to imidacloprid is likely to worsen and without documentation, wide scale remedial action is unlikely.
2. Disclosure of the mechanism(s) involved with neonicotinoid resistance could be beneficial at this early stage of development. This also represents a realistic area for future research that could build upon parallel research currently underway with *B. tabaci.*
3. Although biochemical data from Chapter 5 did not conclusively show that acetylcholinesterases contributed to organophosphate resistance in *T. vaporariorum*, a wider range of insecticidal inhibitors may prove useful in discriminating between the responses of individual strains. A positive correlation between activities and resistance phenotype would confirm any contribution of this established mechanism of defence.
4. Further biochemical work utilising alternative means of quantifying MFO activity could disclose the possible involvement of this mechanism in resistance.
5. Screening for kdr mutations known to cause knockdown resistance to pyrethroids in other pest species would reveal the occurrence and importance of kdr in *T. vaporariorum.*

6.4 INTEGRATED CONTROL STRATEGIES

It is clear that IRM can be an effective tool and that in the context of contemporary glasshouse control of *T. vaporariorum*, IRM strategies should work within over-riding IPM systems. IPM systems combine cultural, physical, biological and chemical control tactics in an integrated approach (Brewer, 2005). From an IRM perspective, insecticides should ideally be used as a final line of defence, to minimise exposure and selection for resistance. A basic, example IPM framework for the control of glasshouse populations of *T. vaporariorum* is presented in Figure 6.2. It is a theoretical approach, based upon best-practice and IRM principles.

The sustainability of both IPM and IRM strategies is heavily dependant upon sound monitoring programmes. Monitoring may entail assessments of plant damage, estimations of pest and beneficial insect numbers, the tracking of insecticide resistance genotypes or phenotypes, or indeed any other informative measurements. The diamond shaped decision boxes within the strategy outlined in Figure 6.2 depend upon accurate monitoring information which underpins any subsequent control tactic (rectangular process box). The strategy assumes that cultural control, in the form of pest and disease tolerant cultivars, has been employed at the start and that all other process boxes are unconstrained by economic thresholds. Best practice would be to remain in the safe zone (green) through effectively employed physical control, proceeding through biological and into chemical control only if essential. In some situations biological control will be a known requirement from the outset, in which case physical defences should also be reinforced wherever possible. If and when chemical control remains as the only alternative, applications should proceed in accordance with a responsive, monitoring-based IRM programme.

