This is to certify that the

dissertation entitled

Characterization, Synergism and Inheritance of Resistance to Azinphosmethyl, Carbofuran and Permethrin Insecticides in the Colorado Potato Beetle (Coleoptera: Chrysomelidae)

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Entomology

Major professor

Date March 29, 1990

CHARACTERIZATION, SYNERGISM AND INHERITANCE OF RESISTANCE TO AZINPHOSMETHYL, CARBOFURAN AND PERMETHRIN INSECTICIDES IN THE COLORADO POTATO BEETLE (COLEOPTERA: CHRYSOMELIDAE)

By

Philippos M. Ioannidis

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Entomology

1990

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ABSTRACT

CHARACTERIZATION, SYNERGISM AND INHERITANCE OF RESISTANCE TO AZINPHOSMETHYL, CARBOFURAN AND PERMETHRIN INSECTICIDES IN THE COLORADO POTATO BEETLE (COLEOPTERA: CHRYSOMELIDAE)

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Colorado potato beetle Leptinotarsa decemlineata (Say) were tested for resistance to azinphosmethyl, carbofuran, and permethrin by topical application bioassays. The research was designed to characterize resistance (resistance levels, cross-resistance, synergist responses, inheritance). Data indicated that most populations in Michigan were heterogeneous for resistance to at least one insecticide. There appeared to be three main patterns of resistance in the field. Pattern I: The resistance ratio was high for permethrin but low for the other two insecticides. Pattern II: High levels of resistance to azinphosmethyl and carbofuran and low to permethrin. Pattern III: Beetles were resistant to all three insecticides.

Synergists studies were conducted to determine resistance mechanisms. Beetles can use almost all the resistance mechanisms known for insects. The effects of the synergists piperonyl butoxide, DEF, and DEM indicated that mainly mixed-function oxidase and esterases were involved in azinphosmethyl resistance. Mixed-function oxidase and acetylcholinesterase were responsible for carbofuran resistance. Another type of mixed-function oxidase was involved in permethrin resistance, plus two other mechanisms, esterases and

the Kdr factor (knock-down resistance), which also gives cross-resistance to DDT.

Synergists can be used in the field to improve insecticide effectiveness and overcome resistance. Cross-resistance exists between aldicarb and permethrin, between azinphosmethyl and carbofuran-imidan, between carbofuran (acetylcholinesterase resistance) and thiodan probably linked genes. No cross-resistance was observed in carbofuran and aldicarb. Use of mixtures are not recommended; rotation of insecticides is preferable. Rotation of crops is absolutely necessary.

Resistance to carbofuran was inherited via a single, autosomal, incompletely dominant factor resulting in acetylcholinesterase insensitivity. Azinphosmethyl resistance appeared to be inherited mainly via two incompletely dominant autosomal linked genes, with about a distance of 20 map units. The two main resistance factors involved in the highest level of resistance were mixed-function oxidase and esterases enzymes. These two resistant factors were synergistic in effect when contained in the same individual. Base line data and discriminating doses for assessment of resistance were established for several insecticides. Strategies for management of resistance in CPB are critical for continued control, resulting in long-term profit and reduced pesticide applications.

To my sister

Your silent memory will

preserve your image

ACKNOWLEDGEMENTS

Conducting this research, including the academic experiences I encountered throughout my studies at Michigan State University, has been both challenging and rewarding. This experience has been the best it could possibly be because of the tireless efforts and academic guidance of my advisor, Dr. Edward Grafius. I sincerely appreciate all he has done for me, including the financial support for this study.

I must share my gratitude with the other members of my committee as well. I thank Dr. Mark Whalon for his useful insights and for the stimulating discussions we had, Dr. Robert Hollingworth, Director of the Pesticide Research Center, for his valuable suggestions and contributions to this study obtained through the formal group meetings we had and through intellectual discussions with him. I thank Dr. Donald Ramsdell for acting on my committee, for the knowledge base I obtained through his excellent class on plant virology and for his suggestions and corrections on this dissertation.

I would also like to thank Dr. Fumio Matsumura and Dr. Frank Drummond, two of my previous committee members who transferred to other universities, for their efforts.

I thank all of the staff members of Dr. Grafius' lab, both past and present. I would also like to thank all of the faculty members and staff of the Department of Entomology, especially Dr. Fred Stehr. Thank you, Beth Bishop for the collaboration and help during all these years. I would also like

to express my thanks to Jan Eschbach for helping with editions and typing this dissertation.

Finally, it is necessary to express my appreciation to my employer, the Hellenic Sugar Industry, not only for the financial support throughout my studies, but also for the opportunity that was granted me to undertake this degree.

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INTRODUCTION

"The word resistance has come to be applied to any population, within a species normally susceptible to a given insecticide, that is no longer controlled by the insecticide in the area concerned" (Brown and Pal 1971).

Resistance arises as a result of selection acting upon natural variation. It is now clear that resistance is invariably "pre-adaptive". The idea that resistance arises from natural variation does not preclude the occurrence of new mutations after the insecticide has been applied (Wood 1981).

Resistance of insects to pesticides has a history since 1910 but its greatest increase and strongest impacts have occurred during the last 40 years, after the extensive use of synthetic organic insecticides. Up to 1986, 447 species (including most major pests) had been reported to be resistant to one or more classes of chemicals (Georgiou 1986). It has been understood that resistance may be caused by one of three mechanisms, acting singly or in combination, decreased sensitivity at the site of action, enhanced metabolic attack within the insect, or decreased transport to the site of action. Past experience and recent studies on resistance modelling showed that reduction of selection pressure in its broadest sense is a key factor in reduction of development of resistance. Colorado potato beetles (CPB) Leptinotarsa decemlineata (Say) spread across North America by the mid-1870's and started to increase in population in Europe after the First World War with the first outbreak in Britain in 1901, and since 1975 have been starting toward Asia (Hurst 1975). The significance of this serious pest was described by Hurst in 1975 in a technical note as

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follows: "The beetle can almost be regarded as a parent of the pesticide industry, because it was largely to stop the devastation of the potato crops that chemicals such as arsenates came into field use and were developed. The beetle can claim to have brought a major European organization into being, for the European and Mediterranean Plant Protection Organization (henceforward abbreviated as EPPO) owed its origin to the committee formed in the 1930's to try to contain the progress of the beetle across Europe."

The CPB colonizes and feeds on several cultivated crops - potato (Solanum tuberosum L.), tomato (Lycopersion esculentum Mill) and eggplant (Solanum melongena L.) and on a number of wild host plants: Buffalo bur (Solanum rostratum Dunal), and common night shade (Solanum nigrum L.). It is known that CPB has demonstrated to be a very adaptable insect (Groden 1986). The CPB can significantly reduce the yields of these crops and often completely defoliates them unless successful control measures are applied. It has been speculated that herbivorous species which have frequently evolved the capacity to deal with plant alkaloids, are in some sense preadapted to dealing with the problems posed by dangerous chemicals in their environment (Croft and Brown 1975). The Allelochemical alkaloids and ketones are toxic to insects. The Leptine glycoalcaloids in Solanum chalcoense are toxic to the CPB (Sinden et al. 1986). Through the co-evolution with the Solanaceous plants, it seems that the beetles have developed effective mechanisms to overcome the alkaloids. This probably has facilitated the development of resistance to insecticides.

The CPB has exhibited substantial resistance to almost all registered insecticides in the eastern United States and has also started to become a problem in the west. It has a remarkable propensity for resistance and it is notorious for the speed with which it has developed resistance to all main

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categories of insecticides, despite only two to three generations completed per year. The CPB has required progressively less time to develop resistance to the subsequently used insecticides: seven years to DDT, five years to azinphosmethyl, two for carbofuran, two for pyrethroids and one for pyrethroids plus the synergist piperonyl butoxide (Georgiou 1986).

Resistance to DDT spread as early as 1953 to spots throughout the Red River Valley (Post 1953). Hofmaster (1956) pointed out that there was considerable evidence that DDT was not adequately controlling CPB on potatoes on the Eastern shore of Virginia. Cutkomp (1958) confirmed resistance of CPB to DDT by determining the toxicity levels of injected DDT to adult beetles collected from two widely separated areas. The synergist chlorfenethol also used in combination with DDT did not produce any detectable difference from DDT alone and he concluded that the results did not provide any clue as to the mechanisms of the resistance. Harris et al. (1976) reported resistance to DDT and cyclodienes in Ontario, 240-fold to Aldrin and 37-fold to Endrin. McDonald (1976) tested CPB from Southern Alberta, Canada and found that 10 years after the DDT was discontinued, susceptibility (1966 23-fold) had returned to those levels recorded in 1957 and mentioned that resistance to this compound was disappearing from this species. Probably the above cases of resistance to DDT were the Kdr type, well known today for its recessiveness. Tests with CPB from Quebec showed that a strain was resistant to most insecticides and especially high for carbofuran (>1600-fold), it was also resistant to DDT, Endrin, endosulfan, azinphosmethyl, phosmet methidathion, methamidophos, phorate, cloethocarb, and carbaryl (Harris et al. 1981). Now it is well documented that populations of CPB with multiple mechanisms of resistance exist in many places in the U.S.

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The objective of this work was to study some of the factors which constitute the whole phenomenon of resistance. In the following chapters we have described techniques and procedures including methods for laboratory and field selection for resistance, with the insecticides azinphosmethyl, carbofuran, and permethrin.

Field monitoring complemented with laboratory bioassays for documentation of resistance using laboratory and field collected strains were also conducted. The data obtained provided baseline data for resistance from the state of Michigan and revealed different patterns of resistance developed in field populations.

Synergists may be used to delay resistance development in addition to their use in overcoming established cases, and to indicate resistant mechanisms (Metcalf 1967). Studies were conducted with the well-known insecticide synergists piperonyl butoxide, MGK 264, DEF, DEM, and chlorfenethol, to define enzymes involved in insecticide detoxification, and to support biochemical analysis data.

Genetic principles and techniques have been of great value in understanding basic mechanisms involved in the development, spread, regression and inheritance of resistance to pesticides. We conducted reciprocal crosses between susceptible and resistant strains and in addition, the F_1, F_2 and several backcrosses, were successfully completed. All this information can help significantly to design resistance management tactics. Colorado potato beetle insecticide resistance is an urgent and alarming problem which may result in considerable economic loss and even in the discontinuation of potato production if allowed to go unchecked.

Chapter 1

General Methods and Materials

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Field Monitoring for Resistance of the Colorado potato beetle

All of the bioassays were conducted on insect populations collected from individual fields at different locations. Most of the populations were summer adults (first generation) or of the F_1 progeny collected just prior to the application of pesticides (about 1 week old).

Synergism studies were applied directly to field resistant populations. Resistance detection and monitoring was conducted as a basis for subsequent resistance studies as well as to establish base-line data of resistance for the state of Michigan.

The insecticides mainly tested are some of the most commonly used in Michigan for controlling potato beetles and belong to the four main categories of pesticides.

INSECTICIDES

Azinphosmethyl	Organophosphorothioate
Carbofuran	Carbamate
Permethrin	Pyrethroid
DDT	Organochlorine

On the basis of laboratory studies, carbofuran, chlorfenvinphos, and azinphosmethyl were the best of registered materials as foliar sprays against a population of CPB of various stages (McClanahan 1975). Harris (1976) tested 40 insecticides against 3rd stage larvae and adults of a susceptible strain and he reported that the most effective larvicides in descending order of efficacy were Carbofuran > Endosulfan > Azinphosmethyl > Carbaryl > Phosmet > Methamidophos. The most effective adulticides were Endosulfan > Carbofuran > Azinphosmethyl > Phosmet > Carbaryl > Methamidophos. The

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superiority of the compounds used in our studies has been also shown by McDonald (1976) and Hare (1980).

For estimation of the Resistance Ratio (RR) we adopted the reported topical toxicities for susceptible adult CPB from northern Maine (Forgash 1980). They were: 0.39 µg/beetle for permethrin, 0.25 µg/beetle for carbofuran, and 0.3 µg/beetle for azinphosmethyl. Susceptible adults from Idaho also had toxicities of insecticides in acetone solutions of equal or lower values to those mentioned above (Johnston et al. 1986). For DDT the value 10 µg/beetle was used (FAO 1974).

Permethrin was given an emergency registration for field use in Connecticut in 1979, because field trials showed permethrin to be the most effective in controlling beetle populations after the development of resistance to carbamates and organophosphates (Hare 1980). It was very important to include permethrin, a type I pyrethroid (Gammon et al. 1981, Scott et al. 1983) in our studies for detection of cross-resistance to DDT. DDT was tested on populations showing permethrin resistance. Specifically with permethrin we performed the following work:

- Standardized the bioassay technique for testing pyrethroids against Colorado potato beetle,
- Characterized permethrin resistance in Michigan,
- Determined mechanism(s) involved in permethrin resistance other than the Kdr factor which confers knockdown resistance (which cannot be overcome by synergists).

The Kdr action is quantitative, since at higher dosages a normal response is observed and there is no recovery. Oppenoorth et al. (1976) concluded that any hypothesis on the resistance mechanism controlled by the Kdr gene has to take into account the following:

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a) the Kdr factor affects the toxicity of DDT and its analogues as well as the pyrethroids, and

b) its action is quantitative since at higher dosages a normal response is observed.

From our studies and the control failures of applying pyrethroids plus synergists after 1-2 years, it appears that the Kdr factor was well spread in certain areas and may have been responsible for some repeated failures of application of pyrethroids.

Farnham (1977) suggested that since Kdr is a recessive character, natural dilution of the population and the removal of selection pressure would help to greatly increase the number of heterozygotes which are susceptible to DDT or pyrethroids. There is, therefore, a need for early detection of this factor in a population, coupled with the use of an alternative means of control in order to keep the frequency of this gene at manageable levels.

Research Approach and Procedures

Colorado potato beetle cultures

Colorado potato beetle cultures were maintained in cages 50 x 50 x 70 cm located in walk-in environmental rooms maintained at 25±1°C, 50±10% RH and a 16:8 L:D light regime.

Larvae and adults throughout their life span were reared on fresh potato plants which had been planted in clay pots under standard techniques used in greenhouses. Attempts were made to rear the beetles on the same suitable susceptible variety of potato. This was essential for maintaining the

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correct biotype populations. Differences in the resistance of cultivars of potatoes to CPB damage have been observed (Mateeva-Radeva 1985). Laboratory testing methods rarely simulate natural conditions. In our experiments, however, we did not consider the role of crop toxicity because the larvae and adults fed on potato plants.

Boiteau (1983) described a method for synchronization of CPB emergence. Eggs can be held at 10°C for up to 12 days without affecting survival. More than 90% of eggs collected daily over a period of up to 12 days and stored at 10°C hatched in 1 day. Using the above technique, we conducted bioassays on beetles (lab cultures) with the same approximate age. Eggs were collected and stored daily. After 10 days eggs were removed from cooling chambers. Hatched larvae were reared in petri dishes on potato leaves for 3 days and were subsequently moved to whole plants in the cages for growth and pupation. Useful information on longevity and fecundity of the CPB and egg development and larvae development can be found in the papers by Peferoen et al. (1981), Logan et al. (1985).

Experimental Conditions

All the bioassays were conducted in a second environmental room maintained in identical conditions with the culture rooms. Attempts were made to test one insecticide on several different strains simultaneously to minimize the influence of various factors affecting the bioassays. Therefore the results were compared directly.

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Topical Applications

Detection of Resistance Using LD_{50} Values and Dose-Mortality Lines

The standard bioassay technique for CPB (FAO 1974) was used. Some changes have been made to the method as a result of preliminary work. Insecticidal toxicity was assessed by topical application of 2 µl of solutions of technical compounds in acetone. Applications of insecticides and synergists were applied using a hand-operated microapplicator to the second abdominal sternite of adults and the lateral portion of the third abdominal segment of larvae. All tests were conducted at 25±1°C. For application of the compounds, the beetles were immobilized by holding them at the tip of a plastic tube to which a vacuum was applied. Concentrations of insecticides were selected based on preliminary trials to give a range of 0-100% mortality. To obtain this information, pesticides were applied in concentrations above (10x), below (0.1x), and at (1x) the recommended field rates. For each bioassay at least four concentrations were used. Control beetles were included with all the tests to which only acetone was applied.

Mortality values were corrected for natural mortality by Abbott's (1925) formula. If control mortality was larger than 20%, we repeated the bioassay (Busvine 1971). In most cases bioassays were performed at least twice.

Toxicity was measured by the log-probit analysis method (Finney 1971) using the MSTAT-C microcomputer program (Eisensmith 1989).

Assessment of Mortality

The timing of the assessment of mortality is extremely important. There are differences between the insecticide groups. For azinphosmethyl and carbofuran maximum mortality (90%) was obtained within 3 days. For permethrin, mortality is usually recorded within 24 hours. Since the knockdown effect of pyrethroids is well known and there is recovery in most of the insects, for a multi-resistant population more than one assessment of mortality was needed in order to obtain useful results. Mortality was estimated as follows:

1/2 hour	knockdown effect
3 days	dead + knockdown effect
5 days	dead + recovered

Standardization of Insects for Testing: Intrinsic and Extrinsic Factors Affecting Toxicity of the Insecticides

It was important to standardize the test procedures because the slope and LD_{50} for a particular population and insecticide can change considerably with the application method, solvent used, sex ratio of insects, time to estimate mortality, temperature, age of insect, and so forth (Busvine 1971, Winney 1973). Bioassays and tests with field beetles were undertaken either directly, on the field material collected before sprays, or on the F_1 colonized generation. Since so many factors influence the validity of the bioassays, a considerable amount of time had been spent on designing appropriate techniques with standard procedures. Great care must always be taken to

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insure that these variables are kept to a minimum when comparing the response of different strains of CPB to insecticides.

Technical Information for Bioassays

Probit analysis: The responses of each genotype (strain) must be linear with low X^2 values (X^2 is a measure of the departure of the actual observations from the fitted probit-log concentration line). Otherwise the following possibilities arise:

- the population is genetically heterogeneous,
- the test method is not sufficiently sensitive,
- the insecticide may contain toxic impurities.

The shallower the slope, the greater was the variation in the susceptibility of the individuals of the population to the poison. The study of slope, LD_{90}, and LD_{10} would increase the value of the information given by the bioassay.

When the population exhibited homogeneous susceptible behavior, or homogeneous resistance behavior, the dose ratio which was most appropriate to use was the geometric series 1 : 2 : 4 : 8 : 16 for estimating the LD_{50}'s.

With heterogeneous populations, which exhibited flat regression lines, we found that it was best to use the ratio 1 : 3 : 9 : 27 : 81 for estimating the LD_{50}'s. With heterogeneous populations it was best to use many doses (6 to 8) and fewer replications per dose when available beetles were limited. Therefore, by inspecting the plotting of the observed probits and the dose-response curve shape, we identified the presence of two types of individuals and we were able to estimate percentages of susceptible and resistant beetles.

The ratio series 1 : 3 : 9 : 27 : 81 worked much better also with pyrethroids. We noticed consistently that the regression lines for pyrethroids were flatter than the ones for azinphosmethyl and carbofuran and observed the same phenomenon working with pyrethroids on houseflies. Occasionally, there are indications that the probit analysis represented by the equation $y = a + bx$ does not adequately express the relationship between mortality probit and quantity of pyrethroids applied topically (Ioannidis 1979).

Substances possessing similar mechanisms of action usually provide parallel regression lines. Before making comparisons between LD_{50}'s of insecticides or treatment, tests for parallelism of the regression lines were necessary. Also data did not always fit the sigmoid curve and care was taken in the interpretation of the results, particularly with heterogeneous populations.

Carbofuran and DDT are quite insoluble in acetone when using large amounts of materials. This can result in some difficulty in the use of topical application in the laboratory. These compounds tended to crystallize on the surface of CPB when high doses were used. Such crystallization had a negative influence in the resulting toxicity since it reduced it. Thus any resistance mechanisms that necessitate a large dose of these compounds are likely to cause a disproportional increase in the LD_{50}'s (Brown et al. 1971). In order to compare our results with the ones already published for carbofuran and DDT, we decided to use acetone. We found the limit of solubility for carbofuran to be about 50 µg/µl and for DDT, 164 µg/µl. To achieve application of larger doses for highly resistant strains a second drop was applied to the same spot.

