# The Genetics of Imidan Resistance in *Drosophila melanogaster*.

By

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**ABSTRACT**

The relationship between the mixed-function oxidase (MFO) system and resistance of Drosophila melanogaster to phosmet (Imidan$^{R}$) an organophosphorus insecticide has been studied. The MFO activity was assayed by 7-ethoxycoumarin-O-deethylase (7-ECD) activity and resistance levels were determined by either adult LC-50 or percent adult survival at 150 uM of Imidan. Two groups of isofemale lines sampled from Vineland, Ontario and a laboratory selected population were used to study variability of the MFO system and its relationship to variability in resistance.

A significant positive correlation was found between 7-ECD specific activity and an antigenic determinant assayed by western blotting. The antiserum used to detect this determinant was prepared against a cytochrome P-450-enriched fraction from a malathion-resistant Drosophila melanogaster strain by J. Pursey (1989). The antigenic determinant was contained on polypeptide P2, believed to be a cytochrome P-450 by its molecular weight and heme content.

Laboratory selection on Imidan resulted in over 2.1-fold increase in resistance. Two MFO activity groups were identified among isofemale lines established from nature. A significant positive correlation was found between 7-ECD specific activity and

resistance to Imidan among the Isofemale lines. The minimum number of genes controlling the difference in 7-ECD specific activity between two isofemale lines was estimated to be about 1.

It was concluded that this natural Drosophila melanogaster population has a major or few genes on chromosome 3 affecting MFO activity and resistance but as well other factors on chromosomes 1 and 2 also contribute to variability in resistance.

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## LIST OF TABLES

## LIST OF FIGURES

## TABLE OF CONTENTS

PART THREE

# INTRODUCTION

## 1.1 Perspectives and Overview of Pesticide Resistance

Despite an ever-growing protest against pesticides, their continued use in agriculture seems inevitable mainly because of the increased need for food world-wide and the loss in productivity when pesticides are not used. Of all pest control methods practiced today only chemicals offer immediate control of sudden outbreaks of pests. Unfortunately the use of pesticides is not always without the accompanying phenomenon of resistance.

Ever since Melander (1914) reported the first case of resistance to pesticide, resistance has increased exponentially. It is now an indispensable consideration in almost all pest control programmes. Georghiou and Mellon (1983) stated that all reported cases of resistance involved at least 428 species of arthropods, 91 species of plant pathogens, 5 species of weeds and two species of plant parasitic nematodes. Forgash (1984) estimated that since 1954 seventeen new species become resistance every year.

Considering the alarming rate at which pests are developing resistance to pesticides and the cost of discovery and commercialization of a new insecticide, from less than $10 million in 1972 to more than $20 million in 1980 as quoted by Braunholtz (1981) the importance of understanding the mechanisms of resistance

and the factors influencing its development cannot be over-emphasized.

## 1.2 Mechanisms of Insecticide Resistance

It has been known for a long time that all organisms have evolved mechanisms by which they tolerate or resist adverse chemicals, both exogenous and endogenous. These mechanisms have been classified as behavioral, physiological and biochemical (metabolic and target sensitivity). Behavioral resistance mechanisms depend on genetic modifications involving avoidance of insecticide, as well as by the systems altered. Mechanisms of physiological resistance to toxic chemicals include diminished penetration, sequestration and excretion. Unlike physiological resistance, biochemical mechanisms only require changes in macromolecules (Brattsten et al, 1986).

The most important documented cases of insecticide resistance mechanisms are biochemical. Typically they involve improved capacity to metabolically detoxify insecticides and/or modifications in the target sites to decrease sensitivity (Brattsten et al, 1986). Although behavioral adaptations are of major importance in insect avoidance of toxic components of their food plants in nature, they are relatively unimportant compared to biochemical adaptations when synthetic insecticides are involved (Brattsten et al, 1986).

Four major kinds of metabolic mechanisms of insecticide resistance have been described. They are :

(1) increased mixed-function oxidase (MFO) activity,

(2) increased glutathione-S-transferase (GSH) activity,

(3) altered ali-esterase and

(4) increased DDT dehydrogenase activity.

The role of the MFO system in organophosphate resistance has received considerable attention in recent years. Detoxification or bioactivation by the cytochrome P-450-dependent MFO system often determines the biological activity or toxicity of a compound.

