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Insecticide Resistance in the Western Flower Thrips, Frankliniella occidentalis

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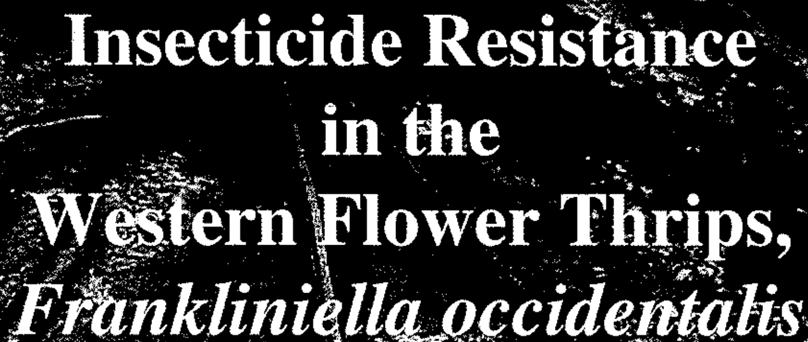

Insecticide Resistance in the Western Flower Thrips, *Frankliniella occidentalis*

Ph.D. Thesis

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January 2000

Foreword

This Ph.D. study was started in 1996. It was part of a project titled "Development and implementation of methods for the determination of insecticide resistance mechanisms and strategies for the prevention of the development of resistance in pest insects". The project was supported by the Danish interministerial research programme on pesticides, and three research institutions have been involved in the project: the Danish Pest Infestation Laboratory (DPIL), Roskilde University (RUC), and the Danish Institute of Agricultural Sciences (DIAS). I was employed at the present Department of Crop Protection, DIAS to work on methods to detect insecticide resistance in thrips and aphids. The work should be done as a Ph.D. study and in October 1996 I was enrolled as a Ph.D. student at Roskilde University. The experimental work has been carried out at Dept. of Crop Protection, DIAS. The department is now placed in a modern research centre at Flakkebjerg, but at the start of this project it was placed in Lyngby, near Copenhagen. The movement of the department caused some discontinuity in the work.

As regards the Ph.D. study, I have concentrated on studying insecticide resistance in the western flower thrips, *Frankliniella occidentalis*. From the start of the project, we had a very precious susceptible population of *F. occidentalis* in the laboratory but no longer any populations resistant to insecticides. Therefore we began to look for resistant populations of *F. occidentalis* to establish new colonies in the laboratory. In that process I contacted several research institutions abroad that are working or had been working with *F. occidentalis*. I was not successful in obtaining any resistant populations, but I received some friendly and encouraging answers. One letter was from Charles O. Knowles, professor of entomology at University of Missouri-Columbia; the head of the group that most thoroughly has studied resistance mechanisms in *F. occidentalis*. He wrote that they had finished their research on *F. occidentalis* and closed the cultures, and he ended his letter this way: "I wish you much success in this project. Based on our experience, I can say that it will be rewarding but not easy research". Now at the end of this project I realise that he was right about the very last statement, it has not been easy research, these thrips do not always behave as you tell them to do. Whether he was right about that it would be rewarding has not always been obvious, but all along it has been very interesting.

Acknowledgements

Supervisor of this study at RUC has been associate professor Søren Achim Nielsen. I wish to thank him for support and guidance during this study. Especially at the start of the study in Lyngby I also received supervision and guidance from senior scientists Merete Albrechtsen and Lars Monrad Hansen. Merete Albrectsen, the Biotechnology Group, DIAS was much helpful with establishing biochemical assays in the laboratory. Another part of my work in the resistance project was to implement a microplate assay for monitoring resistance in the peach-potato aphid. Lars Monrad Hansen has a strong expertise on aphids and I appreciate discussions of this part of the work with him.

The persons involved in the overall project on resistance mechanisms in Danish pest insects were: senior scientists Jørgen B. Jespersen (project manager), Karl-Martin Vagn Jensen, Michael Kristensen, and Andrew Spencer, all of the DPIL, Prof. Jørgen Clausen and associate prof. Søren Achim Nielsen, RUC, and senior scientists Lars Monrad Hansen, Merete Albrectsen, Henrik

Brødsgaard and myself (graduate research fellow), all of DIAS. The meetings of the "resistance group" have been very valuable for me as they gave an opportunity to present and discuss my results with experienced researchers of this field. A special thank to Michael Kristensen and Andrew Spencer for helpful discussions and demonstration of assays to study resistance and resistance mechanisms in insects and for providing synergists for the bioassays.

I further wish to thank the Department of Crop Protection, DIAS (formerly Dept. of Plant Pathology and Pest Management) for providing facilities for this project and for financially supporting the project. The project was mainly funded by the Danish interministerial research programme on pesticides, project PEF-95-131. I hereby acknowledge the support from this research programme.

I am also grateful to persons in the Research Group Entomology, DIAS for discussions and help with various aspects of this study. Especially I thank senior scientists Henrik Brødsgaard, Annie Enkegaard, Gábor Lövei, and Lars Monrad Hansen for critical comments on one or more of the manuscripts, or other parts of this thesis. Particular thanks to Henrik Brødsgaard for sharing his expertise on *F. occidentalis* and resistance in *F. occidentalis* with me and for help with identification of thrips species; furthermore, Henrik started the susceptible laboratory population back in 1990 and also collected the first resistant population used in this study. Further thanks to lab. technician Kirsten Frank for taking care of the susceptible population of *F. occidentalis*, so that I could concentrate on looking after the resistant populations to avoid contamination of populations. I also thank lab. technician Winnie Dam, the Biotechnology Group, DIAS for technical assistance on some of the biochemical assays of the first paper. Also thanks to the New Zealand Institute for Crop & Food Research Ltd. for allowing me to use their figure of *F. occidentalis* life cycle.

Finally, thanks to my "roommates" and other friends and colleagues at the Dept. of Crop Protection, Lyngby/Flakkebjerg for a pleasant time and a friendly atmosphere.

Sten Erik Jensen

January 2000

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PAPERS

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PAPER 2: Jensen, S. E. Mechanisms associated with methiocarb resistance in *Frankliniella occidentalis* (Thysanoptera: Thripidae). (Accepted for publication 20 December 1999 in *Journal of Economic Entomology*). 67

PAPER 3: Jensen, S. E. & Brødsgaard, H. F. Host plant effects on activity of detoxification enzymes and insecticide tolerance in western flower thrips, *Frankliniella occidentalis* (Insecta). (Submitted October 1999 to *ATLA*). 85

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Abbreviations used in the thesis:

AChE: acetylcholinesterase

DEF: *S,S,S*-tributylphosphorotrithioate

GST: glutathione *S*-transferase

OP: organophosphate

PBO: piperonyl butoxide

Front cover: Feeding damage by *Frankliniella occidentalis* on a sweet pepper leaf

SUMMARY

The western flower thrips, *Frankliniella occidentalis* (Pergande) is a serious pest on a wide range of crops throughout the world. In Denmark *F. occidentalis* is a pest in greenhouses. *F. occidentalis* is difficult to control with insecticides because of its thigmokinetic behaviour and resistance to insecticides. Since *F. occidentalis* spread to become a worldwide pest in 1980'es, resistance to a number of different insecticides has been shown in many populations of *F. occidentalis*. This flower thrips has the potential of fast development of resistance owing to the short generation time, high fecundity, and a haplodiploid breeding system.

Although resistance in *F. occidentalis* is a common problem, the underlying mechanisms conferring resistance have only been studied on a few populations. A purpose of this study was to gain more knowledge about possible resistance mechanisms in *F. occidentalis* and, furthermore, to evaluate fast and sensitive biochemical assays for their potential to detect resistance in field populations of *F. occidentalis*. Resistance to the carbamate methiocarb was studied, as this compound is the central insecticide for control of *F. occidentalis* in Denmark. The study was carried out on laboratory populations and populations recently collected in the field from commercial greenhouses in Denmark. Bioassays showed that the populations differed in their susceptibility to methiocarb by 30-fold. The biochemical mechanisms of methiocarb resistance in *F. occidentalis* were studied with synergists in bioassays (piperonyl butoxide, a cytochrome P450-monooxygenase inhibitor, and *S,S,S*-tributylphosphorotrithioate, an esterase inhibitor), assays *in vitro* of activity of detoxification enzymes (general esterases and glutathione *S*-transferases) toward model substrates, and assays *in vitro* of insensitivity and activity of acetylcholinesterase, the target site enzyme for methiocarb.

The results from bioassays with synergists included indicated involvement of cytochrome P450-monooxygenases and esterases in methiocarb resistance in the most resistant populations. Selection with methiocarb on one of the populations to increase the level of resistance resulted in increased activity of acetylcholinesterase and glutathione *S*-transferases. Hence, altered acetylcholinesterase and glutathione *S*-transferases may also contribute to methiocarb resistance. Assays of acetylcholinesterase sensitivity to inhibition by methiocarb, dichlorvos and eserine suggested insensitive acetylcholinesterase in some of the resistant populations. Thus, two alterations of acetylcholinesterase may be possible to confer resistance in *F. occidentalis*: insensitive acetylcholinesterase and increased acetylcholinesterase activity. The results of the study suggest that both metabolic and target-site resistance mechanisms are involved in conferring methiocarb resistance to *F. occidentalis*.

The potential of the enzymatic assays for detecting resistance in *F. occidentalis* was studied by testing if enzyme activities or inhibition of acetylcholinesterase in different resistant populations were significantly and consistently different from those measured in the susceptible population. None of the enzymatic assays showed strong and consistent correlation to the level of methiocarb resistance in the different populations. Therefore, the enzymatic assays used in this study appeared to have modest value for detecting resistance to methiocarb in field populations of *F. occidentalis*.

The particular host plant of a polyphagous insect population may affect activity of detoxification enzymes and tolerance to insecticides. Another part of this study investigated the possible effects of host plant shifts on activity of general esterases, glutathione *S*-transferases, and acetylcholinesterase as well as tolerance to methiocarb in *F. occidentalis*. Thrips from a population adapted to growth on bean plants were transferred to new host plants, sweet pepper and chrysanthemum, to establish two new populations. The size of the adult thrips was decreased on the new host plants, indicating a poorer performance due to the host plant shift. The specific acetylcholinesterase activity differed between the populations, but because the populations also differed in total protein per thrips, no difference was found when acetylcholinesterase activity was expressed as total activity per individual. Specific activity of esterases and glutathione *S*-transferases were largely unaffected by the host plant shifts; the only significant effect observed was a slightly lower level of glutathione *S*-transferase activity in the population cultured on pepper plants. Tolerance to methiocarb was not affected by culturing the thrips on the new host plants.

SAMMENDRAG

Saintpaulia-tripsen, *Frankliniella occidentalis* (Pergande) er et alvorligt skadedyr på mange afgrøder over hele verden. I Danmark er *F. occidentalis* et skadedyr på væksthusafgrøder. *F. occidentalis* er svær at bekæmpe med insekticider pga. dens skjulte levevis og resistens overfor insekticider. Først i 1980'erne spredte *F. occidentalis* sin udbredelse og blev et globalt skadedyr. Siden da er der hos *F. occidentalis* blevet påvist resistens overfor mange forskellige insekticider og i mange populationer. Resistens kan udvikle sig hurtigt i *F. occidentalis* da den har en kort generationstid, høj fekunditet og haplodiploid formering.

Mekanismerne som giver resistens i *F. occidentalis*, er kun blevet undersøgt i få populationer. Et formål med denne undersøgelse var at opnå mere viden om mulige resistensmekanismer i *F. occidentalis* og endvidere at vurdere hurtige og følsomme biokemiske assays for deres potentiale til at påvise resistens i *F. occidentalis* populationer. Resistens over for carbamatet methiocarb blev undersøgt da dette insekticid er det centrale til kemisk bekæmpelse af *F. occidentalis* i Danmark. Undersøgelsen blev lavet på laboratorie-populationer og populationer nyligt opsamlet i danske gartnerier. Bioassays på populationerne viste op til 30 ganges forskel i følsomheden over for methiocarb. De biokemiske mekanismer for methiocarb resistens i *F. occidentalis* blev undersøgt vha. synergister i bioassays (piperonyl butoxid, en hæmmer af cytochrom P450-monooxygenaser, og *S,S,S*-tributylphosphorotrithioat, en esterse-hæmmer), assays *in vitro* af aktivitet af detoksifikations-enzymer (generalle esteraser and glutathion *S*-transferaser) over for modelsubstrater, og assays *in vitro* af insensitivitet og aktivitet af acetylcholinesterase, virkningsstedet for methiocarb.

Resultaterne fra bioassays med synergister indicerede at cytochrom P450-monooxygenaser og esteraser var involveret i at give methiocarbresistens i de mest resistente populationer. Selektion med methiocarb på en af populationerne for at øge niveauet af resistens medførte øget aktivitet af acetylcholinesterase og glutathion *S*-transferaser, et tegn på at ændret acetylcholinesterase og glutathion *S*-transferaser også kan medvirke til at give methiocarbresistens. Assays af hæmning af acetylcholinesterase med methiocarb, dichlorvos og eserine indicerede insensitiv acetylcholinesterase i nogle af populationerne. Dermed er der tegn på to mulige ændringer af acetylcholinesterase til at give resistens i *F. occidentalis*: insensitiv acetylcholinesterase og øget acetylcholinesterase-aktivitet. Undersøgelsen tyder altså på at både detoksifikations-mekanismer og ændringer på virkningsstedet kan medvirke til methiocarbresistens i *F. occidentalis*.

De enzymatiske assays' potentiale til at påvise resistens i *F. occidentalis* blev undersøgt ved at teste om enzym-aktiviter eller acetylcholinesterase-hæmning i de forskellige resistente populationer var signifikant og konsekvent forskellige fra dem målt i den følsomme reference-population. Ingen af de enzymatiske assays var højt korreleret med niveauet af methiocarb-resistens i de forskellige populationer. De enzymatiske assays testet i denne undersøgelse synes derfor kun at have begrænset værdi til med sikkerhed at kunne påvise resistens i *F. occidentalis* populationer.

En given værtsplante for en population af et polyfagt insekt kan påvirke aktiviteten af detoksifikations-enzymer og tolerancen over for insekticider. En anden del af dette studie undersøgte eventuelle effekter af værtsplanteskift på aktiviteten af generelle esteraser, glutathion *S*-transferaser og acetylcholinesterase, og på tolerancen over for methiocarb hos *F. occidentalis*. Trips fra en population tilpasset bønneplanter som værtsplante blev overflyttet til nye værtsplanter, peberplanter og krysantemum, for at starte to nye populationer. Størrelsen af adulte trips blev mindsket efter værtsplanteskiftet hvilket antyder at tripsene generelt klarede sig ringere på de nye værtsplanter. Den specifikke aktivitet af acetylcholinesterase var forskellig i trips fra de forskellige værtsplanter. Imidlertid var den totale proteinmængde pr. trips også forskellig i populationerne hvilket betød at der ikke var nogen forskel mellem populationerne, hvis acetylcholinesterase-aktiviteten blev udtrykt som total aktivitet pr. individ. Den specifikke aktivitet af generelle esteraser og glutathion *S*-transferaser var stort set uændret populationerne imellem; kun populationen opdrættet på peberplanter havde et lidt lavere niveau af glutathion *S*-transferase-aktivitet. Tolerancen over for methiocarb var ikke påvirket af hvilken værtsplante populationen var opdrættet på.

INTRODUCTION

Purpose of the study

Only few studies on few different populations have been carried out to study mechanisms associated with resistance in the western flower thrips, *Frankliniella occidentalis* (Pergande). Research into the mechanisms that confer resistance can give valuable information and tools to be used in resistance management. First, if the resistance mechanism is known, it may be possible to design a highly sensitive biochemical assay to detect and monitor the presence of resistance. Second, identification of resistance mechanisms provides information on potential cross-resistance between insecticides. Third, by knowing the resistance mechanism it may be possible to devise methods for overcoming it.

The goal of this study was to gain more knowledge about possible resistance mechanisms in *F. occidentalis*. As a Danish research programme supported the study, it has focused on Danish populations of *F. occidentalis* and resistance to methiocarb, the central insecticide registered for control of *F. occidentalis* in Denmark.

The aims and scope of this Ph.D. study were to:

- Investigate the biochemical mechanisms of methiocarb resistance in *F. occidentalis*
- Establish rapid and sensitive enzymatic assays on *F. occidentalis* for investigating resistance mechanisms and evaluate these enzymatic assays for use in resistance monitoring
- Investigate the effects of different host plants on methiocarb tolerance and activity of possible resistance-associated enzymes in *F. occidentalis*

Structure of the thesis

The thesis is divided into two parts. The first part is a review of the literature[1] aimed at presenting and discussing topics that are important for understanding the basis and perspective of the experimental work of the study. This part deals with topics such as biology of *F. occidentalis*, *F. occidentalis* as crop pest, resistance in *F. occidentalis*, presentation of the different resistance mechanisms and involvement of these mechanisms in resistance in *F. occidentalis*, and a section on host plant induced enzymatic responses in insects with relation to insecticide resistance. Finally, the conclusions of the study are set out.

The second part of the thesis presents the experimental work of the study. This part contains three original papers or manuscripts that were prepared during the study. They are referred to as *Paper 1*, *2*, and *3* (cf. the table of contents). *Paper 1* is published in Pesticide Biochemistry and Physiology, *Paper 2* is accepted for publication in Journal of Economic Entomology and in press, and *Paper 3* is submitted for publication in ATLA.

At the end of the thesis are enclosed appendices with information relevant for the Ph.D. study.

[1] This review is to be adjusted and submitted to the journal *Integrated Pest Management Reviews*. Editor-in-chief David Dent has informed me that he is looking forward to receiving the manuscript.

Background

The World Health Organization (1957) has defined resistance as "the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals of a normal [susceptible] population of the same species". Implied in this definition is the inheritability of the resistance trait *i.e.* the developed ability is a result of selecting individuals with a heritable capability to withstand the action of the toxicant. Thus resistance develops at the population level.

During the last few decades, populations of many species of insects and mites have acquired resistance to insecticides. Resistance has been recorded in over 500 arthropod species (Georghiou, 1990). Insecticide resistance is a worldwide problem that seriously impairs control of pest insects of agriculture and public health. Resistance often results in increased application frequency or increased dosages of insecticides, thus compounding the resistance problems and causing risks of adverse effects on the environment and human health. In addition, new, safe, and effective insecticides have become increasingly more difficult and costly to discover, develop, register, and manufacture. Therefore, to design strategies to preserve or restore the effectiveness of current and future insecticides it is essential to study factors such as the extent, dynamics, and genetics of resistance and to understand the underlying mechanisms of resistance.

In 1961, the first control failure of the western flower thrips, *F. occidentalis*, was reported (Race, 1961); the chlorinated cyclodiene toxaphene failed to control *F. occidentalis* on cotton in New Mexico. Since then there have been numerous observations of reduced efficacy of insecticides to control *F. occidentalis*, indicating presence of resistance (Robb, 1989). However, the first study to demonstrate insecticide resistance in *F. occidentalis* was carried out by Robb (1989) almost 30 years after the first report on a control failure; at this time *F. occidentalis* had begun to spread to become a worldwide pest.

In Denmark, *F. occidentalis* was first found in the autumn of 1985 in two greenhouses with ornamentals (*Saintpaulia ionantha* (Wendl.)). Comprehensive control measures were taken to prevent the spread of *F. occidentalis* in Denmark, but despite this it had soon spread to a large number of greenhouses in different parts of the country (Brødsgaard, 1989). Before long, *F. occidentalis* became the most serious pest insect in Danish greenhouses with ornamentals; a status that is still valid. *F. occidentalis* proved to be difficult to control with insecticides, and a study including two Danish greenhouse populations suggested that resistance to insecticides could be well-established in Danish populations (Brødsgaard, 1994).

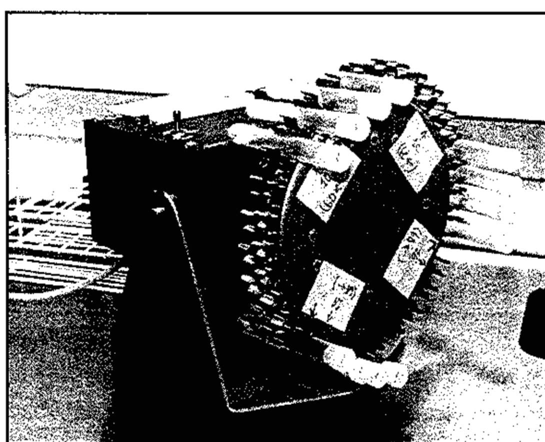
In 1996, when this Ph.D. study was started, two insecticides were registered for control of *F. occidentalis* in Denmark, one carbamate, methiocarb (Mesurol), and one OP, dichlorvos (Vapona). In 1998, the use of dichlorvos for *F. occidentalis* control was prohibited, but in the same year a dispensation was given to use fipronil (Regent) for *F. occidentalis* control. Fipronil proved to be an effective control agent for *F. occidentalis*, and growers report it to be more effective than methiocarb, probably because of resistance to methiocarb in the *F. occidentalis* populations. However, in Denmark fipronil is an expensive insecticide compared to methiocarb, so for many growers methiocarb would be the first choice to treat a *F. occidentalis* infestation. Both insecticides are registered and biologically approved in Denmark for control of *F.*

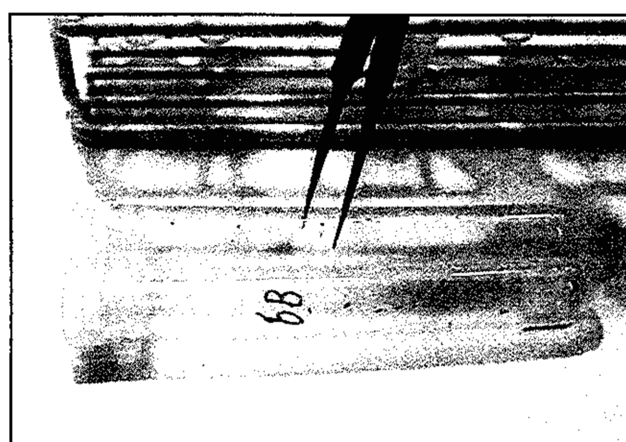
occidentalis, but only for control on ornamental crops (Anonymous, 1999a; Jensen *et al.*, 1999). Methiocarb (Mesurol WP 50, Bayer) was registered in 1990 for control of *F. occidentalis*.

Experimental procedures

The assays used in the experimental work of this study were adapted from other studies on other pest insects and *F. occidentalis*. The assays are now routine assays to detect resistance and study resistance mechanisms in many laboratories. During the study the assays have been further adapted for *F. occidentalis* with reference to the aims of this study. In the individual studies, a lot of effort has been put into standardizing the experimental conditions. Obviously, the key points have been to determine the level of resistance in the different populations and then study resistance associated differences between the susceptible population and the resistant populations.

The most time-consuming part of the experimental work has been the insecticide bioassays. Therefore, the initial work on bioassays of *F. occidentalis* was focused on implementing a relative fast bioassay method. A residual vial bioassay method was tested and implemented. In the bioassays 10-ml polypropylene vials were coated on the inner surface with insecticide, and then thrips were transferred to the coated vials and assessed after 24-h incubation. Disposable vials were used to make the bioassays less labour-intensive. A further significant advantage of the polypropylene vials was that they could be pierced with fine forceps to probe if immobile thrips were dead or alive. During the study, the technique was further refined to make it easier and faster. A piece of wetted insecticide coated filter paper replaced a leaf disk as the water source for the thrips during the incubation period, and a larger rotator made it possible to coat more vials at the same time. As a result, the LC-values in *Paper 1* and *Paper 2* cannot be directly compared. However, in the individual studies, the conditions for the bioassays were standardised as much as possible.



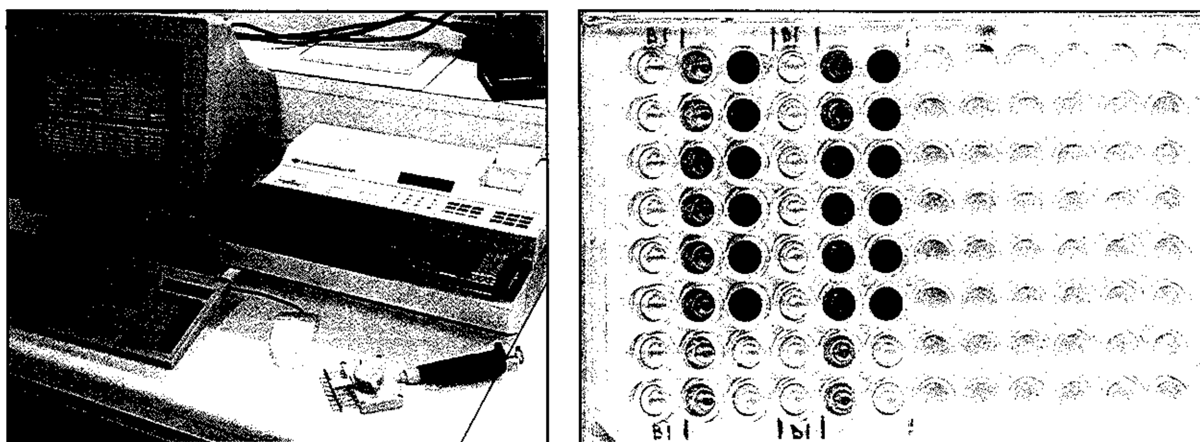


Rotator used to coat vials with insecticides (left), and recording mortality of thrips in coated vials (right). Photos: Sten E. Jensen

The enzymatic assays used in this study were fast and sensitive assays that fulfil criteria for biochemical assays to be used in resistance detection and monitoring in field populations (Brown & Brogdon, 1987). Briefly, the assays should permit analysis of single insects, provide information on likely resistance mechanisms, be fast and accurate, and the equipment should be simple and inexpensive. As stated by Brown & Brogdon (1987): "the purpose of this methodology is not to elucidate or define the enzymology *in vivo* (especially in the case of kinetics) of the resistance enzymes of the species studied, but to recognise resistant phenotypes and the mechanisms likely to produce the resistance". Considering the criteria set up by Brown & Brogdon (1987), enzymatic assays were established on *F. occidentalis* and evaluated for their potential to detect resistance in *F. occidentalis.*

Enzymatic assays *in vitro* to measure activity of general esterases, glutathione *S*-transferases (GSTs), and acetylcholinesterase (AChE) toward model substrates were establish on *F. occidentalis.* The enzymatic assays were established from previous studies of these enzymes on *F. occidentalis* by Zhao *et al.* (1994) and Liu *et al.* (1994).