When resistance was evolving, the R gene conferring that resistance was still at a low frequency of less than 10%. The LD_{50}'s of a field collected

sample could be similar to that of a susceptible strain. The form of the dose response curve was then the best indicator of resistance.

Detection of Resistance Using the LT_{50}'s Values (lethal time)

The diagnostic dose, LT_{50}, also was used when insufficient samples were available to calculate a probit line and magnitude of resistance. In testing for resistance, it was sometimes convenient to expose samples of insects for different periods to the same concentration of insecticide, rather than to different concentrations for a standard time. This procedure is justified when it can be shown that the dose acquired by the insect is directly proportional to the exposure time. Concentration x time is a constant (Wood 1981). For most of the testing of unknown populations we gave a high dose for each insecticide for estimation of LT_{50}'s. Mortality was recorded in time intervals such as 1/2 h, 1 h, 3 h, 9 h and 1 day. The applied dose for azinphosmethyl was 200 µg/beetle, the applied dose for permethrin was 20 µg/beetle, and the applied dose for Carbofuran was 125 µg/beetle. This technique worked reasonably well and gave satisfactory results.

Before a final bioassay was conducted with an unknown population and before setting the proper range of doses that were going to be used, computation of LT_{50}'s with the above method gave a quick estimation of the level of resistance of the population. Several tests were run with well known susceptible and resistant strains; therefore, standard values were established for comparisons.

Symptomatological Observations:

Observations on the type of response of insects after the topical application with different insecticides (paralysis, motionless, recovery, very active movement and lack of incoordination for certain time periods), gave us some information on the mode of action of the poison (Matsumura 1985). In addition, we obtained information from the speed of action of the insecticides. For example, toxicity of azinphosmethyl was expressed after two days of application, while carbofuran killed extremely fast (within two hours). Permethrin paralyzed the beetles in 20 minutes and there was good recovery after 5 days when low doses of less than 5 µg/beetle were used. Beetles with the Kdr resistant factor responded differently.

Selection for Insecticide Resistance

Introduction

Brown and Payne (1988) pointed out in the conclusion of their excellent review paper Experimental Selection for Insecticide Resistance that "a review of the literature indicates that selection for insecticide resistance in the laboratory has provided useful information about the levels of resistance and the rates at which resistance to various insecticides has developed". In the same study, the authors reviewed all the selection studies with the genera Aedes, Culex, Musca, and Heliothis, and concluded that the rates at which resistance development occurred were more rapid for DDT and permethrin than the rates used for malathion, parathion, and other acetylcholinesterase inhibitors.

It is now recognized that resistance is a preadaptive phenomenon and that the insecticide acts as a powerful agent to select those members of the populations that, for one reason or another, have the ability to survive exposure. When the selection pressure is maintained over several generations, substantial levels of resistance are often rapidly attained (Crow 1960, Brown et al. 1971). The idea that resistance arises from natural variation does not preclude the occurrence of new mutations after the insecticide has been applied. There seems to be no reason to doubt that resistance mutations occur naturally, from time to time, at the normal rate of $1\text{-}2x10^{-5}$ gametes or less (Wood et al. 1981).

Selection studies on the common green lacewing predator with carbaryl, showed that larvae collected from widely separated areas of California had different levels of tolerance to six insecticides. The most tolerant larvae selected with carbaryl, exhibited more than 80% survival after four generations, in contrast to three percent survival for the unselected strain (Grafton-Cardwell et al. 1986).

Selection for resistance to methyl parathion for 21 generations on a field-collected strain of tobacco budworm, resulted in 31-fold greater resistance to the insecticide than with an unselected strain. An estimate of heritability of resistance indicated that 78% of the variation was due to additive gene action. The authors started the test with the progeny of 20 insects that were collected in the larval stage (Wolfenbarger et al. 1982). In another study on selection for permethrin resistance in the tobacco budworm, approximately 500 larvae were collected from cotton fields. After 11 generations of continuous selection pressure the LD_{50} increased 37-fold and also cross-resistance to cypermethrin developed (Jensen 1984). Resistances of 33- and 27-fold to fenvalerate and deltamethrin respectively, have been induced in

larvae of the Egyptian cotton leafworm Spodoptera littoralis (Boyd) after selection pressure for 23 successive generations (Riskallah 1983).

Most of the kinds of resistance developed under laboratory selection pressure are probably special cases because what is not known in such cases is the relative survivability of these genotypes under field conditions.

Studies of selection experiments indicate that it is preferable to begin with large samples of insects, gathered from several locations, increasing the likelihood that genes for resistance are present in the initial selection (Roush and McKenzie 1987, Brown and Payne 1988).

A major problem with selection studies is that the selection programs have not had access to major resistance genes. The solution is to modify our selection procedures generating resistant mutations or attempting the first selection in the field, collecting the surviving ones and continuing the selection in the lab. We applied the last technique with great success in our studies, producing unique resistant strains which were utilized further in synergistic, biochemical, and genetic experiments.

Materials - Methods

The Colorado potato beetle is suitable for selection studies for several reasons:

- Techniques for rearing and maintaining lab cultures have been developed, therefore, they are easy to rear.
- CPB do very well under lab conditions.
- CPB have developed resistance to all available insecticides.
- CPB have a well-documented history of resistance in the fields.
- CPB have a short life cycle. Under our lab conditions their life span was 40 days and 7-8 generations per year could be obtained.

Selection Procedure A

Approximately 4000 individuals were collected from volunteer potatoes covering a large area near Vestaburg, Michigan. Both larvae and adults were collected from several locations at various times and mixed to represent the range of genetic potential of natural populations. The total number of collected beetles was subdivided into four groups and selections have been started at 80% mortality levels as follows:

1 Sub-population selection with azinphosmethyl
2 Sub-population selection with carbofuran
3 Sub-population selection with permethrin
4 Sub-population unselected - control

Selection was applied only to adults at a level of 70-80% mortality. Selection was applied first in two groups of beetles keeping a control batch, just in case the selected group had died, to insure the continuation of the interbreeding. Attempts were made for insecticides to be applied before mating started, because if we delivered insecticides after mating had occurred, susceptible sperm could be protected inside resistant females and survive to the next generation.

Selection was conducted over successive generations by topical application of technical material of the insecticides in acetone solution. Pure material must be used because some insecticides are themselves mildly mutagenic and solvents and impurities as well may also increase mutation rates (Wood 1981).

Development of resistance was monitored by calculating a dose-mortality regression (probit analysis) in alternate generations. Details of application techniques and interpretation of results have been given previously.

Selection Procedure B

Selection was started in the field and continued in the laboratory. We began two selections of this type with carbofuran and permethrin. Details of these selections are given in the following chapters (the selected populations are named Montcalm-C and Montcalm-P.). The results were impressive. The advantage of procedure B was that the initial selection was directed on a large population, with a wider gene pool, therefore, resistant genes were more likely to have been included in the selection.

It was useful to make comparisons between the two selection procedures. The change in resistance levels can be calculated if information is available about the initial frequency of R and S genes in a given population, the population size, frequency of inbreeding, strength of selection pressure, number of generations, fitness of R and S genes, etc. (Plapp et al. 1979).

During the project, we got valuable information about the biology of CPB under insecticide selection pressure. Attempts were made to assess the fitness disadvantage that results from resistance. Since we managed to create resistant strains to carbofuran and azinphosmethyl (see Chapters 4 and 5) using the selection Procedure B, we stopped the continuation of selections with the sub-populations 1 and 2 which had not given any indication of increase of resistance after being selected six times. Genetic heterogeneity

present in source colonies was probably insufficient for a resistance potential to be expressed.

When reasonable levels of resistance were obtained we conducted cross-resistance experiments to certain insecticides in order to study the cross-resistance patterns (Table 8, Chapter 3). A specific cross-resistance pattern can often provide information on the resistance mechanisms involved with an insecticide. It was therefore important in determining which alternative insecticides were most suitable for the control of the resistant populations, and to design appropriate protection management tactics.

Chapter 2

Patterns of Insecticide Resistance to Azinphosmethyl, Carbofuran and Permethrin in the Colorado Potato Beetle (Coleoptera: Chrysomelidae).

ABSTRACT:

Twenty-four field-collected and five laboratory strains of Colorado potato beetle, Leptinotarsa decemlineata (Say) were tested for resistance to the insecticides azinphosmethyl (organophosphate), carbofuran (carbamate), and permethrin (pyrethroid) by the dose-mortality response method. Toxicity was assessed by topical application. Log dose-probits of the bioassays indicated that most populations were heterogeneous for resistance to at least one insecticide with shallow regression lines and high variability. Populations with high permethrin resistance tended to have low levels of resistance to azinphosmethyl and carbofuran and vice versa. There appeared to be three main patterns of resistance in the field. Pattern I: the resistance ratio was high for permethrin but low for the other two insecticides. Pattern II: high levels of resistance to azinphosmethyl and carbofuran and low to permethrin. Pattern III: beetles were resistant to all three insecticides and with cross-resistance to DDT. Some negatively correlated resistance between permethrin and azinphosmethyl-carbofuran may exist. The observed patterns and underlying resistance mechanisms are important for insecticide resistance management in CPB.

Key words: Insecta, Colorado potato beetle, Insecticide resistance.

The Colorado potato beetle (CPB) is a major agricultural pest, in part because of its demonstrated ability to rapidly develop resistance to every insecticide that has been used for its control (Forgash 1980). The CPB has been shown to be resistant to all five major classes of insecticides, DDT, cyclodienes, organophosphates, carbamates, and most recently pyrethroids (Forgash 1985, Harris and Turnbull 1986).

Populations of CPB have developed resistance to all nationally registered insecticides and there are no insecticides presently available that effectively control CPB in many parts of the northeastern United States (Ferro 1985). It took 7 years for the beetles to develop resistance to DDT, the first synthetic insecticide against which it was selected, 5 years for resistance to azinphosmethyl, 2 years for carbofuran resistance, and 2 years for pyrethroid resistance (Georghiou 1986). In Long Island, NY, fenvalerate lost effectiveness in 2 years' time with a reported 600-fold resistance level. Although combining fenvalerate with the synergist piperonyl butoxide reestablished control, this combination failed in 1 year (Forgash 1985).

It is obvious from numerous reports of control failures, extensive bioassays and synergistic studies of different field-collected populations that CPB has developed resistance to a variety of insecticides in Michigan (Grafius 1986, Grafius et al. 1987, 1988, Ioannidis and Grafius 1988). To conserve the usefulness of existing insecticides, to be able to develop chemical use strategies, and to design resistance management tactics, it was necessary first to study existing cross–resistance patterns of insecticides. We were able to isolate and retain in culture some of these strains, representing various steps and types of resistance development for further biochemical (Ahammad-Sahib et al. 1989) and genetics of resistance studies and testing of new insecticides.

The objectives of this study were: 1) to assess the current status of insecticide resistance in the Colorado potato beetle in Michigan, and to establish base-line data for resistance; 2) to study the relationship between the commonly-used insecticides azinphosmethyl, carbofuran and permethrin, comparing toxicity data (LD_{50}'s, dosage-mortality curves) and characterizing arising patterns of resistance to these insecticides.

Materials and Methods

Chemicals: The insecticides chosen for the bioassays were some of the most effective and commonly used materials for controlling potato beetles in Michigan and other areas (Hofmaster and Waterfield 1961, Forgash 1980, 1985, Harris and Sveg 1976, McClanahan 1975, McDonald 1976) and represent the three main categories of commercial insecticides: azinphosmethyl, an organophosphate; carbofuran, a carbamate; and permethrin, a synthetic pyrethroid. The insecticides (technical compounds) were azinphosmethyl (90% purity) provided by Mobay Chemical Corporation, Kansas City, MO; carbofuran (98%) and permethrin (94.3%) provided by FMC Corporation, Princeton, NJ.

Test Beetles: Twenty-four populations were tested during 1987-1988. Bioassays were undertaken either directly on the field collected beetles before foliage sprays, or on the F_1 colonized generation, when beetles were collected after foliage sprays. Thus, the tested individuals were representative of the population genotypes. Collected beetles were kept at least 2 days, to ensure that they were in good condition, before tests were conducted. Beetles before and after treatment were maintained in cages (50x50x70cm) with potted potato plants (RH 50% ± 10, temperature 25 ± 1°C, photophase 16:8 L:D).

Knockdown effect and mortality are sensitive to changes in physiological conditions of the insects and bioassays were usually conducted at the same time of day (2-5 pm).

Topical Application of Insecticides: The standard bioassay technique for Colorado potato beetle (FAO 1974) was slightly modified. Instead of using CO_2 to immobilize the adults, they were held on the tip of a glass tube to which a vacuum was applied (3.3 mm diam.) briefly during treatment. This avoided any possible side effects of CO_2 and possible obscuring of early symptoms of knockdown effect for permethrin. Toxicity was assessed by topical application of 2 µl of solutions of the pure insecticide in acetone on the second abdominal sternite of the adult, with a hand operated microapplicator. Doses were selected, based on preliminary trials, to give a range of 10-100% mortality. For each bioassay, 4-7 doses were used to determine a dose-mortality curve. Beetles treated with acetone were used as controls in all tests. Mortality values were corrected for natural mortality by Abbott's formula (Abbott 1925). If control mortality was larger than 20%, we repeated the bioassays (Busvine 1971). In almost all cases, natural mortality was less than 10%. If large amounts of beetles were available, tests were repeated twice and the results were pooled. Toxicity was estimated by log-probit analysis (Finney 1971, SAS 1985).

Carbofuran was quite insoluble in acetone when large amounts were needed and often crystalized on the surface of the beetles at high concentrations (larger than 50 µg/µl). Thus, any resistance mechanism that necessitates a large dose of this compound is likely to give an inflated estimate of the LD_{50} (Brown and Pal 1971). However, to compare our results with data already published for carbofuran, acetone was still used as solvent. To achieve application of larger doses to highly resistant strains a second 2 µl

drop was applied to the same area on the beetle as soon as the first drop was dry.

Assessment of Mortality: Timing of the assessment of mortality is extremely important. There are differences between insecticide groups in the speed of action. For azinphosmethyl and carbofuran, maximum mortality was obtained in 3 days. For permethrin, more than one assessment of mortality was needed to draw useful conclusions. Knockdown effect is common and there may be recovery of most of the insects in some populations. Therefore, mortality for permethrin was estimated in half an hour for knockdown effect, in 3 days for dead beetles + "knocked-down" beetles, and in 5 days for dead + recovered beetles (final mortality). Recovery several days after pyrethroid treatment has also been observed by Grafius (unpubl.) in the field. In this study we report LD_{50}'s for permethrin mortality at 5 days.

Resistance Ratio (RR): As susceptible strain LD_{50}'s for estimation of resistance ratio (RR) (observed LD_{50}/susceptible LD_{50}), we adopted the reported LD_{50} topical toxicities for susceptible adults from Northern Maine; 0.3 µg/beetle for azinphosmethyl, 0.25 µg/beetle for carbofuran and 0.39 µg/beetle for permethrin (Forgash 1985). Idaho adults had similar LD_{50}'s (Johnson and Sandvol 1986) In our study, the most susceptible populations tested also had similar LD_{50}'s.

Results

Eight populations were susceptible to all three insecticides (RR ≤ 6). Most of the tested field populations were to some extent resistant to permethrin, with resistance ratios up to 110-fold. Log-dosage mortality curves

of bioassays for the majority of the examined populations indicated that populations were heterogeneous with shallow regression slopes and high variability. The variability and the shallow slopes would suggest that the next step would be the rapid development of homogeneous resistance if the populations were selected intensely.

Control by the recently introduced synthetic pyrethroids, especially permethrin, is unsatisfactory in many areas (Grafius 1986, Ioannidis and Grafius 1988). Populations with high permethrin resistance tended to have low levels of resistance to azinphosmethyl and carbofuran and vice versa. There appear to be three main patterns of resistance in the field. These patterns reflect the underlying resistance mechanisms. Our results and the proposed patterns are also supported by analyzing data reported by other investigators from different geographic areas and chronological periods.

Pattern I: The resistance ratios for these populations were moderate (10 to 20 fold) to high (20 fold or more) for permethrin but low for azinphosmethyl and carbofuran (Table 1). This tended to occur in fields where permethrin was frequently used, or where aldicarb (a soil applied insecticide) was used. The possible cross-resistance of aldicarb and permethrin has also been discussed by Forgash (1985). Harris and Sveg (1976) also reported results which fit this pattern; no evidence of resistance to azinphosmethyl and carbofuran in four CPB populations, but resistance to DDT and endosulfan. At this time, permethrin had not been introduced into control programs but the similarities and cross resistance between DDT and permethrin are well known (Sawicki 1973, Gammon et al. 1981, 1985, Scott and Matsumura 1983, Scott et al. 1986).

Pattern II: The opposite of pattern I was also observed; high levels of resistance to azinphosmethyl and carbofuran and relatively low (less than 10

fold) resistance to permethrin (Table 2). The mechanisms responsible for resistance to azinphosmethyl and carbofuran seem to be quite specific because the resistance did not extend to permethrin. Forgash (1980), Hare (1980), and Harris and Sveg (1981) reported similar relationships for resistant populations of Colorado potato beetle. The resistance ratio in New Jersey for azinphosmethyl was 100, for carbofuran 400, and for permethrin 5 (Forgash 1980). Connecticut River Valley populations showed resistance ratios of 8,266 for azinphosmethyl, 22,508 for carbofuran and 2.58 for permethrin. In Quebec, in tests where formulated products were applied to potato plants, beetles were resistant to azinphosmethyl and carbofuran and again susceptible to permethrin (Harris and Sveg 1981).

Our results suggest that at least one resistance mechanism is common for azinphosmethyl and carbofuran and that this mechanism is determined by the same or closely linked genes. In laboratory selection studies with carbofuran, the beetles developed 360-fold resistance to carbofuran and 43-fold cross-resistance to azinphosmethyl (without azinphosmethyl selection) after four selections. Genetic analysis of resistance of this strain showed this resistance mechanism to be incomplely dominant and monofactorial in inheritance (Grafius et al. 1988). Boiteau et al. (1987) also reported a high level of resistance to carbofuran with cross resistance to organophosphates and low resistance to permethrin and aldicarb.

Pattern III: Resistance to all tested insecticides was present in areas with heavy spray pressure, using mixtures of different groups of chemicals, 10-15 sprays per season, and little or no crop rotation (Table 3). These beetles were resistant to all three insecticides tested and showed high cross-resistance to DDT. This may be the consequence of one of the resistance mechanisms of permethrin, the Kdr or knockdown resistance factor (Ioannidis and Grafius

1988). Multiple resistance populations are now established in many parts of the United States, for example, Long Island, NY; Michigan; Virginia (Forgash 1985, Grafius 1987, Zehnder 1986).

Discussion

By studying and comparing the resistance patterns of Colorado potato beetle in Michigan and in other areas, one can observe that the rate of development of resistance is much more rapid for carbofuran than for azinphosmethyl. Populations were always either highly susceptible or highly resistant to carbofuran. On the other hand, resistance to azinphosmethyl varied a lot with the tested beetles (5 to 500-fold). The same situation has happened with azinphosmethyl and carbofuran resistance in the green rice leafhopper in Asia (Hamma 1983).