### 1.3 Mode of Action of Organophosphorus Insecticides

Organophosphates (such as malathion) and the carbamates are thought to exert their effect by inhibiting acetylcholinesterase (Baillie and Wright, 1985). In addition malathion may have an inhibitory effect on succinoxidase, glycolysis and Kreb's cycle intermediates (Dahm, 1971). Stereochemical properties that are responsible for high affinity of a more active isomer are also responsible for acetylcholinesterase inhibition (Fukuoto, 1976).

Acetylcholinesterase catalyzes the hydrolysis of acetylcholine, a chemical neurotransmiter acting at synapses in the insect central nervous system. When acetylcholinesterase is inhibited there is an accumulation of acetylcholine, causing hyperexcitation. This starts initially as hyperactivity, followed by convulsion and paralysis

and eventually death. Rate differences observed in enzyme inhibition have been shown to be qualitatively consistent with differences observed in toxicity between enantiomers, that is, the strong acetylcholinesterase inhibitors were more potent toxicants (Ohkawa, 1982).

Organophosphates and the carbamates are known to bind to acetylcholinesterase at is active site, inhibiting its catalytic ability. The binding site of acetylcholinesterase for organophosphates is believed to be "hydrophobic" and different from the anionic binding site for quaternary ammonium chemicals, which are also inhibitors of acetylcholinesterase (Casida, 1973; Aldridge, 1975; and O'Brien, 1976).

## 1.4 The role of Cytochrome P-450 and MFO activity in Metabolism of Xenobiotics

The oxidation of insecticides and other foreign compounds by monooxygenases is of considerable importance and often plays a dominant role in determining their biological activity or toxicity (Wilkinson, 1983). A monooxygenase inserts one atom of atmospheric oxygen into its substrate (Mason et al, 1955 and Hayaishi et al, 1955), the other is reduced to water. These enzymes are by definition classified as mixed-function oxidase. The reactions they catalyze can be represented by the general equation :

$$RH + O^2 + NADPH + H^+ = ROH + H_2O + NADP^+$$

An essential component of the microsomal monooxygenase system is the terminal oxidase, cytochrome P-450. Evolutionary considerations have led to the suggestion that this cytochrome has been adapted to the detoxification of lipophilic dietary toxicants, which would otherwise accumulate to deleterious levels. Its wide distribution among organisms suggest that it has an ancient, monophyletic origin and has been subsequently modified for different substrate specificities (Hodgson, 1983). Cytochrome P-450 is a carbon monoxide binding membrane-bound pigment found in the endoplasmic reticulum of mammals and in the fat body, malpighian tubules and the midgut of insects (Wilkinson, 1983; and Hodgson and Tale, 1983). It derives its name from a major optical absorbance peak at 450 nm when in the reduced form and combined with carbon monoxide. The role of cytochrome P-450 in the mixed-function oxidase system is illustrated in the following diagram :