Preliminary experiments were made with a view to adapt the assays to be capable of measuring enzyme activity in individual *F. occidentalis* on a microplate reader. For example, for the very sensitive esterase end point assay, one of the things tested was the amount homogenate to be used in the assay; to much homogenate may use up the substrate before the reaction is ended, or the amount of product formed may exceed the upper measurement limit of the reader. The kinetic assay of AChE activity was adapted from the extensive study of cholinesterases in *F. occidentalis* by Liu *et al.* (1994); for example, the final concentration of dithiobisnitrobenzoate (DTNB) used in the reaction mixture was low (0.05 mM) as DTNB may inhibit AChE activity in *F. occidentalis.*



Microplate reader used in the Paper 2 & 3 study (left) and microplate from an assay of esterase activity (right). Grey-blue wells in column 2 and 3 of the microplate had α-naphthyl acetate as substrate and red wells in column 5 and 6 had β-naphthyl acetate as substrate; a single experiment from the Paper 2 study (see this study for further details).

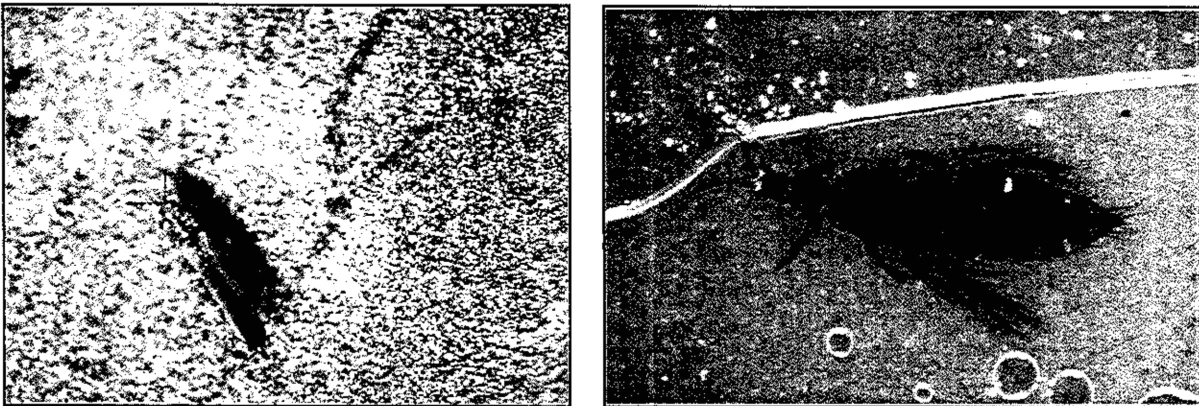
During the study the enzyme assays were further adjusted. For example, the concentration of reduced glutathione in the GST assay was increased from 1 mM in *Paper 1* to 5 mM in the *Paper 2* study. Reduced glutathione has been shown to protect activity of GSTs in housefly homogenates

(Motoyama *et al.*, 1978), and increasing the concentration appeared to result in higher activity measured in *F. occidentalis*. Other experimental conditions may also vary between the individual studies, *e.g.* the homogenisation method, presence and concentration of detergent (Triton X-100) in the homogenisation buffer, and materials and instrumentation. Thus, the activity measured may not be directly compared between the individual studies. However, as in the bioassays, standardisation of the experimental conditions in the individual studies was a key point. In the individual experiments, homogenates of the different populations were assayed simultaneously in one microplate to ensure identical experimental conditions.

THE WESTERN FLOWER THRIPS, *Frankliniella occidentalis*

Thrips belong to the insect order Thysanoptera. About 5,000 species are known and they occur all over the world (Richards & Davies, 1978). The western flower thrips, *Frankliniella occidentalis* (Pergande), belongs to the family *Thripidae*, the family in which most of the economically important thrips species are found (Brødsgaard, 1989).

F. occidentalis is a small insect, adults are about 1 mm long and the females are usually larger than the males. Adults have two pairs of narrow wings that are fringed with long, fine hairs. The wings are held parallel along the back when at rest. The colour of the females can vary from pale yellow to dark-brown or black; males are always yellowish (Tommasini & Maini, 1995).

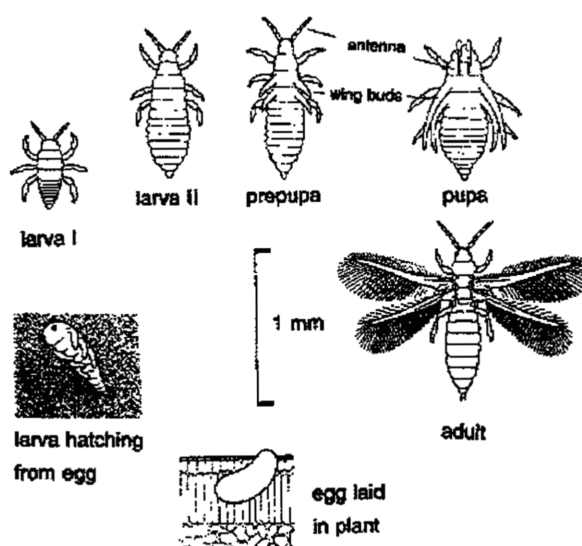


Adult F. occidentalis *on a leaf (left) and trapped on a sticky card (right).*
Photos: Henrik F. Brødsgaard.

Adult female *F. occidentalis* laid eggs singly into the parenchymal tissues of leaves, flowers, or fruits. The eggs hatch into small, white, first instar larvae which immediately begin to feed. The insects pass through two larval stages. Both larval instars are commonly found in tight, protected areas, such as flower buds or foliage terminals. Toward the end of the second larval stage, the insects stop feeding and move down the plant into the soil or leaf litter to pupate. The thrips pass through two "pupal" stages (propupal and pupal), during which no feeding and little movement occur. The pupal stages can be recognised, among other things, by the presence of wing buds (Tommasini & Maini, 1995).

At moderate temperatures, 20-25°C, it usually takes *F. occidentalis* about 2-3 weeks to develop from egg to adult. However, the developmental time is strongly influenced by the temperature. At 15°C the development from egg to adult may take *F. occidentalis* more than a month, but at 30°C and above it may take less than 10 days (Tommasini & Maini, 1995). The fecundity of *F. occidentalis* may also depend on the temperature but it is probably more affected by the host plant and especially availability of pollen as a high quality food source (Brødsgaard, 1989).

Studies on other species of *Frankliniella* have shown that reproduction is facultative parthenogenetic (Brødsgaard, 1989). Female thrips can lay fertilized eggs that develop into females and unfertilized eggs that develop into males, i.e. arrhenotoky. The female thrips are diploid and the male thrips are haploid (Heming, 1995; Moritz, 1997).



Life cycle of F. occidentalis *(from web site Crop & Food Research (www.crop.cri.nz), New Zealand Inst. Crop & Food Research Ltd.).*

F. occidentalis *AS CROP PEST*

F. occidentalis is a serious pest especially on greenhouse crops in many countries in different parts of the world. However, *F. occidentalis* has only recently, in the 1980'es, become a world-wide pest. Originally it was endemic to an area west of the Rocky Mountains in the USA (Brødsgaard, 1989). In Europe, *F. occidentalis* was first found in 1983 in *Saintpaulia* nurseries in the Netherlands. By the end of the of the 1980'es, *F. occidentalis* had been found in most European countries (Tommasini & Maini, 1995). In Denmark, it was first found in 1985 on *Saintpaulia* plants in two greenhouses (Rasmussen & Jakobsen, 1987).

The sudden and rapid dispersal of *F. occidentalis* to become a world-wide pest is brought about by the international trade with plant material. Distribution of plants hosting the thrips is the single most important means of long-distance dispersal of *F. occidentalis* (Brødsgaard, 1993).

F. occidentalis is a polyphagous insect and thus have a wide range of host plants. Two hundred forty four plant species belonging to 62 different families have been registered as host plants for *F. occidentalis* in the USA; the host plants include open-field ornamental, fruit, garden, and agricultural crops (Tommasini & Maini, 1995). In Europe, *F. occidentalis* has mostly been found on protected crops. Permanent field populations of *F. occidentalis* in Europe have become established only in the Mediterranean area; in Northern Europe it may be difficult for *F. occidentalis* to establish permanent field populations because of the cold winters (Brødsgaard, 1993).

The direct damage of *F. occidentalis* on plants is caused by thrips feeding and laying eggs on the plants (Tommasini & Maini, 1995). *F. occidentalis* feeds by penetrating the plant cells with their

stylet-like mouthparts and sucking out the cell sap. This feeding kills plant tissue around the feeding site. Mechanical damage also occurs during oviposition when eggs are inserted into the plant tissue. The damage to plant cells caused by thrips feeding can result in deformation of flowers, leaves, and shoots. There is often silvery streaking and flecking on expanded leaves. Another sign of thrips feeding is deposits of tiny greenish-black faecal specks on the leaves.

Feeding damage by F. occidentalis *on leaves of bean plants (left) and sweet pepper (right). Photos: Sten E. Jensen*

Besides the direct mechanical damage, *F. occidentalis* may also cause severe indirect damage. Pathogens may enter the plant through the punctures made as a result of thrips feeding, and *F. occidentalis* can be a vector for viruses, bacteria, and fungi (Tommasini & Maini, 1995). The most serious indirectly induced damage of *F. occidentalis* is transmitting plant viruses of the genus *Tospovirus.*

The tospoviruses encompasses a group of viruses that cause devastating diseases in many crop plants. These viruses are exclusively transmitted by thrips, and *F. occidentalis* was shown to be the most efficient vector of four different tospoviruses tested in a recent study (Wijkamp *et al.*, 1995). Once *F. occidentalis* has acquired the tospovirus tomato spotted wilt virus from infected plants, it remains a carrier of this virus for the remainder of its life (Wijkamp *et al.*, 1996).

Infestations of crops with *F. occidentalis* can be difficult to control. Multiple tactics is often necessary to obtain an acceptable level of control. Different strategies are possible and the applicability depends on the crop system. Integrated pest management strategies for control of thrips in field, tree and greenhouse crops have recently been reviewed by Parrella & Lewis (1997), Parker & Skinner (1997), and Jacobson (1997), respectively.

Management options to control *F. occidentalis* infestations in greenhouses include cultural control (such as disposal of plant residues, elimination of weeds, and host plant resistance), physical control (such as prevention of thrips from entering the greenhouse), biological control (use of predators, parasitoids and pathogens), and chemical control.

As this Ph.D. study deals with insecticide resistance in *F. occidentalis*, the possibilities and problems with chemical control of *F. occidentalis* is briefly reviewed in the next section.

CHEMICAL CONTROL AND INSECTICIDE RESISTANCE

F. occidentalis is difficult to control with insecticides. The secluded behaviour protects it from many insecticides, and, to further deteriorate chemical control, resistance to insecticides has evolved in many populations and to a number of different insecticides (Table 1). *F. occidentalis* shows a secluded behaviour in all the life cycle stages. Eggs are inserted into plant tissue, the larvae feed in protected areas, the pupal stages are passed in the soil or leaf litter, and the adult thrips also feed in protected areas such as flowers and terminals. Larvae and adults show thigmokinetic behaviour, occupying narrow crevices within or between plant parts. This behaviour makes infestations of *F. occidentalis* difficult to restrain with insecticides.

Table 1. Insecticide resistance in populations of *F. occidentalis*

	Resistance to[a]:				
Origin and number of populations	Organophosphates	Carbamates	Pyrethroids	Others	Reference
4 populations from Californian greenhouses	Chlorpyrifos (4)	Methomyl (4)	Permethrin (4) Bifentrin (4)	Abamectin (4)	Immaraju *et al.* (1992)
3 European and 2 African greenhouse populations	Acephate (5)	Methiocarb (5)		Endosulfan (5)	Brødsgaard (1994)
2 populations from Californian greenhouses	Dimethoate (2) Chlorpyrifos (2)	Methomyl (2)	Fenpropanate (2) Cyfluthrin (1)		Robb *et al.* (1995)[b]
5 populations from greenhouses and 2 laboratory populations from Missouri	Diazinon (7)	Methomyl (7) Bendiocarb (4)	Cypermethrin (7) Permethrin (1)[c] Fenvalerate (1)[c]	DDT (1)[c] Imidacloprid (1)[c] Amitraz (1)[c]	Zhao *et al.* (1995a)[b]
6 populations from greenhouses in Ontario, Canada	Malathion (1) Acephate (2)	Bendiocarb (3)	Deltamethrin (4)		Broadbent & Pree (1997)[b]
4 populations from greenhouses in Israel		Carbosulfan (1) Methiocarb (1)		Abamectin (1)	Kontsedalov *et al.* (1998)[b]
2 populations from greenhouses and 4 laboratory populations from Denmark		Methiocarb (6)			Jensen (*Paper 2 in this Ph.D. thesis)*

[a] Numbers in parentheses following the insecticides show the number of populations resistant to the insecticide in the study.
[b] Not all populations were assayed for resistance to the insecticides tested in the study.
[c] Only tested in the two laboratory populations.

F. occidentalis may eventually come into contact with the insecticide when using the right spray equipment and spray application intervals. However, the chemical control may not be effective because of presence or development of insecticide resistance in the population. Resistance to

insecticides of the three major classes OPs, carbamates, and pyrethroids has been reported in different populations of *F. occidentalis* (Table 1). Furthermore, resistance has been reported to the macrocyclic lactone abamectin (Immaraju *et al.*, 1992; Kontsedalov *et al.*, 1998), the chlorinated cyclodiene endosulfan (Brødsgaard, 1994), the organochlorine DDT, the chloronicotinyl imidacloprid, and the amidine amitraz (Zhao *et al.*, 1995a).

Development and loss of resistance

F. occidentalis has the basis for fast development of resistance. It has a short generation time (see above) and a high fecundity; when reared on chrysanthemum *F. occidentalis* may produce up till 300 eggs per female (Robb & Parrella, 1987), resulting in more than 200 offspring per female (Robb, 1989). Furthermore, the haplodiploid breeding system of *F. occidentalis* can accelerate the development of resistance. Denholm *et al.* (1998) has reviewed the impact of a haplodiploid breeding system on development of resistance. Haplodiploidy means that resistance genes in the haploid males are directly exposed to selection following insecticide treatment, irrespective of intrinsic dominance or recessiveness of the alleles. As a consequence selection for resistance is accelerated compared to insects with only diploid individuals, especially when resistant alleles are recessive. Besides thrips, other important arthropod pests with a haplodiploid breeding system are whiteflies and spider mites (Denholm *et al.*, 1998).

If the resistant alleles are associated with fitness cost for the carriers in an insecticide-free environment, a haplodiploid breeding system implies that the level of resistance in the population may also decline faster in the absence of exposure to insecticides. Resistant alleles are often associated with fitness cost for the carrier (Roush & Daly, 1990). Thus, when carrying resistant alleles associated with significant fitness cost, the decline in the level of resistance in an insecticide-free environment should be fast in *F. occidentalis* because of the short generation time, high fecundity, and haplodiploid breeding system.

Loss of resistance has been followed in a methiocarb resistant population of *F. occidentalis* (DKOdH96) collected for this Ph.D. study. When first tested the DKOdH96 population was more than 20-fold resistant to methiocarb (Jensen, 1997). Seven months later it was tested for the level of methiocarb resistance and resistance had then declined to 10-fold (*Paper 1*). The population had been kept pesticide-free in a separate building away from other thrips populations. Two years after the first bioassays, methiocarb resistance had declined to about 3-fold in the population (*Paper 2*).

In the DKOdH96 population the level of resistance was not constant and declined to a low level, however, in another population the level of resistance has been reported to remain high over a longer period of time. Brødsgaard (1994) kept a population of *F. occidentalis* in pesticide-free culture for about 100 generations (four years) before it was tested for resistance. By the time of testing the population was still 96-fold resistant to acephate; the level of resistance to methiocarb and endosulfan was low, 3.2- and 2.3-fold, respectively.

In insecticide-free environments, the persistence of resistance in isolated populations probably depends on the fitness cost associated with the resistance mechanism and the frequency of resistant alleles in the population. Furthermore, heterogeneity in the population of fitness attributes not related to resistance may select the best-adapted individuals independently of

presence of resistant alleles. The persistence of resistance in a population could then vary depending on the cultural conditions, *e.g.* the particular host plant. In non-isolated populations, persistence of resistance also depends on the immigration of susceptible individuals, which will reduce the frequency of resistant alleles in the population.

Resistance management

Management of resistance to prevent or delay development resistance is necessary for increasing the chances of effective chemical control of *F. occidentalis*. Denholm *et al.* (1998) list three sets of reforms to manage resistance in pests of protected horticultural crops. First, the simplest approach is to use "crop-free" periods, though this approach conflicts with many crop-production programmes. Second, diversification of control tactics is important and should have the greatest extent as possible; minimal use of insecticides is central and chemicals should only be used as the last form of control. Third, when insecticides are applied the way that they are used should be rationalised and optimised to exploit the full diversity of chemicals available and avoid prolonged selection for particular resistance mechanisms.

For optimal use of insecticides to delay development of resistance in *F. occidentalis*, Robb & Parrella (1995) suggest a rotation of insecticide classes every four-six week. At common greenhouse temperatures, then two-three generations only are exposed to insecticides of the same class. Rotation of insecticides is only effective in delaying development of resistance if the insecticides used select for different resistance mechanisms. For instance, a metabolic resistance mechanism may confer resistance to insecticides of different classes. If the resistance mechanisms and cross-resistance spectra are not identified, then rotation schemes should generally encompass as many insecticides with different modes of action as possible. However, in some countries the number of insecticides registered for control of *F. occidentalis* is very limited. For example, in Denmark only two insecticides, methiocarb and fipronil, are currently registered; both insecticides are only registered for use on ornamental crops (Anonymous, 1999b; Jensen *et al.*, 1999). Though methiocarb and fipronil belong to different classes, such a limited number of available insecticides makes the rotation of insecticides less efficacious to manage development of resistance. The growers must then rely more on other control strategies.

The next insecticide to be registered for control of *F. occidentalis* in Denmark is likely to be the new natural macrocyclic lactone, spinosad (Klaus Paaske, personal communication). Spinosad has been reported to be effective for control of *F. occidentalis* and not to have any detrimental effects on the minute pirate bug, *Orius insidiosus* Say (Hemiptera: Anthocoridae), a key predator of *F. occidentalis* used in biological control (Eger *et al.*, 1998). A recent study on houseflies, *Musca domestica* L. (Diptera: Muscidae) indicated that the mechanisms responsible for resistance to the major classes of insecticides are not likely to cause significant cross-resistance to spinosad (Scott, 1998). However, resistance to another macrocyclic lactone, abamectin, has been reported to be present in populations of *F. occidentalis* (Immaraju *et al.*, 1992; Kontsedalov *et al.*, 1998). Thus, resistance may also develop to spinosad, but still it appears to be a promising insecticide for *F. occidentalis* control as it might be combined with biological control in an integrated pest management approach.

Development of resistance in *F. occidentalis* and many other arthropod pests is an international problem. The expanding international trade with plant material not only spreads the pests but also spreads resistance genes associated with pests (Denholm & Jespersen, 1998). For example, Brødsgaard (1994) collected a population of *F. occidentalis* with high resistance to acephate, from a greenhouse in Denmark where insecticides rarely had been used; thus the individuals that started the infestation must have carried resistance genes from previous selection at another locality. Similarly, resistance to the insect growth regulator buprofezin has been detected in whitefly populations in the UK, prior to the official approval of buprofezin in the UK (Denholm & Jespersen, 1998). Clearly, management of resistance is not only a matter to be handled by the individual grower or country but also requires international collaboration.

Research into the mechanisms that confer resistance can give valuable information and tools to be used in resistance management. First, if the resistance mechanism is known, it may be possible to design a highly sensitive biochemical assay to detect and monitor the presence of resistance. Frequently in the field it is only be possible to collect a small number of individuals, then a biochemical assay that allow detection of resistance in single individuals is very useful. Second, identification of resistance mechanisms provides information on potential cross-resistance between insecticides. This may offer the possibility of choosing an alternative insecticide that is not affected by the resistance mechanism and furthermore provides a basis for selection of insecticides to be used in rotations or mixtures of insecticides. Third, by knowing the resistance mechanism it may be possible to devise methods for overcoming it, *e.g.* by the use of synergists to inhibit metabolic resistance mechanisms.

The next part of the thesis intends to give an overview of the different resistance mechanisms and specifically to present what is known about involvement of particular mechanisms in resistance in *F. occidentalis.*

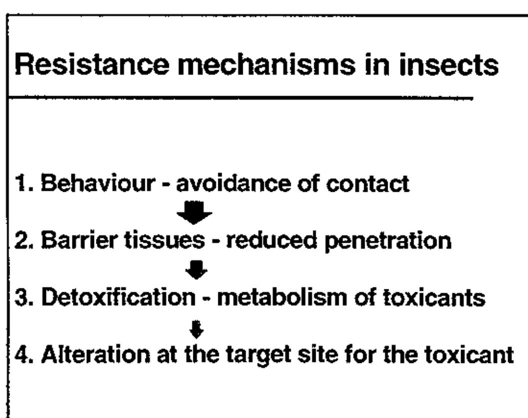
INSECTICIDE RESISTANCE MECHANISMS AND MECHANISMS IN *F. occidentalis*

INTRODUCTION

Development of resistance is an example of 'microevolution'. Variation within a population may include individuals with genetic traits that make them better adapted to survive exposure to an insecticide. If these individuals survive the insecticide exposure, then the resistance traits can be passed on to the next generation, thereby enriching the gene pool with those genes. The subject of this part of the thesis is the resulting mechanisms of the genetic alterations that give rise to resistance.

The intention is to give an overview of the mechanisms involved in insecticide resistance in insects. Several reviews have dealt with this topic, see for example Oppenoorth (1984; 1985) and Price (1991), and reviews in Georghiou & Saito (1983), Roush & Tabashnik (1990) and Mullin & Scott (1992). Other reviews have covered more specific aspects of insecticide resistance such as detection of resistance (Brown & Brogdon, 1987; ffrench-Constant & Roush, 1990; Hemingway *et al.*, 1996), behavioural resistance (Pluthero & Singh, 1984; Sparks *et al.*, 1989), molecular biology of resistance (Feyereisen, 1995), gene amplification in resistance (Devonshire & Field, 1991), modified AChE in resistance (Fournier & Mutero, 1994), population and ecological genetics of resistance (Roush & McKenzie, 1987; Roush & Daly, 1990), management of resistance (Denholm & Rowland, 1992; Denholm *et al.*, 1998), and economics of resistance (Knight & Norton, 1989). So the topic is well-reviewed and the purpose of the next sections is to give the reader not familiar with this topic a basis for understanding the underlying mechanisms of insecticide resistance. At the end of the sections of the different mechanisms, I will try to review what is known about involvement of the particular mechanism in resistance in *F. occidentalis*.

The mechanisms of resistance can be divided into four levels:



The first level, at which resistance rise can arise, is when the insect encounters the insecticide. An altered behaviour can help the insect to avoid coming into contact with the insecticide. Once the insect has come into contact with the insecticide, a delayed penetration through the integument

will reduce the effect of the insecticide at the target-site, yet another level of resistance. Inside the insect, the insecticide may be metabolised and thereby inactivated. At this third level of resistance mechanisms, three systems of detoxification enzymes operate: esterases, glutathione *S*-transferases, and cytochrome P450-dependent monooxygenases. Increased activity of one of these enzyme systems in inactivating insecticides will result in resistance. Alterations at the target-site for the insecticide is the last level of resistance mechanisms. The different classes of insecticides bind to specific target-sites and *e.g.* reduced binding at the target-site or increased number of target-site molecules may confer resistance. The conventional neurotoxic insecticides target only a few sites in insect nervous system:

Targets for conventional insecticides

- **Organophosphates, Carbamates:**
  Acetylcholinesterase
- **Pyrethroids, DDT:**
  Sodium channel
- **Chlorinated cyclodienes, e.g. dieldrin:**
  GABA-receptor
- **Nicotinyl insecticides:**
  Nicotinic acetylcholine receptor

BEHAVIOURAL RESISTANCE

Behavioural resistance mechanisms are the least studied resistance mechanisms in insects, but this is not to say that behavioural resistance is the least significant. Sparks *et al.* (1989) defined behavioural resistance as 'evolved behaviours that reduce an insects exposure to toxic compounds or that allow an insect to survive in what would otherwise be a toxic and fatal environment'. They subdivide behavioural resistance into stimulus-dependent and stimulus-independent mechanisms. Stimulus-dependent behaviour includes repellency and irritancy, mechanisms that enable the insect to avoid the insecticide before and after contact, respectively. Stimulus-independent behaviour includes avoidance of insecticides by occupying habitats that minimise the insects contact with the insecticide (Sparks *et al.*, 1989).

Most of the commonly applied bioassay techniques are not able to detect behavioural resistance, and behavioural mechanisms are difficult to study in the laboratory (Sparks *et al.*, 1989). However, behavioural resistance has been shown in more than 30 species of insects (Sparks *et al.*, 1989) so this kind of resistance may be widespread in insects. Behavioural resistance may coexist with other resistance mechanisms as has been found in *e.g.* the horn fly, *Haematobia irritans* L. (Diptera: Muscidae) (Lockwood *et al.*, 1985), the housefly, *M. domestica* (Golenda & Forgash, 1986), and the German cockroach, *Blattella germanica* L. (Dictyoptera: Blattellidae) (Ross, 1997; Valles & Brenner, 1999). The studies of Mutero *et al.* (1994) and Martinez-Torres *et al.* (1999a)

suggest that when different resistance mechanisms are combined in the same individuals, a synergistic effect, resulting in a high level of resistance, may arise. Therefore, in the field, even a small degree of behavioural resistance may contribute significantly to the overall resistance of the insect. However, from the grower's point of view, behavioural resistance can be desirable in that the insects avoid the insecticide-treated crop. As regards crop pests, the grower has more reason to be concerned about resistance mechanisms that allow the pest to survive on a treated crop.

No studies on resistance due to behavioural mechanisms in *F. occidentalis* have been carried out.

REDUCED PENETRATION

Reduced penetration of insecticides through barrier tissues of insects is one way in which an insect can modify the effective dose of insecticide at the target site. The mechanism may not prevent the insecticide from eventually entering the insect, but it can reduce the rate at which the insecticide reaches the target site. As a single resistance mechanism it usually only confers low levels (< 3-fold) of resistance (Scott, 1990). However, by slowing the penetration rate of insecticides, this mechanism reduces the risk that the insects' detoxification systems become overloaded and the dose of insecticide reaches a fatal level at the target site. Furthermore, by the nature of this mechanism, cross-resistance is often found (Price, 1991).