The main resistance mechanisms for permethrin in Colorado potato beetles, based on synergistic studies, were mixed function oxidases (MFO's) and Kdr factor (Ioannidis and Grafius 1988). Silcox in 1985 showed that oxidative detoxification was only one of the factors involved in CPB pyrethroid resistance because synergistic studies with piperonyl butoxide was unable to entirely overcome CPB pyrethroid resistance. In Pattern I, there was no cross-resistance between permethrin and azinphosmethyl or carbofuran. The MFO's responsible for permethrin resistance therefore appear to be different from those responsible for azinphosmethyl and carbofuran resistance. Further biochemical studies support this (Ahammad-Sahib et al. 1990). It has demonstrated that a field resistant strain (Long Island) which had been further selected with azinphosmethyl shows high resistance to azinphosmethyl, high resistance to carbofuran, and low resistance to

permethrin (Table 2). Synergistic and biochemical analysis with this strain shows that mixed function oxidases were the main mechanisms for resistance to azinphosmethyl for this strain (Ahammad-Sahib et al. 1990). Rose and Brindley in 1984 working with carbamate resistant CPB strains concluded a resistance mechanism in addition to MFO's, which is not sensitive to piperonyl butoxide synergism, may be involved in CPB resistance to carbamates. No cross-resistance between pyrethroids and carbamates has been observed in resistant diamondback moths (Cheng 1988). Cheng assumed that the MFO's responsible for carbamate resistance are not involved in pyrethroid resistance. Genetic analysis in houseflies has shown that there are at least two genes involved in resistance by increasing oxidation (Tate et al. 1974, Plapp and Casida 1969). A gene on chromosome 2 causes oxidation to aldrin, carbamates, organophosphates, and pyrethrins. A gene on chromosome 5 causes oxidation to DDT, DDE, and diazoxon (the oxon form of diazinon). A factor causing pyrethroid resistance on this chromosome may represent the same gene (Oppenoorth 1985).

Useful recommendations can be drawn for CPB management by monitoring resistance. Pattern I suggests the failure of control with permethrin and indicates that a switch to a carbamate or organophosphate should be appropriate. Pattern II suggests the opposite. Because azinphosmethyl resistance gives resistance to phosmet it is not useful to use these two organophosphates in mixtures or sequences. Cross resistance between azinphosmethyl and carbofuran also occurs, at least in the tested populations.

Some negatively correlated resistance between permethrin and azinphosmethyl-carbofuran may exist, where selection of resistance by pressure from permethrin leads to a corresponding selection of susceptibility

to azinphosmethyl and carbofuran and vice versa. This speculation is supported by the observed patterns I and II and by selection studies in our laboratory. Negatively correlated cross-resistance to a synthetic pyrethroid in organophosphorus-resistant Tetranychus urticae (Koch) has been clearly demonstrated; increasing azinphosmethyl resistance confering an increase in fenvalerate susceptibility (Chapman and Penman 1979). A similar negatively correlated cross-resistance has been noted between malathion and fenvalerate in the resistant brown planthoppers N. paparvata, N. lugens Stal and Laodelphax striatellus Fallen, two of the most destructive pests of rice in Japan (Miyata and Saito 1984).

If the negative correlated resistance is clearly demonstrated, then an alternate use of the insecticides can be an effective strategy. Keiding (1963) referred to possible reversal of resistance when insecticides are substituted by another proper insecticide particularly in the early stages of detection of the resistance. He discussed the factors influencing reversal of resistance to DDT, BHC and diazinon in the housefly on farms in Denmark. Also, in the case of negatively correlated resistance, the mixtures used in sprays must not consist of a pair of compounds that display negatively correlated toxicity (Georghiou 1983).

Future studies on the mechanisms and cross-resistance linkages for CPB will be necessary for a more complete understanding of the processes of resistance and potential resistance management tactics. Application of this information to the management of CPB populations before resistance reaches crisis proportions will result in long-term gains in CPB control and reduced pesticide usage.

Table 1. Pattern I. Dose response data for eight populations of Colorado potato beetle with low resistance ratios for azinphosmethyl and carbofuran, and moderate to high resistance ratios for permethrin.

Strains	Number of beetles tested (N)	Slope ± S.E.	LD_{50}'s µg / beetle	95% C.L. lower	95% C.L. upper	Resistance ratio RR
Montcalm 1988						
Azinphosmethyl	74	4.12 ± 1.26	0.79	0.53	1.33	2.60
Carbofuran	36	2.15 ± 0.96	0.40	0.15	1.08	1.60
Permethrin	68	1.96 ± 0.39	3.25	2.15	4.90	10.83
Baker[a]						
Azinphosmethyl	108	2.28 ± 0.40	1.13	0.77	1.66	3.76
Carbofuran	72	3.88 ± 0.84	0.80	0.61	1.03	9.20
Permethrin	60	4.99 ± 1.08	16.10	13.0	19.97	41.28
Leep						
Azinphosmethyl	84	3.80 ± 1.31	2.04	1.55	2.68	6.80
Carbofuran	78	1.55 ± 0.28	2.30	1.31	4.04	3.20
Permethrin	78	0.94 ± 0.34	6.47	3.16	13.24	16.58
TIM						
Azinphosmethyl	82	---	<1.56	---	---	<5.20
Carbofuran	80	3.20 ± 1.44	0.47	0.34	0.67	1.88
Permethrin	76	1.28 ± 0.37	5.19	3.05	8.83	13.30
Vestaburg II[a]						
Azinphosmethyl	96	2.79 ± 0.50	1.7	1.30	2.17	5.70
Carbofuran	96	3.09 ± 0.74	0.2	0.16	10.27	1.00
Permethrin	76	1.73 ± 0.39	10.2	5.36	18.65	26.10
TV						
Azinphosmethyl	118	1.82 ± 0.29	2.98	2.30	3.90	9.90
Carbofuran	110	1.30 ± 0.32	0.19	0.09	0.49	1.00
Permethrin	98	3.20 ± 0.59	12.53	9.79	16.03	32.12
Monroe						
Azinphosmethyl	80	1.70 ± 0.37	1.82	1.19	2.78	6.30
Carbofuran	80	2.39 ± 0.45	0.76	0.51	1.14	3.00
Permethrin	90	0.75 ± 0.28	11.03	8.42	14.50	28.30
SLF						
Azinphosmethyl	64	---	<1.56	—	—	1.00
Carbofuran	69	0.78 ± 0.32	0.29	0.05	1.96	1.00
Permethrin	75	1.59 ± 0.50	21.93	10.25	46.90	56.23

[a] These strains are further maintained in our laboratory and are under selection with permethrin.

Table 2. Pattern II. Dose response data for populations of Colorado potato beetle showing high resistance to azinphosmethyl and carbofuran and low to moderate resistance to permethrin.

Strains	Number of beetles tested (N)	Slope ± S.E.	LD_{50}'s µg /beetle	95% C.L. lower	upper	Resistance ratio RR
Long Island, NY (lab strain)[a]						
Azinphosmethyl	96	2.5 ± 1.00	743.20	321.0	1714.00	2477.0
Carbofuran	80	1.41 ± 0.42	47.80	27.6	82.20	191.0
Permethrin	90	2.71 ± 0.45	3.70	2.8	5.00	9.5
GR Macomb						
Azinphosmethyl	84	1.90 ± 0.37	12.07	6.7	18.30	40.2
Carbofuran	86	---	>200.70	----[b]	---	>400.0
Permethrin	98	2.14 ± 0.35	3.95	2.7	5.70	10.1
BL Macomb						
Azinphosmethyl	92	2.07 ± 0.35	10.72	7.6	15.20	35.7
Carbofuran	96	0.89 ± 0.50	1574.60	32.8	---	>2000.0
Permethrin	100	1.85 ± 0.28	2.70	1.8	3.90	6.9

[a] Long Island strain was under selection with azinphosmethyl in our laboratory.

[b] Not possible to estimate because of very high resistance levels (see Methods).

Table 3. Pattern III. Dose response data for five populations of Colorado potato beetle showing high resistance to all tested insecticides.

Strains	Number of beetles (N)	Slope ± S.E.	LD_{50}'s per/beetle	95% CL lower	95% CL upper	Resistance ratio RR
JP Macomb 1987						
Azinphosmethyl	98	0.80 ± 0.18	36.28	14.7	89.3	120.3
Carbofuran	70	0.64± 0.27	21.98	3.6	131.8	87.9
Permethrin	80	1.05± 2.68	8.40	4.1	17.3	21.5
JP Macomb 1988[a]						
Azinphosmethyl	102	0.79 ± 0.79	272.90	64.5	1146.0	907.0
Carbofuran	80	---	>400.00	—	—	>1000.0
Permethrin	87	0.95 ± 0.44	42.40	8.4	213.3	108.7
U.B.						
Azinphosmethyl	64	1.14 ± 035	4.02	1.8	9.2	13.4
Carbofuran	69	---	>100.00	---b	—	>400.0
Permethrin	75	1.49 ± 0.50	17.53	8.8	35.5	45.0
Shoe						
Azinphosmethyl	69	1.95 ± 0.41	7.22	4.7	11.0	24.1
Carbofuran	80	---	>100.00	—	—	>400.0
Permethrin	94	0.73 ± 0 0.51	13.90	2.8	70.5	35.6
Mayer						
Azinphosmethyl	96	2.67 ± 0 0.43	12.60	8.7	16.6	42.0
Carbofuran	96	---	>100.00	—	—	>400.0
Permethrin	96	1.43 ± 0 0.42	23.14	11.0	47.5	59.4

[a] This strain is now maintained in our laboratory.

[b] Not possible to estimate because of very high resistance levels (see Methods).

Chapter 3

Synergist Studies

SYNERGISTS

One approach to elucidate the physiological mechanisms of insecticide resistance involves the use of synergists. Synergists are also of practical importance to the entomologist (Metcalf 1967):

a) in the more economical or efficient control of insects by a mixture,
b) in increasing the spectrum of activity of an insecticide,
c) in restoring the activity of an insecticide against resistant strains.

Synergists have potential uses in insecticide resistance management as analytical tools, control of resistant populations, delay of resistance development, and preservation of natural enemies (Raffa et al. 1983). But as they have pointed out, the most promising use of synergists are as research tools. Partly because of a failure in the above assumptions, the theoretical potential of synergists has been a commercial disappointment until now, with the exception of pyrethroid-synergist aerosols. The extensive investigation of synergism and synergists has led to a much better understanding of the mechanisms of detoxification in insects, of the basic biochemical processes involved in insecticide resistance.

A synergist will be defined as any chemical which in itself is not toxic to insects as dosage used, but when combined with an insecticide, greatly enhances the toxicity of the insecticide. This, of course, differs from potentiation, which occurs when two toxic materials applied together elicit a response greater than that expected from the sum of the individual toxicants (Kuhr 1976).

When tolerance or resistance is mainly metabolic, the synergist may play a useful role in overcoming resistance even temporarily without loading the environment with extensive use of toxicants.

Wilkinson (1968) described the role of insecticide synergism in resistance problems. He pointed out that it becomes immediately obvious that insecticide synergists constitute a group of compounds with considerable theoretical potential for the control of a large number of resistant strains of insects.

Although considerable variation is observed in the relative potency of the synergists with different insecticides and in different species and strains of insects, some degree of synergism can be expected to occur with all insects which depend on microsomal oxidation for their survival after insecticide exposure (Wilkinson 1971). A great amount of useful information on the toxicology of an insecticide chemical has been and can be gained by appropriate investigations with synergists.

Regarding insecticide synergists and their mode of action, the reviews by Casida (1970) and Wilkinson (1971) are very detailed and comprehensive. The synergist studies conducted were of great practical value because most of them were applied directly on field resistant strains.

The use of insecticide synergists fits with IPM (Integrated Pest Management) because mortality of the beneficial and non-target species will be lower and the insecticide rates needed for the control of the pest will be reduced. We can also use synergists to control resistant populations to some extent.

The use of PB and DEF as synergists with carbamates, organophosphates, and pyrethroids has been well established with many insecticides and species (Plapp et al. 1967, Ishaaya et al. 1983, Liu et al. 1984, Dai

et al. 1984, Hemingway et al. 1984). PB and DEF are widely used to indicate which type of detoxification mechanism is involved (Attia 1980, McCord 1987, Prabhaker 1988).

From the logdose-mortality regression equation apart from the estimation of synergistic ratio (SR) at 50% mortality level, we could estimate the SR in 90% mortality for the purpose of field efficacy. The SR_{90} is very important since in most cases, 90% mortality is required for acceptable control in the field.

The high synergistic effect of PB with azinphosmethyl, which we found consistently with several populations, may prove useful in controlling resistant populations and reducing the rate of selection of resistance in the field or decreasing the frequency of resistant genes already present in field populations.

Available Information about Synergists on Resistance of the Colorado potato beetle

Research that has been conducted with synergists on CPB is almost exclusively with piperonyl butoxide (PB). Studies with other groups of synergists have not been reported (Table 1) and synergistic studies with azinphosmethyl are completely absent.

Forgash (1985) showed that PB reached a maximum effect when applied 2 hours prior to pyrethroid treatment. In the same studies, rotenone was synergized very well by PB suggesting that PB had two effects, i.e., it increased penetration of rotenone and interferred with detoxification. This interference could be through inhibition of MFO. In addition, PB was also quite effective in combination with aldicarb or oxamyl but slightly so with carbaryl and carbofuran. This is true, but in our study we found very good

synergism of carbofuran by PB also in strains with MFO's increasing activity. It is well known that target insensitivity is another main resistant mechanism for carbofuran (see details in Chapter 4). Increasing the PB treatment from 10 µg to 40 µg greatly increased the degree of synergism of aldicarb (aldicarb alone, LD_{50} = 14.0 µg/beetle, aldicarb + 10PB = 4.50, aldicarb + 40PB = 0.31). It seems that the ratio 1 : 4, toxin : synergist, is the optimal. The results with carbofuran are in agreement with our findings (low synergism with PB) in some cases. Rose (1985) also found low synergism with PB and carbofuran.

In the following table we have summarized all the synergist studies available in the literature on research conducted on the CPB:

Table 1. Synergism studies with Colorado potato beetle.

Author/Synergists	Insecticides	Results
R.L. Rose (1985) Piperonyl Butoxide	Carbofuran Carbamates	A. Resistance of the New Jersey beetles (larvae) was less dependent upon the enzymes monoxygenases L % S = 29% B. Significant differences between resistant and susceptible Colorado potato beetle larvae were not observed in the penetration of [^{14}C] carbaryl.
C.A. Silcox, et al. (1985) Piperonyl Butoxide	Permethrin Fenvalerate Also conducted field experiments	Larvae and adults However oxidative detoxicates is only one of the factors involved in CPB pyrethroid resistance because PB was unable to entirely overcome CPB pyrethrins resistance. During the experiments the beetles used were field collected with small resistance.
Forgash (1980) Piperonyl Butoxide	Carbofuran Azinphosmethyl Chlorfenviphol Aldicarb Lindate Phosmet Carbaryl	Only slight synergism None
Harris (1986) Piperonyl Butoxide	Fenvalerate " " Deltametrin "	2:1 5-fold More toxic with R-strain 8:1 12-fold More toxic with R-strain 8:1 1.6-fold More toxic with S-strain 8:1 2.5-fold More toxic with S-strain 8:1 3.0-fold More toxic with R-strain

Use of Insecticide Synergists to Indicate Resistance Mechanisms

Research Approach and Procedures

Synergist studies were conducted to complete the already available information and to determine whether metabolic resistance mechanisms were involved using lab resistant strains and different field collected populations. Raffa and Priester (1983) emphasized that the simplest use of synergism in resistance management is direct application to resistant populations.

Instead of testing extensively one strain with all the available synergists, we considered it would be better to test the well known synergists (PB, MGK 264, DEF, DEM, Chlorfenethol) with different populations collected from places with different spray histories. Therefore, we were able to carry out comparisons to better elucidate the results, and to figure out different patterns of resistance mechanisms.

Synergists used were:

Synergists	Inhibition of
Piperonyl Butoxide (PB)	mixed-function oxidases
MGK 264	mixed-function oxidases
DEF	esterases
DEM	gluthathione-S transferase
Chlorfenethol (DMC)	DDT dehydrochlorinase

References to the above synergists are given in the following pages.

To produce better effect in CPB, it was necessary to apply the synergist in advance of the insecticide (two-hour pretreatment). The synergistic ratio

(SR) was calculated as the LD_{50} of the insecticide alone over the LD_{50} of the mixture. It was considered that SR > 1 means we had synergism, and SR < 1 antagonism.

Two methods for applying synergists were used:

Method A: The use of ratio 1 : 1 or 1 : 2 or 1 : 4 (ratio insecticide : synergism for example):

1 : 1	1 : 2	1 : 4
2 : 2	2 : 4	2 : 8
4 : 4	4 : 8	4 : 16
8 : 8	8 : 16	8 : 32

In the control process the largest dose of synergist was applied.

Method B: Keeping the synergist constant. Applying the largest dose of synergist (x) which gives less than 10% mortality (preliminary work for estimation of x was necessary). Therefore, we designed experiments as follows:

Control

Synergist x

Insecticide a + x

Insecticide 2a + x

Insecticide 4a + x

Insecticide 8a + x

Since the tolerance of synergists by the beetles depends on the level of resistance (the more resistant the beetles, the more synergist they can tolerate)

we adopted both the methods but favor the insecticide : synergist ratio technique which gives more accurate results, particularly with moderate levels of resistance. We used method B with highly resistant populations, because it was technically impossible to apply 2000 µg/beetle with one drop of 2 µl when the level of resistance to an insecticide is 500 µg/beetle. After the insecticide-synergist combination yields a presumed mechanism, specific biochemical experiments were needed to confirm the resistance mechanisms. It is worthwhile to note that a solid agreement between synergistic studies in vivo and biochemical analysis in vitro were obtained in the case of the Colorado potato beetle. Details about the synergists, techniques, and considerations are given in the following passage.

Piperonyl Butoxide (PB)

Methyllenedioxyphenyl group. Mixed-function oxidase inhibitors (Brown 1971). Studies with synergists such as PB and Sesamex have now become an established and widely used technique for indicating the in vivo importance of oxidative degradation.

The methyllenedioxyphenyl (MDP) compounds are the most important synergists from the standpoint of historical development, current use, and effectiveness (Casida 1970). The best established mixed-function oxidase (MFO) inhibitors are the MDP compounds. Through the use of synergistic ratio the effects of PB provide a fairly precise indication of the relative contribution of oxidative metabolism to the observed toxicity.

Sesamex, an MDP compound, is more active than the PB but it is not used commercially because, in the presence of sunlight and moisture, it decomposes to yield objectionable colored products. Generally, a highly

potent cholinesterase inhibitor and toxic compound such as carbofuran is poorly synergized by PB (Casida 1970, Metcalf 1967). This is probably the case with susceptible populations.

MGK 264

Alkylamines and Amides group. Mixed-function oxidases inhibitor.

The inhibition of MFO activity is not restricted to MDP compounds, it also includes alkylamine derivatives such as MGK 264, and SKS 525-A. MGK 264's main use is as an insecticide synergist for pyrethroids (Worthing 1979). Recently, Cochran (1987) demonstrated that resistance to pyrethroids and bendiocarb in the German cockroach can be overcome by the use of PB or MGK 264 as synergists. The results were rather similar in terms of their ability to reduce resistance. The optimum ratio of synergists to bendiocarb was 1 : 3 and for Pyrethrin 1 : 10 (insecticide : synergist).

SKF 525-A and MGK 264 inhibits the conversion of thiophosphoryl into phosphoryl by mammalian microsomes and cockroach guts but in houseflies SKF 525-A synergized azinphosmethyl.

DEF

DEF (S,S,S-tributylphosphorothionite) is a plant defoliant that inhibits esterases and oxidases (Plapp 1986). Data with P. interpuitella larvae indicates that DEF is probably an esterase synergist as well as an oxidase synergist (Attia et al. 1980).

Synergism by DEF is normally considered as indicative of the involvement of esterases and/or glutathione S transferases in insecticide

metabolism (Hemingway and Georgiou 1984). Dimethyl and triphenyl phosphate synergists, like DEF, behave as competitive inhibitors and would have to be applied in the ratio of at least 1 mol inhibitor to 1 mol synergist (Fest 1982).

The highest synergistic activity of DEF was obtained when it was given to mice 6 hr before treatment with malathion; certain metabolic transformation might be involved in the manifestation of the activity (Eto 1979). Moreover, against certain resistant insect strains DEF synergized to some extent paraoxon, azinphosmethyl, the carbamate insecticides, and DDT.