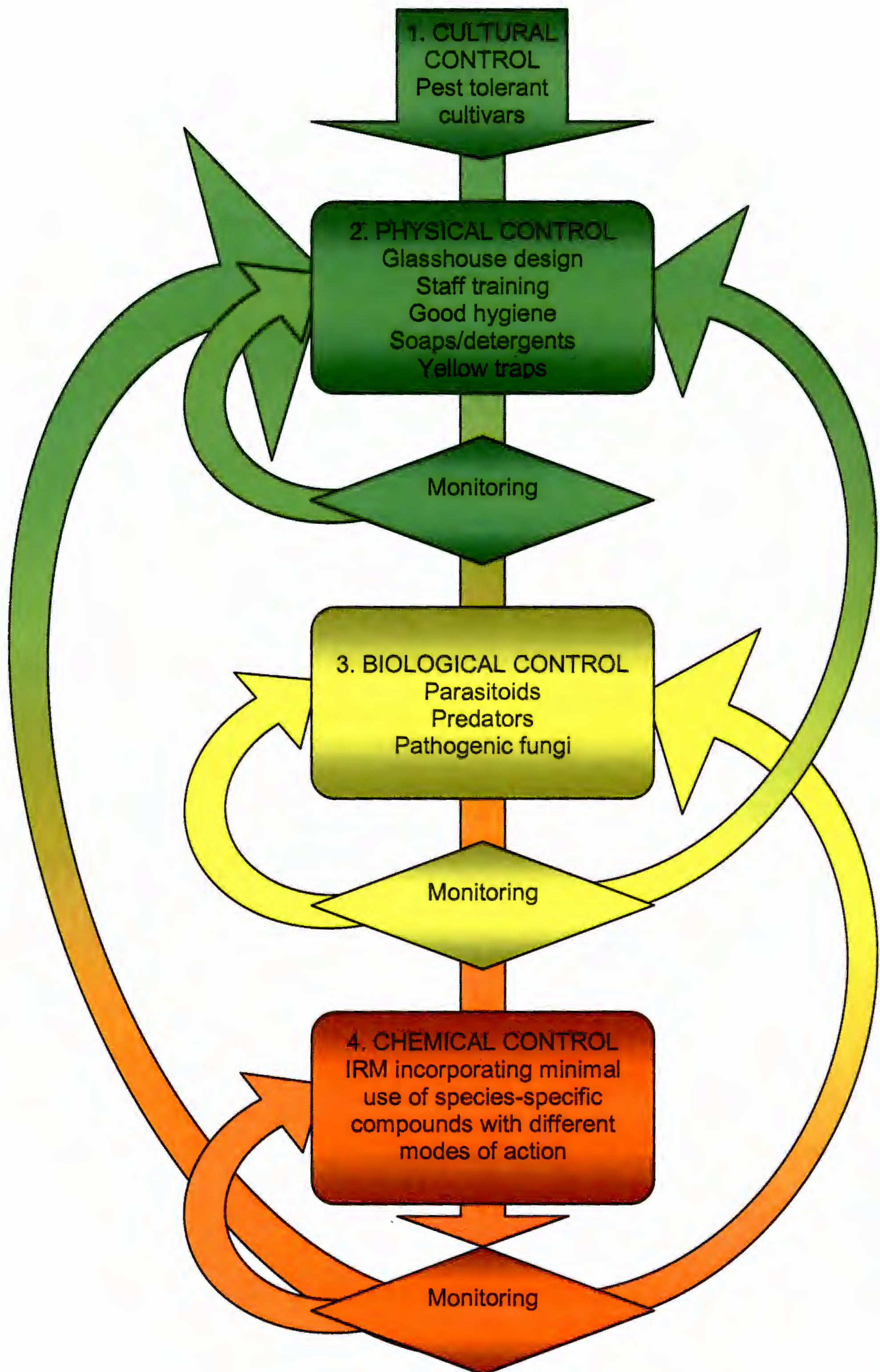


Figure 6.2 Flowchart demonstrating IPM for the control of indoor *T. vaporariorum*. Green = safe zone; yellow = warning zone; orange = danger zone.

This project has revealed new, generic information relating to commonly used products that influences both their suitability for UK use and how they should be used. Either within IPM programmes or not, if targeted at *T. vaporariorum* pyrethroids should only be used towards the end of the season, when any likely selection for resistance has the chance to be negated by selection against resistance over winter, fallow and long unexposed periods. Organophosphate insecticides should be avoided; susceptible genes are rare and so environmental incompatibilities and risk outweigh the potential benefits. IGR's should be used sparingly. The selection for resistance may be rapid where efficacy of buprofezin and teflubenzuron remains. Increased doses have no additional effect and where present, resistance could be stable for protracted periods without further selection. The neonicotinoid class of chemistry is threatened by insecticide resistance and usage should be moderated accordingly. Resistance monitoring over the coming years is vital and should to some extent, dictate future recommendations. Nevertheless, the development of neonicotinoid resistance in other species has demonstrated the perils of negligence and the advantages of early IRM. Behavioural influences may also require some consideration.

Combining all available information into IRM that is confined within an IPM strategy most often leads to a rotational, chemical control approach that employs a minimum number of applications of the most effective, species-specific insecticides available, whilst simultaneously ensuring that they do not select for the same mechanisms of resistance. Correct dosage, delivery and timing of applications are also essential to optimise performance. Perhaps most importantly, it should be remembered that dynamic environments, such as those found in agriculture and horticulture, necessitate an equally dynamic approach to pest management if an acceptable level of control over the economics of crop production is to be maintained.

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Appendix A

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