The rate of penetration of insecticides through the cuticle or other barriers depends on the physicochemical properties of the insecticide and the barrier. Thus, reduced penetration of insecticides is caused by change in physicochemical properties of the barrier tissues. For example, reduced penetration can contribute to DDT resistance in the tobacco budworm, *Heliothis virescens* F. (Lepidoptera: Noctuidae). Vinson & Law (1971) showed that DDT resistant larvae had an altered composition of the cuticle; the protein and lipid content were greater in the cuticle of resistant larvae and, furthermore, the cuticle of the resistant larvae probably had a higher degree of sclerotization. In *M. domestica*, two resistant strains, with reduced penetration as one of the resistance mechanisms, also showed increased cuticular lipid content; more total lipids, monoglycerides, fatty acids, sterols and phospholipids were present in the resistant strains compared to a susceptible strain (Patil & Guthrie, 1979). Reduced penetration has been documented as a resistance mechanism only at the level of the insect cuticle, but any biological membrane may serve as a barrier and thereby give resistance (Scott, 1990).

Reduced penetration has been shown to function as a resistance mechanism to many different insecticides, including insecticides of the three major classes, OPs, carbamates, and pyrethroids. Reduced penetration of OPs through the cuticular barrier has reported for *e.g.* diazinon in *M. domestica* (Forgash *et al.*, 1962), azinphos-methyl in the pear psylla, *Psylla pyricola* Foester (Hemiptera: Psyllidae) (van de Baan & Croft, 1991), and profenofos in *H. virescens* (Kanga & Plapp, 1995). For carbamates, reduced penetration in resistant insects has been reported for *e.g.* carbaryl and propoxur in *B. germanica* (Ku & Bishop, 1967; Siegfried & Scott, 1991), and carbofuran in the oriental fruit moth, *Grapholita molesta* Busck (Lepidoptera: Tortricidae) (Kanga *et al.*, 1998). Several examples of reduced penetration involved in resistance to pyrethroids have also been reported, *e.g.* cypermethrin and fenvalerate in cotton bollworm, *Helicoverpa armigera*

Hübner (Lepidoptera: Noctuidae) (Gunning *et al.*, 1991), permethrin in *B. germanica* (Anspaugh *et al.*, 1994), and fenvalerate in bollworm, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae) (Abd-Elghafar & Knowles, 1996). In most of the above-mentioned studies, the usual method for estimating the rate of penetration of insecticides was employed: radiolabelled insecticide was topically applied to the insects and then, at fixed times after application, unpenetrated insecticide was washed off with an appropriate solvent and quantified. This technique has some limitations. The choice of solvent can influence the result as it may force applied insecticide deeper into the cuticle or the solvent may extract insecticide already penetrated into the cuticle (Price, 1991). However, other methods such as dissection of insects after insecticide exposure can be time-consuming (Gunning *et al.*, 1991) and this method would be impractical with small insects.

Reduced penetration and Frankliniella occidentalis

Reduced penetration as a resistance mechanism in *F. occidentalis* has only been studied in one population. A population selected for resistance to diazinon and cross-resistant to several other insecticides (Zhao *et al.*, 1995a) has been compared to a more susceptible reference population. The diazinon-selected population was 14-fold more resistant to diazinon at the LC_{50} than the reference population. However, reduced penetration of diazinon did not contribute to diazinon resistance in the selected population; in fact, diazinon penetrated faster in the selected population than in the more susceptible population (Zhao *et al.*, 1994). However, it must be noted that the more diazinon susceptible population used as reference showed resistance to some insecticides, including diazinon (Zhao *et al.*, 1995a). The diazinon-selected population showed a low level (7-fold at the LC_{50}) of cross-resistance to fenvalerate as compared to the reference population. Fenvalerate penetrated more slowly into the diazinon-selected population thus indicating that reduced penetration of fenvalerate was involved in conferring resistance to fenvalerate (Zhao *et al.*, 1995c). As often observed when reduced penetration is present as a mechanism of resistance, reduced penetration was not the sole resistance mechanism. Enhanced metabolism of fenvalerate, probably caused by oxidases, could also be demonstrated and target site insensivity (knockdown resistance) may also be involved (Zhao *et al.*, 1995c). The diazinon-selected population was also cross-resistant to bendiocarb; at the LC_{50}, the selected population was 14-fold more resistant of bendiocarb than the reference population. No difference in penetration of bendiocarb was found between the two populations. So bendiocarb resistance in the selected population was caused by other mechanisms, probably mainly by enhanced oxidative metabolism (Zhao *et al.*, 1995b). From the few studies on only a single resistant population, it is difficult to draw any general conclusions about reduced penetration as a resistance mechanism in *F. occidentalis*. It can merely be stated that reduced penetration has also been demonstrated as a possible resistance mechanism in *F. occidentalis*, and that reduced penetration of fenvalerate do not entail reduced penetration of diazinon or bendiocarb, the two other insecticides studied.

DETOXIFICATION

Metabolic detoxification of insecticides is an important toxicokinetic mechanism for insects to reduce the toxic effects of insecticides. OPs, organochlorines, carbamates and pyrethroids are generally hydrophobic compounds and detoxification enzymes transform the insecticides to more hydrophilic and less biologically active compounds that can be eliminated by excretion. Increased detoxification of insecticides has often been reported in resistant populations. Three enzyme systems are generally recognised as the major detoxification systems involved in insecticide resistance in insects. These are esterases, glutathione *S*-transferases, and cytochrome P450-dependent monooxygenases (Soderland & Bloomquist, 1990).

Esterases

Many insecticides contain carboxyl, phosphate, or carbamate ester bonds. Hydrolytic cleavage of ester bonds is an important mechanism in detoxification of insecticides. The enzymes hydrolysing these bonds are esterases and encompass a diverse group of enzymes. The esterases involved in metabolism of organophosphorous insecticides have been divided into carboxylesterases and phosphoric triester hydrolases ("phosphatases") (Price, 1991), but this division may be artificial in insects (Soderland & Bloomquist, 1990).

Specific nomenclature of esterases is problematic because of the diversity in this group of enzymes and their overlapping substrate specificity. Ahmad *et al.* (1986) divide esterases into carboxylesterases (B-esterases or aliesterases) and arylesterases (A-esterases or paraoxonases) based on their sensitivity to inhibition by organophosphorous insecticides; carboxylesterases are inhibited by organophosphorous triesters whereas arylesterases are not. The division of esterases into B- and A-esterases based on OP sensitivity was originally suggested by Aldridge (1953a,b). Heymann (1980) recognise the division of esterases into B- and A-esterases as valuable; however, he regards both B- and A-esterases as carboxylesterases, thereby, aryl-(A-)esterases are a subgroup of carboxylesterases.

In the Swiss-Prot Enzyme nomenclature database, more than 70 EC entries of carboxylic ester hydrolases (EC 3.1.1.-) are listed (Anonymous, 1999c). In this database it is noted that carboxylesterases (EC 3.1.1.1, alternative names ali-esterase or B-esterase) may catalyse reactions of arylesterases (EC 3.1.1.2, alternative names A-esterase or paraoxonase) and enzymes of seven other EC entries. Furthermore, the phosphoric triester hydrolase, aryldialkylphosphatase (EC 3.1.8.1, alternative names A-esterase, paraoxonase, phosphotriesterase and paraoxon hydrolase) has its own EC entry, but it is noted that this enzyme was previously regarded as identical with EC 3.1.1.2, the arylesterase (Anonymous, 1999c).

The problems with classification of esterases reflect the great versatility of these enzymes and their very broad substrate specificity. However, often in insect studies, no attempt is made to specify the esterases assayed. In many cases, esterase activity is assayed without specific inhibitors present and with a model substrate that may be hydrolysed by many different esterases. Therefore, in this section I will generally refer to this group of detoxification enzymes simply as esterases.

Esterases in resistance

Esterases involved in detoxification of insecticides have often been reported to be present in resistant insect populations. A very well studied example of esterase-based resistance is the amplified esterase of the peach-potato aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae). An overproduction of a specific carboxylesterase isoenzyme, E4, is responsible for the marked increase in esterase activity measured in resistant clones (Devonshire, 1977). The E4 esterase hydrolyses different insecticides, but generally it is not very effective in hydrolysing insecticides because of inhibition by OPs and carbamates and slow recovery. However, the vast amounts of the E4 esterase in some clones (up to 3% of total protein) can confer resistance by sequestration, i.e. the insecticides phosphorylates or carbamylates the E4 esterase thereby preventing the active insecticides from reaching the target site (Devonshire & Moores, 1982). The E4 esterase may also confer a low level of resistance to pyrethroids on its own, but it has recently been shown that the major mechanism conferring pyrethroid resistance in *M. persicae* is target site insensitivity (knockdown resistance); coexistence of the two mechanisms can confer a very high level of pyrethroid resistance (Martinez-Torres *et al.*, 1999a). The overproduction of E4 esterase in *M. persicae* arises from amplification of the structural gene for E4. The degree of amplification correlates with both the levels of resistance and the E4 esterase activity (Field *et al.*, 1988). Amplification of esterase genes resulting in overproduction of esterases that detoxify or sequester insecticides has also been shown in resistant populations of *Culex pipiens* L. (Diptera: Culicidae) mosquito complex (see reviews of Devonshire & Field (1991) and Feyereisen (1995)).

Esterase-based resistance in *M. persicae* is an example of a quantitative change, an increased amount of an esterase isoenzyme. Qualitative changes of esterases may also give rise to resistance, and a well-studied example is the malathion-specific carboxylesterase of the housefly, *M. domestica*. Early studies of OP-resistant housefly strains showed that OP resistance was associated with a decrease in esterase activity to simple general esterase substrates (Oppenoorth, 1959a,b; van Asperen & Oppenoorth, 1959; van Asperen & Oppenoorth, 1960). On the basis of these findings the "mutant ali-esterase" theory was put forward; the theory suggests that a mutation in the gene for a non-specific esterase had taken place to produce a more specific esterase that metabolises OPs more rapidly but has a concomitant lower activity to simple general substrates (Oppenoorth & van Asperen, 1960).

Qualitative changes of esterases to hydrolyse malathion at a higher rate with a concomitant decrease in esterase activity to α-naphthyl acetate have also been found in malathion-resistant populations of other species *e.g.* the Indian mealmoth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) (Beeman & Schmidt, 1982) and the rust red grain beetle, *Cryptolestes ferrugineus* Stephens (Coleoptera: Cucujidae) (Spencer *et al.*, 1998). However, presence of malathion-specific esterase do not necessarily result in reduced esterase activity to simple general substrates as has been found in a population of malathion-resistant mosquitoes, *Culex tarsalis* Coquillett (Diptera: Culicidae) (Ziegler *et al.*, 1987), and even in a strain of *M. domestica* (Picollo de Villar *et al.*, 1983).

In the sheep blowfly, *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae), a point mutation has been shown in the open reading frame of an esterase gene associated with OP resistance

(Newcomb *et al.*, 1997). The resistance-associated point mutation caused an amino acid substitution at the active site of the esterase. Expression *in vitro* of the wildtype and OP-resistant alleles showed that the amino acid substitution is responsible for both the loss of esterase activity to simple substrates and the acquisition of hydrolase activity to OPs. These findings at the molecular level are in support of the "mutant ali-esterase" theory.

Methods for studying esterase-based resistance

Indications that esterases are involved in conferring resistance can be obtained from bioassays and biochemical studies. For bioassay studies several synergists have been described that are able to inhibit esterases involved in detoxification. These include DEF (*S,S,S*-tributylphosphorotrithioate), TPP (*O,O,O*-triphenylphosphate), and IBP (*O,O*-bis[1-methylethyl]*S*-phenylmethylphosphorothioate) (Raffa & Priester, 1985).

The synergists themselves usually only have a low level of toxicity but when applied in combination with an insecticide they may markedly increase the toxicity of the insecticide. When the synergist inhibits detoxification enzymes involved in resistance to a particular insecticide, the difference in tolerance to the insecticide is reduced between the resistant and susceptible insects (Scott, 1990). The use of synergists in bioassays is useful to obtain preliminary information on possible resistance mechanisms; however, this approach has some pitfalls as discussed by Scott (1990). For example, DEF, a widely used synergist for indicating esterases involved in resistance, is not completely specific for esterases; DEF may also inhibit oxidases at high concentrations.

Nevertheless, positive results with DEF in synergist bioassays are a good indication of insecticide detoxification by esterases in the insects (Soderland & Bloomquist, 1990). In many studies, insecticide bioassays with DEF included as synergist have indicated esterases involved in resistance, and subsequent biochemical assays have supported this relationship. For instance, studies showing esterase-based resistance to carbofuran and azinphosmethyl in the oriental fruit moth, *G. molesta* (Kanga *et al.*, 1998), resistance to malathion caused by malathion-specific carboxylesterase in the rust red grain beetle, *C. ferrugineus* (Spencer *et al.*, 1998), and cypermethrin resistance in several field populations of the German cockroach, *B. germanica* (Valles, 1998).

In many resistant insect populations, increased esterase activity to model substrates has been shown in enzymatic assays in vitro. Widely used model substrates are naphthyl acetates and nitrophenyl acetates. These substrates may be hydrolysed by various esterases of the insects; therefore, increased esterase activity in a single resistant population can be caused by interpopulation differences in esterase activity that are not associated with resistance. If increased esterase activity is associated with resistance, bioassays with a synergist that inhibits esterases may support this. However, as mentioned above, including synergists in bioassays may not always have an effect on the resistance mechanism. Correlation of esterase activity to model substrates with the level of resistance in different populations is a good indication of esterases involved in resistance. This approach has suggested involvement of esterases in *e.g.* resistance to trichlorfon in the lygus bug, *Lygus hesperus* Knight (Hemiptera: Miridae) (Xu & Brindley, 1993), esfenvalerate resistance in the cotton bollworm, *H. armigera* (Gunning *et al.*, 1996), profenofos

resistance in the tobacco budworm, *H. virescens* (Harold & Ottea, 1997) and parathion resistance in the greenbug, *Schizaphis graminum* Rondani (Hemiptera: Aphididae) (Zhu & Gao, 1998). Such studies also indicate if the enzymatic assay could be suitable as a detection tool in resistance monitoring.

Other biochemical methods such as native electrophoresis of esterases may also indicate esterases involved in resistance. However, the best indication of esterases or other detoxification enzymes involved in resistance comes from insecticide metabolism studies with resistant insects. Detection of higher levels of insecticide hydrolysis products in resistant insects is a strong indication of esterase-based resistance, but this method requires more time and effort than the simple biochemical assays with model substrates (Soderland & Bloomquist, 1990).

Esterase-based resistance and Frankliniella occidentalis

Involvement of esterases in insecticide resistance in *F. occidentalis* has only been studied in a few different populations. The most thoroughly studied resistant population is the diazinon-selected population originally collected from a greenhouse in Missouri (Zhao *et al.*, 1994). Synergism of insecticide toxicity by DEF has not indicated involvement of esterases in detoxification of insecticides in this population. In the presence of DEF, the toxicity of diazinon, bendiocarb, or fenvalerate was not different from that of the insecticide alone (Zhao *et al.*, 1995a). A similar result has been reported from a Canadian population of *F. occidentalis*; DEF did not synergize the toxicity of malathion (Broadbent & Pree, 1997). However, lack of synergism by a known enzymatic inhibitor does not rule out the involvement of the metabolic pathway in detoxification or resistance (Scott, 1990).

In the study of Zhao *et al.* (1994), the possible involvement of esterases in resistance to diazinon in the diazinon-selected population of *F. occidentalis* was studied with biochemical methods. Metabolism of radiolabelled diazinon indicated that diazinon metabolism was mainly oxidative and not hydrolytic in nature. Nor did enzymatic assays *in vitro* indicate esterases involved in diazinon resistance. Esterase activity toward α-naphthyl acetate was assayed and esterase activity was found to be slightly higher in the more susceptible reference population. Furthermore, staining for esterases with α-naphthyl acetate after electrophoretic separation revealed more esterase bands in the more susceptible population. On the basis of these findings it was concluded esterases were not involved in diazinon resistance in the selected population. The diazinon-selected population was also more resistant to bendiocarb than the reference population. Studies on metabolism of radiolabelled bendiocarb showed enhanced metabolism of bendiocarb in the more resistant population (Zhao *et al.*, 1995b). However, it was inferred that the enhanced metabolism was probably mainly due to oxidases rather than hydrolases.

In the first paper of this Ph.D. study (*Paper 1*), a population of *F. occidentalis* resistant to methiocarb showed slightly increased esterase activity to α-naphthyl acetate compared to the susceptible population. Involvement of esterases in methiocarb resistance was not investigated further in this study, so the difference in esterase activity between the two populations could be the result of different expression of esterase activity not related to resistance. In *Paper 2* the esterase inhibitor DEF was included as synergists in the methiocarb bioassays. DEF lowered the level of methiocarb resistance in the three most resistant populations. Assays of esterase activity

in vitro showed that two of these populations (LiDK98 and AIDK98) had increased esterase activity to α-naphthyl acetate and ß-naphthyl acetate compared to the susceptible population. Thus the results of *Paper 2* are indicative of esterases involved in methiocarb resistance in at least some of the populations. However, the results are not demonstrative, it remains to be demonstrated that esterases can hydrolyse methiocarb at a higher rate or sequester methiocarb to a greater extent in methiocarb resistant populations.

Cytochrome P450-dependent monooxygenases

Cytochrome P450-dependent monooxygenases (EC 1.14.14.1) are also called mixed function oxidases, polysubstrate monooxygenases, microsomal monooxygenases, P450s and other names. In the Swiss-Prot Enzyme nomenclature database the official name for EC 1.14.14.1 is referred to as unspecific monooxygenase (Anonymous, 1999c). In this section I will refer to this group of enzymes as P450-monooxygenases.

The P450-monooxygenases are found in a wide variety of organisms from bacteria to vertebrates as well as in invertebrates and higher plants (Nelson *et al.*, 1993). In eukaryotes, these enzymes are found primarily in the endoplasmic reticulum (*i.e.* microsomal) but are also present in mitochondria (Guengerich, 1991). The P450-monooxygenase system consists of two components, the flavoprotein NADPH-cytochrome P450 reductase and the heme-thiolate protein cytochrome P450; the latter exits as multiple isoenzymes often displaying partially overlapping and broad substrate specificity. In the reaction catalysed, cytochrome P450 binds the substrate, then two electrons are transferred from NADPH to cytochrome P450 via the reductase and are used in the reduction of molecular oxygen to water with the co-oxidation of the substrate. Many different chemical reactions may be catalysed and the enzymes act on a wide range of substrates (Guengerich, 1991).

Insect P450-monooxygenases have recently been reviewed by Feyereisen (1999). For a comprehensive orientation of insect P450-monooxygenases see this up-to-date review of Feyereisen and see the reviews of Hodgson & Kulkarni (1983), Agosin (1985), and Hodgson (1985).

In insects, P450-monooxygenases are involved in many processes including roles in growth, development and reproduction, in the metabolism of plant allelochemicals by herbivores, and in detoxification of insecticides. However, insecticides may also be metabolised to more active forms by the P450-monooxygenases; bioactivation of some phosphorothioates to their corresponding "oxons" and cyclodiences to their epoxides is accomplished by P450-monooxygenases. Furthermore, some classes of insecticide synergists are also activated by P450-monooxygenases, *e.g.* the monooxygenase inhibitor piperonyl butoxide (PBO) (Feyereisen, 1999). As in mammals, an important feature of insect P450-monooxygenases is that they are inducible by many different endogenous and exogenous compounds including insecticides and plant allelochemicals (Terriere, 1984; Feyereisen, 1999). Often the P450-monooxygenases are induced by the xenobiotics they metabolise.

P450-monooxygenase-based resistance studied with synergists in bioassays

Detoxification of harmful xenobiotics is one important function of P450-monooxygenases in insects. Hence, it follows that increased activity of P450-monooxygenases in detoxifying insecticides can confer resistance in insect populations. In many studies, the use of insecticide synergists in bioassays has indicated involvement of P450-monooxygenases in insecticide resistance. The methylenedioxyphenyl synergists, such as PBO and Sesamex, are generally considered reasonably specific inhibitors of P450-monooxygenases (Casida, 1970; Keseru *et al.*, 1999). Studies with methylenedioxyphenyl synergists have indicated involvement of P450-monooxygenases in resistance to organophosphates, carbamates, pyrethroids, DDT, chitin synthesis inhibitors, and juvenile hormone mimics (Wilkinson, 1983). However, use of synergists in bioassays may indicate involvement P450-monooxygenases in resistance when this mechanism is not involved and vice versa (Soderland & Bloomquist, 1990). Therefore, subsequent biochemical confirmation of results from bioassays with synergists is worth having.

P450-monooxygenase-based resistance studied with biochemical methods

Different biochemical methods have been used to study P450-monooxygenase activity in insects. The early studies often relied on single substrates, such as aldrin, to measure P450-monooxygenase activity. However, there may be many P450-monooxygenase isoenzymes with different substrate specificity within an organism. Therefore, to characterise P450-monooxygenase activity accurately, it is necessary to use a variety of substrates (Rose *et al.*, 1991).

Recently, molecular biology techniques have been used in studying P450-monooxygenases associated with insecticide resistance. For example, by use of these techniques, P450-monooxygenase-mediated resistance in a strain of *M. domestica* has been shown to be caused by increased transcription of a gene for a specific P450-monooxygenase (Liu & Scott, 1998). Other examples of constitutive overexpression of P450-monooxygenase genes in resistant populations are listed and discussed in Feyereisen (1999).

Another method to indicative P450-monooxygenases involved in resistance is to measure the content of cytochrome P450 in resistant and susceptible populations. Total cytochrome P450 in a sample can be quantified by measuring the absorption at 450 nm before and after complexing P450 with carbon monoxide (Price, 1991). Spectral characterisation of P450-monooxygenases may also detect qualitative differences between cytochrome P450 of resistant and susceptible populations (Hodgson & Kulkarni, 1983).

Biochemical assays *in vitro* of insect P450-monooxygenases can be problematic. Hodgson & Kulkarni (1983) list some of the features that complicate investigations of P450-monooxygenases in insects. Especially it may be difficult to assay P450-monooxygenases in smaller insects. With smaller insects it is often necessary to use whole individuals when preparing samples for assays *in vitro*; the size of the individuals makes it difficult to prepare isolated tissues. However, the widespread presence of endogenous inhibitors of P450-monooxygenases and of powerful proteases give difficulties in stabilising P450-monooxygenases prepared from whole insects (Hodgson & Kulkarni, 1983; Hodgson, 1985; Price, 1991).

Despite the difficulties in studying P450-monooxygenases, numerous studies have indicated and demonstrated involvement of P450-monooxygenases in resistance to a variety of insecticides and in several insect species. Detoxification by P450-monooxygenases is considered generally to be an important mechanism in conferring resistance in insects (Hodgson & Kulkarni, 1983; Agosin, 1985; Oppenoorth, 1985; Soderland & Bloomquist, 1990).

P450-monooxygenase-based resistance and Frankliniella occidentalis

Immaraju *et al.* (1992) were the first to report on possible involvement of P450-monooxygenases in resistance in *F. occidentalis*. In four populations of *F. occidentalis* collected from greenhouses in California, PBO lowered the level of resistance to permethrin. In none of the populations PBO completely suppressed resistance, but resistance to permethrin was lowered up to 20-fold at the LC_{50} and up to 50-fold at the LC_{90}.

As with esterases, involvement of P450-monooxygenases in resistance in *F. occidentalis* has been studied most thoroughly in the diazinon-selected population originally collected from a greenhouse in Missouri. Enhanced metabolism of diazinon, bendiocarb and fenvalerate contributed to resistance to these insecticides in the diazinon-selected population. Analysis of the products from metabolism *in vivo* of the radiolabelled insecticides indicated involvement of P450-monooxygenases in resistance to diazinon and bendiocarb (Zhao *et al.*, 1994; Zhao *et al.*, 1995b); it was not possible to determine with certainty whether metabolism of fenvalerate was due to action of oxidases or hydrolases or both (Zhao *et al.*, 1995c).

In *Paper 1* and *Paper 2* of this Ph.D. study, PBO was included as synergist in the methiocarb bioassays. PBO appeared to lower the level of methiocarb resistance in the *F. occidentalis* populations that had more than 10-fold resistance to methiocarb. One of the populations, LiDK98, had 11-fold resistance to methiocarb at LC_{50} and 16-fold resistance to methiocarb at LC_{90}. In LiDK98, PBO appeared to lower the level of resistance only when comparing resistance ratios at the LC_{90} (*Paper 2*). One might speculate that the apparent lack of effect of PBO on methiocarb resistance in populations with a lower level of resistance could be due to detection limitations of the bioassay method used in the study. Still, the bioassay results with PBO indicate that P450-monooxygenases is involved in conferring resistance to methiocarb in *F. occidentalis*.

All in all, the few studies done so far suggest that detoxification by P450-monooxygenases could be an important resistance mechanism in *F. occidentalis* to many insecticides. Implementation of an assay for measuring P450-monooxygenase activity *in vitro* in *F. occidentalis* could probably add significant information on the role P450-monooxygenases in resistance in *F. occidentalis*. However, as whole insect homogenates are rarely suitable for examination of P450-monooxygenase activity (Hodgson & Kulkarni, 1983; Hodgson, 1985), a simple P450-monooxygenase assay on insects as small as *F. occidentalis* may be difficult to implement.

Glutathione S-transferases (GSTs)

The glutathione S-transferases (GSTs, EC 2.5.1.18) are a group of enzymes catalysing the conjugation of hydrophobic compounds with the tripeptide glutathione. In the reaction, the thiol group of glutathione reacts with an electrophilic site on the compound thereby forming the conjugate (Jakoby & Habig, 1980). The glutathione conjugation product may be further metabolised or excreted as such. The GSTs thus serve to solubilise, detoxify, and initiate the catabolism of a wide variety of hydrophobic compounds.

GSTs are found throughout the animal kingdom but also in certain bacteria, algae and higher plants (Jakoby & Habig, 1980). Clark (1989) has reviewed GSTs in non-vertebrate organisms, and it was concluded that the non-vertebrate enzymes generally resemble very closely those from mammals. Studies on insects indicate that insect GSTs, like esterases and P450-monooxygenases, exist as multiple isoenzymes with broad and partially overlapping patterns of substrate specificity (Clark, 1989; Soderland & Bloomquist, 1990). GST activity is inducible in insects and various xenobiotics, such as plant allelochemicals and pesticides, have been shown to induce GSTs (Terriere, 1984; Clark, 1989).

GSTs in resistance

The involvement of GSTs in resistance to insecticides is well reviewed. As GSTs are recognised as important in insecticide resistance, sections on GSTs are found in many reviews of resistance mechanisms in insects. For extensive sections of GSTs and resistance see reviews of Dauterman (1983), Oppenoorth (1985), and Soderland & Bloomquist (1990).

GSTs are of particular importance in the detoxification of OPs; several compounds of this class have been reported to be metabolised by GSTs (Dauterman, 1985). In several insect species, high levels of GST activity have been associated with resistance to OPs (see above-mentioned reviews).