Because DEF is a defoliant, mixtures of this synergist and insecticides cannot be used in the field to overcome resistance due to esterases. We found populations of CPB resistant partly due to esterase activity. Probably of practical use are the mixtures with sprayable esterase inhibitors, such as kitazin P, a systemic fungicide used to control Piricularia oryzae in rice (Worthing 1979). Inhibition of carboxyesterase by kitazin P was the main factor responsible for synergism between malathion and kitazin P against the green rice leafhopper (Ozaki 1983). Hemingway and Georgiou (1984) used kitazin P in synergistic and selection studies with Culex quinquefasciatus.

Diethyl Maleate (DEM)

DEM is an inhibitor of glutathione s-transferase but can inhibit both mixed-function oxidases and esterases. It is unfortunate that there are no specific inhibitors of glutathione S-transferases (G-S-T) that can be tested in vivo like the more specific inhibitors of enzymes as PB, DEF, and Chlorfenethol. Therefore, negative results in synergism with DEM does not necessarily mean absence of glutathion S-transferase action.

DEM inhibits both MFO and transferase and Welling et al. (1985) suggested that only if commonly used inhibitors of the MFO, like Sesamex and PB, fail to synergize does it seem indicated to try DEM and analogs. It also seems that thiono compounds are less synergized than their oxon analogs.

The importance of G-S-T as a possible resistance mechanism seems to have been neglected due to the fact that the products of its action on organophosphorous (OP) compounds are identical to those formed by other enzymes. G-S-T is probably of more importance in OP metabolism in the dealkylation of dimethyl phosphates and phosphorothionates (Oppenoorth 1976).

Chlorfenethol (DMC)

The compound 4,4'-dichloro-a-methylbenzhydrol used to have the trademark name Dimite. The new trademark name is Chlorfenethol. It is a non-systemic acaricide with a pronounced ovicidal activity, for use on fruit, vegetables, and cotton at 50-75 g./100L (Worthing 1979). It is reported to act as a synergist for DDT against DDT-resistant insects (Sumerford et al. 1951). The ratio used was a water emulsion of 5% DDT and 1% DMC. Metcalf (1967) summarized the investigation about the synergist DMC and analogs as inhibitors of DDT dehydrochlorinase enzyme which is one of the mechanisms of resistance to DDT (Oppenoorth 1984). DMC proved to be a most effective synergist in resistant houseflies and mosquitos.

Insecticide-Synergist Ratio

Another factor that should be weighed in order to obtain maximum insecticide synergism was the relative quantity of each ingredient needed for treatment. Usually, the higher the synergist-insecticide ratio, the greater the effectiveness of the combination. Eventually a maximum ratio is reached beyond which additional synergist no longer enhances toxicity. Generally, routine screening of synergists with carbamates is 1 : 5 to 1 : 10 insecticide-synergist ratio (Kuhr 1976).

With carbaryl the ratio 1 : 5 gives the best synergism. The difference between 1 : 5 and 1 : 10 was very small, when applying topically 26 different methyllenedioxyphenyl compounds on Musca domestica (Hewlett et al. 1967). In other studies with carbamates, the ratio 1 : 5 was used (Moorefield 1960, Fukuto et al. 1962, Plapp et al. 1967). Brattsten and Metcalf in 1970 and 1973 made an extensive and careful evaluation of synergistic effects of carbaryl with PB (ratio 1 : 5). They tested 74 insect species representing 40 families, and eight orders. In general, Coleoptera species, especially chrysomelidae, showed high susceptibility and low synergistic ratio. This information was very valuable for conducting comparisons since CPB is in the family chrysomelidae.

With carbamates, maximum inhibition of the enzyme system by methyllenedioxyphenyl synergists (PB, Sesamex, Sulfoxide) occurs at a carbamate-synergist ratio of 1 : 2 to 1 : 5 (Metcalf et al. 1966).

Pyrethroid Synergism

The importance of insect esterases in pyrethroid metabolism is that esterases are likely to be of greater relative significance in detoxifying the trans-isomers and oxidases with the cis-isomers (Jao et al. 1974). Chang and Whalon (1985) found that pyrethroid esterases were very sensitive to inhibition by DEF in the resistant predator mite Amblyseius fallacis and esterases hydrolyzed trans-Permethrin two times faster than cis-Permethrin. Casida and Ruzo (1980) concluded that synthetic pyrethroids with primary alcohols (permethrin) were mostly cleaved by esterases that were sensitive to inhibition by a variety of organophosphorous compounds while those with secondary alcohols (with a cyano group like cypermethrin, deltamethrin, fenvalerate) were only slowly attacked by esterases. Similar results were found by Dai et al. (1984). A follow-up survey surprisingly indicated that brown planthopper was highly resistant to permethrin with only low or moderate resistance to a-cyano containing pyrethroids.

The optimal ratio of mixtures of PB with pyrethroids for the control of Diamond-back moth were 1 : 1 to 1 : 5 insecticide: PB (Liu et al. 1984).

NPC (1-napthyl N-propyl carbamate) (which is another inhibitor of esterases apart from the classical DEF) applied to milkweed bugs was essentially the same when applied simultaneously, 8 hours, or 24 hours prior to the pyrethroids.

In some cases, the synergist is much more effective when it is applied several hours before the insecticide. From a research standpoint, this is the best way to study synergists because competitive uptake is eliminated.

Mixed-Function Oxidases

Since age, sex, and stage of development play an important role in enzyme activity and the levels of MFO change dramatically between larvae molting, it had been decided to conduct most of the bioassays with adults 1 week old and, when possible, testing the sexes separately depending on the available number of individuals. Wilkinson and Brattstein (1973) have summarized investigations on factors affecting microsomal activity (MFO), such as age, sex, stage, strain differences, effect of nutrition and diet, and pointed out that the problem is exacerbated by the fact that the entire activity may occur within days or even hours.

Ahmad and Forgash (1975), working with gypsy moth, clearly demonstrated that the increase in tolerance to carbaryl and diazinon with larvae growth, as well as increase in PB efficacy, correlates with increases in mixed-function oxidases in advanced instars. Tests were conducted using a 1 : 5 insecticide : synergist ratio.

A reduction of the fat-body fraction occurring in certain stages results also in a reduction of the activating oxidation mechanisms which are localized in the fat-body in favor of the detoxifying hydrolysis mechanisms. The application of insecticides must, therefore, be directed against particularly susceptible stages in the development of a pest (Fest 1982).

Because the microsomal enzymes are also responsible for the oxidative activation of the organophosphorothionate insecticides P = S $\rightarrow$ P = O, synergists often show an antagonistic effect on the toxicity. In our studies, azinphosmethyl, despite the fact that it is a phosphorothionate, was demonstrated to be synergized very well by PB and we have not observed any antagonistic effect.

There are also reports of synergism of phosphorothionates (fenitrothion, diazinon) by PB with the resistant moth Plodia interpunitella (Attia 1980). O'Brien (1960) tried to elucide this paradox further in his studies with the synergist SKF 525-A, an inhibitor of MFO. He found that SKF 525-A synergized Guthion and Diazinon and inversely had an antagonistic effect with parathion. This apparent contradiction, the synergistic and antagonistic action, is well demonstrated by Sun et al. (1967) who conducted experiments using Sesamex (MFO inhibitor) as a synergist and several organophosphorothionates or chlorinated insecticides. They pointed out that these apparent differences between different phosphorothionates may be due to the degree of stability and rate of penetration of P → S and P → O analogs as well as to possible toxicity of some P = S compounds without converting to P = O analogs.

The most important oxidase for xenobiotic metabolism is Cytochrome P-450, a component of the microsomal mixed-function oxidase system. This is called Cytochrome P-450 because an intense absorption at 450 nm appears as a reaction of the reduced pigment with carbon monoxide (Eto 1979).

Recently there have been several reports about the existence of multiple forms of P-450's. Wilkinson (1983) suggest that these are under separate genetic control. Ronis et al. (1988), working with an insecticide resistant housefly, was able to characterize four different isozymes of P-450. It is interesting that two forms of P-450 were found in Drosophila, the P-450A ~ 59 kΔa which was widespread among strains, whereas P-450 B-56 kΔa was expressed only in strains resistant to insecticides. Levi et al. in 1988 reported that fenitrothion yields the corresponding fenitroxon by oxidative desulfuration, an activation reaction, and 4-nitro-m-cresol by oxidative dearylation, a detoxification reaction. They also found that four P-450

isozymes can be induced by phenobarbital in mice, with different effects in the production of the oxon form of the fenitrothion.

Results

First results are given from the resistant field strain of beetle (collected from the Macomb Co. area) in 1987.

Resistance to azinphosmethyl was found to be 81-fold (Table 2). The population was heterogeneous, containing both susceptible and resistant individuals, as indicated by the high X^2 value. With the addition of PB, resistance was reduced approximately 15-fold, indicating mixed-function oxidase activity. The resistance ratios were calculated by dividing the LD_{50} of an insecticide by the LD_{50} of the insecticide plus synergist. The response of the beetles to azinphosmethyl plus PBO was homogeneous, a more uniform level of resistance, as indicated by the low X^2 value. This suggests that the main mechanism for resistance to azinphosmethyl is mixed-function-oxidase.

Actual resistance levels to carbofuran could not be determined for the Macomb JP population because levels of technical grade material could not be applied in high enough amounts to cause mortality. LD_{50} values were estimated as approximately 1000 fold higher (Table 2). The synergism of carbofuran with both PB and DEF was low. This lack of synergist activity plus other observations discussed below suggest a change in the target site of the insecticide as the mode of resistance.

With permethrin, a fairly effective synergistic ratio was found with PB (6.5 times) (Table 3). With addition of the synergist PB returns the highly

Table 2. Resistance and insecticide synergism of JP Macomb population of the Colorado potato beetle to azinphosmethyl and carbofuran using topical application bioassays.

	LD50 (µg/beetle)	Synergism Ratio	χ^2 (df 2)
azinphosmethyl	24.3[a]	--	7.0**
+ PB	1.6	14.9	0.6
+ DEF	12.2	2.0	1.1
+diethyl maleate	22.3	1.1	4.9*
carbofuran	>1000.0[a]	--	
+ PB	198.1	>5.0	6.24**
+ DEF	422.8	>2.0	0.67

[a] Susceptible LD_{50}: carbofuran = 0.2µg; azinphosmethyl 1.25µg.

** Significantly different; this value is higher than figure at χ^2 for the 5% level.

Table 3. Resistance and insecticide synergism of JP Macomb population of Colorado potato beetle to permethrin and DDT using topical application bioassays.

	LD50 (μg/beetle)	Synergism Ratio	χ^2 (df2)
permethrin	16.80[a]	--	13.30***
+ PB	2.50	6.90	1.90
+ DEF	6.20	2.70	6.30**
DDT	>1000.0[a]	--	0.45
+ PB	253.0	--	3.60
+ DMC	898.0	--	6.30**

[a] Susceptible LD_{50}: DDT = 10.0 μg; Permethrin 0.39 μg.

** Significantly different; these values are higher than figure of χ^2 for the 5% level.

*** Highly significantly different; these values are higher than figure of χ^2 for the 1% level.

heterogeneous responses with permethrin alone to a homogeneous response (low X^2 value), also suggesting the involvement of mixed-function oxidases. However, resistance of Colorado potato beetle to permethrin cannot be explained sufficiently by the detoxification mechanisms using PB and DEF. Resistance levels to DDT are also high for this population (Table 3). Therefore, a possible additional resistance mechanism to permethrin is the kdr factor (knock-down resistance). The LD_{50} for DDT was larger than 1000 µg/beetle (Table 3). Bioassays with DDT and PB indicated that the synergistic ratio was about 3.9 and for DDT plus chlorfenethol (DMC) it was 1.2 The permethrin-DDT cross resistance and the low synergism with DDT leads us to the conclusion that another possible mechanism for these two insecticides was probably the kdr factor. This may help explain populations that in some cases were tolerant to pyrethroids when these materials were first used in the field.

Conclusions for synergist studies

The conclusions drawn are based on several field strains tested in our laboratory. With all tested strains, the resistance to azinphosmethyl can be reversed significantly by applying piperonyl butoxide, strong evidence that mixed-function oxidases are primarily responsible for resistance to azinphosmethyl.

The field populations resistant to azinphosmethyl were also highly resistant to carbofuran. In one case 85-fold resistance to azinphosmethyl was accompanied by more than 300-fold resistance to carbofuran.

Table 4. Synergism of azinphosmethyl of GRM Colorado potato beetle strain from piperonyl butoxide or DEF or DEM using topical application bioassays.

Insecticide-Synergist	LD50 (µg/beetle)	Synergism Ratio[a]	χ^2 (3 Δf)
azinphosmethyl	14.26[b]		6.30*
azinphosmethyl + PB	0.65	21.9	0.65
azinphosmethyl + DEF	4.45	3.2	6.34*
azinphosmethyl + DEM	8.18	1.8	9.14**

[a] Synergistic ratio LD_{50} insecticide/LD_{50} insecticide synergist.

[b] Resistant ratio 47.5, LD_{50} = 0.3 for susceptibles.

** Significantly different; these values are higher than figure of χ^2 for the 5% level.

In another field collected population (GRM) with a moderate level of resistance ratio of 47.5 to azinphosmethyl the synergism with PB was extremely high, returning the toxicity of azinphosmethyl back to susceptible. The synergism with DEF and DEM was very low without affecting the heterogenicity of the original population (Table 4). These results indicated that MFO's were the only resistance mechanism. Synergist studies with two highly resistant strains to azinphosmethyl, the Long Island (RR=380) and Macomb (RR=186) indicated that beside the MFO's, esterases must be involved in the resistance to azinphosmethyl (Table 5). It is interesting that application of both the synergists (2 minute time interval) two hours before the application of azinphosmethyl gave an extremely high synergistic ratio of 132, reducing the toxicity almost to susceptible levels, but the synergists produced 20% mortality to the control.

For carbofuran, we found low synergism with piperonyl butoxide. We also tested another inhibitor of mixed-function oxidase II, MGK 264. We did not find any synergism. The synergism by DEF was also low. These results suggest that acetylcholinesterase insensitivity was mainly responsible for resistance to carbofuran. Further synergistic studies revealed three possible cases of resistance to carbofuran can exist (Table 6): First, biochemical analysis with the Long Island strain did not indicate the mechanism of acetylcholinesterase to be present, and the resistance ratio (RR) to carbofuran was 224.9-fold. Applications of carbofuran plus piperonyl butoxide reduced the RR to 15.85 (Table 4, Chapter 4). This was evidence that MFO's were the main resistance mechanism in this strain. Second, with the JP strain almost immune to the carbofuran and resistant as well to azinphosmethyl, the carbofuran synergism by piperonyl butoxide had an intermediate SR less than 5, in contrast to the first (SR=14.9). Biochemical analysis with this strain

suggested that both the resistance mechanisms were present, MFO's and acetylcholinesterase insensitivity. Thirdly, in the MNT-C selected strain for resistance to carbofuran (600-fold), the synergism of carbofuran by piperonyl butoxide was almost zero. Biochemical analysis confirms that only the acetylcholinesterase insensitivity mechanism was present (Table 4, Chapter 4). Rose and Brinley (1985) working on carbamates resistance with Colorado potato beetle, did not find significant differences between susceptible and resistant beetles in the penetration rates with ^{14}C labeled carbaryl.

Table 5. Toxicity and synergistic ratios of azinphosmethyl in mixtures with piperonyl butoxide (PB) or DEF or DEM with a susceptible and two multiple highly resistant strains of Colorado potato beetle (Long Island and Macomb).

CPB Strain	Pesticide and/ or Synergist (1:4 ratio)	LD_{50} (µg/beetle)	95% c.l.	Synergist Ratio
Arizona	azinphosmethyl (AZ)	1.46	0.86 - -1.85	--
	AZ + PB	0.32	0.40 - 0.24	4.56
Long Island	azinphosmethyl	556.00	306.00 - 921.00	--
	AZ + PB	27.60	20.20 - 36.90	20.10
	AZ + DEF	63.10	39.90 - 99.70	8.80
	AZ + DEM	288.70	132.00 - 617.00	1.90
	AZ + PB + DEF	4.20	0.34 - 6.20	132.00
Macomb (GRM)	AZ	272.20	64.5 - 1133.00	--
	AZ + PB	16.50	9.40 - 25.30	16.50
	AZ + DEF	19.50		14.00
	AZ + DEM	36.60	25.70 - 51.40	7.40

Different Types of Mixed Function Oxidases for Resistance in the Colorado potato beetle

The Baker strain possesses a high level of resistance to permethrin while it is susceptible to azinphosmethyl and carbofuran (Table 7). The synergism of permethrin by piperonyl butoxide was 22.87-fold, a strong indication that mixed-function oxidases are involved in permethrin resistance, and esterases based on the fact that synergism by DEF was 10.73-fold. The absence of cross resistance to other insecticides leads us to believe that in Colorado potato beetle more than one type of P-450 occurs. It is very interesting that Baker strain shows cross-resistance to aldicarb (Table 9). This explains the observation in the fields of control failures of permethrin where aldicarb was used as a soil treatment.

The possibility of the existence of different kinds of MFO's in three Colorado potato beetle strains was also biochemically supported. The levels of resistance were as follows:

	Azinphosmethyl	Permethrin
Long Island (RL)	High	Low
Macomb (GRM)	High	High
Baker (RB)	Susceptible	High

In all three strains, the microsome NADPH oxidation representing the MFO activity was about 50 to 100% greater than susceptibles. However, in the strains RL and GRM, the oxidation enzyme aminopyrene N-demethylase was about 100% greater while P-nitroanisole o-demethylase was 40% higher than for the susceptible Arizona strain. Interestingly in the Baker strain aminopyrene N-demethylase did not show any change, while the P-

Table 6. Three different cases of resistance of the Colorado potato beetle to carbofuran. Synergistic studies with piperonyl butoxide (PB) or DEF indicate possible resistance mechanisms involved.

Strain	LD_{50} µg/beetle	Synergistic Ratio[a]	Resistance Mechanism
Long Island			
carbofuran	47.3	--	
carborufan + PB	3.2	14.9	MFO's
+ DEF	30.0	1.6	
MNT-C			
carbofuran	120.9		
carbofuran + PB	no	--	Ache.
carbofuran + DEF	no	--	
JP Macomb			
carbofuran	>400.0	--	
carbofuran +PB	198.0	>5.0	MFO's & Ache.

[a] Synergistic ratio: LD_{50} insecticide/LD_{50} insecticide + synergist.

Table 7. Toxicity of azinphosmethyl, carbofuran and permethrin on Baker strain of Colorado potato beetle only resistant to permethrin. Synergist ratios for mixtures of permethrin and piperonyl butoxide (PB) or DEF.

Insecticide Synergism	LD_{50} (µg/beetle)	95% C.L.	Synergist Ratio RR[a]
permethrin	24.48	8.90 - 67.37	--
permethrin + PB	1.07	0.79 - 1.44	22.87
permethrin + DEF	2.28	1.73 - 2.90	10.73
azinphosmethyl	0.77	0.49 - 1.21	--
carbofuran	0.80	0.61 - 1.03	--

RR = LD_{50} permethrin/LD_{50} permethrin + synergism
LD_{50} for susceptible 0.39 µg/beetle.

nitroanisole o-demethylase showed about 100% greater activity in comparison with the Collins Road strain (susceptible strain), suggesting that the MFO's types in RL and GRM are different compared to RB. (Amammad Sahib and Whalon, unpublished data).

Finally, it seems that Colorado potato beetles can use all the available resistance mechanisms to resist insecticides (Table 8). Therefore, according to the type of insecticide and to different field selection pressures, beetles can develop one or multiple resistance mechanisms. Physiological and biochemical studies have been conducted in vitro which satisfactorily support the above results (Ahammad Sahid et al. 1990).