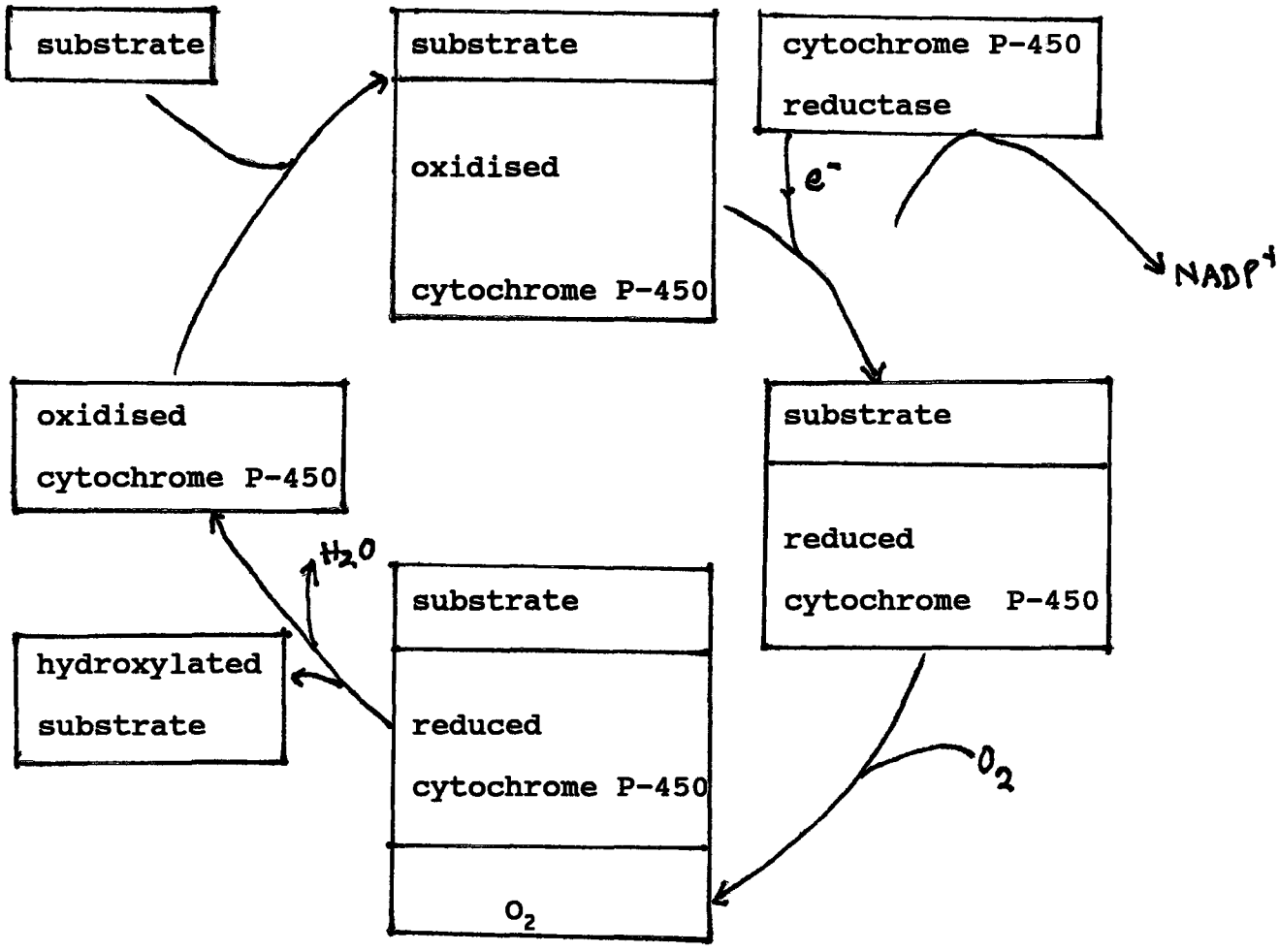



Figure 1: Role of Cytochrome P-450 in the Oxidative Metabolism of Xenobiotics (Adapted from Hodgson and Tale, 1976).

The hydroxylated product can then be further processed by additional metabolism step(s).

Organophosphorus compounds undergo various metabolic reactions in living organisms. The metabolic conversion of thiophosphoryl (P=S) esters to the corresponding phosphoryl (P=O) esters by mixed-

function oxidase (MFO) is a well known activation reaction occurring in many organisms. Since P=S esters are generally poor anti-cholinesterases, MFO-mediated activation to the P=O ester is required for intoxication by thiophosphoryl insecticides (Ohkawa, 1982).

### 1.5.1 Evolution of Insecticide Resistance

Insecticide resistance is a dynamic, multidimensional phenomenon, dependent on species, population and geographic location. Georghiou and Taylor, 1976 categorized factors influencing the development of resistance as genetic, biological and operational. The genetic factors include frequency, number, dominance and penetrance of alleles. The biological factors on the other hand include generation turn-over, offspring per generation, isolation, mobility and accidental survival. The operational factors are those related to the chemical and its application such as the chemical nature of pesticide, relationship to earlier used chemicals, persistence of residues, application threshold, life stages selected and mode of application. All these factors combined affect the rate of resistance development. Insect resistance to insecticides via enhanced enzyme detoxification is an accelerated version of the natural selection process that has been operating since life first began (Wilkinson, 1983). Selective pressure exerted by the insecticide sharply increases frequency of genetic variants expressing resistance within the exposed population. These variants

increase in number by the differential survival and reproduction of individuals carrying mutations that increase resistance such as those enhancing degradation after exposure to insecticides. The mechanisms of resistance are believed to be the same developed by insects through their inter-relationships with other organisms.

Herbivorous insects are believed to have co-existed with higher plants for over 250 million years. Plants produce many allelochemicals, such as alkaloids, terpenes, and phenols for defense against insects and pathogens (Fraenkel, 1959). These chemicals favour the evolution of co-adaptations in plant-feeding insects. Insects often rely on a complex of general-purpose defense enzymes to overcome the potential toxicity of the plants they eat. In sharp contrast to the slow evolution of resistance to natural toxicants, resistance to synthetic insecticides developed extremely rapidly, probably because insects use some of the same mechanisms that have evolved in defense against plant allelochemicals (Brattsten et al, 1986).