The association between GST activity and resistance is especially studied in *M. domestica*; a number of resistant populations have been shown to have increased GST activity, not only to model substrates but also to several OPs (see Table 5 in Oppenoorth (1985)). In fact, most studies on the role of GSTs in resistance involve various OP-resistant strains of *M. domestica*. In one resistant strain of *M. domestica* (the Cornell-R strain), a GST isoenzyme involved in insecticide detoxification has been found to be overproduced as a result of gene amplification (Syvanen *et al.*, 1996).

As GSTs are a group of detoxification enzymes with broad substrate specificity, they may also act in the metabolism of insecticides other than OPs. At least two studies have indicated an association between GSTs and resistance to carbamates. In a population of tobacco budworms, *H. virescens*, selection for resistance to thiodicarb was accompanied by an increased activity of GST to the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Rose *et al.*, 1995), and in worker honey bees, *Apis mellifera* L. (Hymenoptera: Apidae), the level of propoxur susceptibility in different colonies was positively correlated with the level of GST activity to CDNB (Smirle, 1990).

A major resistance mechanism to DDT in *M. domestica* is metabolism of DDT to the non-toxic DDE; a reaction catalysed by the enzyme DDT-dehydrochlorinase or DDT-ase. This enzyme has

been studied for more than 40 years but for a long time its origin remained obscure (Soderland & Bloomquist, 1990). However, studies on *M. domestica* by Clark & Shamaan (1984) strongly indicate that DDT-dehydrochlorinase is a GST catalysing the glutathione-dependent dehydrochlorination of DDT to DDE.

Methods for studying GST-based resistance

In many OP-resistant populations, increased GST activity to model substrates has been shown in enzymatic assays *in vitro*. Widely used model substrates are 1-chloro-2,4-dinitrobenzene (CDNB) and 3,4-dichloronitrobenzene (DCNB). These substrates may be hydrolysed by various GSTs of the insects; therefore, increased GST activity in a single resistant population can be caused by interpopulation differences in GST activity that are not associated with resistance. Positive correlation of GST activity to model substrates with the level of resistance in different populations is a better indication of GSTs involved in resistance. This approach has suggested involvement of GSTs in *e.g.* resistance to diazinon in the sheep blowfly, *L. cuprina* (Wilson & Clark, 1996), profenofos resistance in the tobacco budworm, *H. virescens* (Harold & Ottea, 1997), and azinphosmethyl resistance in the tufted apple bud moth, *Platynota idaeusalis* Walker (Lepidoptera: Tortricidae) (Carlini *et al.*, 1995). Such studies also indicate if a GST assay could be suitable as a detection tool in resistance monitoring.

Direct evidence for the involvement of GSTs in resistance is often difficult to obtain. First, no highly reliable synergists for GSTs exist (Scott, 1990). To give information on possible resistance mechanisms present, insecticide bioassays with synergist included require that the synergist is specific for the mechanism in question. Diethylmaleate (DEM) has been used in bioassays as an inhibitor of GST activity, but DEM may also strongly inhibit P450-monooxygenases (Price, 1991); furthermore DEM is water-soluble and may not penetrate the cuticle. Second, results from insecticide metabolism studies may be difficult to interpret. For example, the metabolites resulting from the action of GSTs on OPs are often identical to those produced by the action of P450-monooxygenases or esterases on the same insecticide (Soderland & Bloomquist, 1990). Thus, evidence for GSTs involved in metabolism of insecticides *in vivo* requires demonstration of the formation of glutathione conjugates of the cleaved moieties, using appropriately radiolabeled insecticides.

GST-based resistance and Frankliniella occidentalis

The least studied metabolic resistance mechanism in *F. occidentalis* is GST mediated detoxification of insecticides. Zhao *et al.* (1994) have measured GST activity to the model substrate CDNB in the diazinon-selected population originally collected from a greenhouse in Missouri and compared it with GST activity of a more susceptible population. No difference was found in activity to CDNB between the two populations. As the diazinon-selected population was cross-resistant to several insecticides of different classes (Zhao *et al.*, 1995a), the result may suggest that GSTs are not contributing to resistance in this population.

In the first paper of this Ph.D. study (*Paper 1*), a population of *F. occidentalis* resistant to methiocarb showed slightly increased GST activity to CDNB compared to the susceptible population. Involvement of GSTs in methiocarb resistance was not investigated further in this

study, so the difference in GST activity between the two populations could be the result of different expression of GST activity not related to resistance.

In *Paper 2*, GST activity to two model substrates CDNB and DCNB was measured in seven populations of *F. occidentalis* with different levels of methiocarb resistance. The most resistant population had significantly higher GST activities compared to the susceptible population. Furthermore, methiocarb selection on one of the populations increased the level of methiocarb resistance with a concomitant increase in GST activity to CDNB and DCNB. These findings suggest a possible association between methiocarb resistance and GSTs in at least one of the populations. However, it remains to be demonstrated that GSTs contributed to methiocarb resistance. The possibility exists that selection with methiocarb selected for some other resistance mechanism in the population and that GST activity was genetically linked to that mechanism. Studies on *M. domestica* have indicated that a single gene plays a major role in metabolic resistance and it is hypothesized that the product of this gene induces the synthesis of the major detoxification enzymes in *M. domestica* (Plapp, 1984). Thus, as both esterases and P450-monooxygenases may be involved in methiocarb resistance in *F. occidentalis* (*Paper 2*), the role of GSTs is uncertain but it should be considered in further studies.

TARGET SITE RESISTANCE

The insecticides of the three major classes, OPs, carbamates, and pyrethroids, have proteins in the nervous system as the site of action. OPs and carbamates have a single enzyme as target, acetylcholinesterase, and the pyrethroids and DDT have the voltage-gated sodium channel of the nerve membrane as target. Indeed a very limited number of target sites. Other target sites for neurotoxic insecticides are the γ-aminobutyric acid (GABA)-receptor (Casida, 1993; Sattelle, 1990; Narahashi, 1996) and the nicotinic acetylcholine receptor (Sattelle, 1985; Narahashi, 1996). The GABA-receptor is the target for cyclodienes (*e.g.* dieldrin and endosulfan), γ-HCH (lindane) and fipronil, and the nicotinic acetylcholine receptor is the target for nicotinyl insecticides (*e.g.* imidacloprid and nicotine).

Alteration at the target-site, to less a sensitive target for neurotoxic insecticides, is an important toxicodynamic resistance mechanism in insects. Resistance at the two most important target sites for conventional insecticides, acetylcholinesterase and the voltage-gated sodium channel, are discussed in the next sections. Altered GABA-receptor as a resistance mechanism has been reviewed by *e.g.* Feyereisen (1995) and Soderland & Bloomquist (1990).

Altered acetylcholinesterase (AChE)

The target for OPs and carbamates is the enzyme AChE (EC 3.1.1.7). The function of AChE is to break down acetylcholine in the synaptic cleft (Zubay, 1983). Acetylcholine is an excitatory neurotransmitter that transmits a signal from a presynaptic to the postsynaptic neurone. Arrival of an action potential to the presynaptic membrane of a cholinergic synapse triggers the release of acetylcholine into the synaptic cleft. Acetylcholine diffuses to the postsynaptic membrane and binds to a specific receptor. The insect acetylcholine receptors has been reviewed by *e.g.* Sattelle

(1985) and Gundelfinger (1992). Upon binding to the receptor, acetylcholine ultimately causes the development of an action potential in the postsynaptic neurone (Zubay, 1983).

For proper function at the cholinergic synapse, acetylcholine must be rapidly broken down in the synaptic cleft to restore the resting potential of the postsynaptic membrane. This is achieved by AChE that hydrolyses acetylcholine to acetate and choline (Zubay, 1983). In insects, AChE is mainly located in the central nervous system. Localisation, properties and structure of insect AChE have been reviewed by Eldefrawi (1985) and Toutant (1989).

OPs and carbamates are potent inhibitors of AChE. These compounds act by forming a stable covalent intermediate with AChE thus preventing the enzyme from hydrolysing acetylcholine. An accumulation of acetylcholine leaves the cation channel of the receptor permanently open, which eventually is lethal. OPs and carbamates are quasi-irreversible inhibitors of AChE; OPs phosphorylates and carbamates carbamylates the active-site serine in AChE (Main, 1979; Eldefrawi, 1985). The time of reactivation of phosphorylated or carbamylated AChE is generally long. However, half-lives of reactivation varies considerably, from minutes to several days, depending on the compound interacting with AChE (Aldridge & Reiner, 1972; Main, 1979). Carbamylated AChE generally reactivates faster than phosphorylated AChE.

Insensitive AChE

Reduced sensitivity of AChE to inhibition by OPs and carbamates is an important resistance mechanism in insects; the mechanism is often referred to as altered or insensitive AChE. This mechanism has been found in several insect populations resistant to these compounds and several reviews have covered this topic. For extensive review of altered AChE as a resistance mechanism see reviews of Hama (1983), Oppenoorth (1985), and Fournier & Mutero (1994).

The presence of insensitive AChE conferring resistance was first suggested by Smissaert (1964) from a study on OP-resistant mites, *Tetranychus urticae* Koch (Acari: Tetranycidae). Inhibition of AChE activity *in vitro* by diazoxon and paraoxon was assayed in resistant and susceptible mites, and it was found that AChE activity of resistant mites had reduced sensitivity to inhibition by the two OPs.

Assays *in vitro* of AChE sensitivity to inhibition by OPs or carbamates are now a routine method in many laboratories and compare AChE activity in individual homogenates in the presence and absence of the insecticides. This method has indicated insensitive AChE involved in resistance to different OPs and carbamates in more than 30 species of arthropods. Fournier & Mutero (1994) list 25 examples of insensitive AChE in species of Hemiptera, Coleoptera, Diptera, and Acarina. More recently, insensitive AChE has been reported to be present in *e.g.* the greenbug, *S. graminum* (Siegfried & Ono, 1993a,b), the peach-potato aphid, *M. persicae* and the tobacco aphid, *Myzus nicotianae* Blackman (Hemiptera: Aphididae) (Moores *et al.*, 1994), the pear psylla, *Cacopsylla pyri* L. (Hemiptera: Psyllidae) (Berrada *et al.*, 1994), and the lesser grain borer, *Rhyzopertha dominica* Fabricius (Coleoptera: Bostrichidae) (Guedes *et al.*, 1997b).

Most insects species appear only to have a single gene encoding AChE (Fournier & Mutero, 1994). A notable exception is the mosquito, *C. pipiens*, in which two distinct genes are thought to code for AChEs (Bourguet *et al.*, 1996a; Malcolm *et al.*, 1998); however, only one of the genes

have an allele that codes for an insensitive form of AChE (Bourguet *et al.*, 1996b). In five other mosquito species, only a single gene encoding AChE seems to exist (Bourguet *et al.*, 1997a).

Multiple forms of insensitive AChE exist in several insect species; the different forms can be distinguished on the basis of their kinetic features and their sensitivity to various inhibitors. Thus it appears that existence of several resistant alleles for AChE is a common feature in many insect species (Fournier & Mutero, 1994).

Concerning insensitive forms of AChE, some point mutations of AChE have been reported to be associated with reduced sensitivity to insecticide inhibition. Five possible point mutations of AChE associated with resistance have been identified in the fruit fly, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Mutero *et al.*, 1994), and a serine to glycine point mutation of AChE is associated with azinphosmethyl resistance in the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) (Zhu *et al.*, 1996; Zhu & Clark, 1997; Zhang *et al.*, 1999).

Increased AChE activity

The level of AChE activity may differ in susceptible and resistant populations. In several cases insensitive AChEs in resistant populations have a lower activity compared to the susceptible ones (Oppenoorth, 1985). A lower level of AChE activity may reflect that the alterations giving reduced sensitivity to inhibition has resulted in a catalytically less efficient enzyme or reduced amounts of the enzyme. Decreased AChE activity implies that a smaller proportion of AChE activity would need to be inhibited to reach a critical threshold for disruption of synaptic function (Byrne & Devonshire, 1997). Thus decreased AChE activity counteracts reduced sensitivity to inhibition, and the balance between AChE activity and sensitivity determines the toxicological effect of anticholinesterases on the synaptic function.

Hence, it follows that increased activity of AChE can offer some degree of protection against OP and carbamate toxicity and could be a possible resistance mechanism. Several examples of increased AChE activity in OP-resistant insects have already been described, *e.g.* in the California red scale, *Aonidiella aurantii* Mask. (Hemiptera: Coccoidae), increased AChE activity is associated with chlorpyrifos resistance (Levitin & Cohen, 1998); in the greenbug, *S. graminum*, three resistant populations all showed increased AChE activity (Zhu & Gao, 1999); in the lesser grain borer, *R.. dominica*, all of the 15 resistant populations tested showed higher AChE activity than found in the susceptible population (Guedes *et al.*, 1997a); in the tobacco budworm, *H. virescens*, nine of 10 resistant strains showed significantly increased AChE activity (Harold & Ottea, 1997); in the fruit fly, *D. melanogaster*, selection for resistance to parathion and fenthion was accompanied by a correlated increase in AChE activity (El-Abidin Salam & Pinsker, 1981), and in several resistant strains of the housefly, *M. domestica*, increased V_{max} (maximum initial rate of an enzyme reaction) of AChE has been shown (*e.g.* Tripathi & O'Brien, 1973; Voss, 1980; Yeoh *et al.*, 1981; Oi *et al.*, 1990).

These examples of increased AChE activity were found in homogenates of whole insects or heads and may reflect a modification of catalytic properties of AChE or an increased amount of AChE or both. Modification of AChE to a catalytically more efficient enzyme may protect the organism against toxicity of AChE inhibitors by reducing the accumulation of acetylcholine at the

synapse. Resistance to OPs and carbamates may also develop from an overproduction of AChE (Fournier & Mutero, 1994) thereby providing additional sites for phosphorylation or carbamylation. Some examples of increased amounts of AChE conferring insecticide resistance have been reported. In the olive fruit fly, *Dacus oleae* Gmelin (Diptera: Tephritidae), selection with organophosphates increased the gene dosage of the AChE gene (Tsakas, 1976), and in *D. melanogaster*, a direct proportionality between the flies' resistance to malathion and the amount of AChE present has been shown (Fournier *et al.*, 1992). Another study on *D. melanogaster* showed that flies genetically transformed to express additional AChE in the haemolymph acquired resistance to parathion (Fournier *et al.*, 1993); the mechanism of resistance may be sequestration of the insecticide by soluble AChE in the haemolymph, however, this specific mechanism remains to be demonstrated in naturally resistant populations.

To summarise, alterations of AChE to less sensitivity to inhibition, a catalytically more efficient enzyme, or higher amounts of the enzyme may confer resistance to OP or carbamate insecticides. However, OP and carbamate interactions at cholinergic synapses may be even more complex. Bourguet *et al.* (1997b) has recently suggested that the enzyme that synthesises acetylcholine, choline acetyltransferase, may also be involved in resistance. Reduced activity of choline acetyltransferase results in decreased production of acetylcholine thereby delaying a toxicologically critical accumulation of the neurotransmitter following insecticide inhibition of AChE. This relationship has been suggested from a study on *D. melanogaster* mutants with reduced activity of choline acetyltransferase and *C. pipiens* mosquitoes with insensitive AChE; choline acetyltransferase could be another target site for OPs and carbamates, counteracting the effect of AChE inhibition (Bourguet *et al.*, 1997b). The authors of this study emphasised that the pivotal step for OP/carbamate toxicology is not the acetylcholinesterase activity but the amount of the neurotransmitter acetylcholine present at the synapse.

Altered AChE and Frankliniella occidentalis

Presence of altered AChE has been studied in the Missouri diazinon-selected population of *F. occidentalis*. The diazinon-selected population was 14-fold resistant to diazinon compared to a more susceptible reference population (Zhao *et al.*, 1995a). Altered AChE with reduced sensitivity to diazoxon contributed to diazinon resistance in the selected population (Zhao *et al.*, 1994). Relative inhibition of AChE in the selected and the reference population was assayed with five OPs and four carbamates in another study (Liu *et al.*, 1994). The selected population was less inhibited by two of the OPs and one of carbamates and more inhibited by one OP and one carbamate compared to the reference population. Thus based on the insecticide inhibition profiles, altered AChE was present in the selected population but sensitivity to inhibition depends on the anticholinesterase insecticide. Although the selected population was also 14-fold more resistant to the carbamate bendiocarb, insensitive AChE did not appear to contribute to bendiocarb resistance as no difference in sensitivity to bendiocarb inhibition was found between AChEs of the resistant and reference population (Zhao *et al.*, 1995b).

The level of AChE activity did not differ between the diazinon-selected and the reference population (Zhao *et al.*, 1994). Interestingly, butyrylcholinesterase-like activity is present in *F.*

occidentalis (Liu *et al.*, 1994). Butyrylcholinesterase is an enzyme usually found in plasma of vertebrates (Walker & Thompson, 1991), and it has been considered not to be present in insects (Toutant, 1989). The enzyme having butyrylcholinesterase-like activity in *F. occidentalis* meets the criteria to be classified as a butyrylcholinesterase (Liu *et al.*, 1994). The function of butyrylcholinesterase in *F. occidentalis* is unknown; Liu *et al.* (1994) suggest that it may function as a scavenger enzyme providing alternative phosphorylation or carbamylation sites for OPs and carbamates. However, this sequestration function does not seem to contribute to diazinon resistance in the diazinon-selected population as butyrylcholinesterase of this population was markedly less sensitive to diazoxon inhibition (Zhao *et al.*, 1994); thus apparently butyrylcholinesterase leaves more active diazoxon in the selected population than in the more susceptible reference population. The level of butyrylcholinesterase activity was the same in the two populations (Zhao *et al.*, 1994).

Insensitive AChE did not appear to contribute to methiocarb resistance in the methiocarb resistant population studied in *Paper 1* of this Ph.D. study. In fact, AChE activity of this population was more sensitive to inhibition by the anticholinesterases methiocarb, dichlorvos, and eserine. However, AChE activity was 2.6-fold higher in the resistant population, a result that suggests increased AChE activity involved in resistance in this population. Butyrylcholinesterase of *F. occidentalis* has no apparent activity to the AChE substrate analog, acetylthiocholine, used to assay AChE activity in this study (Liu *et al.*, 1994).

Possible involvement of increased AChE activity in methiocarb resistance in the resistant population of *Paper 1* was supported by findings of *Paper 2*. When assayed for the *Paper 2* study, the resistant population of *Paper 1* had been in culture without exposure to pesticides for nearly two years. In this period, the level of resistance to methiocarb had declined in the population, and the level of AChE activity had also declined to the level of the susceptible population. However, selection with methiocarb on the population enhanced the level of methiocarb resistance and was accompanied by an in increase in the level of AChE activity (*Paper 2*). This result suggests involvement of increased AChE activity in methiocarb resistance of this population.

The other methiocarb resistant populations included in the *Paper 2* study did not show increased AChE activity. However, the AChE activity in two of the populations showed reduced sensitivity to inhibition by methiocarb, dichlorvos, and eserine, indicating presence of insensitive AChE as a resistance mechanism in these populations (*Paper 2*). Interestingly, in *Paper 2*, a positive correlation was found between relative AChE inhibition and the basal levels of AChE activity in the populations. This correlation suggests presence of AChE with different catalytic properties in the populations (see the discussion in *Paper 2*).

Thus, two alterations of AChE may be involved in resistance to OPs and carbamates in *F. occidentalis*. One is increased AChE activity as suggested for the resistant population of *Paper 1*, the second is insensitive AChE as suggested for two of the resistant populations of *Paper 2* and shown in the Missouri diazinon-selected population (Zhao *et al.*, 1994).

Knockdown resistance (kdr)

The major site of action of DDT and pyrethroids is the voltage-gated sodium channel, an ion channel in membranes of electrically excitable cells (Zlotkin, 1999). The electrical signals in the nervous system are the action potentials. These signals are conducted along axons of the neurones as waves of depolarisation-repolarisation events that involve the opening of the voltage-gated sodium channels. For a general description of the action potential and the involvement of the voltage-gated sodium in propagation of action potentials see *e.g.* Alberts *et al.* (1983) pp. 1018-1034 or Zubay (1983) pp.1148-1157.

Pyrethroids and DDT are potent neurotoxic insecticides that induce repetitive activity in the nervous system. Neurophysiological studies have shown that the major action of these compounds is an altering of the gating kinetics of the voltage-gated sodium channel. The binding of these compounds to the sodium channels causes prolonged opening of the channels, which leads to repetitive firing of action potentials. The repetitive activity may eventually block neuronal transmission. The action of pyrethroids and DDT on the voltage-gated sodium has been reviewed by *e.g.* Sattelle & Yamamoto (1988), Soderlund & Bloomquist (1989), Narahashi (1992 and 1996), and Bloomquist (1996).

Zlotkin (1999) has recently reviewed the voltage-gated sodium channel of insects. The structure, function, and pharmacology of the insect sodium channel resemble closely those found in the vertebrate counterpart, however, at least some pharmacological differences are found. In *Drosophila* the *para* (paralysis) gene encode the voltage-gated sodium channel, and a *para*-type sodium channel gene has been identified in the housefly, *M. domestica* (Williamson *et al.*, 1996) and the German cockroach, *B. germanica* (Dong, 1997). Marked homology between the *para* gene and *para*-type sodium channel gene sequences from other insects has also been reported (Doyle & Knipple, 1991; Martinez-Torres *et al.*, 1997).

Target site resistance to DDT and pyrethroids was known before the major target for these insecticides was known. The mechanism first observed in *M. domestica* in 1951 (Busvine, 1951) and has been termed knockdown resistance (*kdr*). Knockdown resistance is characterised by a reduced sensitivity of the nervous system to the action of DDT and pyrethroids and involves modification of sodium channel pharmacology (Bloomquist, 1996). As the major molecular target for these insecticides has been shown to be the voltage-gated sodium channel, this resistance mechanism is now also referred to as insensitive sodium channel. Recent reviews of this resistance mechanism are found in *e.g.* Oppenoorth (1985), Soderland & Bloomquist (1990), Scott & Dong (1994), and Zlotkin (1999).

Knockdown resistance may be a widespread resistance mechanism. Toxicological and electrophysiological studies have demonstrated the presence of this resistance mechanism in species of Diptera, Lepidoptera, Coleoptera and Dictyoptera (Soderland & Bloomquist, 1990; Schuler *et al.*, 1998).

However, in many species, nerve insensitivity to DDT and pyrethroids cannot easily be demonstrated. Soderlund & Bloomquist (1990) emphasised two types of experiments that must be done to demonstrate presence nerve insensitivity. First, metabolism studies are required to rule out completely the involvement of metabolic mechanisms, and, second, appropriate

neurophysiological assays of insecticide actions on nerve preparations must be established, and these preparations must exhibit intrinsic reduced sensitivity in resistant populations. These criteria can be hard to meet: first, the metabolic studies may show presence of enhanced metabolism of pyrethroids or DDT in the resistant population, and, second, on smaller insects an electrophysiological experiment with electrodes to record neuronal activity may be difficult to implement. Therefore, knockdown resistance has often in the past been inferred from indirect evidence such as no indication of involvement of metabolic mechanisms, behavioural symptoms of intoxication, and cross-resistance to other pyrethroids and DDT.

Molecular studies of knockdown resistance

Recently, genetic evidence has been presented that shows that mutation in the *para*-type sodium channel gene is associated with target-site resistance to pyrethroids and DDT at the sodium channel. In *B. germanica*, Dong & Scott (1994) showed a tight genetic linkage between the *para*-type sodium channel locus and the locus for knockdown resistance. A fragment of the *para*-type sodium channel gene was amplified, cloned and used as a probe to detect a restriction fragment length polymorphism (RFLP) between the susceptible and knockdown resistant strains. A linkage analysis showed tight genetic linkage between the trait for knockdown resistance and the *para*-type sodium channel gene and it was concluded that the gene for knockdown resistance is very likely to be the same as the *para*-type sodium channel gene. Linkage analysis have also showed tight genetic linkage between molecular markers for the *para*-type sodium channel gene and knockdown resistance in the tobacco budworm, *H. virescens* (Taylor *et al.*, 1993) and *M. domestica* (Williamson *et al.*, 1993; Knipple *et al.*, 1994).

In *M. domestica*, two point mutations in the *para*-type sodium channel, that is associated with knockdown resistance, have recently been identified (Williamson *et al.*, 1996). Both mutations are located in domain II of the sodium channel. A leucine to phenylalanine replacement in the IIS6 transmembrane segment is associated with *kdr* resistance and an additional methionine to threonine replacement in the IIS4-S5 loop is associated with *super-kdr* resistance. *M. domestica* expressing the *super-kdr* phenotype show greatly enhanced resistance to some pyrethroids (Farnham *et al.*, 1987).

A leucine to phenylalanine substitution within the IIS6 segment of the *para*-type sodium channel has also been found to be associated with knockdown resistance in other insects. An association between this substitution and resistance to pyrethroids and DDT has been found in *B. germanica* (Dong, 1997), in the mosquitoes, *Anopheles gambiae* Giles *s.s.* (Diptera: Culicidae) (Martinez-Torres *et al.*, 1998) and *C. pipiens* (Martinez-Torres *et al.*, 1999b), in the Colorado potato beetle, *L. decemlineata* (Clark *et al.*, 1999; Lee *et al.*, 1999), in the peach-potato aphid, *M. persicae* (Martinez-Torres *et al.*, 1997; Martinez-Torres *et al.*, 1999a), in the horn fly, *H. irritans* (Guerrero *et al.*, 1997), and the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) (Schuler *et al.*, 1998). Furthermore, in *H. virescens*, a leucine to histidine (rather than leucine to phenylalanine) substitution in the IIS6 segment has been reported to be associated with pyrethroid resistance (Park & Taylor, 1997). Other mutations in the sodium channel may be contributing to pyrethroid resistance in *H. virescens*.

The methionine to threonine substitution in the IIS4-S5 loop associated with *super-kdr* resistance in *M. domestica* may also be present in other insects. In *H. irritans* this mutation has been found in a strain with very high pyrethroid resistance (Guerrero *et al.*, 1997). A *super-kdr*-like mutation may also be present in *P. xylostella*; a threonine to isoleucine substitution located near the met-thr substitution of *super-kdr M. domestica* has been suggested to be associated with very high pyrethroid resistance in a strain of *P. xylostella* (Schuler *et al.*, 1998).

The consistent association between knockdown resistance and the leucine to phenylalanine substitution within the IIS6 segment of the para-type sodium channel found in a range of insect species, provides a strong body of evidence that this mutation is responsible for resistance to pyrethroids and DDT and this target-site. This has recently been confirmed by functional expression of rat and *M. domestica* sodium channels in *Xenopus* oocytes; sodium channels containing the *kdr*-like mutation are less sensitive to pyrethroids compared with the wild-type channel (Smith *et al.*, 1997; Vais *et al.*, 1997). Thus, in future studies the use of molecular biology techniques is likely to be very useful tools to indicate presence of target-site resistance to pyrethroids and DDT.