Table 8. Resistance levels and resistance mechanisms of different Colorado potato beetle strains for the resistance to azinphosmethyl, carbofuran, permethrin and DDT insecticides based on synergist studies.

Strains	Insecticides	LD_{50} µg/beetle	RR[a]	Resistance Mech.
Long	azinphosmethyl	556.00	449.00	MFO's, Esterase
Island	carbofuran	47.50	237.00	MFO's
	permethrin	3.70	9.00	Esterase
JP	azinphosmethyl	272.20	217.00	MFO's,Ach-ase,Esterase
Macomb	carbofuran	>400.00	>1000.00	MFO's,Ach-ase
	permethrin	42.00	108.00	MFO's-P,Esterases,kdr
	DDT	>1000.00		MFO's-P, kdr
MNT-C	azinphosmethyl	11.43	9.14	Ach-ase
	carbofuran	120.95	605.00	Ach-ase
	permethrin	10.00	8.30	
Baker	azinphosmethyl	1.13	1.00	
	carbofuran	0.80	4.00	
	permethrin	16.10	41.00	MFO's-P, Esterase

Table 9. Cross-resistance spectrum of different resistant strains of Colorado potato beetle. LD_{50}'s µg/beetle with the 95% confidence limits are given for the tested insecticides.

	Susceptible strains		Strains with different types of resistance		
Compounds	**Vestaburg**	**MNT-C**	**Baker**	**L. Island**	**JP Macomb**
	Suscept.	Achet.	MFO's-P	MFO's[1] Esterases[2]	MFO's[1] Esterases[2]cAchet.
Azinphosmethyl	1.25 1.01 - 1.50	11.43 9.10 - 14.40	1.13 0.77 - 1.66	743.20 321.00 - 1714.00	272.90 64.00 - 1146.00
Carbofuran	0.21 0.18 - 0.26	120.95 49.20 - 279.90	0.80 0.61 - 1.03	47.80 27.60 - 82.00	>400.00
Permethrin			24.48 13.00 - 19.97	3.70 3.00 - 5.00	42.40 8.00 - 213.00
Temik	1.97 (1.55 - 2.59)	2.36 (1.86 - 3.01)	7.32 4.93 - 11.01	7.17 (5.00 - 10.00)	3.09 (2.15 - 4.42)
Thiodan	1.38 0.48 - 3.90	>100.00	3.61 1.68 - 7.73	244.37	
Rotenone		2.10 1.50 - 3.10	5.60 3.70 - 8.50	9.96 6.40 - 14.90	13.07 7.70 - 22.20

[1] Probably different isozymes of P-450.

[2] Total esterases.

Chapter 4

Selection, Inheritance and Characterization of Carbofuran Resistance

ABSTRACT:

The objective was to select for resistance to carbofuran in a susceptible field population of Colorado potato beetles, Leptinotarsa decemlineata (Say), and assess the progression of resistance and characterize the inheritance and possible mechanism of resistance. Initial selection in the field resulted in over 99.9% mortality of treated larvae but high resistance in the survivors. Subsequent laboratory selections at ca. 80% mortality increased resistance to > 100-fold by the fourth generation. Resistance appeared to be inherited via a single, autosomal, incompletely dominant gene, resulting in decreased acetylcholinesterase sensitivity. Other Michigan populations tested also appeared to exhibit reduced cholinesterase sensitivity. Resistance to carbofuran in a population from Long Island NY appears to involve primarily mixed-function oxidase enzymes. The history of insecticide use and resistance development probably affects which primary resistance mechanism appears in response to carbofuran treatment.

Key words: Insecta, Colorado potato beetle, insecticide resistance, carbofuran, acetylcholinesterase

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Colorado potato beetle (CPB), Leptinotarsa decemlineata (Say), was one of the very first pests to exhibit resistance to DDT (1952) and has subsequently developed resistance to all synthetic insecticides on Long Island, New Jersey, and other areas of the northeastern United States (Gauthier et al. 1981). Apparently because of cross-resistance, insecticides often fail within 1 or 2 years of introduction (Forgash 1981, Grafius 1986). Michigan may be on the geographic edge of severe problems. Within Michigan, potato beetle populations range from virtual non-pest status to highly resistant to all major insecticide groups (Ioannidis & Grafius 1990). This presents the opportunity to collect and study populations in many different stages of resistance development and to observe the development of resistance in individual populations over time.

Insecticides have been relied upon for control of CPB since their very first introduction. Selection for resistance can be reduced with IPM techniques (e.g. Wright et al. 1987), but no effective natural enemies act on the Colorado potato beetle in commercial situations (Harcourt 1964) and cultural practices are often not practical, at least in the short term. Thus, effective insecticide resistance management is essential for continued economic control of CPB. Strategies for resistance management are dependent for their success upon accurate understandings of resistance mechanisms, the genetics and inheritance of resistance and gene flow between populations. However, there is little information available on the mechanisms and genetics of resistance (Ioannidis & Grafius 1988, Ahamaad et al. 1990) or Colorado potato beetle gene flow and dispersal (Caprio & Grafius 1990).

Carbofuran, a carbamate insecticide, was chosen for study because it is highly effective for controlling Colorado potato beetles under normal situations (e.g., Grafius et al. 1988), creating intense selection pressure.

Insecticide resistance to carbofuran in CPB appears to develop unusually quickly - often within one growing season (Gauthier et al. 1981). Thus, an understanding of insecticide resistance management strategies seems essential for this material. Information might also be applicable to management of resistance for other insecticides and CPB or for other pest species.

The objectives were to select for resistance to carbofuran in a susceptible field population of Colorado potato beetles and: 1) assess the progression of resistance and 2) characterize the inheritance and possible mechanism of the resulting resistance.

Materials and Methods

CPB populations. The initial field selection for carbofuran resistance was conducted in 1987 at the Michigan State University Montcalm County Potato Research Farm (Entrican MI). The CPB at the research farm were susceptible to carbofuran (LD_{50} = 0.63 µg/beetle) and organophosphate insecticides (e.g., LD_{50} for azinphosmethyl = 1.27 µg/beetle) and moderately resistant to pyrethroids (e.g., LD_{50} for permethrin = 6.86 µg/beetle). Beetles at this site had been exposed to a range of commercial and experimental insecticides in previous years, particularly endosulfan (a chlorinated hydrocarbon), methamidophos (an organophosphate), and the carbamates, carbaryl and aldicarb. To the best of our knowledge, no carbofuran had been sprayed on the research farm prior to the study. Small plot studies of carbofuran as a soil insecticide had been conducted previously, but these never involved more than 0.3% of the potato acreage.

The laboratory-susceptible culture was started with ca. 300 individuals collected from unsprayed volunteer potatoes next to a commercial farm near Vestaburg MI (Montcalm Co.) in the summer of 1987 and kept in continuous laboratory culture. This was the most susceptible population to carbofuran of 29 field populations tested (unpublished data). The LD_{50} for the Vestaburg strain to carbofuran was 0.18 µg/beetle and 1.24 µg/beetle to azinphosmethyl, with a homozygous response to both compounds. These values are in agreement with those for susceptible strains reported by Forgash (1985) and Johnston and Sandvol (1986). The Vestaburg strain has been regularly tested to ensure continued susceptibility to carbofuran and shows no loss of vigor due to inbreeding.

Laboratory techniques. Technical grade carbofuran (98% purity, courtesty of FMC Corporation) in acetone was used for all laboratory selections and assays. Adults were immobilized using a 3.3 mm diam. vacuum hose and 2 µl of solution was placed on the underside of the abdomen of each adult. Mortality was assessed 72 h post-treatment. Fresh potato leaves from greenhouse plants were provided to the beetles daily during the testing. For LD_{50} determinations, at least four doses of carbofuran with 16 beetles per dose (2 replications of 8 each), plus an untreated group (acetone only), were used. In the back-cross tests up to eight doses were used in order to help recognize any distinct plateau (Tsucamoto 1963). Results were corrected for mortality in the controls (acetone alone - usually less than 10% mortality) using Abbott's (1925) formula and standard log-probit analyses were performed (SAS Institute 1982). All laboratory rearing and experiments were conducted at 25°C±2°C, 16 h light : 8 h dark photophase. Adults were tested ca. 1 week after emergence from pupation.

Field selection. Selection for carbofuran resistance was initiated in the field on 16 July 1987. Carbofuran was applied at a 16 row x 168 m plot of potatoes at 0.55 kg active ingredient per hectare using a commercial ground insecticide sprayer. CPB was in the first generation larval stage at the time of treatment and the pre-treatment density was estimated as 37 ± 10 (mean ± SE) larvae per plant based on data from adjacent untreated plots (unpublished data). The total number of CPB larvae treated in the field was approximately 488,400 (37 x 13,200 plants estimated in the plot). After treatment (24h) the plot was searched visually for surviving larvae. All surviving larvae were collected and returned to the laboratory.

Laboratory selection. A sample of the field-collected larvae was tested for carbofuran resistance using a dose of 5 µg/beetle (all survived.) The rest of the larvae were fed untreated potato foliage and reared to the adult stage in the laboratory. Progeny were reared to the adult stage and tested for LD_{50} for carbofuran as described above. The adults that survived the two highest doses (12.5 and 2.5 µg/beetle) were kept in culture. Progeny were reared to the adult stage and a sample was again tested for LD_{50} to carbofuran. The untested adults were selected with carbofuran to cause ca. 80% mortality. This process was repeated on the next 2 generations. These beetles were designated as the "Montcalm-C" strain and maintained in culture and subjected to a dose of 100 µg carbofuran/beetle every generation to assure maintenance of resistance.

Resistance mechanism studies. The synergists piperonyl butoxide (PBO) and s,s,s,tributylphosphorothionate (DEF), a plant defoliant, were used as indicators of possible resistance mechanisms. PB is a specific inhibitor of mixed-function oxidase enzymes (MFO's) (Brown 1971, Casida 1970). DEF inhibits esterases and MFO's (Plapp 1986). Synergists were applied at 100

μg/beetle to Montcalm-C or 50 μg/beetle to susceptible beetles, 2 h prior to insecticide treatment of the beetles. 100 μg of PB caused no mortality to the Montcalm-C beetles but more than 20% mortality to susceptible beetles. 50 μg caused <10% mortality to susceptible beetles. DEF caused no mortality.

Resistance ratios with and without synergist were calculated from LD_{50} values for resistant and susceptible strains. Susceptibility of the Montcalm-C strain to azinphosmethyl was also estimated using the standard LD_{50} method and results were compared to LD_{50} values for the unselected (field-collected) Montcalm Farm population.

Inheritance studies. Adults from the Montcalm-C and the Vestaburg strains were sexed within 48 h after emerging from pupation. Male/female reciprocal crosses were conducted. Pairs were kept together until they began mating (to insure that no male/female errors had been made) and then grouped with other pairs of the same cross in cages and fed potato foliage. Adult offspring (F_1 females and males) from the reciprocal crosses were tested separately for estimation of log-probit lines. A subsample of the F_1 adults were treated with a discriminating dose (6.25 μg/beetle) to be sure that there were no accidents involving susceptibles. The survivors (all of them resistant heterozygotes) were back-crossed (B_1) to the susceptible strains and adult progeny were tested for segregation of genotypes using both the technique determination of dose-mortality lines for appearance of a distinct plateau and with the discriminating dose technique, as before, and the ratio of susceptibles to resistant beetles was calculated. (Tsucamoto 1963, Georgiou 1965, 1969, Hama and Iwata 1978). This process was continued for studying the second backcross (B_2) to Vestaburg males. Backcrossing was continued with another susceptible strain (Arizona) to further confirm the segregation of the genotypes and the monofactorial type of inheritance of this resistant

mechanism. The F_2 adults (progeny of F_1 x F_1 crosses) were also tested.

Results. Initial field selection resulted in survival of ca. 80 larvae (>99.90% mortality). Survivors occurred in groups of 5 to 10 larvae on a plant, rather than as individual larvae, as might have occurred from random effects of factors such as spray coverage. Analysis of adults developing from the surviving larvae indicated that they were approximately 52 times more resistant to carbofuran than the initial population (Table 1). Subsequent laboratory selection increased the resistance ratio to 175 fold, within four generations. Reports from commercial potato fields and on-farm resistance monitoring also support the rapid change from carbofuran susceptible to highly resistant, often within one growing season (Grafius et al. 1988).

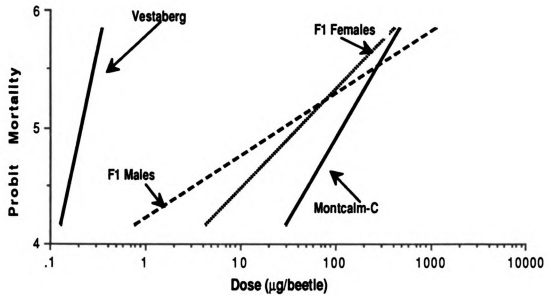
Inheritance studies indicated that F_1 offspring of resistant x susceptible crosses were uniformly resistant. Resistance levels were nearly as high as parental levels and showed no significant difference between sexes, suggesting near complete dominance and autosomal inheritance (Fig. 1). The degree of dominance (Δ) was estimated using the formula by (Stone 1968). Values of Δ are presented in (Table 2).

$$\Delta = \frac{2X_2 - X_1 - X_3}{X_1 - X_3}$$

X_1 ,X_2 ,X_3 are the $\log_{10}$ of the LD_{50}'s of:

X_1 = Resistant,
X_2 = Heterogenous,
X_3 = Susceptible.

A.



B.

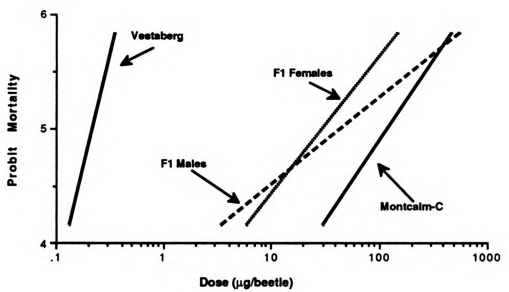


Figure 1. Dose-mortality responses to carbofuran of adults of susceptible (Vestaberg), and resistant (Montcalm-C) strains of Colorado potato beetle and their F1 offspring, as a result of topical application bioassays. A. Vestaberg males (ss) x Montcalm-C females (RR). B. Vestaberg females (ss) x Montcalm-C males (RR).

The values of Δ can be:

$\Delta = 1$	complete dominance
$0<\Delta<1$	incomplete dominance
$\Delta = 0$	intermediate
$-1<\Delta<0$	incomplete recessivity
$\Delta = -1$	complete recessivity

Values of 0.55 to 0.69 for Δ indicate incomplete dominance (Table 2).

F_1 backcrosses to susceptible beetles and $F_1 \times F_1$ crosses showed nearly perfect Mendelian segregation, indicating a single gene or closely linked genes (Figure 2, 3 and Table 3). The level of resistance to carbofuran of the MNT-C strain was very high and ranges of the dosage-mortality curve for susceptibles and resistant strain did not overlap (Figure 1). Therefore, it was possible to draw effective discriminating doses between (SS) and (RR), (RS).

Cross-resistance and synergist experiments showed moderate cross-resistance to azinphosmethyl and almost no synergism for carbofuran or azinphosmethyl with either PB or DEF (Table 4). Lack of synergism and the absence of low and intermediate levels of resistance in the Montcalm-C strain or other populations from commercial fields in Michigan (Ioannidis and Grafius 1989) suggests target site insensitivity. The extremely rapid mortality of susceptible beetles in response to carbofuran treatment (100% mortality in <2 h) also suggests that detoxification enzymes would be of limited usefulness unless they were present at very high levels. Preliminary data from <u>in vitro</u> studies on the Montcalm-C strain and on another Michigan carbofuran-resistant population showed altered acetylcholinesterase activity (Weirenga and Hollingworth, unpublished data). It is interesting that this acetyl-cholinesterase related resistant mechanism to carbofuran gives no cross resistance to aldicarb (LD_{50} = 2.36 µg/beetle). Aldicarb is an aliphatic carbamate compared with carbofuran, an aromatic carbamate (Kuhr 1976).

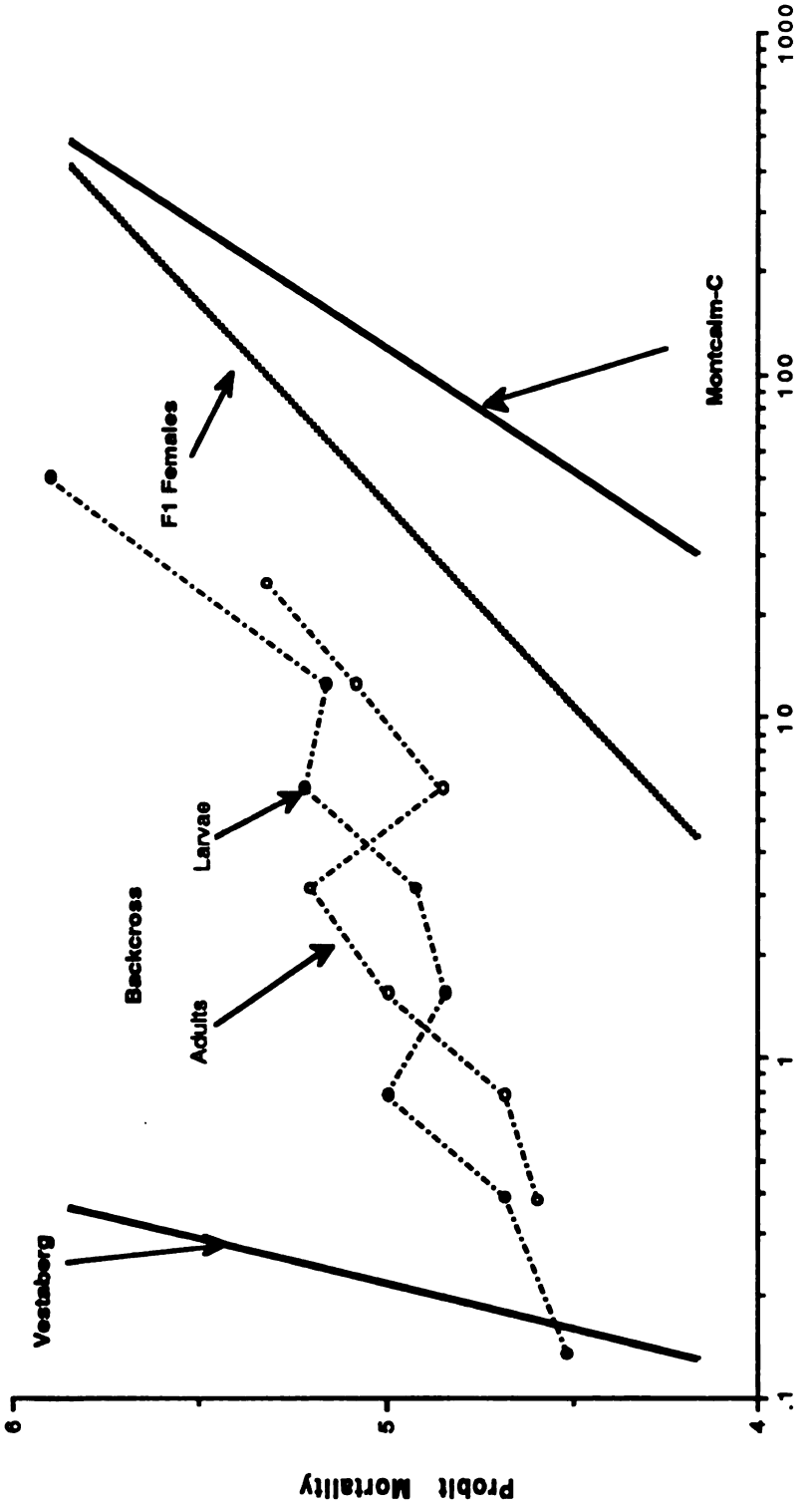


Figure 2. Dose-mortality responses to carbofuran of susceptible (Vestaberg--s), and resistant (Montcalm-C--R), strains of Colorado potato beetle, and their F1 female offspring, and backcross offspring (BC1, ([Vestaberg {s} females x Montcalm-C {R} males] x Vestaberg {s} males). Symbols: o Larvae, o Adults.