### 1.5.2 Genetic Mechanisms Producing Resistance

Resistance mechanisms arise through heritable changes - mutations - in the genome of individual insects. Mutations may include base substitutions in DNA, amplification of pre-existing genes, translocations, chromosome inversions or other DNA re-arrangements. Gene amplification may be an especially important genetic mechanism

conferring resistance that involves sequestration of toxins by binding to lipids, or proteins or on a reduced rate of penetration through the integument because of overproduction of a gene product. Base pair substitutions may be a likely cause of target site resistance where the macromolecules are modified so that they no longer bind the insecticide (Brattsten et al, 1986). However both gene amplification and base pair substitutions may be important in resistance that is due to increased metabolic detoxification. Support for the gene amplification hypothesis has been from the organophosphate resistance of the green peach-aphid Myzus persicae (Sulzer) (Devonshire and Sawicki, 1979; and Devonshire and Moore, 1982). It was possible to show that carboxylesterase was present in various strains in different amounts that could be arranged in a geometrical series with a factor of 2, from 1 to 64 (Devonshire and Sawicki, 1979). In the mosquito, Culex pipiens although the amount of esterase was not determined, the level of resistance itself was suggestive of a series of gene duplications (Pasteur and Georghiou, 1980). Work using molecular genetic techniques also support the gene amplification hypothesis. Adults of OP-resistant Culex quinquefasciatus Say (Tem-R strain) were found to possess at least 250 times more copies of an esterase gene responsible for resistance than adults of a susceptible strain (S-Lab) (Mouches et al, 1986). Insecticide resistance in peach-potato aphids (Myzus persicae Sulz.) was also shown to result from amplification of an esterase gene (Field et al, 1988).

Target resistance to organophosphorus compounds and carbamates are examples resulting from mutant enzymes since they have different properties and occur in equal or even lower quantity than their susceptible counterparts. The same is true for hydrolytic enzymes ("mutant ali-esterase") present in some OP-resistant houseflies, which are present in about the same quantity as the original ali-esterase in susceptible strains (Oppenoorth and Welling, 1977). Several factors including insecticides could be responsible for producing new mutant enzymes resistant to insecticides. Although many insecticides are known to be genotoxic in laboratory experiments it would be difficult to determine if field exposure to insecticides increases the frequency of mutations (Brattsten et al, 1986). Mutation rates may also be increased by allelochemicals, linear furanocoumarins pyrrolizidine alkaloids and aflatoxins which can alter DNA (Pearlman et al, 1985; and Clark, 1959).

Little is known about regulatory mutations affecting the amount of detoxifying enzymes but they may be especially important for oxidase like cytochrome P-450. Work in mammalian systems like mouse has shown the possibility of regulatory mutations affecting P-450 activity (reviewed by Nebert and Gonzalez, 1987).

### 1.5.3 Origin of Increased MFO activity

Cytochrome P-450 is an essential component of the oxidative

metabolism pathway of xenobiotics. In light of cytochrome P-450's function a correlation between P-450 levels and MFO activity is expected. An increase in MFO activity could be produced either by a more active mutant enzyme or by an increase in the amount of previously existing enzyme. The level of a 56K immunoreactive P-450 has been shown to be 10 to 20 times higher in strains selected for DDT resistance and having high MFO activity than in their susceptible counterparts (Sundseth et al, 1989). Selection could operate at the level of the structural gene, a regulatory gene or both. Any heritable increase in the amount of an enzyme can be attributed to either gene amplification or regulatory mutation. If amplification is responsible for increased activity, the amplification could be of the structural gene or of the structural gene plus its regulatory region. According to Terriere (1983), since resistant strains (house flies) are readily induced, both the structural and the regulatory region must be amplified together. This is because of the expected larger amount of repressor and hence the greater response to induction. However regulation of cytochrome P-450 expression is probably via a separate gene, as in the mouse system where a regulatory gene codes for an inducer protein (Karenlampi et al, 1983) and therefore induction may not be limited by the number of structural genes. The suggestion that increased P-450 activity could be the results of a more active enzyme is supported by the discovery of multiple forms of cytochrome P-450 by Lu et al (1976) and Coon et al (1977). These two studies demonstrated that different inducing agents cause the

synthesis of qualitatively different forms of cytochrome P-450 with different catalytic and structural properties.