Knockdown resistance and Frankliniella occidentalis

Resistance to pyrethroids has been reported in several populations of *F. occidentalis*; in European and African populations (Brødsgaard, 1994), in North American populations (Immaraju *et al.*, 1992; Zhao *et al.*, 1995a; Broadbent & Pree, 1997), and in populations from Israel (Kontsedalov *et al.*, 1998), and probably also from Australia (Herron *et al.*, 1996).

Involvement of knockdown resistance in pyrethroid resistance has not been directly studied in *F. occidentalis*. However, in two studies it has been suggested that knockdown resistance may have contributed to pyrethroid resistance. Cross-resistance to pyrethroids and DDT indicate possible existence of the knockdown resistance mechanism in the Missouri diazinon-selected population (Zhao *et al.*, 1995c), and cross-resistance among pyrethroids could indicate presence of knockdown resistance in Californian populations of *F. occidentalis* (Immaraju *et al.*, 1992).

Thus, presence of knockdown resistance in *F. occidentalis* has only been inferred from indirect evidence. As the *kdr*-like mutation in the *para*-type sodium channel has been identified in a range of insects of different orders, future studies of knockdown resistance in *F. occidentalis* should investigate if this point mutation also is present and associated with knockdown resistance in *F. occidentalis*. If the *kdr*-like mutation also occurs in *F. occidentalis*, a simple PCR-based diagnostic test for rapid identification of this mutation may be developed. Such tests have already been developed for *e.g.* mosquitoes (Martinez-Torres *et al.*, 1998; Martinez-Torres *et al.*, 1999b) and they are likely to be valuable tools in monitoring for presence of knockdown resistance in insect populations.

HOST PLANT INDUCED ENZYMATIC RESPONSES IN INSECTS WITH RELATION TO INSECTICIDE RESISTANCE

Plants defend themselves from attack by herbivores in several ways. One important way is the chemical defence by toxic allelochemicals in the plants. Many different groups of phytotoxic allelochemicals exist, *e.g.* organic cyanides, some terpenoids, and alkaloids, and they are commonly present in plants; for example, it has been estimated that allelochemical alkaloids occur in about 20% of all plant species (Wink *et al.*, 1998). For herbivorous insects to survive on plants containing toxic allelochemicals, they must have the capacity to reduce the toxic effects to a non-fatal level. Detoxification of harmful allelochemicals is an important way for insects to reduce the toxic effects (Krieger *et al.*, 1971; Brattsten & Ahmad, 1986; Ahmad, 1992; Yu, 1992; Feyereisen, 1999; Johnson, 1999). The detoxification systems in insects that metabolise plant allelochemicals may also be involved in detoxification of insecticides (Gordon, 1961; Terriere, 1984; Ahmad *et al.*, 1986; Brattsten, 1988a,b; Rose *et al.*, 1992); hence, detoxification responses in insects caused by host plants may affect, increase or decrease, tolerance to insecticides in the insects. Thus, information on host plant associated detoxification responses in insects could prove valuable in increasing the effectiveness of pest control with insecticides.

P450-monooxygenases

Cytochrome P450-dependent monooxygenases have often been reported to be involved in detoxification of plant allelochemicals (Ahmad *et al.*, 1986; Brattsten, 1988a; Feyereisen, 1999; Johnson, 1999). It is well documented that different plant allelochemicals can induce P450-monooxygenases (several examples listed in reviews of Terriere (1984), Yu (1986), and Feyereisen (1999)). For instance, nicotine and 2-tridecanone induce P450-monooxygenases in the tobacco budworm, *H. virescens* (Rose *et al.*, 1991) and several different allelochemicals can induce P450-monooxygenases in the fall armyworm, *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) (Yu, 1983; Yu, 1987).

Insects with host plant or allelochemical induced P450-monooxygenases may be more tolerant to insecticide exposure. Examples of this relationship come from studies on larvae of different lepidopterans. It was first demonstrated by Brattsten *et al.* (1977); they showed that induction of P450-monooxygenases by allelochemicals resulted in increased tolerance to nicotine in larvae of the southern armyworm, *Spodoptera eridania* Cramer (Lepidoptera: Noctuidae). Similary, peppermint leaves induce P450-monooxygenases in the variegated cutworm, *Peridroma saucia* Hübner (Lepidoptera: Noctuidae) (Yu *et al.*, 1979; Berry *et al.*, 1980); microsomal aldrin epoxidase activity was increased up to 45-fold in larvae fed on peppermint leaves compared to larvae fed on a control diet. When tolerance to carbaryl, acephate, malathion, and methomyl was bioassayed, the peppermint-fed larvae were more tolerant of the insecticides as larvae fed on bean leaves, a weaker inducer of monooxygenase activity (Yu *et al.*, 1979; Berry *et al.*, 1980). Peppermint leaves also increased P450-monooxygenase activity in the alfalfa looper, *Autographa californica* Speyer (Lepidoptera: Noctuidae) and the cabbage looper, *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) (Farnsworth *et al.*, 1981). For both species, rearing on peppermint increased tolerance to the carbamates carbaryl and methomyl compared to larvae reared on plants

without inducing effect. However, in contrast to *P. saucia*, tolerance to the OP acephate was not affected by rearing the larvae on peppermint. In *S. frugiperda* larvae, maize leaves induce P450-monooxygenase activity and tolerance to eight different insecticides (five OPs, one carbamate and two pyrethroids) was increased when the larvae were fed maize compared to larvae fed on leaves of non-inducing soybeans (Yu, 1982a). Furthermore, *H. virescens*, selected for resistance to the allelochemical quercetin, showed increased tolerance to methyl parathion/paraoxon, methomyl and fenvalerate; the quercetin resistant larvae had increased P450-monooxygenase activity to two substrates (Rose *et al.*, 1992).

GSTs

GSTs can also be induced as well as depressed by plant allelochemicals (reviewed in Terriere (1984), Yu (1986), Clark (1989), and Yu (1992)). Considerable induction of GST activity has been reported in some systems, *e.g.* indole-3-carbinol, indole-3-acetonitrile and flavone increased GST activity by 4-, 18- and 7-fold, respectively, in larvae of *S. frugiperda* when fed on diets containing the respective allelochemicals (Yu, 1983). As with P450 enzymes, induction of GSTs by host plants or allelochemicals derived from host plants may increase the level of tolerance to insecticides in the induced insects. Yu (1982b) found that *S. frugiperda* larvae fed on cowpeas has elevated level of GST activity compared to larvae fed on soybeans. When tolerance to the OPs diazinon, methamidophos, and methyl parathion was assayed, the cowpea-fed larvae were twice as tolerant at the LC_{50} to the OPs as the soybean-fed larvae. The result suggests that cowpea-induced GSTs caused the increased OP tolerance in the *S. frugiperda* larvae (Yu, 1982b).

Esterases

Host plants or allelochemicals derived from host plants may also affect esterase activity in herbivorous insects. Extracts from soybean leaves affect esterase activity to *p*-nitrophenyl acetate in *T. ni* and the soybean looper, *Pseudoplusia includens* Walker (Lepidoptera: Noctuidae). When *T. ni* was fed leave extract from a resistant variety of soybean, esterase activity increased in the midgut compared to larvae fed on the control diet; in *P. includens* the opposite effect was found, leave extract from the resistant variety decreased midgut esterase activity (Dowd *et al.*, 1983). Several host plants and allelochemicals induce esterase activity in *S. frugiperda.* Eleven allelochemicals and three host plants increased esterase activity to α-naphthyl acetate in the larvae (Yu & Hsu, 1985); most of the inducers of esterase activity were also inducers of P450-monooxygenases or GSTs in *S. frugiperda* (Yu, 1983). Neem oil from seeds of the neem tree decreases esterase activity in the obliquebanded leafroller, *Coristoneura rosaceana* L. (Lepidoptera: Tortricidae). Larvae reared on a diet containing neem oil showed decreased activity to α-naphthyl acetate and α-naphthyl butyrate compared to larvae reared on control diet (Smirle *et al.*, 1996). Another example of allelochemicals decreasing esterase activity has been observed in the Colorado potato beetle, *L. decemlineata*; limonoid allelochemicals decreased esterase activity to α-naphthyl acetate in the larvae (Ortego *et al.*, 1999).

Allelochemical or host plant effects on insect esterase activity may affect tolerance to insecticides in the insects. However, to my knowledge, no studies have yet been carried out to

address this issue and it would be of special interest to explore this in populations with esterase-based insecticide resistance.

Frankliniella occidentalis

F. occidentalis may perform differently when reared on different host plant species or cultivars. Reproduction, survival, feeding behaviour and size of individuals have been reported to be affected by the host plant (van Dijken *et al.*, 1995; de Jager *et al.*, 1996; Harrewijn *et al.*, 1996; de Kogel *et al.*, 1997a,b,c; de Kogel *et al.*, 1998; de Kogel *et al.*, 1999). However, no studies appear to have examined if host plants can affect activity of detoxification enzymes or insecticide tolerance in *F. occidentalis*. Therefore, a preliminary study was included in this Ph.D. project to investigate this subject (see *Paper 3*). Thrips from a laboratory population reared on bean plants for nine years were transferred to new host plants sweet pepper or chrysanthemum. The thrips appeared to perform poorer on the new hosts as the level of total protein of individual adults was lowered in the new populations. However, no difference was found in general esterase activity or tolerance to methiocarb between the three populations. The only significant effect observed was a lower level of GST activity in the population reared on sweet pepper (*Paper 3*). Activity of P450-monooxygenases was not assayed in this study. Obviously, development of assays for monooxygenase activity in *F. occidentalis* would be valuable to examine possible effects on this detoxification system.

CONCLUSIONS

Insecticide resistance in field populations of *F. occidentalis* appears to be common and it is likely to be the primary cause of many failures to control *F. occidentalis* infestations with insecticides. *F. occidentalis* has the potential for fast development of resistance and to develop resistance to a range of different compounds. At present, only few studies on few different populations have been carried out to study the underlying mechanisms conferring resistance in *F. occidentalis*. From these studies it appears that resistance in *F. occidentalis* is polyfactorial; different mechanisms can confer resistance in different populations and different mechanisms may coexist in the same population. Possible resistance mechanisms in *F. occidentalis* are discussed in the previous section on resistance mechanisms and include:

1) Reduced penetration (Zhao *et al.*, 1995c)
2) Increased detoxification by P450-monooxygenases (Immaraju *et al.*, 1992; Zhao *et al.*, 1994; Zhao *et al.*, 1995b; *Paper 2*)
3) Increased detoxification or sequestration by esterases (*Paper 2*)
4) Increased detoxification by GSTs may also contribute (*Paper 2*)
5) Altered AChE: insensitive AChE (Liu *et al.*, 1994; Zhao *et al.*, 1994; *Paper 2*) and increased AChE activity (*Paper 1 & 2*)
6) Knockdown resistance (Immaraju *et al.*, 1992; Zhao *et al.*, 1995c)

Thus, *F. occidentalis* appears to have a large potential for developing resistance to different insecticides.

The aims and scope of this Ph.D. study were to:

- Investigate the biochemical mechanisms of methiocarb resistance in *F. occidentalis* (*Paper 1 & 2*)
- Establish rapid and sensitive enzymatic assays on *F. occidentalis* for investigating resistance mechanisms and evaluate these enzymatic assays for use in resistance monitoring (*Paper 1 & 2*)
- Investigate the effects of different host plants on methiocarb tolerance and activity of possible resistance-associated enzymes in *F. occidentalis* (*Paper 3*)

The biochemical mechanisms of methiocarb resistance in *F. occidentalis* were studied with synergists in bioassays (PBO and DEF), assays *in vitro* of detoxification enzyme (esterase and GST) activities toward model substrates, and assays *in vitro* of insensitivity and activity of AChE. Laboratory populations and populations recently collected in the field from commercial greenhouses in Denmark were included in the study.

The results from bioassays with synergists included, indicated involvement of P450-monooxygenases and esterases in methiocarb resistance in some of the populations (*Paper 2*). Selection with methiocarb on one of the populations to increase the level of resistance resulted in increased activity of GSTs and AChE (*Paper 2*). Hence, GSTs and altered AChE may also contribute to methiocarb resistance. Two alterations of AChE may be possible to confer resistance in *F. occidentalis*; insensitive AChE (*Paper 2*) and increased AChE activity (*Paper 1 & 2*). Thus, this study suggests that both metabolic and target-site resistance are involved in conferring methiocarb resistance to *F. occidentalis*.

The biochemical assays used in this study were fast and sensitive assays that have been successfully used in detection of resistance in other insect pests. Thrips were assayed *in vitro* for esterase activity to the model substrates α- and β-naphthyl acetate, GST activity to two chloronitrobenzene model substrates, and AChE activity and inhibition by insecticides.

In *Paper 2*, the utility of these tests in detecting resistance in *F. occidentalis* was studied by testing if activities of these enzymes in different resistant populations were significantly and consistently different from those measured in the susceptible population. None of the enzymatic assays showed strong and consistent correlation to the level of methiocarb resistance in the different populations. GST activity to CDNB showed the best correlation with the level of resistance ($r = 0.77$ in analysis with LC_{50} values), but in a multiple comparison analysis only one of six resistant populations had significantly higher GST activity to CDNB than measured in the susceptible population (*Paper 2*). Therefore, the enzymatic assays used in this study appeared to have modest value for detecting resistance to methiocarb in field populations of *F. occidentalis*.

The influence of host plant shift on activity of putative detoxification enzymes and insecticide tolerance was studied in *Paper 3*. As discussed in the preceding section, host plants and specifically allelochemicals of the host plants may affect activity of detoxification enzymes in insects and thereby affect tolerance to insecticides in the insects. A relationship that could be exploited in integrated pest management.

F. occidentalis from a population adapted to growth on bean plants were transferred to new host plants, sweet pepper and chrysanthemum, to study possible effects on esterase, GST, and AChE activity and tolerance to methiocarb. The size of the adult thrips was decreased on the new host plants, indicating a poorer performance due to the host plant shift. The specific AChE activity differed between the populations, but because the populations also differed in total protein per thrips, no difference was found when AChE activity was expressed as total activity per individual. This result may merely indicate that the nervous system was equally developed in adults of the three populations.

Specific activity of esterases and GSTs were largely unaffected by the host plant shifts; the only significant effect observed was a slightly lower level of GST activity in the population reared on pepper plants. Tolerance to methiocarb was not affected by the host plant shift; thus the apparently poorer performance on the new host plants may not have any impact on the effectiveness of applied insecticides. Other host plants than the ones included in this study may of course affect insecticide tolerance in *F. occidentalis*.

Knowledge of resistance mechanisms can form the basis for developing rapid and sensitive biochemical assays to detect resistance in insect pest populations. Despite the small size of *F. occidentalis*, the biochemical assays used in this study were sensitive enough to allow measurements on single individuals. Assays for discrimination between susceptible and resistant phenotypes on individual insects offer the capability of an early detection of resistance and to study population dynamics of resistant insects. However, in future studies of tools to detect resistance in *F. occidentalis*, the value of biochemical assays that are *not* both rapid and sensitive should also be considered.

Increased detoxification by P450-monooxygenases may be an important metabolic resistance mechanism in *F. occidentalis*. Implementation of an assay for P450-monoxygenase activity in *F. occidentalis* could have practical value in resistance monitoring. However, even assays of P450-monooxygenase activity to model substrates may not be simple to implement on *F. occidentalis*. Difficulties with P450-monooxygenase assays are mentioned in the previous section on cytochrome P450-monooxygenases, and especially the small size of *F. occidentalis* can make it difficult to prepare homogenates free of endogenous inhibitors and proteases. Other biochemical methods to study resistance in *F. occidentalis* are also worth considering. For example, if assays of general enzyme activity to specific model substrates fail to indicate presence of a metabolic resistance mechanism, then separation of isoenzymes, *e.g.* by native electrophoresis, may reveal specific isoenzymes associated with resistance. Production of antibodies to resistance-associated isoenzymes could form the basis for developing immunoassays, *e.g.* ELISA tests, to detect resistance. As regards detection of target-site resistance to pyrethroids, molecular biology techniques have proved to be useful tools as discussed in the section on knockdown resistance. Recently, a molecular-based diagnostic test to detect insensitive AChE in the Colorado potato beetle has been developed (Clark *et al.*, 1999), and in the future, molecular biology-based diagnostic tests to detect other resistance mechanisms are likely to be developed; these tests may even be designed to detect resistant genotypes. However, more sophisticated biochemical assays are generally more laborious, costly, and require special equipment and skilled personnel, factors that diminish their usefulness in routine monitoring for resistance.

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Acetylcholinesterase Activity Associated with Methiocarb Resistance in a Strain of Western Flower Thrips, *Frankliniella occidentalis* (Pergande)

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A strain of western flower thrips (*Frankliniella occidentalis*) with resistance to methiocarb was collected from a Danish greenhouse for investigation of possible resistance mechanisms. Bioassays with methiocarb showed moderate (10-fold) resistance at LC_{50} in the greenhouse-collected strain compared to a susceptible laboratory strain. Piperonyl butoxide, a cytochrome P450 monooxygenase inhibitor, showed no synergistic effect on the tolerance level to methiocarb. *In vitro* assays of enzyme activities showed significantly increased activity of acetylcholinesterase (2.6-fold), general esterases (1.3-fold), and glutathione *S*-transferases (1.2-fold) in the resistant strain. Assays of acetylcholinesterase sensitivity to inhibition by methiocarb, dichlorvos, and eserine showed no indications of insensitive acetylcholinesterase in the resistant strain. These results indicated that increased activity of acetylcholinesterase contributed to methiocarb resistance in the resistant strain. The slightly increased activity of general esterases and glutathione *S*-transferases may also have contributed to methiocarb resistance. ©1998 Academic Press

INTRODUCTION

Thrips are important pests of a number of crops throughout the world. The western flower thrips, *Frankliniella occidentalis* (Pergande), has recently invaded Western Europe, and in Denmark *F. occidentalis* was introduced in 1985 and it is now a common pest in Danish greenhouses (1). *F. occidentalis* is a polyphagous pest that causes damage to crops by feeding and egg laying and as an efficient vector of several tospoviruses (2).

F. occidentalis is capable of developing resistance to many different insecticides including organophosphates, carbamates, and pyrethroids (3–6). The mechanisms of insecticide resistance in *F. occidentalis* have, however, only been studied in a few strains. Enhanced oxidative metabolism conferred resistance to diazinon, bendiocarb, and fenvalerate in a diazinon-selected strain of *F. occidentalis* from a greenhouse in Missouri; for diazinon and fenvalerate, insensitive acetylcholinesterase (AChE,[1] EC 3.1.1.7) and decreased penetration, respectively, also contributed to resistance (7–10). Another study on four strains of *F. occidentalis* from Californian greenhouses has shown that the synergist piperonyl butoxide (PBO) reduced permethrin resistance in these strains, indicating involvement of cytochrome P450-dependent monooxygenases in the resistance (3).

As has been shown in several insect species, different resistance mechanisms can exist in different strains, even in strains resistant to the same insecticides; moreover, different resistance mechanisms may also coexist in a single strain (e.g., 11–13). To evaluate the potential for multiple resistance mechanisms to be present in *F. occidentalis*, strains from different populations with resistance to different insecticides must be studied. For this purpose, a strain of *F. occidentalis* was collected from a Danish greenhouse in which efforts to control thrips infestation with insecticides had failed. This study has focused

[1] Abbreviations used: AChE, acetylcholinesterase; PBO, piperonyl butoxide; ASChI, acetylthiocholine iodide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); α-NA, α-naphthyl acetate; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione *S*-transferase.

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on the biochemical mechanisms of resistance to methiocarb as this is the only approved insecticide for control of *F. occidentalis* in Denmark.

MATERIALS AND METHODS

Insects

F. occidentalis was reared on bean plants (*Phaseolus vulgaris* L.) at 22°C with a 16:8 (L:D) h photoperiod. The susceptible laboratory strain, UCD90, was collected in 1990 in the botanical garden of the University of California, Davis (4). The resistant strain, DKOdH96, was collected in 1996 from hibiscus plants in a commercial greenhouse in Denmark. The greenhouse had been treated regularly with methiocarb (Mesurol, Bayer) and dichlorvos (Vapona, Cyanamid) for thrips control. Occasionally endosulfan (Thiodan, AgrEvo) and aldicarb (Temik, Rhône-Poulenc) had been used. The DKOdH96 strain was collected after a control failure in the greenhouse; 1 month before collection an intensive treatment with Mesurol and Vapona every fourth day was not sufficient to control the thrips infestation. Since collection the strains have been kept isolated and pesticide free.

Chemicals

Technical grade methiocarb (99.5% pure) and dichlorvos (99.6% pure) were provided by Bayer (Leverkusen, Germany). PBO (99% pure) was provided by Cheminova Agro (Lemvig, Denmark). Acetylthiocholine iodide (ASChI), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), α-naphthyl acetate (α-NA), *o*-dianisidine (tetrazotized), eserine hemisulfate, and bovine serum albumin (fraction V) were purchased from Sigma Chemical Company (St. Louis, MO). Reduced glutathione and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Merck (Darmstadt, Germany). The Bradford dye reagent was purchased from Bio-Rad, (Richmond, CA). All other chemicals were of analytical quality and purchased from commercial suppliers.

Insecticide Bioassays

Solutions of methiocarb in acetone were made to coat the inner surface of 10-ml polypropylene vials (Nunc, Denmark). Methiocarb solution (500 μl) was added to the vials which were then rotated on a roller (Heto, Denmark) until the acetone had evaporated. Adult female thrips (age unknown) were transferred to the coated vials (10 thrips per vial) and incubated at 22°C with a 16:8 (L:D) h photoperiod. Three hours after start of incubation a small piece (0.5 cm^2) of a bean (*Phaseolus vulgaris* L.) leaf was added to each vial as a food/water source. After 24 h mortality (no response to probing) was recorded in the vials. No control mortality was observed in vials treated with only acetone. Five concentrations causing >0% and <100% mortality were assayed with at least four replications (10 individuals per replication) for each concentration. For assays of possible synergism with PBO, methiocarb solutions containing 5.0 ppm PBO were used to coat the vials; at this concentration of PBO, control mortality was 2.5 and 4% in bioassays on UCD90 and DKOdH96, respectively. Data for methiocarb mortality in bioassays with PBO were corrected for control mortality with Abbott's formula (14). The dose–mortality data were analyzed by probit analysis (15) using statistical software (16).

Homogenization of Thrips for Enzyme Assays in Vitro

For analyses *in vitro*, adult female thrips (age unknown) were used; the thrips had been stored at −80°C after collection. The thrips were homogenized in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.0025% Triton X-100. To obtain homogenate from individual thrips, the thrips were placed in buffer in individual wells in a microplate and homogenized with a multiple homogenizer (Buckard Scientific, UK). Homogenate of pooled thrips was prepared from thrips homogenized together in buffer on ice with a hand-held pellet homogenizer (Kontes, New Jersey); one sample of homogenate was prepared from at least 20 pooled thrips. This homogenate

was centrifuged at 10,000*g* for 5 min at 4°C and the supernatant was used in the assays.

Enzyme Assays

All incubations and measurements in the enzyme assays were made at an ambient temperature (20–25°C) and absorbance was recorded using a Multiskan MCC/340 microplate reader (Labsystems, Finland).

AChE activity was measured with ASChI as substrate by the method of Ellman *et al.* (17) modified for a microplate. The level of AChE activity was measured in individual thrips. Homogenate (100 μl) equivalent to one-half thrips was added to each well in a microplate. Then 100 μl of ASChI (0.5 mM final concentration) and DTNB (0.05 mM final concentration) in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.1% Triton X-100 was added to each well; DTNB was applied in a low concentration as it has been shown to have an inhibitory effect on the hydrolysis of ASChI in *F. occidentalis* (18). The microplate was left 5 min to equilibrate and then absorbance at 414 nm was recorded continuously. Reaction rates were calculated by linear regression analysis on the linear portion of the curves after plotting absorbance against time.

Assays of methiocarb and dichlorvos inhibition of AChE activity were done on homogenate made from pooled thrips. Homogenate (50 μl) was added to each well. However, because of difference in AChE activity between the two strains, UCD90 homogenates equivalent to one and three thrips per 50 μl were assayed and DKOdH96 homogenates equivalent to one-half (only in methiocarb assay) and one thrips per 50 μl were assayed. Assays with eserine hemisulfate as inhibitor were made only on homogenate equivalent to one-half thrips per 50 μl for both strains. To wells with homogenate were added 50 μl of the insecticide dissolved in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.1% Triton X-100, and the samples were left to incubate for 15 min. After incubation 100 μl ASChI/DTNB was added and absorbance recorded as described above. At least three replications, each with duplicate determinations, were conducted for all insecticide concentrations assayed. For estimation of I_{50} values, the average values of percentage of activity remaining were transformed to probit values and plotted against the log inhibitor concentration. Then linear regression analysis was done and I_{50} calculated.

General esterase activity was measured with α-NA as substrate in individual thrips by the method of van Asperen (19) modified for a microplate. Homogenate (25 μl) equivalent to 0.125 thrips was added per well in a microplate. As substrate 75 μl of α-NA (0.75 mM final concentration) in 0.1 M sodium phosphate buffer (pH 7.5) was added to each well and after 5 min the reaction was ended by adding 50 μl of *o*-dianisidine (0.1% final concentration) and sodium dodecyl sulfate (1.0% final concentration) in H_2O to each well. The plate was left to equilibrate for 5 min and absorbance was read at 595 nm. Change in absorbance was converted to rate of product formation from a standard curve of α-naphthol. Assays of eserine hemisulfate inhibition of general esterase activity were performed on homogenate made from pooled thrips. Homogenate (12.5 μl) equivalent to 0.125 thrips was added per well and incubated with 12.5 μl of eserine hemisulfate in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.0025% Triton X-100. After incubation with eserine hemisulfate for 15 min, α-NA substrate was added and the esterase assay was carried out as described above. Three replications, each with duplicate determinations, were conducted for all eserine hemisulfate concentrations assayed.