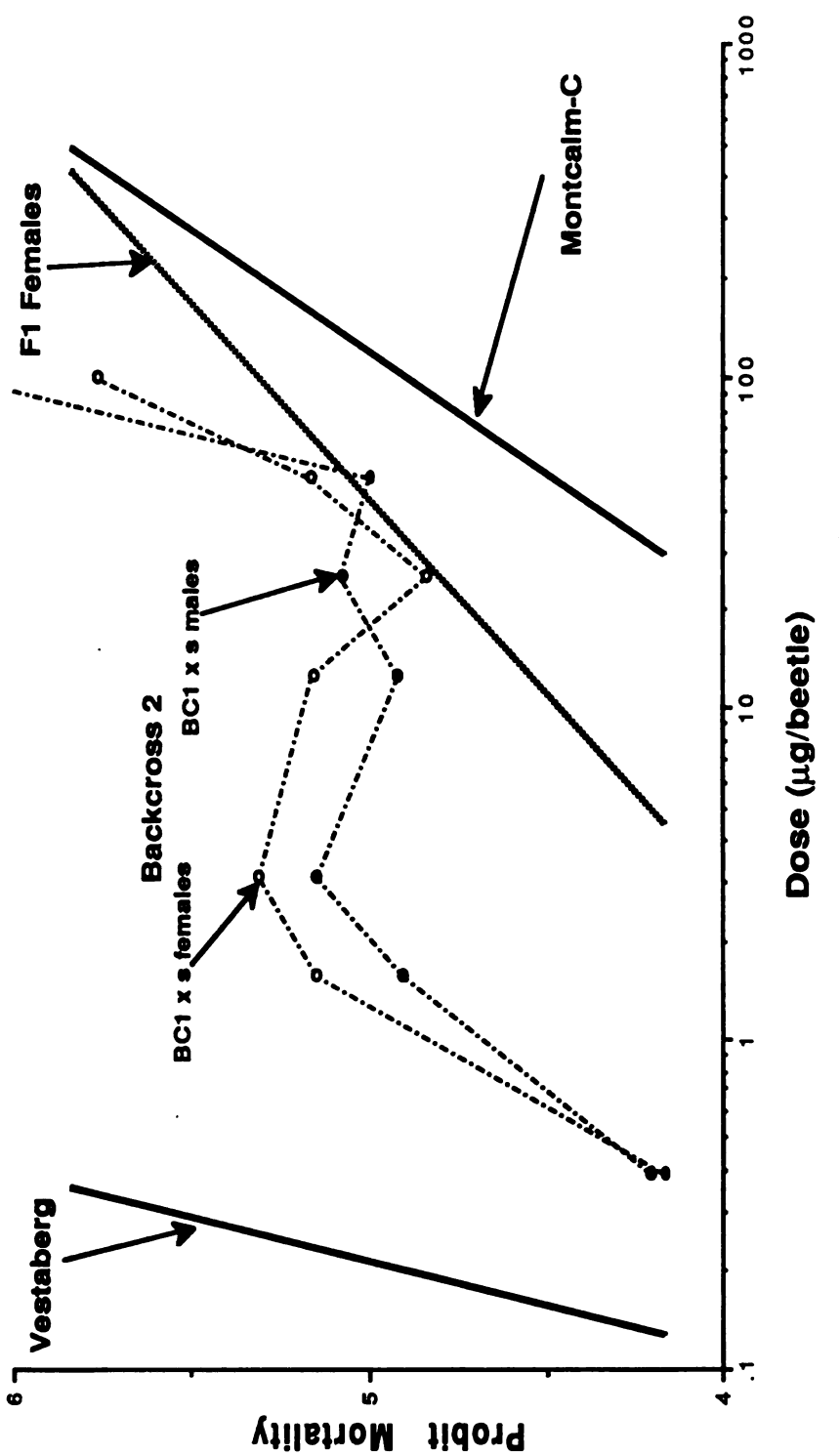


Figure 3. Dose-mortality responses to carbofuran of susceptible (Vestaberg--s), and resistant (Montcalm-C--R), strains of Colorado potato beetle, and their F1 female offspring, and offspring of two backcrosses to susceptible males and females (Vestaberg). Symbols: o Backcross 2 = BC1 females x Vestaberg males. o Backcross 2 = BC1 males x Vestaberg females.

Toxicity of aldicarb for the susceptible Vestaburg strain was LD_{50} =1.97 µg/beetle.

The low levels of cross resistance to azinphosmethyl (Table 1) suggests alteration of the target enzyme. If this acetylcholinesterase site insensitivity is mainly a qualitative change, it should be possible to search for compounds which will be more toxic or only toxic to resistant beetles and not to the susceptible CPB or beneficial insects, like the green rice leafhopper where alteration of the target enzyme is the main factor for resistance to carbamates (Hama 1983). Alternation between compounds affecting different acetylcholinesterase types could be an excellent control technique for this specific kind of resistance.

Acetylcholinesterase insensitivity has been documented for several other species (Devonshire and Moore 1984, Oppenoorth 1985). As in our case, acetylcholinesterase insensitivity reported for carbamate resistance in the green rice leafhopper, is mainly controlled by an incompletely dominant autosomally inherited factor (Hama and Iwato 1978).

In contrast to the Michigan populations studied, a population from Long Island NY, selected for azinphosmethyl resistance in our laboratory, showed significant synergism of carbofuran by PB and a lower level of resistance, probably indicating detoxification as the primary mechanism of resistance (Table 4). In vitro studies with this strain did not show any altered acetylcholinesterase activity, but high P-450 oxidation activity (Ahamaad et al. 1990).

Historically, carbofuran was only used very late in the resistance history on Long Island, after failure of numerous organophosphates and carbamates (Forgash 1981). In Michigan, and specifically at the Montcalm site, carbofuran resistance was selected much earlier in the resistance history. We

hypothesize that this difference in resistance history explains the different mechanisms that have manifested themselves. At the time of selection, detoxification enzymes in the Montcalm population were probably not near the very high levels required to detoxify the rapidly-acting carbofuran, thus leading to selection for target-site insensitivity.

Management of carbofuran resistance in CPB appears to be a difficult proposition. The single gene dominant nature of the inheritance and high selection pressure exerted by carbofuran treatment has resulted in the occurrence of exceptionally rapid resistance. Resistant populations are, for all practical purposes, immune to carbofuran treatment. The resistance factor appears to be present at very low frequencies in many or perhaps all Michigan populations, judging from the widespread and rapid buildup of resistance in the field (Ioannidis & Grafius 1989). Rapid buildup of resistance to insecticides is characteristic of mono-factorial inheritance (Wood and Bishop 1981; Roush and Croft 1986). We do not know of any cases of continued carbofuran use in commercial fields that have not resulted in rapid resistance. Limited use of carbofuran (<once per CPB generation) and alternation of carbofuran treatment with non-cross resistance materials (e.g., permethrin, where effective, or aldicarb), along with non-chemical management tactics, may increase the effective life of carbofuran in the field.

Table 1. Resistance of the "Montcalm-C" strain of CPB to carbofuran over four generations of selection, beginning with selection of larvae in the field.

		carbofuran		azinphosmethyl[1]	
Selecting agent carbofuran	**Gen. tested**	**LD_{50} µg/beetle**	**Degree of heterogenity x^2 (Δf)**	**LD_{50} µg/beetle**	**Degree of heterogenity x^2 (Δf)**
before selection	P	0.63	0.019(3)	1.27	3.9(2)
Selection (in field) 99.9% level-larvae	F_1	46.51	10.3 (6)	11.21	6.1(3)
Selection (in lab) 70-80% level-adults	F_2	93.87	4.68(2)	9.10	8.3(3)**
Selection (in lab) 70-80% level-adults	F_3	120.95	3.52(2)	18.54	6.7(2)**
Selection (in lab) 70-80% level-adults	F_7	>100.00	—	11.43	1.1(2)

[1] Cross resistant to azinphosmethyl without selection.

** Significantly different from expected ($P<0.05$).

Table 2. Responses to carbofuran of adults of Colorado potato beetle from the homozygous susceptibles (SS), resistant (RR) parental strains, and the F1 (SS x RR) hybrids of the reciprocal crosses.

Strain or cross	Slope (SE)	LD_{50} µg/beetle	Confidence Intervals 95%	Resistance Ratio 1	Degree of dominance 2Δ
Vestaburg (SS)	3.96 (0.81)	0.21	0.18 - 0.26	–	
Arizona (SS)	1.86 (0.47)	0.34	0.21 - 0.55	–	
MNT-C (RR)	1.39 (0.49)	120.95	49.20 - 297.90	575.90	
F1 (SS♀ x RR♂)♀	0.86 (0.36)	42.90	15.50 - 118.40	204.30	0.67
F1 (SS♀ x RR♂)♂	0.52 (0.20)	29.30	8.00 - 107.90	139.50	0.55
F1 (SS♂ x RR♀)♂	0.75 (0.21)	44.92	16.40 - 122.80	213.90	0.69
F1 (SS♂ x RR♀)♀	1.20 (0.23)	30.35	17.50 - 52.70	144.50	0.56

[1] Resistance ratio determined by dividing the LD_{50} of the resistant strain by the LD_{50} of the susceptible Vestaburg.

[2] Degree of dominance formula Stone 1968 $\Delta = -1$ complete recessiveness, $\Delta = 0$ intermediate, $\Delta = 1$ complete dominance.

Table 3. Colorado potato beetle percentage mortality from carbofuran: Ratio expected if resistance is monofactorial inherited and observed mortalities of susceptibles (SS) by applying discriminating doses.

Crosses	Stages/Number Tested	Expected Ratio SS:RS:RR	Observed Mort. SS Indivds.	X^2 1 degree of frdm.
Cross to obtain F_2				
F_1 (vest[a]♂ x MNTC[a]♀)) x F_1	Larvae 98[a]	1 : 2 : 1	23.5%	0.12
F_1 (vest[a]♂ x MNTC[a]♀) x F_1	Females 109[a]	1 : 2 : 1	23.8%	0.80
F_1 (vest[a]♂ x MNTC[a]♀) x F_1	Males 75[a]	1 : 2 : 1	32.0%	1.97
F_1 (vest[a]♂ x MNTC[a]♀) x F_1	Adult mix 160[b]	1 : 2 : 1	24.4%	0.03
Backcrosses series 1				
B1: (vest♀ x MNT♂)x vest♂	Larvae 146[a]	1 : 1 : 0	48.7%	0.11
B1: (vest♀ x MNT♂) x vest♂	Females 95[a]	1 : 1 : 0	50.6%	0.01
B1: (vest♀ x MNT♂)xvest♂	Males 96[a]	1 : 1 : 0	48.9%	0.04
B2: B_1 x vest♂	Larvae 64[a]	1 : 1 : 0	62.5%	4.00*
B2: B_1 x vest♂	Females 80[a]	1 : 1 : 0	48.7%	0.05
B2: B_1 x vest♂	Males 123[a]	1 : 1 : 0	43.9%	1.82
B3: B2 x AR[a]♂	Adult mix 54[b]	1 : 1 : 0	64.8%	4.74*
B4: B3 x AR♂	Adult mix 72[a]	1 : 1 : 0	52.8%	0.27
B5: B4 x AR♂	Adult mix 84[b]	1 : 1 : 0	44.0%	1.19
Backcrosses series 2				
B1 (vest♀) x MNT♂) X vest♂				
B2 B1 x vest ♀)	Larvae 64[a]	1 : 1 : 0	53.1%	0.25
B2 B1 x vest ♀)	Female 80[a]	1 : 1 : 0	52.5%	0.20
B2 B1 x vest ♀)	Males 80[a]	1 : 1 : 0	55.0%	0.80
Backcrosses series 3				
B1 (vest♂ x MNT ♀) x vest ♀	Adult mix 80[b]	1 : 1 : 0	47.5%	0.20
B1 (vest♂ x MNT ♀) x vest ♂	Adult mix 66[b]	1 : 1 : 0	42.4%	1.51

* Values of X^2 smaller than 3.84 (probability 0.05) considered to represent satisfactory agreement between observed and expected results.

[a] Topical application bioassays (pooled results of all the doses within the discriminating range, killing the ss).

[b] Segregation of susceptibles applying a distinct discriminating dose 3.12 µg/beetle.

Table 4. Synergisms of carbofuran and azinphosmethyl by piperonyl butoxide (PB) and DEF in susceptible Vestaburg (SS) and in two strains resistant to carbofuran with different resistance mechanisms.

	Vestaburg (SS)				MNT-C (R)				L-Island (R)			
Compounds	Slope (SE)	LD_{50} µg/beetle (95% fl)	SR[a]	RR[b]	Slope (SE)	LD_{50} µg/beetle (95% fl)	SR[a]	RR[b]	Slope (SE)	LD_{50} µg/beetle (95% fl)	SR[a]	RR[b]
carbofuran	3.96 (0.8P)	0.21 (0.18 - 0.26)	--	--	1.39 (0.49)	120.95 (49.16 - 297.90)	--	575.9	2.39 (0.57)	47.24 (33.51 - 66.57)	--	224.9
PB[c] + carbofuran	not tested				--	91.90 (6.25 - 13.30)	1.3	--	1.68 (0.45)	3.17 (33.51 - 66.57)	14.90	
DEF[c] + carbofuran	not tested				--	94.64 (34.66 - 258.40)	1.3	--	5.73 (1.26)	29.95 (24.71 - 36.29)	1.57	
azinphosmethyl	5.84 (1.18)	1.25 (1.06 - 1.49)	--	--	4.10 (0.88)	11.43 (9.08 - 14.40)	--	9.14	3.35 (0.78)	556.1 (402.00 - 710.00)	--	444.9
PB[c] + azinphosmethyl	1.35 (0.77)	0.28 (0.07 - 1.05)	4.49		2.30 (0.53)	7.29 (4.30 - 10.84)	1.6	26.00	1.92 (0.43)	27.54 (20.5 - 36.9)	20.19	98.4
DEF[c] + azinphosmethyl	not tested				3.75 0.73	2.44 1.92 - 3.10		4.7	1.41 (0.41)	63.10 (39.98 - 99.7)	8.8	

[a] SR synergist ratio LD_{50} of unsynergized/LD_{50} of synergized treatment.

[b] RR resistance ratio LD_{50} of resistant strain/LD_{50} of susceptible.

[c] Dose of the synergists 50µg/beetle for susceptibles. 100µg/beetle for resistant strains.

Chapter 5

Mechanisms and Genetics of Azinphosmethyl Resistance

ABSTRACT:

Resistance to the organophosphate insecticide azinphosmethyl was studied in the Colorado potato beetle (CPB), Leptinotarsa decemlineata (Say). High levels of azinphosmethyl resistance appeared to be transmitted by autosomal chromosomes. The mode of inheritance was incomplete in dominance. Two main resistance factors were responsible for this level of resistance: mixed-function oxidase enzymes (MFO's) and esterases. Esterase enzymes were shown to have more activity in the Long Island resistant strain than in Michigan beetles. Based on toxicity and synergism data with the parental strains, the hybrids from the reciprocal crosses and on the offspring of the backcrosses, it seemed that these two resistance factors were not simply additive, but had synergistic action when contained in the same organism. These beetles can exist just as well in the field. The rapid expansion of resistance in the field and the fact that these highly resistant strains performed very well under laboratory culture conditions indicate that there is no reproductive disadvantage associated with them. The LD_{50} of the presumed AABB genotype was 556 µg azinphosmethyl/beetle, for AAbb (MFO's) 65 µg/beetle, and for aaBB (esterases) 28 µg/beetle. Therefore, the synergistic action of these two resistance genes was six times more than the additive result. Application of piperonyl butoxide as a synergist with azinphosmethyl on the F_1 hybrids partially suppressed the resistance while also producing evidence of difactorial inheritance.

Key words: Insecta, Colorado potato beetle, insecticide resistance, azinphosmethyl, genetics.

It is generally agreed that resistance to insecticides is controlled by heredity and that transmission of the characters for resistance conforms to Mendelian patterns (Crow 1960, Georgiou 1965, Tsukamoto 1983, Plapp 1986). Resistance of the houseflies to either parathion or malathion is inherited through single dominant gene pairs. The fact that the genes for parathion resistance and those for malathion resistance lie on the same chromosome indicates that these two types of gene are either alleles or closely linked (Nguy et al. 1960). Genetic studies on Dieldrin-resistance in Musca domestica L. and Lucilia cuprina (Wied.) strongly indicate monofactorial inheritance (Guneidy et al. 1964). The same results have been found for Dieldrin-resistance in houseflies by Georgiou et al. (1963). Houseflies are diploid organisms, showing classical Mendelian inheritance characteristics as far as resistance is concerned. Plapp (1983) pointed out that what is true for houselifes and other higher Diptera in the way of resistance genetics is probably true for other insects. That is, genetic mechanisms involved as probably ubiquitous and rather specific. Malathion resistance in the Triboloium castaneum (Herbst) is inherited as a simple autosomal semidominant trait (Beeman 1983). There are also several reports on the inheritance of resistance, not only from the well known genetic studies on flies of medical importance, but also on agricultural pests and predators (Towgood and Brown 12962, Telford and Brown 1964, Croft et al. 1976, Croft and Whalon 1983, Roush 1986).

Resistance to the organophosphate insecticide azinphosmethyl was first reported in populations of the Colorado potato beetle (CPB) in 1971, when azinphosmethyl started to show a decrease in activity in controlling larvae in Long Island, NY farms. As a consequence, failure of azinphosmethyl led to devastating losses in 1973 in Long Island, with the beginning of resistance being reported in Virginia in 1974 (Gauthier et al.

1980). Topical toxicity of azinphosmethyl for a New Jersey population was 30 µg/beetle, and application of piperonyl butoxide (PB) on this population did not synergize azinphosmethyl (Forgash 1985). Indications are that for this level of resistance, another resistance mechanism was responsible aside from MFO's. Toxicity data from 24 different resistant populations in Michigan indicated that resistance to azinphosmethyl can range from 5 to 600 µg/beetle (Ioannidis et al. 1990).

Synergistic and biochemical studies in vivo and in vitro with a highly homogeneously resistant strain to azinphosmethyl (LD_{50} = 556 µg/beetle) and with another Michigan field collected resistant strain to azinphosmethyl (LD_{50} - 358 µg/beetle) revealed that more than one resistance mechanism contributed to this high level of azinphosmethyl resistance, due mainly to the mixed function oxygenase (MFO's) and the esterases (Ahammad-Sahid et al. 1990).

Argentine et al. in 1989 reported that a strain of CPB from Massachusetts was 435 times more resistant to azinphosmethyl than to a susceptible strain. Based on synergist studies in vivo, they concluded that esterases probably were involved in azinphosmethyl metabolism although esterases could not be solely responsible for the observed resistance.

In our laboratory, the Long Island strain had been selected with azinphosmethyl for six succeeding generations at 80% mortality level, before being used in the crosses. This strain, after the selections, shows a homogenous response, with steep log-dose mortality lines to the selecting agent azinphosmethyl.

The objectives of this study were to: (1) investigate the mode of inheritance of this high level of resistance of the Long Island strain, (2) to determine whether the resistance to azinphosmethyl was carrying on

autosomal chromosomes and the existance of any kind of sex-linked influence, and (3) to figure out from the backcross and $F_1 \times F_1$ crosses how many resistance factors were involved.

Materials and methods

Insecticides and synergist used

The insecticides used were technical grades of azinphosmethyl (90% purity). The synergist, s,s,s,tributylphosphorotrithioate DEF (99%), was provided by Mobay Corporation, Kansas City, MO. The piperonyl butoxide (PB) (80%) was a gift of Mclaughlin Gormley King, Minneapolis, MN.