Glutathione *S*-transferase (GST, EC 2.5.1.18) activity was measured with CDNB as substrate as described by Habig *et al.* (20) and modified for a microplate. Assays were done on homogenate from pooled thrips. Homogenate (20 μl) equivalent to one thrips was added per well in a microplate and 180 μl of glutathione (1.0 mM final concentration) and CDNB (1.0 mM final concentration) in 0.1 M sodium phosphate buffer (pH 6.5) was added to each well. The microplate was left 2 min to equilibrate and then absorbance at 340 nm was recorded continuously. Reaction

TABLE 1
Toxicity of Methiocarb and Methiocarb/PBO to Susceptible, UCD90, and Resistant, DKOdH96, Adult Female *F. occidentalis* Exposed 24 h in a Residual Assay

Insecticide	Strain	*n*	Slope ± SE	LC_{50} (95% FL)[a]	χ^2	RR_{50}[b]
Methiocarb						
	UCD90	238	1.48 ± 0.23	3.26 (2.50–4.51)	5.69	
	DKOdH96	246	1.10 ± 0.20	31.1 (22.1–48.9)	2.21	9.5
Methiocarb + PBO						
	UCD90	200	1.78 ± 0.25	3.06 (2.38–4.11)	0.077	
	DKOdH96	247	1.06 ± 0.21	33.1 (21.4–74.5)	1.93	11

[a] LC_{50} in ppm (95% fiducial limits).
[b] Resistance ratio, LC_{50} of DKOdH96/LC_{50} of UCD90.

rates were calculated by linear regression analysis on the linear portion of the curve after plotting absorbance against time. An extinction coefficient of 9.6 mM^{-1} cm^{-1} (20) was used to convert change in absorbance to rate of conjugate formation. Three replications, each with duplicate determinations, were made for each strain.

Protein content in homogenates was determined by the method of Bradford (21), as modified in the Bio-Rad microassay for microplates, with bovine serum albumin as a standard.

RESULTS

Results of methiocarb and methiocarb/PBO bioassays on adult female *F. occidentalis* are summarized in Table 1. The strain DKOdH96 collected from a commercial greenhouse was 10-fold more tolerant of methiocarb as the susceptible laboratory strain UCD90. The synergist PBO showed no synergistic (or antagonistic) effect on the tolerance level to methiocarb in either strains.

AChE activity (ASChI hydrolysis) assayed in individual thrips showed that the methiocarb-resistant strain DKOdH96 on average had a 2.6-fold higher activity than found in the susceptible strain UCD90 (Table 2). The frequency distributions of the AChE activity in the two strains showed some overlap (Fig. 1). However, a Mann-Whitney U test shows significant difference ($P < 0.001$) in AChE activity between the two strains.

Inhibition of AChE activity with methiocarb or dichlorvos showed that AChE in the resistant strain DKOdH96 was *more* sensitive to methiocarb or dichlorvos inhibition than AChE in the susceptible strain UCD90; I_{50}s for methiocarb inhibition were 0.43 and 2.1 μM for DKOdH96

TABLE 2
Enzyme Activity of AChE (ASChI), General Esterases (α-NA), and Glutathione *S*-Transferases (CDNB) in the Susceptible Strain UCD90 and Resistant Strain DKOdH96 of *F. occidentalis*

	Enzyme activity [a]		
	ASChI[b] (nmol min^{-1} mg $protein^{-1}$)	α-NA[b] (nmol min^{-1} μg $protein^{-1}$)	CDNB[c] (nmol min^{-1} mg $protein^{-1}$)
UCD90 (S)	28.8 (14.9)	4.05 (2.96)	124 (5.0)
DKOdH96 (R)	73.7 (24.7)*	5.19 (1.84)*	145 (8.1)*
Ratio (R/S)	2.6	1.3	1.2

[a] Mean activity with SD in parentheses.
[b] Activity measured in individual thrips; $n = 51$ and $n = 96$ for activity to ASChI and α-NA, respectively.
[c] Activity measured in homogenate of 20 pooled thrips, $n = 3$.
* Significantly different from UCD90, $P < 0.05$.

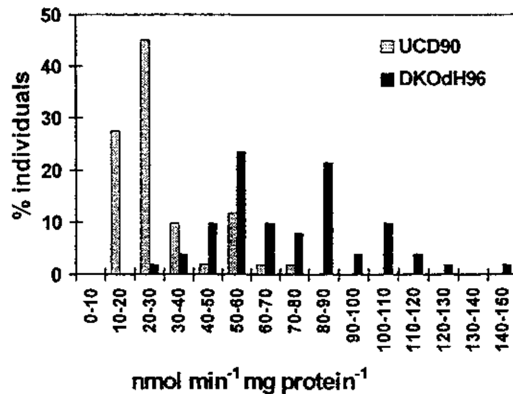

FIG. 1. The frequency distribution of AChE activity (ASChI hydrolysis) in individual *F. occidentalis* of the susceptible strain UCD90 and resistant strain DKOdH96 (n = 51 thrips for each strain).

and UCD90, respectively, and for dichlorvos, 0.33 and 1.7 μM (Fig. 2). The relative inhibition was not affected by adjusting to equal AChE activity in the two strains. In Fig. 2 the values for UCD90 are means of data measured in homogenate equivalent to one or three thrips per well in the microplate. The AChE activity measured with three UCD90 thrips per well correspond approximately to the activity measured with one DKOdH96 thrips per well; the values for DKOdH96 in Fig. 2 are means of data measured in homogenate equivalent to one-half (Fig. 2A only) or one thrips per well. Therefore, the difference in relative inhibition of AChE activity between the two strains is not caused by interstrain difference in the level of AChE activity. However, because of the increased AChE activity in the DKOdH96 strain, inhibition with about 20 μM methiocarb or 15 μM dichlorvos in the assay was required to have equal AChE activity in the two strains (Fig. 3). The AChE activity in the DKOdH96 strain was also more sensitive to inhibition with another carbamate, eserine hemisulfate; I_{50}s for eserine inhibition were 0.0094 and 0.048 μM for DKOdH96 and UCD90, respectively.

General esterase activity was assayed in individual thrips with α-NA as substrate. The resistant strain DKOdH96 showed a slightly (1.3-fold) increased average esterase activity compared to the susceptible strain (Table 2). A considerable overlap in esterase activity was found between the two strains (Fig. 4), however, a Mann-Whitney U test shows significant difference (P = 0.0047) in esterase activity between the two strains. The esterase activity in DKOdH96 remained higher than in UCD90 when 1.0 μM eserine hemisulfate was included in the assay (Fig. 5); 1 μM eserine gave about 90% inhibition of the AChE activity in both strains (% AChE activity remaining (± SE) after incubation with 1.0 μM eserine was 11.9 ± 3.8% and 10.3 ± 2.7% for UCD90 and

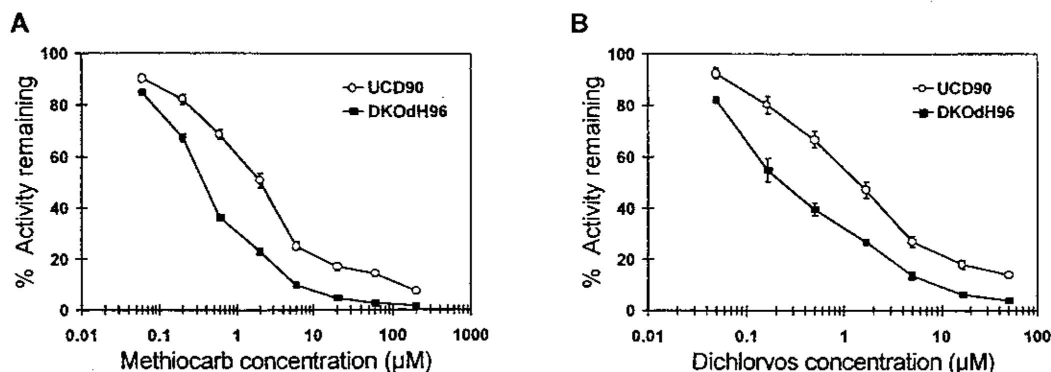

FIG. 2. The relative inhibition of AChE activity in the susceptible strain UCD90 and resistant strain DKOdH96 of *F. occidentalis* by methiocarb (A) and dichlorvos (B). Percentage of activity remaining after inhibition. Values are means of at least three replications for each concentration. Bars represent SE.

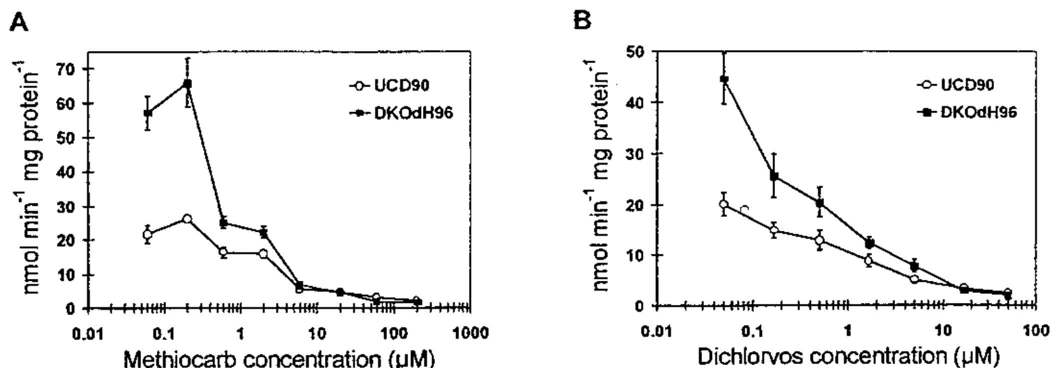

FIG. 3. The level of AChE activity in the susceptible strain UCD90 and resistant strain DKOdH96 of *F. occidentalis* after inhibition by methiocarb (A) and dichlorvos (B). Values are means of at least three replications for each concentration. Bars represent SE.

DKOdH96, respectively). Like the AChE activity of DKOdH96, the general esterase activity of DKOdH96 was more sensitive to inhibition with eserine than esterase activity in UCD90 (Fig. 5).

GST activity was assayed with CDNB as substrate. The resistant strain DKOdH96 showed a slightly (1.2-fold) increased average GST activity compared to the susceptible strain (Table 2); a t test shows significant difference ($P = 0.020$) in GST activity between the two strains.

DISCUSSION

The resistant strain, DKOdH96, collected from a commercial greenhouse in Denmark showed moderate (10-fold) resistance to methiocarb compared with a susceptible strain UCD90. The toxicity of methiocarb was not increased by including the synergist PBO in the bioassays. PBO is a known inhibitor of cytochrome P450-dependent monooxygenases; however, lack of synergism with PBO does not rule out the involvement of cytochrome P450-dependent monooxygenases in resistance (22). PBO increased the toxicity of bendiocarb, diazinon,

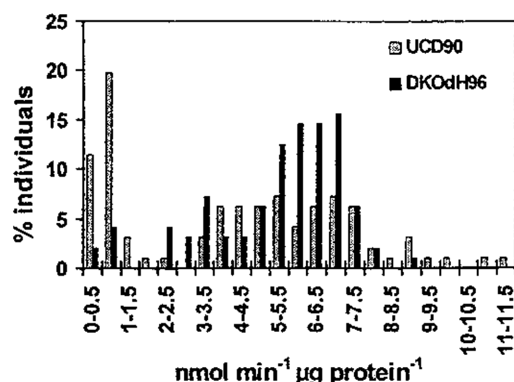

FIG. 4. The frequency distribution of general esterase activity (α-NA hydrolysis) in individual *F. occidentalis* of the susceptible strain UCD90 and resistant strain DKOdH96 (n = 96 thrips for each strain).

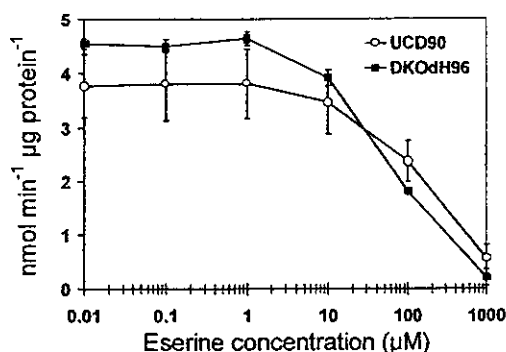

FIG. 5. Inhibition by eserine of general esterase activity in the susceptible strain UCD90 and resistant strain DKOdH96 of *F. occidentalis*. Values are means of three replications for each concentration. Bars represent SE.

and fenvalerate in a diazinon-selected strain of *F. occidentalis* from a greenhouse in Missouri (10), and studies of the insecticide metabolites confirmed that the selected strain possessed enhanced oxidative metabolism to these insecticides (7–9).

In the enzyme assays, the higher level of AChE activity in the resistant strain was the most pronounced difference between the two strains. Several examples of increased AChE activity in resistant insects have already been described; e.g., in the lesser grain borer, *Rhyzopertha dominica*, all of the 15 resistant populations tested showed higher AChE activity than found in the susceptible strain (23); in the tobacco budworm, *Heliothis virescens*, 9 of 10 resistant strains showed significantly increased AChE activity (24); in the fruit fly, *Drosophila melanogaster*, selection for resistance to parathion and fenthion was accompanied by a correlated increase in AChE activity (25); and in several resistant strains of the house fly, *Musca domestica*, increased V_{max} of AChE has been shown (e.g., 26–29). These examples of increased AChE activity were found in homogenates of whole insects or heads and may reflect a modification of catalytic properties of AChE or an increased amount of AChE or both. Modification of catalytic properties of AChE may protect against toxicity of AChE inhibitors as hypothesized by Byrne and Devonshire (30); e.g., AChE that is more catalytically efficient would be able to have a larger proportion of the activity inhibited before reaching a critical threshold for disruption of synaptic function. Insecticide resistance may also develop from an overproduction of the target-site enzyme AChE (31) and examples of increased amounts of AChE conferring insecticide resistance have been reported. In the fruit fly, *Dacus olea*, selection with organophosphates increased the gene dosage of the AChE gene (32), and in *D. melanogaster*, a direct proportionality between the flies' resistance to malathion and the amount of AChE present has been shown (33). Another study on *D. melanogaster* showed that flies genetically transformed to express additional AChE in the haemolymph acquired resistance to parathion (34); the mechanism of resistance may be sequestration of the insecticide by soluble AChE in the haemolymph; however, this specific mechanism remains to be demonstrated in naturally resistant populations. Still, the increased AChE activity found presently in the resistant strain DKOdH96 may be a factor that contributed to methiocarb resistance in this strain; however, further studies to clarify the role of AChE activity in resistance in the DKOdH96 strain are necessary. In the study of diazinon resistance in *F. occidentalis* by Zhao *et al.* (7), no difference in AChE activity between the susceptible and resistant strain was found.

Modifications of AChE, with the AChE of the resistant strain being less sensitive to inhibition with relevant insecticides, are frequently associated with resistance to organophosphates and carbamates (35). The resistant strain DKOdH96 showed no indications of insensitive AChE; in fact, the AChE of DKOdH96 was more sensitive to inhibition with the tested inhibitors methiocarb, eserine, and dichlorvos than AChE of the susceptible strain. In the greenhouse from which the DKOdH96 strain was collected, methiocarb and dichlorvos have been used for thrips control; therefore, the control failure before collection of the DKOdH96 thrips is not likely to be due to AChE insensitivity to methiocarb or dichlorvos. In contrast, the diazinon-resistant strain in the study of Zhao *et al.* (7) showed diazoxon-insensitive AChE; however, this strain was also resistant to the carbamate bendiocarb, but the AChE of the resistant and susceptible strains were equally sensitive to bendiocarb inhibition (8). Other modifications of AChE like faster reactivation of carbamylated enzyme in resistant insects may also be worth considering in resistance to carbamates (31).

In a number of insect pest species, elevated level of general esterase activity has been correlated with insecticide resistance (35). When general esterase activity (α-NA hydrolysis) was measured in this study the two strains showed a different distribution of esterase activity. The individuals of both strains had widely differing esterase activities with the most widely differing activities found in the susceptible strain. This

indicated heterogeneity of the strains which was supported by the low slopes in the bioassays, although the steepness of the slope of the concentration–mortality line does not necessarily reflect the genetic variation in the strains (36). However, the resistant DKOdH96 strain showed a slightly higher average activity of general esterases so it is possible that elevated esterase activity contributed to methiocarb resistance in this strain. The elevated general esterase activity in DKOdH96 was not caused by higher AChE activity hydrolyzing the α-NA substrate; when most of the AChE activity was inhibited with eserine, the DKOdH96 strain still showed a higher average esterase activity. In the study of Zhao *et al.* (7) on diazinon resistance in *F. occidentalis*, the resistant strain did not show elevated esterase activity; in fact, the susceptible strain had a significantly higher esterase activity than the resistant strain. However, general esterases have been found to be associated with resistance in strains of the citrus thrips, *Scirtothrips citri* (37, 38).

Enhanced activity of GSTs has mainly been associated with resistance to organophosphates (35); however, at least one example of an association between carbamate resistance and enhanced GST activity have been reported. In a strain of tobacco budworms, *Heliothis virescens*, selection with thiodicarb was accompanied by an increased activity of GST to CDNB (39). Although significant, the GST activity in the resistant strain DKOdH96 was only slightly increased (1.2-fold). Thus, if increased GST activity is involved in methiocarb resistance in DKOdH96, the contribution is presumably of minor importance. However, the study of Mutero *et al.* (40) on *D. melanogaster* suggests that different factors, each conferring weak resistance, can result in high resistance when combined in the same individuals. In the study of diazinon resistance in *F. occidentalis* by Zhao *et al.* (7), no difference in GST activity to CDNB between the susceptible and resistant strain was found.

In summary, methiocarb resistance in the DKOdH96 strain appeared to be associated with increased activity of AChE. Other factors, like the slightly increased activity of general esterases and GSTs, may also have contributed to the 10-fold resistance. There was no indication of the presence of the same resistance mechanisms as found in the North American greenhouse strains of *F. occidentalis* (3, 7–10). Therefore, different strains of *F. occidentalis* can possess different resistance mechanisms and resistance may be polyfactorial. Thus, *F. occidentalis* probably has a large potential for developing resistance to different insecticides, and in combination with the short generation times of *F. occidentalis*, resistance may develop fast in this pest. For further studies of the mechanisms of resistance to methiocarb in *F. occidentalis*, selection for higher methiocarb resistance in *F. occidentalis* strains has been undertaken.

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Jensen: Resistance Mechanisms in

F. occidentalis

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Insecticide resistance and resistance

management

Mechanisms Associated with Methiocarb Resistance in *Frankliniella occidentalis* (Thysanoptera: Thripidae)

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Abstract

Biochemical mechanisms associated with methiocarb resistance were examined in laboratory-selected and field populations of the western flower thrips, *Frankliniella occidentalis* (Pergande). Seven populations were examined and they differed in their susceptibility to methiocarb by 30-fold. Including the synergists piperonyl butoxide, a cytochrome P-450 monooxygenase inhibitor, or *S,S,S*-tributylphosphorotrithioate, an esterase inhibitor, in the methiocarb bioassays partially suppressed resistance in the most resistant populations. In vitro assays of general esterase, glutathione *S*-transferase, and acetylcholinesterase activities showed increased activity in some of the resistant populations and increased activity of the enzymes after methiocarb selection on one of the populations. Assays of acetylcholinesterase sensitivity to inhibition by methiocarb, dichlorvos, and eserine suggested insensitive acetylcholinesterase in two of the resistant populations. These results indicate that methiocarb resistance in *F. occidentalis* was polyfactorial and involved detoxification and altered target site. None of the biochemical assays showed interpopulation enzymatic differences strongly correlated with the level of methiocarb resistance. The possibilities for developing rapid biochemical diagnostic assays to detect methiocarb resistance in *F. occidentalis* are discussed.

Key words *Frankliniella occidentalis,* insecticide resistance, methiocarb, resistance mechanisms

Introduction

THE WESTERN FLOWER thrips, *Frankliniella occidentalis* (Pergande), is a serious pest on a wide range of crops throughout the world and causes growers substantial economic losses (Lewis 1998). *F. occidentalis* is difficult to control with insecticides. First, the hidden habitats of *F. occidentalis* protect it from many insecticides; adults and larvae feed within flowers and buds, eggs are oviposited in leaf tissue, and pupal stages are passed in soil or leaf litter. Second, insecticide resistance has evolved in many populations and to a number of different insecticides (Immaraju et al. 1992, Brødsgaard 1994, Robb et al. 1995, Zhao et al. 1995a, Broadbent and Pree 1997, Jensen 1998, Kontsedalov et al. 1998). Third, development of resistance in *F. occidentalis* may be fast because of the short generation time and high fecundity (Brødsgaard 1989). In addition, the haplodiploid breeding system of *F. occidentalis*, in which resistance genes in the haploid males are directly exposed to selection following insecticide treatment, can accelerate the development of resistance (Denholm et al. 1998).

Methiocarb can be effective for control of *F. occidentalis* (Cook et al. 1995, Herron et al. 1996) and it is registered for use on different crops in several countries (Australia [Herron et al. 1996], Israel [Kontsedalov et al. 1998], and Denmark [DEPA 1999]) and in most states of the United States (Gowan Company 1999). However, resistance to methiocarb exists in different populations of *F. occidentalis* (Brødsgaard 1994, Jensen 1998, Kontsedalov et al. 1998), and even in populations never exposed to methiocarb before testing, most likely because of cross-resistance among insecticides (Brødsgaard 1994).

Although resistance in *F. occidentalis* is a common problem for many growers, the underlying mechanisms conferring resistance have only been studied on a few populations (Jensen 1998). Previous studies have shown that decreased penetration (Zhao et al. 1995b), detoxification by monooxygenases (Immaraju et al. 1992, Zhao et al. 1995a), increased activity of acetylcholinesterase (AChE) (Jensen 1998) and insensitive AChE (Zhao et al. 1994) are all possible mechanisms of resistance.

Information on resistance mechanisms is valuable for predicting cross-resistance spectra and for developing rapid and sensitive biochemical diagnostic assays to detect resistance (Brown and Brogdon 1987, Scott 1990). In a previous study on methiocarb resistance in *F. occidentalis* (Jensen 1998), higher enzyme activity of AChE, the target site enzyme for methiocarb, and higher enzyme activities of detoxification enzymes (general esterases and glutathione *S*-transferases [GSTs]) were found in a population of *F. occidentalis* resistant to methiocarb. The higher enzyme activities in the resistant population indicate that these enzymes may be involved in methiocarb resistance in *F. occidentalis* and could form the basis for developing biochemical diagnostic assays. For the present study, additional populations of *F. occidentalis* resistant to methiocarb have been included to investigate if enzymatic differences between the populations were correlated with the level of methiocarb resistance. Rapid and sensitive enzymatic assays were used and assessed as methods to detect methiocarb resistance. The mechanisms of methiocarb

resistance in *F. occidentalis* were studied with synergists in bioassays, assays of detoxification enzyme activities toward model substrates, and assays of insensivity and activity of AChE.

Materials and Methods

Insects. The *F. occidentalis* populations were maintained in culture on bean plants (*Phaseolus vulgaris* L. 'Montano') at 22°C, 60% RH, and a photoperiod of 16:8 (L:D) h. The susceptible population, UCD90, was collected in 1990 in the botanical garden of the University of California, Davis (Brødsgaard 1994). The resistant population, DKOdH96, was collected in 1996 from a commercial greenhouse in Denmark (Jensen 1998). To increase methiocarb resistance in the UCD90 population and to restore methiocarb resistance in the DKOdH96 population, the populations were selected with methiocarb, by the residual bioassay technique described below; the surviving adults were used to start a new selected population. The UCD-II population was established after two selection steps with 5 and 10 ppm methiocarb on the UCD90 population; the population DKOdH-III was established after three selection steps with 20, 50, and 50 ppm methiocarb on the DKOdH96 population. Another population, named DKOdH-IV, was established after selection with 20 ppm methiocarb on the DKOdH-III population. A further resistant population, LiDK98, was collected in 1998 from *Hedera helix* (L.) plants from a commercial greenhouse in Denmark. The greenhouse had been treated four times with dichlorvos (Vapona, Cyanamid, Glostrup, Denmark) and once with methomyl (Lannate, Du Pont, Albertslund, Denmark) over a 3-mo period. The latest treatment (Vapona) was 2 mo before collection. Resistant population AlDK98 was collected in 1998 from rose (*Rosa* sp.) plants from a commercial greenhouse in Denmark. The greenhouse had been treated three times with methiocarb (Mesurol, Bayer, Lyngby, Denmark) and once with deltamethrin (Decis, AgrEvo, Hvidovre, Denmark) over a 2.5-mo period. The latest treatment (Mesurol) was 1 wk before collection. New populations of *F. occidentalis* were propagated on bean plants and were in culture for at least 1 mo until collected for bioassays or biochemical assays. The characteristics of the different populations are summarized in Table 1.

Chemicals. Technical grade methiocarb (99.5% pure) and dichlorvos (99.6% pure) were provided by Bayer (Leverkusen, Germany). Piperonyl butoxide (PBO, 99% pure, Cheminova Agro, Lemvig, Denmark) and *S,S,S*-tributylphosphorotrithioate (DEF, 98.7% pure, Cheminova Agro, Lemvig, Denmark) were provided by the Danish Pest Infestation Laboratory, Denmark. Acetylthiocholine iodide (ASChI), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), α-naphthyl acetate (α-NA), ß-naphthyl acetate (ß-NA), α-naphthol, ß-naphthol, *o*-dianisidine (tetrazotized), reduced glutathione, and eserine hemisulfate were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Merck (Darmstadt, Germany) and 3,4-dichloronitrobenzene (DCNB) was purchased from Aldrich (Milwaukee, WI). The Bradford dye reagent (Coomassie Plus) and bovine serum albumin (fraction V) were purchased from Pierce (Rockford, IL). All other chemicals were of analytical quality and purchased from commercial suppliers.

Insecticide Bioassays. Solutions of methiocarb in acetone were made to coat the inner surface of 10-ml polypropylene vials (Nunc, Roskilde, Denmark). Methiocarb solution (500 μl) was added to the vials containing a 1-cm^2 Whatman no. 1 filter paper. The vials with filter paper were then rotated on a Roto-torque rotator (Cole-Parmer Instrument, Vernon Hills, IL) until the acetone had evaporated. Adult thrips were transferred to the coated vials (c. 10 thrips per vial), the coated filter papers were wetted with 8 μl of H_2O (MilliQ deionized) and placed in their vials. The wetted filter paper was added as a water source. In a previous study (Jensen 1998) a leaf disk was added as the water source, but it could be replaced by the wetted filter paper, thereby rendering the bioassay method simpler. The vials were closed with a cap and incubated at 22°C with a photoperiod of 16:8 (L:D) h. Mortality (no response to probing) was recorded after 24 h. For each population, at least five concentrations causing > 0% and < 100% mortality were assayed and each concentration was assayed with at least four coated vials with c. 10 individuals per vial; control vials treated with only acetone were included in each assay. For assays of possible synergism with PBO or DEF, methiocarb solutions containing 50 ppm PBO or DEF were used to coat the vials. In each assay with synergists, control vials treated with only 50 ppm PBO or DEF in acetone were included.

Enzyme and Protein Assays. All incubations and measurements in the enzyme assays were made at room temperature (22-24°C). Absorbance was recorded with a Multiskan MS microplate reader (Labsystems, Helsinki, Finland).

For enzyme assays, adult thrips stored at -80°C after collection were used. They were homogenized in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.01% Triton X-100. One homogenate was prepared from 24 thrips homogenized together in a microtube on ice with a hand-held pellet homogenizer (Kontes, Vineland, NJ). The homogenate was centrifuged at 10,000*g* for 5 min at 4°C and the supernatant was used in the assays.

General esterase activity was measured by the method of van Asperen (1962), modified for a microplate. Homogenate (25 μl) equivalent to 0.125 thrips was added per well in a microplate; the homogenization buffer contained 1.0 μM eserine to inhibit AChE activity in the sample. As substrate 75 μl of α-NA or ß-NA (0.75 mM final concentration) in 0.1 M sodium phosphate buffer (pH 7.5) was added to each well. After 5 min the reaction was ended by adding 50 μl of *o*-dianisidine (0.1% final concentration) and sodium dodecyl sulfate (1.0% final concentration) in H_2O to each well. The plate was left to equilibrate for 5 min and absorbance read at 600 nm for α-NA activity and at 540 nm for ß-NA activity. Three experiments, each with freshly prepared homogenates, eserine and *o*-dianisidine solutions, were conducted in the esterase assays; the naphthyl acetate solutions used were prepared from stock solutions of the substrates dissolved in acetone. Each sample was assayed with both esterase substrates; duplicate determinations were done on each sample.

GST activity was measured with CDNB and DCNB as substrates as described by Habig *et al.* (1974), modified for a microplate. For assay of GST activity to CDNB, homogenate (20 μl)

equivalent to 1 thrips was added per well in a microplate and 180 μl of CDNB (1.0 mM final concentration) and reduced glutathione (5.0 mM final concentration) in 0.1 M sodium phosphate buffer (pH 6.5) was added to each well. For assay of GST activity to DCNB, homogenate (100 μl) equivalent to 5 thrips was added per well in a microplate and 100 μl of DCNB (1.0 mM final concentration) and glutathione (5.0 mM final concentration) in 0.1 M sodium phosphate buffer (pH 7.5) was added to each well. The microplate was left 2 min to equilibrate and then absorbance at 340 nm was recorded continuously. Reaction rates were calculated by linear regression analysis on the linear portion of the curve after plotting absorbance against time. Extinction coefficients of 9.6 mM^{-1} cm^{-1} for the CDNB conjugate and 8.5 mM^{-1} cm^{-1} for the DCNB conjugate (Habig et al. 1974) were used to convert change in absorbance to rate of conjugate formation. Three experiments, each with freshly prepared homogenates, were conducted in the GST assays. Aliquots of stock solutions of CDNB and DCNB dissolved in 96% EtOH and GSH dissolved in H_2O were used for preparing CDNB/GSH and DCNB/GSH assay mixtures; the stock solution aliquots had been stored at -20°C. Each sample was assayed with both GST substrates; duplicate determinations were done on each sample.

AChE activity was measured with ASChI as substrate by the method of Ellman et al. (1961), modified for a microplate as described previously (Jensen, 1998). Absorbance was recorded at 405 nm. For assays of methiocarb, dichlorvos, and eserine inhibition of AChE activity, homogenate (50 μl) equivalent to two thrips was added to each well in a microplate. Fifty microliters of the insecticide dissolved in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.1% Triton X-100 were then added to wells with homogenate. Final concentrations of the insecticides were 20 μM methiocarb, 20 μM dichlorvos, and 1.0 μM eserine corresponding to ≈90% inhibition of the AChE activity in the susceptible UCD90 population after preincubation. The samples were left to incubate for 15 min and after incubation 100 μl ASChI/DTNB was added and absorbance recorded. Percentage of inhibition after preincubation was calculated as: 100 – (AChE activity with inhibitor/AChE activity without inhibitor) × 100. Three experiments, each with freshly prepared homogenates, ASChI, DTNB and eserine solutions, were conducted in the AChE assays; the methiocarb and dichlorvos solutions used were prepared from stock solutions of the insecticides dissolved in acetone. Duplicate determinations were done in each experiment.

Protein content in homogenates was determined by the method of Bradford (1976), as modified in the Pierce protein assay protocol for microplates, with bovine serum albumin as a standard.

Statistical Analysis. Bioassay results were analyzed by probit analysis (Finney 1971) with the SAS PROC PROBIT procedure (SAS Institute 1990); the OPTC option in the procedure was used to correct for control mortality. Data from the enzyme assays were subjected to analysis of variance (ANOVA) followed by Tukey's multiple comparison test (Zar 1996). Correlations between activities of different enzymes and between enzyme activities and methiocarb resistance were tested by Pearson product-moment correlation analysis (Zar 1996). Significance level, α =

0.05. The sequentially rejective Bonferroni test procedure by Holm (1979) was used to adjust the significance level in correlations between enzyme activities and methiocarb resistance.

Results

Insecticide Bioassays. Results of methiocarb bioassays on the different *F. occidentalis* populations are summarized in Table 2. The resistance ratio at the LC_{50} ranged from 3.2- to 30-fold and at the LC_{90} from 2.1- to 34-fold in the six resistant populations compared with the susceptible UCD90 population. Probit analysis on the data for methiocarb resistance in the AlDK98 population showed that the data did not fit the probit model ($P = 0.011$). The lack-of-fit indicated that this population was heterogeneous with respect to methiocarb resistance.

When PBO was included in the methiocarb bioassays, resistance ratios were lowered in the two most resistant populations, DKOdH-IV and AlDK98; PBO also lowered the resistance ratio at LC_{90} in the LiDK98 population. DEF likewise lowered methiocarb resistance ratios in the DKOdH-IV, LiDK98, and AlDK98 populations. Neither PBO nor DEF completely suppressed methiocarb resistance in the affected populations.

Enzyme Assays. Activities of general esterases, GSTs and AChE were measured in adult *F. occidentalis* of the different populations (Table 3). General esterase activities toward α-NA and ß-NA in the LiDK98 and AlDK98 populations were significantly higher than those measured in the other populations, including the susceptible UCD90 population. GST activities toward CDNB and DCNB were significantly higher in the AlDK98 population and significantly lower in the DKOdH96 population. However, selection with methiocarb on the DKOdH96 population significantly increased the level of GST activity as measured in the DKOdH-III and DKOdH-IV populations. AChE activities were significantly higher in the DKOdH-III and DKOdH-IV populations than found in the susceptible UCD90 population. Selection with methiocarb on the DKOdH96 population increased AChE activity; a 1.9-fold increase was measured in the DKOdH-III population. Further selection with methiocarb on the DKOdH-III population decreased the level of AChE activity although the level was still higher in the DKOdH-IV population than in the DKOdH96 population (Table 3). Activities of general esterases, GSTs or AChE in the different populations were not correlated with the level of methiocarb resistance in the populations.

General esterase and GST activities in the populations showed a positive correlation: populations that had a higher level of general esterase activity also tended to have a higher level of GST activity (Table 3). Both CDNB activity versus both naphthyl acetate activities ($r = 0.76$) and DCNB activity versus both naphthyl acetate activities ($r = 0.79$) were correlated (df = 5, $P < 0.05$ for all correlations). Neither the level of general esterase activity nor GST activity was correlated with the level of AChE activity in the populations.

Inhibition of AChE activity in the populations was assayed after preincubating thrips homogenates with methiocarb, dichlorvos, or eserine (Table 4). In two of the populations, LiDK98 and AlDK98, AChE activities were significantly less inhibited by the three inhibitors

than found in the susceptible UCD90 population. AChE activity of the resistant population DKOdH-III was significantly more inhibited by the three inhibitors than AChE activity of the susceptible population. Correlation analyses showed that the relative AChE inhibition was not correlated with the level of methiocarb resistance in the populations. Significant positive correlation was found between uninhibited AChE activity and AChE inhibition of the populations (Fig. 1): populations with a higher basal level of AChE activity generally showed a higher degree of AChE inhibition in assays with the three inhibitors.

Discussion

Resistance to methiocarb was evident in the field-collected and in the laboratory-selected populations of *F. occidentalis*. By selection with methiocarb on the susceptible population and on the resistant population DKOdH96, the level of methiocarb resistance was increased. The DKOdH96 population was previously more than 20-fold resistant to methiocarb (Jensen 1997) but in this study, 2 yr later, the resistance had declined to ≈3-fold in the absence of exposure to pesticides. However, the high level of resistance could be restored after successive selection with methiocarb to obtain the DKOdH-IV population. Decline in resistance in a pesticide free environment is expected as resistant alleles often are associated with fitness cost for the carrier (Roush and Daly 1990). When carrying resistant alleles associated with high fitness cost, the decline in resistance should be fast in *F. occidentalis* because of the short generation time, high fecundity, and haplodiploid breeding system.

Methiocarb bioassays with PBO, a monooxygenase inhibitor, or DEF, an esterase inhibitor, lowered the level of methiocarb resistance in the three most resistant populations. These results indicated involvement of cytochrome P-450-dependent monooxygenases and esterases in resistance. Methiocarb bioassays without synergists indicated that the most resistant population, AlDK98, was genetically heterogeneous with respect to methiocarb resistance. Including the synergists PBO or DEF in the methiocarb bioassays made the data for methiocarb resistance in AlDK98 fit the probit model. As both synergists lowered the level of methiocarb resistance in AlDK98, this population was probably heterogeneous with respect to expression of cytochrome P-450-dependent monooxygenases and esterases contributing to methiocarb resistance. At lower levels of resistance, bioassays with the synergists PBO or DEF did not indicate involvement of cytochrome P-450-dependent monooxygenases or esterases in methiocarb resistance. However, lack of synergism by a known enzymatic inhibitor does not rule out the involvement of the metabolic pathway in resistance (Scott 1990).

The resistant populations LiDK98 and AlDK98 showed increased esterase activities compared to the susceptible population and in both populations methiocarb bioassays with DEF indicated involvement of esterases in resistance. Methiocarb bioassays with DEF also indicated esterase-based resistance in the DKOdH-IV population, but this population had the same esterase activity as found in the susceptible population. Esterase-based resistance in insects is caused by specific isozymes hydrolyzing or sequestering the insecticides (Soderland and Bloomquist 1990).

The activity of these specific isozymes toward model substrates may well be masked by activity of esterases not related to resistance, thus assays of general esterase activity may not correlate with the level of resistance.

GSTs are another group of detoxification enzymes often reported to be involved in insecticide resistance, especially to organophosphates (Soderland and Bloomquist 1990). In this study, the most resistant population, AlDK98, had significantly higher GST activities compared to the susceptible population and methiocarb selection on the DKOdH96 population increased GST activities in the DKOdH-III and DKOdH-IV populations. However, in the multiple comparison analysis only two populations were significantly different from the susceptible population. One of these populations, DKOdH96, had a lower level of activity to CDNB although this population was slightly more tolerant of methiocarb toxicity. As with general esterase activities, increased activity of GST isozymes specifically associated with resistance may be masked by GST activities not related to resistance in assays with model substrates (Soderland and Bloomquist 1990).

Insensivity of AChE to inhibition by organophosphates and carbamates is a common target site resistance mechanism in insects (Soderland and Bloomquist 1990). In the LiDK98 and AlDK98 populations, the AChE activity was significantly less inhibited by all three inhibitors compared with the susceptible UCD90 population. Consequently, insensitive AChE may contribute to resistance in these two populations. Interestingly, a positive correlation was found between relative AChE inhibition and the basal levels of AChE activity in the populations. This correlation indicated the presence of AChE with different catalytic properties in the populations. Modification of AChE to less sensitivity to inhibition by insecticides often results in altered kinetic properties of the enzyme decreasing AChE activity in the insects (Fournier and Mutero 1994). The correlation between relative AChE inhibition and the basal levels of AChE activity in the populations was probably not an artifact caused by interpopulation differences in AChE activity. In a previous study on the UCD90 and DKOdH96 populations, adjusting to equal AChE activity in homogenate samples of the two populations did not affect the relative inhibition by methiocarb and dichlorvos (Jensen 1998). The DKOdH96 population had then 2.6-fold higher AChE activity as UCD90. Since the previous study on DKOdH96 (Jensen 1998), the methiocarb resistance and AChE activity of the population have declined. Selection on the population to increase its methiocarb resistance was accompanied by an increase in the level of AChE activity. This result confirmed that increased AChE activity was associated with methiocarb resistance in the DKOdH populations. As discussed in Jensen (1998), increased AChE activity is a possible resistance mechanism. Thus, two different mechanisms for resistance at the molecular target AChE may be possible in *F. occidentalis*. One is increased AChE activity as suggested for the DKOdH populations, the second is insensitive AChE as suggested for the LiDK98 and AlDK98 populations and shown in a diazinon resistant population of *F. occidentalis* (Zhao et al. 1994).

Biochemical diagnostic assays for resistance can be useful tools in monitoring for resistance. These assays can be fast, give information on resistance mechanisms and level, and can be done on single insects or even fractions of single insects to allow different biochemical

assays to be done on the same individual (Brown and Brogdon 1987). Examples of simple, rapid biochemical assays used in detecting resistance are assays for insensitive AChE in the aphid *Myzus persicae* (Sulzer) (Moores et al. 1994), the whitefly *Bemisia tabaci* (Gennadius) (Byrne and Devonshire 1993), and the noctuid *Heliothis virescens* (F.) (Brown et al. 1996) and assays for increased esterase activity in *Myzus persicae* (Devonshire et al. 1992) and the mosquito *Anopheles albimanus* (Wiedemann) (Brogdon et al. 1988). None of the simple biochemical assays used in this study showed a strong correlation between enzymatic differences and level of methiocarb resistance in *F. occidentalis*. In the biochemical assays of AChE, neither the relative inhibition by insecticides nor the basal level of AChE activity could be used to detect methiocarb resistance in all the resistant populations. In the biochemical assays of esterases or GSTs, simple assays with model substrates may not be sufficiently specific to detect resistance in *F. occidentalis*. Further studies on esterases or GSTs could be directed toward studying isozyme polymorphisms to look for resistance markers. However, more sophisticated biochemical assays are generally more laborious and costly, thereby have diminished usefulness in routine monitoring for resistance. Simple biochemical assays for cytochrome P-450-dependent monooxygenase activity can be difficult to implement on small insects such as thrips (Price 1991). However, the bioassays with PBO suggested involvement of monooxygenases in methiocarb resistance in *F. occidentalis* and studies on North American populations of *F. occidentalis* have also indicated involvement of monooxygenases in resistance (Immaraju et al. 1992, Zhao et al. 1995a). Thus, development of an assay for monooxygenase activity in *F. occidentalis* may have practical value in detecting resistance.

To summarize, enhanced detoxification by cytochrome P-450-dependent monooxygenases, esterases and possibly GSTs, insensitive AChE and increased AChE activity are all possible mechanisms conferring methiocarb resistance in *F. occidentalis*. Thus, methiocarb resistance in *F. occidentalis* appeared to be polyfactorial. The enzymatic assays used in this study were rapid and sensitive assays that have been used in detection of resistance in other pest species (ffrench-Constant and Roush 1990). The potential of these assays in routine monitoring for methiocarb resistance in *F. occidentalis* appeared to be modest as none of the biochemical assays correlated strongly and consistently with the level of resistance in the different populations.

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Table 1. Origin of *F. occidentalis* populations

Population[a]	Field collected (host plant)	Selected in the laboratory[b]	Derived from population[c]
UCD90 (S)	June 1990 (*Rosa* sp.)	-	-
UCD-II (R)	-	April 1998 and Jan. 1999 (2)	UCD90
DKOdH96 (R)	May 1996 (*Hibiscus* sp.)	-	-
DKOdH-III (R)	-	April, May, and Oct. 1998 (3)	DKOdH96
DKOdH-IV (R)	-	April, May, Oct. 1998, and Jan. 1999 (4)	DKOdH96
LiDK98 (R)	Dec. 1998 (*Hedera helix*)	-	-
AIDK98 (R)	Sept. 1998 (*Rosa* sp.)	-	-

[a] S, susceptible; R, resistant to methiocarb.

[b] Number of selection steps with methiocarb in parentheses.

[c] Origin of the methiocarb selected populations.

Table 2. Toxicity of methiocarb, methiocarb mixed with 50 ppm PBO, and methiocarb mixed with 50 ppm DEF to populations of adult *F. occidentalis* exposed 24 h in a residual assay

Treatment	Population	N[a]	Slope (SE)	LC_{50} (95% FL)[b]	LC_{90} (95% FL)[b]	χ^2 (df)[c]	RR_{50} (95% CL)[d]	RR_{90} (95% CL)[d]
Methiocarb								
	UCD90	80	2.12 (0.17)	0.810 (0.689–0.934)	3.27 (2.70–4.16)	5.66 (4)	-	-
	UCD-II	40	2.24 (0.24)	3.14 (2.29-4.03)	11.7 (9.22-15.7)	5.18 (3)	3.9 (2.8-5.3)	3.6 (2.6-5.1)
	DKOdH96	34	3.01 (0.28)	2.60 (2.26-2.98)	6.94 (5.76-8.91)	3.40 (4)	3.2 (2.6-4.0)	2.1 (1.6-2.9)
	DKOdH-III	59	2.15 (0.17)	7.97 (6.82-9.18)	31.4 (25.9-40.1)	6.79 (5)	9.8 (7.9-12)	9.6 (7.1-13)
	DKOdH-IV	40	2.00 (0.20)	18.5 (15.4-22.2)	80.9 (60.3-122)	5.71 (4)	23 (18-29)	25 (16-37)
	LiDK98	35	1.67 (0.20)	9.14 (7.07-11.5)	53.3 (37.4-90.8)	5.05 (3)	11 (8.5-15)	16 (10-26)
	AIDK98	78	1.95 (0.25)	24.6 (17.8-32.7)	111 (75.1-214)	18.2 (7)*	30 (23-41)	34 (21-54)
Methiocarb +PBO	UCD90	80	2.15 (0.17)	0.529 (0.449-0.612)	2.09 (1.73-2.65)	5.76 (3)	-	-
	UCD-II	20	2.54 (0.35)	1.67 (1.16-2.21)	5.35 (4.03-7.82)	3.98 (3)	3.2 (2.2-4.5)	2.6 (1.7-3.8)
	DKOdH96	20	2.17 (0.34)	1.74 (1.30-2.26)	6.78 (4.66-12.8)	5.61 (3)	3.3 (2.4-4.5)	3.2 (1.9-5.4)
	DKOdH-III	55	1.85 (0.16)	4.88 (4.12-5.74)	24.1 (18.5-34.3)	5.67 (3)	9.2 (7.3-12)	12 (8.0-17)
	DKOdH-IV	40	2.70 (0.26)	5.80 (4.88-6.75)	17.3 (14.3-22.2)	2.69 (3)	11 (8.8-14)	8.3 (6.1-11)
	LiDK98	20	2.56 (0.39)	5.11 (3.81-6.47)	16.2 (12.1-25.6)	2.91 (3)	9.7 (7.2-13)	7.8 (5.1-12)
	AIDK98	55	1.51 (0.16)	4.84 (3.67-6.04)	34.0 (25.2-51.7)	3.35 (5)	9.1 (6.8-12)	16 (11-25)
Methiocarb +DEF	UCD90	20	2.23 (0.44)	0.734 (0.468-0.996)	2.76 (1.91-5.49)	0.620 (3)	-	-
	UCD-II	20	2.34 (0.38)	2.79 (1.89-3.69)	9.83 (7.22-15.9)	1.96 (3)	3.8 (2.4-6.1)	3.6 (1.9-6.5)
	DKOdH96	40	1.82 (0.29)	1.59 (1.03-2.12)	7.99 (5.75-13.6)	5.72 (3)	2.2 (1.3-3.5)	2.9 (1.6-5.4)
	DKOdH-III	20	2.09 (0.37)	8.33 (6.20-11.0)	34.1 (22.5-74.5)	1.06 (3)	11 (7.3-18)	12 (6.0-25)
	DKOdH-IV	20	2.29 (0.30)	8.95 (7.04-11.2)	32.4 (23.8-51.7)	3.76 (3)	12 (8.0-18)	12 (6.4-21)
	LiDK98	20	2.03 (0.28)	5.72 (4.44-7.32)	24.4 (16.9-43.7)	4.32 (3)	7.8 (5.1-12)	8.8 (4.6-17)
	AIDK98	33	2.21 (0.28)	15.2 (12.0-18.7)	58.0 (43.4-89.4)	0.919 (4)	21 (14-31)	21 (12-38)

[a] Number of methiocarb coated vials (c. 10 thrips per vial).

[b] LC values in ppm (95% fiducial limits).

[c] Pearson χ^2 statistic (degrees of freedom); *, departure from the expected model ($P < 0.05$).

[d] Resistance ratio at the LC_{50} and LC_{90} with 95% confidence limits calculated by the method of Robertson and Preisler (1992).

Table 3. Enzyme activity (mean ± SEM) of general esterases, glutathione *S*-transferases (GST) and acetylcholinesterase (AChE) in populations of *F. occidentalis*

Population	Esterase[a]		GST[b]		AChE[c]
	α-NA	ß-NA	CDNB	DCNB	ASChI
UCD90	2.60 ± 0.40b	2.94 ± 0.46b	417 ± 16.3b	13.5 ± 0.43b	49.0 ± 6.06cd
UCD-II	2.53 ± 0.23b	2.91 ± 0.29b	468 ± 30.6b	15.6 ± 1.70b	41.0 ± 2.16cd
DKOdH96	1.64 ± 0.30b	1.81 ± 0.30b	289 ± 4.9c	7.39 ± 0.38c	54.1 ± 2.02c
DKOdH-III	1.79 ± 0.24b	2.00 ± 0.23b	502 ± 39.6b	17.1 ± 2.05b	101 ± 3.87a
DKOdH-IV	2.54 ± 0.45b	2.84 ± 0.47b	472 ± 13.9b	14.9 ± 1.00b	79.4 ± 6.63b
LiDK98	5.54 ± 0.54a	6.11 ± 0.72a	514 ± 3.3b	18.4 ± 0.40ab	37.4 ± 4.94cd
AIDK98	6.03 ± 0.16a	6.61 ± 0.23a	663 ± 14.3a	24.1 ± 1.18a	29.7 ± 3.70d

Mean activity ± SEM of three experiments each with duplicate determinations. Means followed by the same letter within a column are not significantly different (Tukey multiple comparison test, $\alpha = 0.05$).

[a] α-Naphthyl acetate (α-NA) and ß-naphthyl acetate (ß-NA) as substrates (nmol min^{-1} µg $protein^{-1}$).

[b] 1-Chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) as substrates (nmol min^{-1} mg $protein^{-1}$).

[c] Acetylthiocholine iodide (ASChI) as substrate (nmol min^{-1} mg $protein^{-1}$).

Table 4. Percent inhibition (mean ± SEM) of AChE activity in populations of *F. occidentalis* after preincubation with methiocarb (20 μM), dichlorvos (20 μM), or eserine (1.0 μM)

Population	Methiocarb	Dichlorvos	Eserine
UCD90	89.5 ± 0.72cd	90.9 ± 0.64cd	87.5 ± 0.30bc
UCD-II	87.5 ± 0.67bc	89.3 ± 0.63bc	85.6 ± 0.23ab
DKOdH96	91.4 ± 0.32cde	93.0 ± 0.19de	89.2 ± 0.05c
DKOdH-III	94.6 ± 0.22e	95.7 ± 0.07e	90.2 ± 0.14c
DKOdH-IV	92.5 ± 0.64de	94.1 ± 0.44de	88.6 ± 0.54bc
LiDK98	83.3 ± 2.13ab	86.8 ± 1.33ab	82.8 ± 1.18a
AlDK98	82.6 ± 0.94a	85.0 ± 1.07a	83.3 ± 1.24a

Mean percentage of inhibition ± SEM of three experiments each with duplicate determinations. Means followed by the same letter within a column are not significantly different (Tukey multiple comparison test, $\alpha = 0.05$).

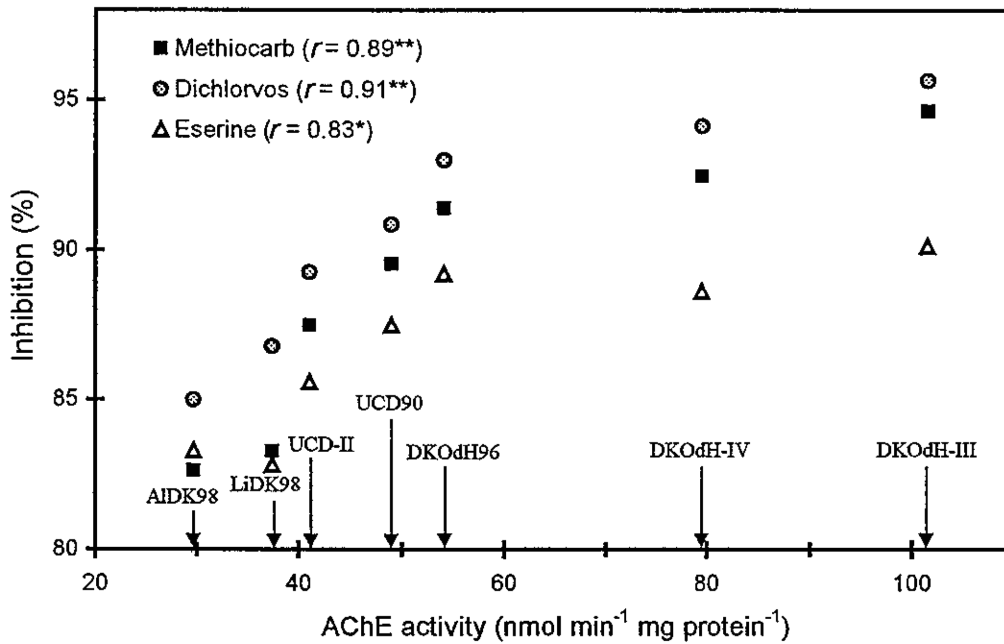

Fig. 1. Relationship between uninhibited AChE activity and relative inhibition after preincubation with methiocarb (20 μM), dichlorvos (20 μM) and eserine (1.0 μM) in seven *F. occidentalis* populations (r, correlation coefficient; *, $P < 0.05$; ** $P < 0.01$; $n = 7$). Arrows indicate the level of uninhibited AChE activity in the populations.