Bioassay tests - measurements of resistance

The successful identification of phenotypes depends to a large extent on the proper choice of a sensitive bioassay method. We used a dose-response test, the standard bioassay technique for CPB (FAO 1974). Some changes were made in the method as a consequence of our experience with preliminary work. The character being investigated cannot be seen, it can only be recognized by challenging groups of beetles to a series of doses of insecticides, i.e., quantal response methods. Conclusions on segregation of resistance in progeny of various types of hybrids were made from data from dose-mortality studies or by using distinct discriminating doses.

Treatments were conducted by topical application of 2 µg/beetle on the abdominal sternite. The insecticides were of technical grade and stock solutions were made up in acetone. For the establishment of dose mortality relationships, we used at least four doses. In the tests with backcrosses up to

nine concentrations were used because when the number of observed mortality plots is not enough (for example only four to five plots), no distinct plateau may be recognized even though the resistance is inherited in a simple monofactorial system (Tsucamoto 1963).

Statistical Analysis

Dosage-mortality lines for all parental F_1, F_2 and backcross populations were determined by the probit analysis method of Finney (1971) using the MSTAT computer program Eisensmith (1989). The chi-square (X^2) value, a measure of the departure of the actual observations from the fitted probit-log dose line, was also calculated. The value of X^2 was compared with tables for this statistic for n-2 degrees of freedom (where n is the number of doses used per bioassay). If the calculated value is found to be higher than the tabular X^2 for the 5% level, this will be an indication of heterogeneity.

To evaluate a genetic hypothesis, we need a test which can convert deviations from expected values into the probability of such inequalities occurring by chance. A particularly useful test is the chi-square (X^2) method when the probability is smaller than 0.05 (1 in 20), the difference between the results observed and those expected is said to be significant (Snyder et al. 1985). The value of X^2 is given by the formula:

$$X^2 = \frac{(ob_1 - ex_1)^2}{ex_1} + \frac{(Ob_2 - ex_2)^2}{ex_2}$$

The above test was applied with the discriminating dose technique which can separate two phenotypes: dead (susceptibles) and live (resistants).

In this formula, susceptibles refer to observed (Ob_1) and expected (ex_1) whereas resistant refers to observed (Ob_2) and expected (ex_2).

The progeny from F_2 and backcross generations is always heterogeneous, with high x^2 values because of the production of different genotypes. Therefore straight log-probit lines are given only for the parental and F_1 hybrids.

Establishment of homozygous strains

Vestaburg susceptible

This strain was collected from an unsprayed volunteer potato next to a commercial farm near Vestaburg, MI. This has been interbreeding for 3 years before the crosses started and its susceptibility has remained remarkably stable. (LD_{50} = 1.25 µg azinphosmethyl/adult and for larvae, 0.89). Dose-mortality data has shown a straight line with a reasonably steep slope indicating homogeneity (Table 1). Beetles were not affected by any interbreeding depression.

Long Island Resistant

This field-collected strain was heterogeneously resistant to azinphosmethyl when it was collected five years ago. After repeated selection at 80% mortality and intercrossing for five generations, a resistant homozygote had been produced. This gave rise to a steeper log-probit dose line. This is clearly demonstrated by comparing the slopes and X^2 values in the following regression equations. After the selections, b = 3.01 (steeper slope) and x^2 = 0.625 (very small), meaning a strong indication for

homogeneity was revealed. These beetles have been exposed to a dose of 200 µg azinphosmethyl/beetle for every generation to confirm maintenance of resistance and homogeneity. The resistance ratio of this strain was 444-fold and did not show any reproductive disadvantage.

Before selection

Heterogeneous $y = 1.80 + 1.18x$, ($x^2 = 7.14$ D.F. 5) LD_{50} 489 µg/beetle

After selection

Homogenous (RR) $y = -3.260 + 3.01x$, ($x^2 = 0.625$ D.F. 5) LD_{50} 556 µg/beetle

Genetical techniques

Descriptions for these techniques and methods can be found in: Milani 1960, Georgiou 1965, Tsucamoto 1964, 1965, 1969, Plapp 1976, 1983.

The use of virgin females in crossing experiments is essential. The maximum permissible period before the first emerged male reaches sexual maturity depends on the temperature. Preliminary studies under our laboratory conditions at 25° C show that the permissible period for separation of sexes was less than 3 days after emergence. For synchronization of the emergence of adults we used the technique by Boiteau (1983). This facilitated the bioassays and allowed testing beetles at approximately the same age, an important intrinsic factor for accuracy and consistency.

Mass cross-mating (for all the experiments) was arranged by putting together groups of about 20 males of one strain with the same number of

females of another strain. Beetles were maintained in the same way as has been described in Chapter 1.

Reciprocal crosses

To determine the mode of resistance inheritance of azinphosmethyl, reciprocal crosses were conducted between the Resistant and Susceptible strains. Establishment of regression lines for the F-hybrids provided information on the degree and type of resistance. The reciprocal crosses were also used to determine whether resistance to azinphosmethyl was sex-linked or autosomal. In the Colorado potato beetle the sex determination mechanism is the xo type and the chromosome number is 2n = 34 + xo in the males and 2n = 34 + xx in the females (Hsiao et al. 1983). In the males, the x chromosome has no homologous pairing partner. Therefore, if any resistant factor exists in the x chromosome of males, resistance occurs only to the hybrid females from the cross S♀ x R♂.

For more confidence in the data, the interpretation of results was based on the study of the complete dosage-mortality lines for the F_2 and backcross generations and, additionally, the discriminating dose technique was used to confirm the results and separate the resistant phenotypes.

Backcrosses and F2

F_1 progenies were backcrossed to the susceptible parent and dose-mortality curves for the segregated phenotypes for offsprings were established (Figure 2). Before each backcross, the F_1 beetles were selected with a discriminating dose for killing any accidentally involved susceptibles and leaving the heterozygous resistant beetles alive.

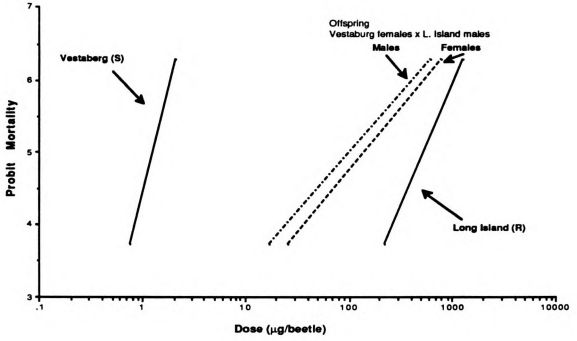
Discriminating dose technique

With the high resistance level and the incomplete dominance of the F_1's, this technique was convenient because effective discriminating doses could be easily determined between the phenotypic susceptibles and the resistants.

Results and Discussion

The high level of resistance is transmitted to the hybrids by either the male or the female parent (Figure 1). Therefore, the resistance factors must be located on the autosomes. The observed probit-log dose line was widely separated and distinct from that of the susceptible strain, indicating incomplete dominance. In both reciprocal crosses the females were slightly more resistant than males. This portrays the possibility of slight cytoplasmic or maternal influence with no sex linkage involvement. Of course, sex linkage may also produce differences in the results of reciprocal crosses but in those cases the phenotype can be predicted from the sex of the parents. With regard to the Colorado potato beetle, female hybrids should show more resistance only from the cross S♀xR♂ as was previously explained. This, however, was not the case. In the maternal effect, phenotype changes appear because of differences in egg cytoplasm rather than differences in sex chromosomes and often (although not always) affects both male and female offspring equally. The characteristics of the probit-log dose lines of the parental strains and of their F_1 hybrids are given in Figure 1 and Table 1.

A.



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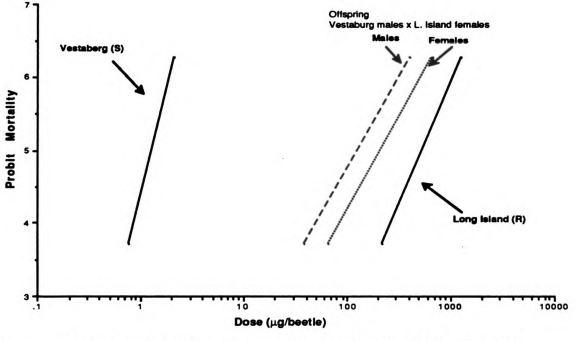
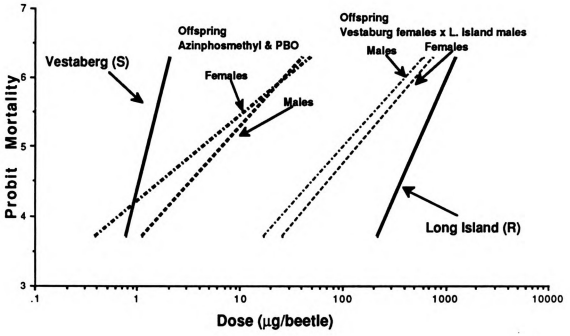


Figure 1. Dose-mortality responses to azinphosmethyl of adults of Colorado potato beetle susceptible (Vestaberg- s) and resistant (Long Island- R) strains and F1 offspring (males and females). A. Vestaberg (s) females x Long Island (R) males. B. Vestaberg (s) males x Long Island (r) females.

A.



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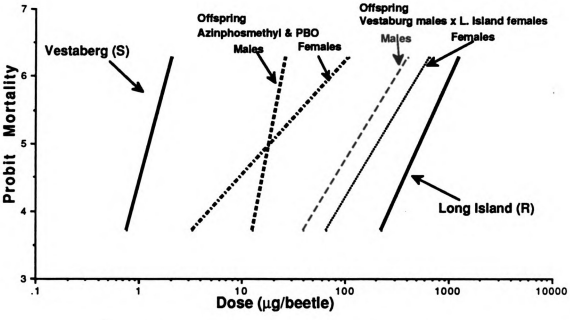

Figure 2. Dose-mortality responses of adults of Colorado potato beetle susceptible (Vestaberg- s) and resistant (Long Island- R) strains and F1 offspring (males and females) to azinphosmethyl and application of azinphosmethyl and piperonyl butoxide to F1 offspring only. A. Vestaberg (s) females x Long Island (R) males. B. Vestaberg (s) males x Long Island (r) females.

Reciprocal crosses in preliminary studies with another field collected susceptible population showed F_1 hybrids with LD_{50}'s about the same as the F_1 hybrids from the crosses with Vestaburg (unpublished data). This strongly supports the autosomal incomplete dominance of the inheritance of the azinphosmethyl resistance.

Application of piperonyl butoxide on the F_1 hybrids partially synergized azinphosmethyl (Figure 2, Table 1) indicating that another resistance factor exists. The synergism is less with the hybrids from the S♂xR♀ cross because the LD_{50}'s of 18.43 for males and 19.18 for females are larger than the corresponding values from the cross S♀ x R♂. It is interesting that the level of resistance of the hybrids from S♂ x R♀ are higher than the opposite cross (Figure 1, Table 1). Despite that, by considering the confident limit values, there are no real significant differences.

The main factor for resistance to azinphosmethyl involves MFOs and also esterases play an important role: these two enzymatic resistance mechanisms give cross resistance to several other insecticides. This was supported from synergistic studies with piperonyl butoxide and DEF with different resistant strains (Chapter 3) and also from biochemical work with two highly resistant strains (Ahammad-Sahid et al. 1990). Argentine (1989) reported that esterase probably was the main factor for resistance to azinphosmethyl, working with a 435-times resistant azinphosmethyl strain. In their synergist studies, the dose of piperonyl butoxide used was the same for susceptibles and resistants (5 µg/larva). In our opinion, based on extensive synergist work in our laboratory, 5 µg/larva is too small of a dose to produce synergistic effects with resistant beetles. In our synergistic experiments, we used stable doses of at least 50 µg/larva and even 100 µg/adult to the resistant strains, gave almost zero mortality. In the same

report, despite the fact that the backcrosses to susceptibles did not produce distinct plateaus, Argentine et al. 1989 found that azinphosmethyl resistance was largely monofactorial for their CPB, but its dominance or expressiveness was influenced by other genes.

We have shown that the resistance to azinphosmethyl in the Long Island strain of the CPB is inherited as two incomplete dominant genes. Therefore, if the two genes independently segregated in the backcross, a plateau at the 25% mortality level should be observed. If the two genes were tightly linked, segregation should be exhibited as one factor with a distinct plateau at the 50% mortality level. In our case, a very clear plateau can be seen around the 40% level (Figure 3) indicating two distantly linked genes. If more than two main resistant factors had been involved in the expression of the resistance in the Long Island strain, the plateau in the backcrosses would not have been present, but the plotted dose-mortality line would have been straighter (no segregation) (Tsucamoto 1963, Snyder et al. 1985). The linkage of the two resistant factors was supported also from the results of the F_2 progenies. A discriminating dose of 3.12 µg/beetle of azinphosmethyl applied to the F_2 progenies killed only the susceptibles. The estimated mortality was 13.9%. This was more in agreement with the expected mortality of 16% for two linked genes with about a distance of 20 map units (Table 2), than the value of 6.25% which should be expected for two independent segregating genes. Furthermore, in a careful examination of the backcross lines in (Figures 3 and 4) two plateaus can be shown at 40% and 60% mortality levels, instead of 25% and 75% values as expected if two independent segregating genes existed.

It is interesting that in all the backcrosses, the dose of 3.12 µg/beetle (which kills only the susceptibles) gave a mortality around 40%, which was in

agreement with the percentage of susceptibles produced by two linked genes. The evidence for difactorial mendelian inheritance is based on (1) the presence of continuous distribution of the character of resistance in the F_2 and backcross progeny, and (2) the statistical agreement of the observed responses to those which may be expected in case of difactorial inheritance with two main linked genetic factors with around 20% recombination plus synergist results. If other minor important genes contributed to this high level of resistance they should be closely linked to these two main resistant factors described above. Backcross to the resistant parent ($F_1 \times R♂$) produced genotypes with resistance between the F_1 and the parent. Application of a discriminating dose of 100 µg/beetle gave no mortality as it was expected while 200 µg/beetle killed about 50%. This was as expected with mendelian inheritance.

We have successfully completed six generations of backcrossing with the susceptible strain Vestaburg followed by mild selection with azinphosmethyl. The surviving heterozygotes of the 6th backcross progeny (after giving a discriminating dose which kills the susceptible aabb and the Aabb, aabB heterozygous beetles) were mass-inbred. The resulting progeny were under selection to produce a homozygous resistant strain. Therefore, the genetic background of the resistant strain became essentially equivalent to the 98.44% of the susceptibles. The new strain can be utilized for further genetic studies and biochemical work.

Based on synergist studies with the parental resistant strain, the F_1 and backcross progenies, it was possible to estimate the amount of resistance given by each allele to azinphosmethyl (Table 2). The AABB stands for: AA for MFO's and BB for esterases, the two main enzymatic resistance mechanisms. Therefore, these two linked genes in the backcross with the susceptibles, and

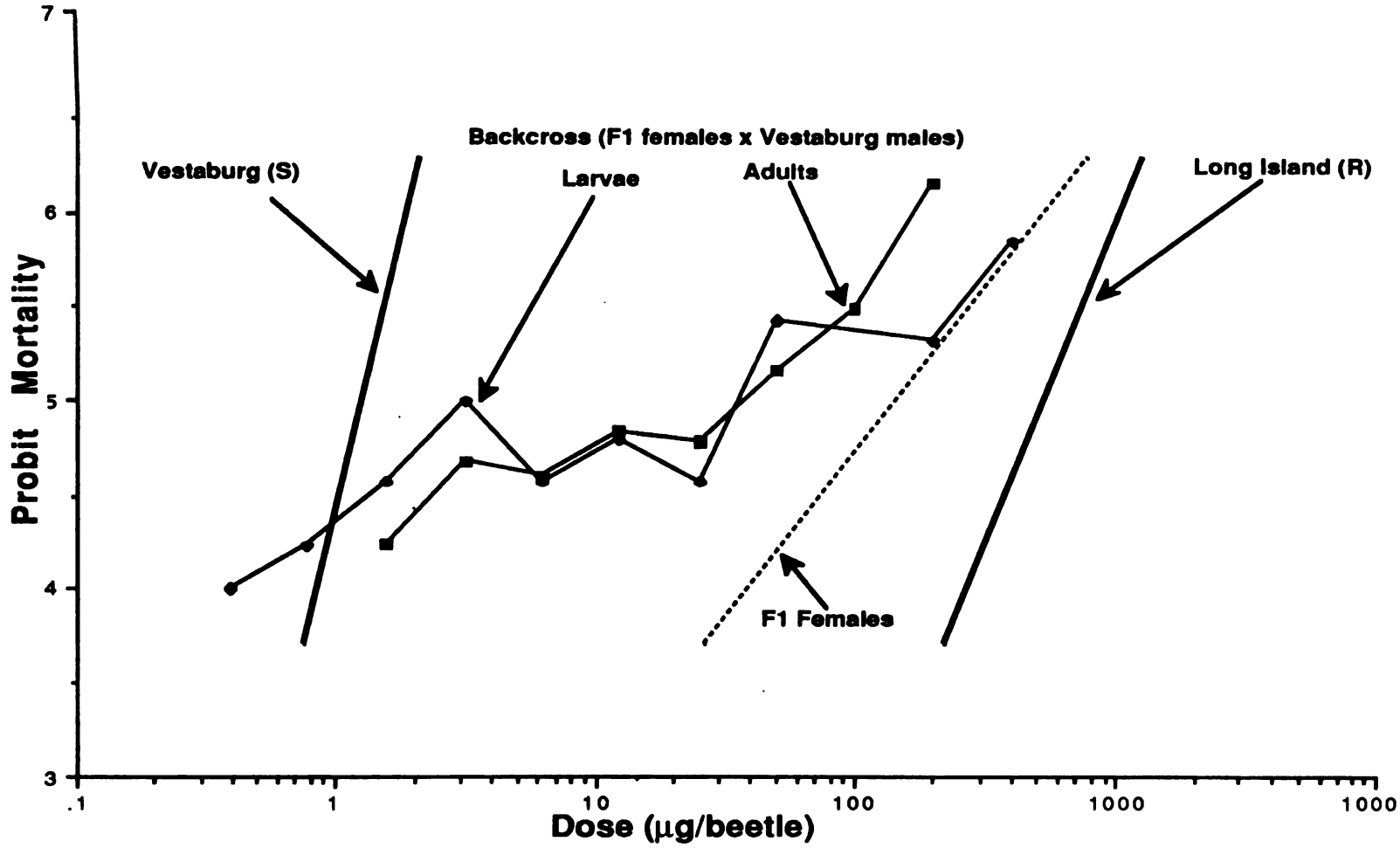

Figure 3. Dose-mortality response to azinphosmethyl in adults of susceptible (Vestaberg) and resistant (Long Island) strains of Colorado potato beetle and in their F1 offspring (Vestaberg [s] females x L. Island [R] males) and in offspring of a backcross (BC1) of F1 females with Vestaberg (s) males.