Host Plant Effects on Activity of Detoxification Enzymes and Insecticide Tolerance in Western Flower Thrips, *Frankliniella occidentalis* (Insecta)

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Summary

The polyphagous western flower thrips, *Frankliniella occidentalis*, is a severe pest of horticultural crops. Individuals from a laboratory population adapted to bean plants, were transferred to new host plants, sweet pepper or chrysanthemum, to establish two new populations. The thrips appeared to perform poorly on the new host plants as the total protein content of individual adults was lowered in the new populations. The specific activities of two insect detoxification enzyme systems, esterases and glutathione *S*-transferases, were assayed *in vitro* in the three populations. Host plant shifts had no effect on the level of general esterase activity to α-naphthyl acetate and only a minor effect on the level of glutathione *S*-transferase activity to 1-chloro-2,4-dinitrobenzene. The new population on pepper plants had slightly lowered glutathione *S*-transferase activity. The level of tolerance to the insecticide methiocarb was not affected by culturing the thrips on new host plants, nor was the total activity per individual of acetylcholinesterase, the target site enzyme for methiocarb.

Keywords: Frankliniella occidentalis, detoxification enzymes, host plant shift, insecticide tolerance, esterase, glutathione S-transferase

Introduction

Insect enzymes involved in detoxification of xenobiotics are important for insects to survive in a chemically unfriendly environment. The ability of herbivorous insects to detoxify plant allelochemicals is a basic determinant for the selection and range of host plants (1). Furthermore, many insect herbivores are pests of agricultural crops where they may be exposed to synthetic insecticides. Detoxification of insecticides is one important mechanism for insects to tolerate applied insecticides (2).

Generally three enzyme systems are recognised in detoxification of xenobiotics in insects. These are esterases, glutathione *S*-transferases (GST) and cytochrome P450-dependent monooxygenases (3). In all three enzyme systems, multiple forms of the enzymes exist and may metabolise many different substances. Enzymes of the three systems may be inducible in individual insects upon exposure to xenobiotics such as plant allelochemicals or insecticides (3). Furthermore, in the presence of a toxic agent, higher basal level of enzyme activity of one or all of the detoxification systems can evolve in a population. Several examples of higher activity of detoxification enzymes are described in populations resistant to insecticides (2). Studies on induction of detoxification enzymes caused by host plant allelochemicals suggest that insects with induced detoxification enzymes may metabolise insecticides faster than non-induced insects (3). Hence, tolerance of host plant allelochemicals and insecticides may be related.

The western flower thrips, *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae), is a serious pest on a wide range of crops throughout the world (4). The polyphagous nature of the western flower thrips may have predisposed it to detoxify xenobiotics such as plant allelochemicals in the different host plants and insecticides applied as control agents (5). Studies on insecticide resistance in the western flower thrips have indicated involvement of esterases, GST and cytochrome P450-dependent monooxygenases in detoxification of insecticides (6-8).

The aim of the present study was to examine possible effects of host plant shifts on activity of the detoxification enzymes esterases and GST in western flower thrips. The host plant induced detoxification responses in insects may contribute to information on intrinsic resistance in host plants to insect pests. Assays of detoxification enzymes may also give information on the level of tolerance to insecticides and are useful tools in monitoring for resistance to insecticides in insect populations (9). The biochemical assays used in resistance monitoring require a much lower number of insects to be tested than the traditional bioassays; however, the practical value of these assays may be limited if the enzyme activity assayed is affected by the recent host plant of the population. Furthermore, in case of a host plant induced effect on activity of detoxification enzymes, an effect on insecticide tolerance may also show. Therefore, tolerance to a common insecticide used for thrips control, methiocarb, and the level of activity of the target site enzyme for methiocarb, acetylcholinesterase (AChE), were also examined in the thrips populations cultured on different host plants.

Materials and Methods

Insects

Populations of western flower thrips cultured on three different host plants were studied. Originally, a population of western flower thrips was established in the laboratory in 1990 (10). This population, named UCD90-Bean, has been cultured on bean plants (*Phaseolus vulgaris* L.; last two years on cv. Montano) since establishment. Adult thrips from the UCD90-Bean population were transferred to sweet pepper plants (*Capsicum annuum* L. cv. California Wonder) or chrysanthemums (*Dendranthema grandiflora* Tzvelev cv. Purple Cindy) to start two new populations. The population cultured on pepper was named UCD-Pepper and the population cultured on chrysanthemum was named UCD-Mum. The two new populations were propagated on the new host plants for at least 1½-4½ months until collected for assays. UCD90-Bean and UCD-Pepper were cultured at 22°C and UCD-Mum was cultured at 25°C.

Enzyme and protein assays in vitro

All incubations and measurements in the enzyme assays were made at 21-23°C and absorbance was recorded using a Multiskan MS microplate reader (Labsystems, Finland).

For assays *in vitro*, adult thrips stored at -80°C after collection were used. They were placed individually in buffer (cf. below) in wells of a microplate and homogenised with a multiple homogeniser (Buckard Scientific, UK). For esterase and AChE assays, thrips were homogenised in 0.1 M sodium phosphate buffer (pH 7.5) containing 0.01% Triton X-100 and for GST assays, thrips were homogenised in 0.1 M sodium phosphate buffer (pH 6.5).

General esterase activity was measured with α-naphthyl acetate as substrate in individual thrips by the method of van Asperen (11) modified for a microplate as described by Jensen (8). Briefly, homogenate (equivalent to 0.125 thrips) was incubated with α-naphthyl acetate (0.75 mM final concentration) and after 5 min the reaction was ended by adding *o*-dianisidine (0.1% final concentration) and sodium dodecyl sulphate (1.0% final concentration). Absorbance was read at 600 nm.

GST activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate in individual thrips as described by Habig *et al.* (12) and modified for a microplate. Homogenate (90 µl) equivalent to 0.45 thrips was added per well in a microplate and 90 µl of CDNB (1.0 mM final concentration) and reduced glutathione (5.0 mM final concentration) in 0.1 M sodium phosphate buffer (pH 6.5) was added to each well. The microplate was left for 2 min to equilibrate and then absorbance at 340 nm was recorded continuously. An extinction coefficient of 9.6 mM^{-1} cm^{-1} (12) was used to convert change in absorbance to rate of conjugate formation.

AChE activity was measured with acetylthiocholine iodide as substrate in individual thrips by the method of Ellman *et al.* (13) modified for a microplate as described by Jensen (8). Briefly, homogenate (equivalent to 0.45 thrips) was incubated with acetylthiocholine iodide (0.5 mM final

concentration) and 5,5'-dithio-bis(2-nitrobenzoic acid) (0.05 mM final concentration) and absorbance recorded continuously at 405 nm.

Protein content in homogenates was determined by the method of Bradford (14), as modified in the Pierce (Rockford, IL, USA) protein assay protocol for microplates, with bovine serum albumin as a standard.

Assay of methiocarb tolerance

Solutions of methiocarb in acetone were made to coat the inner surface of 10 ml polypropylene vials (Nunc, Denmark). Methiocarb solution (500 μl) was added to the vials containing a 1 cm^2 Whatman no. 1 filter paper. The vials with filter paper were then rotated on a Roto-torque rotator (Cole-Parmer Instrument, Vernon Hills, IL, USA) until the acetone had evaporated. Adult thrips were transferred to the coated vials (10 thrips per vial), the coated filter papers were wetted with 8 μl water (MilliQ deionised) and placed in their vials. The vials were closed with a cap and incubated at 22°C with a 16:8 (L:D) h photoperiod. Mortality (no response to probing) was recorded after 24 h. For each population, at least five concentrations of methiocarb were assayed and each concentration was assayed with at least four coated vials with 10 individuals per vial; control vials treated with only acetone were included in each assay.

Statistical analysis

Data from the enzyme assays were subjected to analysis of variance followed by Tukey multiple comparison test (15). If considerable deviations from normality or equal variances in the populations, the nonparametric Kruskal-Wallis analysis of variance was employed (15).

Bioassay results were analysed by probit analysis (16) with the SAS PROC PROBIT procedure (17); the OPTC option in the procedure was used to correct for control mortality. Parallelism between probit lines was tested by the Z-test to test for differences between slopes (18). The 95% confidence limits for the ratio of LC_{50} values were calculated by the method of Robertson and Preisler (19); if the 95% confidence interval for the ratio includes 1, the LC_{50} values for the two populations compared are not considered significantly different (19).

Results

Activities of esterases, GST and AChE were measured in individual adult thrips of the populations cultured on three different host plants (Table I). Esterase activities toward α-naphthyl acetate were not significantly different in the three populations (Kruskal-Wallis ANOVA; $p = 0.060$). GST activities toward CDNB were significantly lower in UCD-Pepper compared to UCD90-Bean (Tukey multiple comparison test; $p < 0.05$); although the UCD-Mum population also showed lower GST activity compared to UCD90-Bean, this difference was not significant. The specific AChE activity in UCD-Pepper was significantly higher than measured in the two

other populations (Table I). However, the level of total protein differed in the three populations so when AChE activities were expressed as total activity per individual, the three populations were not significantly different (ANOVA; $p = 0.39$). Total AChE activity per individual was 0.131 ± 0.008, 0.116 ± 0.007 and 0.120 ± 0.008 nmol/min/individual (± SEM) for UCD90-Bean, UCD-Pepper and UCD-Mum, respectively ($n = 72$ thrips for each population). For each individual thrips assayed in the enzyme assays, the protein concentration of the homogenised thrips was determined. All three populations differed significantly with respect to the amount of total protein per individual (Table I).

Results of the assays for tolerance to methiocarb in the different thrips populations are summarised in Table II. At the LC_{50}, the level of tolerance to methiocarb in UCD-Pepper and UCD-Mum were not different from the level of tolerance in UCD90-Bean. The 95% confidence intervals for the ratio included 1 for LC_{50}'s of UCD-Pepper/UCD90-Bean and UCD-Mum/UCD90-Bean. The slopes of the probit lines for UCD-Pepper and UCD-Mum were not different from the probit line slope for UCD90-Bean. Comparing the slope of the UCD-Pepper line to that of the UCD90-Bean line gave $p = 0.053$ ($Z = 1.94$) and comparing the slope of the UCD-Mum line to that of the UCD90-Bean line gave $p = 0.47$ ($Z = 0.728$).

Discussion

In this study, western flower thrips from a laboratory population cultured on bean plants for nine years were transferred to new host plants. New populations were established on sweet pepper and chrysanthemum. The adult thrips used in the assays had completed a full life cycle on their host plants. All three populations differed with respect to the level of total protein of individual thrips. Both populations cultured on new host plants had lowered level of total protein, i.e. smaller individuals. Recent studies have shown that western flower thrips cultured on thrips resistant cultivars of cucumber are smaller and have lower reproduction than when cultured on a susceptible cucumber cultivar (20, 21). Thus, the smaller individuals of the pepper and chrysanthemum populations may indicate that the thrips adapted to bean plants performed poorer when cultured on the new host plants.

Although the thrips appeared to perform poorly when cultured on new host plants, no large effects were evident on specific activity of the detoxification enzyme systems esterases and GST. The esterase activity did not differ between the three populations. So either this enzyme system was not affected by the host plant shift or the assay employed was not able to detect the response. Esterase activity was assayed with α-naphthyl acetate as substrate. α-Naphthyl acetate is a commonly used substrate for measuring esterase activity associated with detoxification in insects. Esterase-based detoxification in insects is caused by specific isozymes hydrolysing the toxicant (2). So, as naphthyl acetate is a general substrate for a variety of esterases (9), the general esterase activity in the populations may mask any specific esterase responses. GST activity was assayed

with the general model substrate CDNB. The GST activity in the new population cultured on pepper plants was slightly lower than in the original population on bean plants. So, bean plants may have induced higher GST activity in the thrips or pepper plants inhibited GST activity in the thrips. Plant allelochemicals have been reported to induce as well as inhibit GST activity in insects (e.g. 22, 23).

AChE is the target site enzyme for the carbamate insecticide methiocarb (8). Specific AChE activity expressed as activity per μg protein of the thrips homogenate was markedly higher in the population cultured on pepper plants. However, because the populations differed in total protein per individual thrips, no difference was found when AChE activity was expressed as total activity per individual. As AChE is mainly located in the central nervous system of insects (24), our findings indicate that the nervous system was equally developed in adults of the three populations and not affected by the host plant shifts. Increased level of AChE activity or detoxification are possible mechanisms associated with increased tolerance to methiocarb in western flower thrips (8). The host plant shifts did not affect the level of methiocarb tolerance in the new populations. A result in accordance with the findings of no major effects on esterase or GST activity or total AChE activity in the new populations. Whether further adaptation to the new host plants gives larger effects on the detoxification enzymes studied or methiocarb tolerance, warrants further investigation. For further studies, development of an assay for cytochrome P450-dependent monooxygenase activity in western flower thrips would be valuable to examine possible effects on this detoxification system.

Acknowledgments

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Table I: Enzyme activity of esterases, glutathione *S*-transferases (GST) and acetylcholinesterase (AChE) and total protein of individual thrips of populations cultured on different host plants

Population	Esterase[a] (nmol/min/µg protein)	GST[b] (nmol/min/mg protein)	AChE[c] (nmol/min/mg protein)	Total protein[d] (µg/thrips)
UCD90-Bean	1.72 ± 0.21a	252 ± 10.0a	29.6 ± 1.93a	4.52 ± 0.085a
UCD-Pepper	1.83 ± 0.27a	215 ± 11.4b	48.2 ± 3.64b	2.56 ± 0.064c
UCD-Mum	1.96 ± 0.28a	223 ± 10.6ab	30.2 ± 2.42a	3.30 ± 0.072b

Mean activity ± SEM. Means followed by the same letter within a column are not significantly different (Tukey multiple comparison test, $\alpha = 0.05$).

[a] *α-naphthyl acetate as substrate; n = 104 for each population.*

[b] *1-chloro-2,4-dinitrobenzene as substrate; n = 66, 70 and 71 for UCD90-Bean, UCD-Pepper and UCD-Mum, respectively.*

[c] *acetylthiocholine iodide as substrate; n = 72 for each population.*

[d] *protein concentration determined in homogenates of individual thrips; n = 242, 246 and 247 for UCD90-Bean, UCD-Pepper and UCD-Mum, respectively.*

Table II: Methiocarb tolerance of thrips populations cultured on different host plants

Population	n[a]	Slope (SE)	LC_{50} (95% FL)[b]	χ^2 (df)[c]	$Ratio_{50}$ (95% CL)[d]
UCD90-Bean	800	2.12 (0.17)	0.810 (0.689–0.934)	5.66 (4)	-
UCD-Pepper	219	1.56 (0.23)	1.03 (0.753-1.43)	4.22 (4)	1.3 (0.90-1.8)
UCD-Mum	213	1.86 (0.31)	0.722 (0.351-1.15)	2.28 (3)	0.89 (0.50-1.6)

[a] *Number of insects on which the probit analysis was performed.*

[b] *LC values in ppm (95% fiducial limits).*

[c] *Pearson χ^2 statistic (degrees of freedom); goodness-of-fit of the probit analysis.*

[d] *Ratio of UCD-Pepper/UCD90-Bean or UCD-Mum/UCD90-Bean at the LC_{50} with 95% confidence limits.*

Ministry of Food, Agriculture and Fisheries
Danish Institute of Agricultural Sciences
Department of Crop Protection

To whom it might concern

21 January 2000

Re. joint manuscript entitled 'Host plant effects on activity of detoxification enzymes and insecticide tolerance in western flower thrips, Frankliniella occidentalis *(Insecta)'*

The planning of the experiment was done in co-operation between the two authors Sten E. Jensen and Henrik F. Brødsgaard.

Sten E. Jensen carried out the experiment, analysed the data, presented the results at the 17th SSCT workshop, and wrote the manuscript. Henrik F. Brødsgaard made critical comments to the manuscript.

Yours sincerely

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SP rapport no. 8 (1997), pp. 159-167.

14. Danske Planteværnskonference 1997
Sygdomme og skadedyr

Påvisning af insekticidresistens med biokemiske metoder

Detection of insectide resistance with biochemical methods

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Summary

Mechanisms and level of insecticide resistance can be studied by biochemical methods. The biochemical assays are applied on the mechanism that confer resistance in the insect, e.g. the activity of an enzyme involved in resistance can be determined.

In this study, the western flower thrips (*Frankliniella occidentalis*) is used as model organism to test biochemical assays for assessment of insecticide resistance. Assays for the enzymes carboxylesterase and cholinesterase are tested.

A resistant strain of *F. occidentalis* showed elevated esterase activity, the carboxylesterase activity was 1.5 times higher and the cholinesterase activity was 2.6 times higher than found in an insecticide susceptible strain; increaced activity of both enzymes can give resistance. On the other hand, the cholinesterase activity in the susceptible strain was more resistant to inhibition with the insecticide methiocarb than was cholinesterase from the resistant strain; however, because of the higher cholinesterase activity in the resistant strain it had to be inhibited with 20 μM methiocarb in the assay before the cholinesterase activity was at the same level in the two strains. The results indicate that elevated esterase activity contributes to organophosphate/carbamate resistance in the insectide tolerant strain of *F. occidentalis*.

SukkerroeNyt no. 2 (1999), p. 18.

Ny mekanisme giver ferskenbladlus resistens mod Perimor (pirimicarb)

Af kandidatstipendiat Steen Erik Jensen, Danmarks JordbrugsForskning - Flakkebjerg

Brug af insekticider spiller en vigtig rolle ved bekæmpelsen af bladlusangreb. Imidlertid kan behandling med insekticider føre til udvikling af resistens overfor insekticiderne – et fænomen som er velkendt fra ferskenbladlus. Den væsentligste resistensmekanisme hos ferskenbladlus skyldes et bestemt enzym, et esterase-enzym, som giver ferskenbladlus bred resistens overfor mange forskellige insekticider. Et højt niveau af dette esterase-enzym giver ferskenbladlus en høj grad af resistens overfor organofosfat-midler og pyrethroider, og det giver også en mindre grad af resistens mod carbamat-insekticider heriblandt bladlus-midlet Pirimor med aktivstoffet pirimicarb.

Indtil for nylig var den eneste kendte resistensmekanisme i ferskenbladlus ovennævnte esterase-enzym. I 1990 blev der imidlertid fundet en ny resistensmekanisme i ferskenbladlus i Grækenland, en resistensmekanisme, som specifikt giver resistens over for aktivstofferne pirimicarb (Pirimor) og triazamate (Aztec).

Denne resistensmekanisme skyldes en ændring på det sted i nervesystemet, hvor pirimicarb og triazamate skal virke i bladlusene. Det er en genetisk bestemt ændring, og den kan således nedarves til kommende generationer af ferskenbladlus.

Pirimicarb-resistensmekanismen er nu veletableret i det sydlige Europa, og den har siden hen spredt sig nordpå i sin europæiske udbredelse; således blev den i 1993 fundet i hollandske ferskenbladlus, og i 1995 blev de første to ferskenbladlus med specifik pirimicarb-resistens fundet i England. Allerede året efter, 1996, i England kunne der konstateres bekæmpelseproblemer adskillige steder, hvor bekæmpelse med Pirimor de seneste sæsoner havde været eneste effektive middel; efterfølgende blev det påvist, at bekæmpelsesproblemerne skyldtes tilstedeværelsen af den pirimicarb specifikke resistensmekanisme. Det, som især bekymrer i England, er at pirimicarb-resistensmekanismen som oftest findes i ferskenbladlus, der tillige har et højt niveau af esterase-enzymet; altså begge resistensmekanismer i samme bladlus, hvilket gør dem meget svære at bekæmpe.

3 ud af 8 prøver fra Storestrøms Amt viste pirimicarb-resistens

I nu over 10 år har vi ved Danmarks JordbrugsForskning fulgt resistensniveauet i ferskenbladlus ved at måle niveauet af esterase-enzym i indsamlede bladlus; ferskenbladlusene er hovedsageligt blevet indsamlet fra sukkerroeområderne i Storstrøms Amt. I alle årene har en vis del af de indsamlede ferskenbladlus kunne betegnes som svagt - stærkt resistente ud fra esterase-enzymmetoden. Sidste sæson var der kun få indsamlede prøver af ferskenbladlus, hvilket skyldtes at forekomsten var forholdsvis ringe; efter bestemmelse af esterase-enzymniveauet i de indsamlede prøver, blev prøver af bladlusene sendt til Rothamsted, England, hvor de har indkørt en metode til at undersøge for tilstedeværelse af pirimicarb-resistensmekanismen. Det viste sig, at i tre ud af de otte prøver, som blev sendt til England, var der bladlus med specifik pirimicarb-resistens; de specifikt pirimicarb-resistente bladlus havde alle tillige et højt niveau af esterase-enzymet. De undersøgte ferskenbladlus-prøver var indsamlet i sukkerroe-marker.

Hvor udbredt den nye pirimicarb specifikke resistensmekanisme er i danske ferskenbladlus-populationer, er svært at afgøre ud fra de forholdsvis få prøver, som er blevet undersøgt; det kan kun slås fast, at den er tilstede i Danmark.

På Danmarks JordbrugsForskning i Flakkebjerg har vi planer om at undersøge udbredelsen af pirimicarb-resistensmekanismen i ferskenbladlus fra danske roemarker. Hvis denne nye resistensmekanisme er eller bliver udbredt i danske ferskenbladlus-populationer kan den, i kombination med den gammelkendte esterase-resistensmekanisme, give bekæmpelsesproblemer i år med mange ferskenbladlus.

Brødsgaard, C.J., Jensen, S.E., Hansen, C.W. & Hansen, H. (1999). Spring treatment with oxalic acid in honeybee colonies as varroa control. *DIAS report Horticulture* no. 6, 16 pp.

SUMMARY

In late March 1998, 30 honeybee colonies (*Apis mellifera*) in four apiaries were treated for the parasitic mite (*Varroa jacobsoni*) with either spraying or trickling of oxalic acid. Four colonies were not treated and served as controls. Prior to the treatment, eight days after the treatment, and at the first honey harvest in June one food sample was taken in each colony. Of these samples five from sprayed, five from trickled, and the four from control colonies were chosen and the oxalic acid residue level was determined by means of liquid chromatography. The results showed that the maximum residue level was found eight days after treatment in the sprayed group ($\bar{x}$=0.0062%) but also that there was no significant difference in oxalic acid concentration between the groups at any of the sampling dates.

In another apiary, the glutathione *S*-transferase (GST) activity was measured in individual pupae and adult bees from trickled and control colonies. The result showed that 15 days after treatment the GST activity in pupae and adult bees from the trickled colonies was not different from the GST activity found in non-treated colonies indicating that trickling treatment of colonies with oxalic acid does not seem to have an effect on the level of GST activity in pupae or newly emerged adult bees.

The varroa mortality was recorded after the spring treatments with oxalic acid trickling and spraying and again in the autumn after an oxalic acid trickling treatments. Furthermore, the bee colony strength and brood amount were recorded prior to the spring treatment and again a year after the treatments. A significant difference in varroa mortality was seen after the spring treatment between the treated colonies and the controls. In the trickling group the total mite drop-down per colony was in average 61.53, in the sprayed group it was 145.47 and in the control group 1.50. After the autumn treatment, no significant difference was found between the three groups and the mite drop-down ranged between 936 and 1,400 mites. In 1998, the mean bee colony strength was approximately 5.5 comb gates before the treatment. At the same time the mean brood amount ranged from 1.77 to 3.25 dm^2. During the 1998 season, no difference in colony development was observed among the three trial groups. One year after the treatments the mean colony strength ranged from 4.93 to 6.25 comb gates. The brood amount ranged from 0.89 to 1.53 dm^2. There was no significant difference between the treated groups and the control group at any time.

ACTIVITIES WITH RELATION TO THE PH.D. STUDY

Conferences, symposia, and workshops

- 14th Danish Plant Protection Conference, 4-5 March 1997, Nyborg, Denmark. Oral presentation: *Påvisning af Insekticid-resistens med Biokemiske Metoder [Detection of insecticide resistance with biochemical methods].*
- Resistance '97, international conference, 14-16 April 1997, IACR-Rothamsted, UK. Poster and abstract: *Methiocarb Resistance in Western Flower Thrips (Frankliniella occidentalis) Collected from a Danish Greenhouse.*
- Science Day, 23 April 1997, Roskilde University, Denmark. Poster: *Insecticide resistance in western flower thrips (Thysanoptera: Thripidae).*
- 15th Scandinavian SSCT Workshop on *In vitro* Toxicology, 4-7 September 1997, Roskilde University, Denmark. Oral presentation, poster and abstract: *Insecticide resistance in western flower thrips (Insecta).*
- IOBC/EUCARPIA workshop meeting "Breeding for Resistance to Insects and Mites", 14-17 September 1998, Dundee, Scotland.
- 17th SSCT Workshop on *In Vitro* Toxicology, 23-26 September 1999, Röstanga, Sweden. Oral presentation and abstract: *Host plant effects on activity of detoxification enzymes and insecticide tolerance in western flower thrips (Insecta: Thysanoptera).*
- Symposium "Bisygdomme før, nu og i fremtiden [Honeybee diseases past, present and future]", 24 November 1999, Flakkebjerg, Denmark. Oral presentation: *Oxalsyre-behandling af bifamilier – Test for påvirkning af et afgiftningssystem hos bier [Oxalic acid treatment of honeybee colonies – Testing effects on a detoxification system in honeybees].*

Courses completed with satisfactory result

- "Conceptual and Analytical Approaches to Problem Solving in Ecotoxicology". Ph.D. course 3-14 March 1997 at Roskilde University, Denmark.
- "Molecular Markers in Plant Genetics and Plant Breeding". Ph.D. course 8-16 June 1998 at Risø National Laboratory, Denmark.
- "Logical Framework Analysis". Course 7-8 April 1997 at Carl Bro Management, Glostrup, Demark.
- "SAS System Orlando II". Course 18-20 November 1997 at Danish Institute Agricultural Sciences, Foulum, Denmark.

Before this Ph.D. study was started, I had completed courses on:
"Modern Microscopy Techniques". Ph.D. course at Panum Institute, University of Copenhagen.
"Confocal Microscopy". Biomedical Centre, Uppsala University, Sweden.
"Academic Writing in English – Natural Sciences". DME, Lyngby, Denmark.
Seminar series for Ph.D. students on "Membrane transport and ATPases", "Cell Cycle Control and Apoptosis", and "The Cytoskeleton" at Panum Institute, University of Copenhagen.

Teaching

In 1995, when employed at the Protein Laboratory, University of Copenhagen, I was a teacher on the Ph.D. course "Molecular Cell Biology". At the Protein Laboratory, I also gave seminar lectures.

During this Ph.D. study, I have held seminars on mechanisms of insecticide resistance at Roskilde University (two seminars) and at the Danish Institute of Agricultural Sciences (three seminars).

As part of the "Overseas training programme in sugar beet development" at Research Centre Flakkebjerg, I have taught Chinese agronomists about mechanisms of insecticide resistance (lecture) and detection of insecticide resistance with biochemical methods (demonstration in the laboratory).