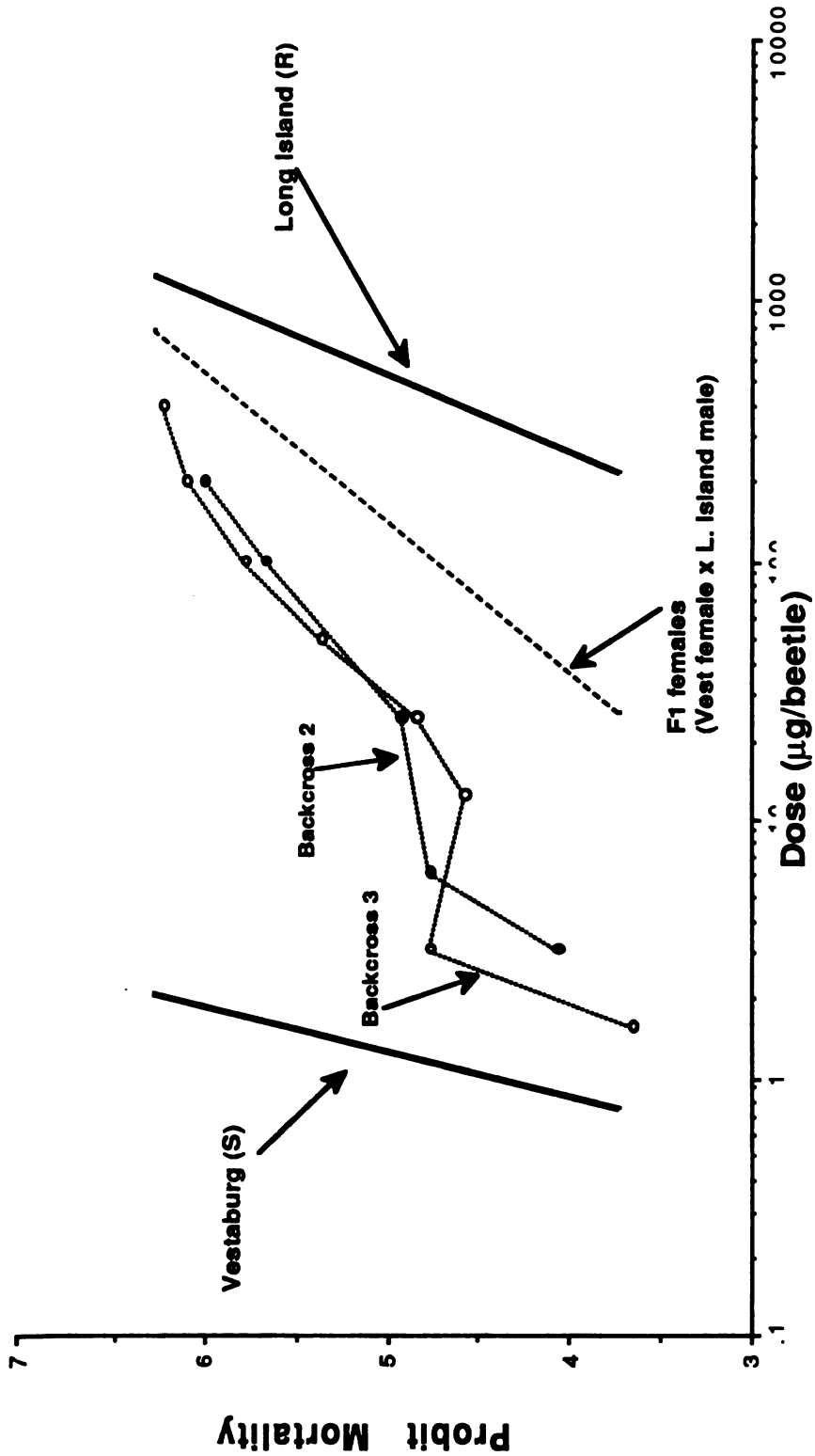

Figure 4. Dose-mortality response to azinphosemtheyl in adults of susceptible (Vestaberg) and resistant (Long Island) strains of Colorado potato beetle and in offspring of a second backcross (BC2 = BC1 male x Vestaberg [s] female) and third backcross (BC3 = BC2 male x Vestaberg [s] female]. BC1 = F1 females (from Vestaberg female x L. Island male) x Vestaberg [s] male.

in the F_2, yielded the genotypes presented in Table 2. The phenotypic expression of resistance is presented in Table 3 based on the confidence limits of the corresponding LD_{50} values.

The procedure of backcrossing while selecting for resistance to one compound is a very effective way of isolating a single gene if one exists (Crow 1957). Since we have estimated the discriminating doses of the LD_{50}'s which kill the corresponding genotypes (Table 2) it would be possible, by applying the following technique, to separate the two main resistance mechanisms, establishing different pure strains with one of the resistant factors. Although genetic markers are unknown as yet for the Colorado potato beetle, the specific inhibitor of MFO's (piperonyl butoxide) can be used like a marker. Progeny from the backcross with the susceptible are maintained in pairs and eggs collected, keeping them separated from eggs from other pairs. A discriminating dose of 3.12 µg azinphosmethyl/beetle can be applied which kills only the susceptible aabb. After five days the beetles again receive the application of piperonyl butoxide 20 µg/beetles followed after two hours with the same discriminating dose of azinphosmethyl. Only the pairs with the Aabb genome should be dead; therefore, we raise only the eggs which were produced from the beetles before they died. The new beetles will interbreed; therefore these must have only the A_ genotype. If the same procedure is repeated with application of azinphosmethyl with piperonyl butoxide on the new beetles, all the pairs should die, which is clear evidence for a pure strain.

The same technique can be applied using DEF, which inhibits esterases and is not a specific inhibitor of but probably also inhibits oxidation (Attia et al. 1980, Plapp 1986). The above procedure should necessarily be repeated several times to purify the strain with only the esterase resistance mechanisms. It is also possible that DEF inhibits MFO activity slightly in the

Colorado potato beetle, since microsomal preparations from beetles treated with DEF show only a small reduction in ability to oxidize NADPH (Ahammad-Sahid et al. 1990).

A potential marker with the Colorado potato beetle is limited only to a white-body color mutant larva, which has been established. This trait has proved to be stable for at least nine generations (Boiteau 1980). This mutant trait is recessive to the wild type and is due to single autosomal genes (Hsiao 1983). It is interesting that we have noticed this mutant only in the Vestaburg strain selected for permethrin resistance in our laboratory. Attempts to establish a colony by interbreeding albino beetles were not successful. We noticed rare mating between them, while albinos mated successfully with normal ones. Further attention and examination may raise some useful correlation to the permethrin resistance.

It is interesting that there are reports for inheritance of resistance of organophosphates in other species concluding that more than one gene is involved in the resistance. Harris et al. (1970) early in 1961, reported that two sets of alleles affect the inheritance of malathion resistance in housefly, exhibiting incomplete duplicate dominance epistasis. Oppenoorth (1959) in genetic studies with the diazinon resistant housefly found that one gene is responsible for low esterase activity and responsible for part of the resistance and at least one other resistance factor is present (MFO's, Oppenoorth 1985).

The genetics of malathion resistance in two strains of the flour beetle Tribolium castaneum (Herbst) show that resistance in one strain was polygenic for the other one, the resistance was due to a dominant allele at a single autosomal focus (Wool et al. 1982). Raymond et al. (1987) reported that the number of additive and independent genes contributing to chlorpyrifos resistance in Culex pipiens (L.) was at least two and perhaps more, and they

had found in previous studies the presence of an insensitive acetyl cholinesterase and a modified oxidative detoxification. It is very interesting that one of the strains in our laboratory, the J-P strain of Colorado potato beetle, possesses both of the above resistance mechanisms. This has been demonstrated by the use of synergists which were unable to eliminate the total level of resistance and from biochemical studies (Ahammad-Sahid et al. 1990, Wierenga and Hollingworth - unpublished data).

It is obvious now that several resistance mechanisms might be involved in different strains. Therefore, to obtain specific knowledge for resistance mechanisms for each gene it is necessary to select special resistant strains in which only a single resistance gene will be involved.

These results and results of future studies will provide essential information for new approaches in the control of the CPB and to better elucidate the insecticide resistance problem which should be considered as one of the subjects of population genetics (Roush et al. 1986). A better understanding of the detailed genetic mechanisms should help us to confront the Colorado potato beetle, one of the most threatening pests in the U.S. and other parts of the world for potato plantations.

Table 1. Toxicity of azinphosmethyl to susceptible (S), resistant (R), parental strains and to F_1 (S♀ x R♂ and S♂ x R♀) hybrids. Synergism of azinphosmethyl by piperonyl butoxide (PB) with the F_1 hybrids.

Strain	LD_{50} µg/beetle	95% CL	RR[a]	Slope (SE)	x2 (df)
azinphosmethyl					
S	1.25	1.01 - 1.49	--	5.84 (1.18)	0.40 (2)
R	556.00	402.00 - 710.00	445.00	3.35 (0.78)	
F_1 (S♀ x R♂) males	103.03	69.00 - 154.00	82.00	1.65 (0.36)	6.88 (4)
F_1 (S♀ x R♂) females	141.61	94.00 - 214.00	113.00	1.74 (0.39)	5.80 (4)
F_1 (S♂ x R♀) males	125.71	93.00 - 170.00	101.00	2.50 (0.43)	1.89 (4)
F_1 (S♂ x R ♀) males	207.77	143.00 - 289.00	116.00	2.58 (0.52)	4.56 (4)
azinphosmethyl + PB					
F_1 (S♀ x R♂) males	6.69	3.70 - 12.20	5.30	1.61 (0.62)	1.70 (2)
F_1 (S♀ x R♂) females	7.00	4.10 - 11.90	5.60	1.50 (0.52)	8.47 (2)**
F_1 (S♂ x R♀) males	18.43	12.50 - 23.50	14.70	7.73 (2.34)	0.30 (2)
F_1 (S♂ x R♀) females	19.18	8.90 - 41.10	15.40	1.67 (0.82)	1.20 (2)

[a] Resistance Ratio = $LD_{50}R/LD_{50}$'s.
** Significantly different from expected ($P<0.05$).

Table 2. Theoretical genotypic expression in dihybrid crosses and backcrosses with two autosomal resistance factors to azinphosmethyl, the enzymes MFO's (AA) and esterases (BB).

Genotype $F_1 \times F_1$	LD_{50}'s µg/beetle	Two Linked Genes [20% recombination expected ratios]	Two independent genes [independent segregation expected ratios]
AABB	556.00	16.00%	6.25%
AABb	--	8.00%	12.50%
AaBB	--	8.00%	12.50%
AaBb	144.00[1]	34.00%	25.00%
AAbb	65.00[2]	1.00%	6.26%
aaBB	28.00[3]	1.00%	6.25%
Aabb	--	8.00%	12.50%
aabB	13.00[4]	8.00%	12.50%
aabb	1.250	16.00%	6.25%

Backcross to susceptible			
AaBb		40.00%	25.00%
Aabb		10.00%	25.00%
aabB		10.00%	25.00%
aabb		40.00%	25.00%

1 Mean of F_1 offsprings of the reciprocal crosses to azinphosmethyl.

2 Resistance of the resistant strain to azinphosmethyl plus DEF synergist from Table 4.

3 Resistance of the resistant strain to azinphosmethyl plus PB synergist from Table 4.

4 Mean of F_1 offsprings of the reciprocal crosses to azinphosmethyl plus PB from Table 1.

Table 3. Phenotype expressing of resistance to azinphosmethyl in the F_2 progenies (F_1 x F_1) from two autosomal linked factors for MFO's and esterases with 20 map distance.

Phenotypes	Number of alleles for resistance	Phenotypic range of resistance[a] µg/beetle	Theoretical Fraction of F_2 %
AABB	4	Highly resistant <402.00 - 710.00	16
AABb, AaBB	3	Highly resistant 300.00- 400.00[b]	16
AaBb	2	Intermediate 80.00- 290.00	2
AAaa,aaBB	2	Medium 40.00- 80.00[c]	34
Aabb,aabB	1	Low 3.70- 41.00[d]	16
aabb	0	Susceptible 1.01- 1.49	16

[a] The 95% confidence limits from the corresponding LD_{50}'s (Table 2).

[b] Arithmetical estimation.

[c] Application of azinphosmethyl with PB or DEF on the resistant parents.

[d] Application of azinphosmethyl with PB on the F1 offsprings.

General Discussion

General Discussion

Past experience and recent studies on resistance modeling show that reduction of selection pressure in its broadest sense is a key factor for slowing the development of resistance.

An important requirement for developing strategies for avoiding or delaying the development of resistance is the study of the factors which influence the evolution of these phenomena. These factors are classified as follows (Georgiou and Taylor 1986):

A. Genetic: (1) frequency of R alleles; (2) number of R alleles; (3) dominance of R alleles; (4) penetrance, expressivity; interactions of R alleles; (5) past selection of other chemicals; (6) extent of integration of R genome with fitness factors.

B. Biological:
   a. Biotic potential: (1) generation turn-over; (2) offspring per generation; (3) monogamy/polygamy; parthenogensis.
   b. Behavioral: (1) isolation; mobility; migration; (2) monophagy/polyphagy; fortuitous survival; refugia.

C. Operational:
   a. The chemical: (1) chemical nature of pesticide; (2) relationship to earlier used chemicals; (3) persistence of residues; formulation.
   b. The application: (1) application threshold; (2) selection threshold; (3) life stage(s) selected; (4) mode of application; (5) space-limited selection; (6) alternating selection.

Factors in the genetic and biological categories are inherent qualities of the species and, therefore, are beyond management. However, knowledge of how these contribute to selection pressure is important. In the following discussion some factors are summarized from the information obtained through this study which can help in designing control tactics.

Use of synergists in these practices and particularly piperonyl butoxide can significantly increase the toxicity of certain insecticides, since it is well documented now that mixed-function oxidases is the main defense mechanism of the Colorado potato beetle.

The high synergistic effect of PB with azinphosmethyl, which we have found consistently with several populations, may prove useful in controlling resistant populations and reducing the rate of selection of resistance in the field or decreasing the frequency of resistant genes already present in field populations.

Since DEF is a defoliant, mixtures of this synergist and insecticides cannot be used in the field to overcome resistance due to esterases. We have found resistant populations of CPB partly due to esterase activity. Post-harvest treatment or non-crop site treatment may be possible. Probably of practical use are the mixtures with sprayable esterase inhibitors, such as kitazin P, a systemic fungicide used to control Piricularia oryzae in rice (Worthing 1979), in combination with insecticides (Ozaki 1983).

As was mentioned before apart from the estimation of synergistic ratios (SR) at 50% mortality level, we can estimate the SR in 90% mortality for the purpose of field efficacy. The SR_{90} is very important since in most cases, 90% mortality is required for acceptable control. Argentine et al. 1989 reported that permethrin resistance to Colorado potato beetle has a recessive character. From our studies and the control failure of applying pyrethroids plus synergists after 1-2 years, it appears that the Kdr (knockdown-resistance) factor was well spread in certain areas and may well be responsible for some reported failures of application of pyrethroids.

Two types of resistance to permethrin exist. There are cases in the field where the Colorado potato beetle populations developed resistance to

permethrin relatively fast. This is typical when resistance is dominant or semidominant (enzymatic resistant mechanism), where the RR homozygotes and Rr heterozygotes are able to survive the application of normal rates of insecticides. If, on the other hand, the resistance is recessive (Kdr), the population remains susceptible until the quantitatively smallest fraction of rr homozygotes appear.

Farnham (1977) suggested that because Kdr is a recessive character, therefore natural dilution of the population and the removal of selection pressure would help to increase greatly the number of heterozygotes which are susceptible to DDT or pyrethroids. There is, therefore, a need for early detection of this factor in a population, coupled with an alternative means of control in order to keep the frequency of this gene at manageable levels.

There are indications and some evidence that between the insecticides azinphosmethyl and carbofuran on one side and permethrin on the other side, negative correlated resistance exists, where selection of resistance by pressure from insecticide A leads to a corresponding selection of susceptibility to insecticide B, and vice versa. Therefore, when A is no longer effective in in controlling the CPB, a switch to B may give control again for a few years. Alternate use of insecticides A and B can be repeated. Georgiou (1963) suggested that the mixtures used in sprays must not consist of a pair of compounds that display negatively correlated toxicity. Examples of negatively correlated resistance are few in the literature; there are probably more undocumented cases. We tried to document further this hypothesis for CPB with selection studies, by searching the literature for cases of resistance to CPB, and, mostly, by conducting bioassays directly to field collected populations. Therefore, some very useful conclusions were drawn, affecting recommendations for the use of mixtures of azinphosmethyl and permethrin

or carbofuran and permethrin with resistant populations, a tactic used so far by some farmers. Valuable information for resistance management tactics were obtained (Chapter 2).

Carbofuran kills extremely fast, (<1 hour), compared with azinphosmethyl which may take 3 days to express its toxicity. Carbofuran is profoundly more toxic to the larva than adults, therefore less amount of chemical can be applied to the larvae stages.

Some fungicides affect the development of the CPB. Hare (1984) reported suppression of CPB on solanaceous crops with a copper-based fungicide. Therefore fungicides might be chosen not only for their ability to control plant diseases, but also for their capacity to help manage the beetles.

Migratory flight of Colorado potato beetles is merely a particular prolonged flight which, it seems, is ontogenetically controlled and adapted to occur at a certain stage in adult development (Johnson 1967). Generally, CPB are pests with relatively low dispersal tendencies. Therefore, insecticide pressures favor the development of resistance and cultural practices are very important. Crop rotation is fundamental to solve and retard resistant problems particularly with Colorado potato beetle, since we now know that the resistant mechanisms are inherited almost as dominant genes and migration of susceptibles does not seem to be a factor.

Detection and monitoring of resistance is fundamental. During this study, discriminating doses have been established for several insecticides, therefore they can be used effectively to determine resistant portions in field populations.

Dose-mortality lines have been calculated for more than 30 populations from different areas of Michigan and can be used to track

resistance over time and make comparisons with development of resistance in other areas and even other countries.

One of the principles for resistance management is to apply the least insecticide amount on the population to conserve susceptibility. It must be enough to eliminate the susceptibles (SS) and heterozygotes (RS), in the case of permethrin resistance. Because of the recessiveness of permethrin resistance, this is easier than for azinphosmethyl and carbofuran, where resistance is inherited as incompletely dominant.

Application of mixtures of insecticides is another resistance management technique. Information in the literature is contradictory. The idea behind this technique is that it is difficult for individuals to carry more than one resistant mechanism. We believe that the use of mixtures in resistance management of the Colorado potato beetle is very risky. Field observations and our laboratory studies with several different multiple resistance strains suggests that beetles have a propensity to develop resistance and resistant genes preexist in the populations, in significant frequencies. Rotation of insecticides seems more advantageous since our information for resistance patterns and cross resistance, essentially increases. Insecticides where resistance depends on the interaction of several resistance factors should be avoided or used last, since these give a broader spectrum of cross-resistance, particularly for those in which resistance development comes from enzymatic mechanisms.

For estimation of resistance levels to a certain insecticide comparisons should be made with populations that recently were controlled by this insecticide. It used to be that resistance in field-collected strains as compared with susceptible strains that had been kept in isolation for long periods of time in an insecticide-free environment. This may have given wrong

estimations. Field populations have acquired some degree of resistance, and the genomes of these populations adapted to new agricultural environments under the continuous pressure of insecticides used. Development of resistance is a natural process and must be considered as an evolutionary phenomenon from the genetic consideration point of view.

Resistance management strategies are currently being adopted for the Colorado potato beetle in an attempt to extend the potential and usefulness of the insecticides, but for how long? The design of an integrated pest management system for this threatening pest is a necessity.

Oppenoorth 1985 mentions that as entomologists and toxicologists, our knowledge of resistance mechanisms is increasing, but the development and molecular genetics of resistance has just been touched. Whether chemical control of a majority of pest species will remain possible seems difficult to predict at present.

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Appendix 1

APPENDIX 1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 1990-02

Title of thesis or dissertation (or other research projects):

Characterization, Synergism and Inheritance of Resistance to azinphosmethyl, carbofuran and permethrin insecticides in the Colorado potato beetle (Coleoptera: Chrysomelidae)

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

none

Investigator's Name (s) (typed)
Philippos M. Ioannidis

Date March 26, 1990

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24:141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.
Copies: Included as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

APPENDIX 1.1

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Leptinotarsa decemlineata (Say) Colorado potato beetle									
Strains:									
Vestaburg susceptible	MI Montcalm Co. Vestaburg Aug.1987					8	8		
Baker permethrin resistant	Lab Entom. MSU Summer 1988					8	8		
Macomb multiple resistant	MI Macomb Co. near Macomb July					8	8		
MNT-C carbofuran resistant	MI Montcalm MSU 1987					8	8		
	Potato Exp. Sta. 1/16/1987								
Long Island azinphosmethyl resistant	Lab Entom. MSU 1985					8	8		

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Philippos M. Ioannidis

Date March 27, 1990

Voucher No. 1990-02

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator

29 Mar 1990

Date