### 1.6.1 Organophosphorus Resistance in Drosophila melanogaster

Resistance to insecticides in Drosophila melanogaster has received considerable attention in the past decade. Both reduced acetylcholinesterase sensitivity and increased MFO activity have been associated with insecticide resistance in Drosophila. In 1982, Morton and Singh investigated the relationship between acetylcholinesterase activity and insecticide resistance. A negative correlation was found between activity and malathion KT50 (the time required to knockdown 50% of the flies) among isofemale lines. There was also some variability in the electrophoretic mobility of the acetylcholinesterase enzyme, suggesting that these altered enzyme forms may have had a decreased affinity for the insecticide and therefore reducing its effect. The study also mapped the altered acetylcholinesterase activity to the Ace structural locus at position 50 on chromosome three.

However target resistance due to the acetylcholinesterase enzyme was not totally responsible for increased malathion resistance in Drosophila. The variability of Drosophila to metabolize a wide variety of chemical compounds was demonstrated about a decade ago (Hallstrom and Grafstrom, 1981; and Waters et al, 1982). Morton and Holwerda (1985) found that resistant flies had increased cytochrome

P-450 content and that resistant fly microsomes degraded both malathion and malaoxon more rapidly. Malathion resistance has also been associated with
7-ethoxycoumarin-O-deethylase (7-ECD) activity but this was also the result of increased P-450 content (Houpt et al, 1989).

### 1.6.2 Localization of MFO genes in Drosophila melanogaster.

Studies have shown that cytochrome P-450 dependent enzyme activities in Drosophila melanogaster are controlled by genes on both autosomes. Halpern and Morton (1987), reported that increased MFO activity and malathion resistance in D. melanogaster were controlled by genes on chromosomes 2 and 3. High p-nitroanisole (PNA) N-demethylation and biphenyl 3-hydroxylation activity which are both P-450-dependent reactions and an increased amount of a protein with an apparent M.W. of 54K after SDS-gel electrophoresis of microsomes in insecticide-resistant Drosophila strains were found to be inherited as dominant second chromosome traits by Hallstrom (1985). She also found that a low capacity for benzo[a]pyrene (BP) hydroxylation and 7-ethoxycoumarin-O-deethylation (a P-450-dependent reactions) in the Hikone R strain was semidominantly inherited. In both cases, activities were determined by one or two genes on the third chromosome. Houpt et al (1989) found that microsomes from Drosophila strains carrying the chromosome 3 factors contained more cytochrome P-450 and increased amounts of two heme-staining protein bands (Mr = 50K and 54K).

Genes controlling these proteins were closely linked to the recessive marker striped (3-62). These were probably identical to loci near 3-58 producing malathion resistance and increased mixed-function oxidase activity. However resistance genes on both chromosome 2 and 3 as well as target resistance were required for the full expression of malathion resistance in the selected Drosophila population studied by Houpt et al (1989). Using monoclonal antibodies to resistance-related forms of cytochrome P-450 in Drosophila melanogaster, Sundseth et al (1989) showed that the expression of a 50K cytochrome P-450 polypeptide was regulated by resistance genes on the second and third chromosomes. The level of P-450 was correlated with resistance to phenylurea, DDT, and malathion and with N-nitrosodimethylamine demethylase activity.

## 1.7 Monogenic versus Polygenic Inheritance of Resistance

Genetic factors influencing the development of resistance in a population include the frequency and the nature of the genes controlling resistance. Of importance to the nature of genes controlling resistance are the number, dominance, penetrance, expressivity and interactions of genes (Georghiou and Taylor, 1976). Previous exposure of the population to other xenobiotics and allelochemicals and the pleiotropic effect of resistance genes on fitness also affect the rate of development of resistance. While allele frequency, dominance, the relative fitness of the various genotypes and the population structure have a profound influence on

the evolution of resistance, the number of genes controlling resistance will determine the limits of progress of recurrent selection with pesticide. The importance of the number of genes determining the limits of selection however may be dependent on other factors such as dominance. Falconer (1981) stated that, with a given amount of initial variation and in a situation where genes act additively, a small number of genes will produce less response to selection than a larger number. If a given amount of variation is produced by few genes, their individual effects are greater than if many genes are involved. Therefore knowing the number of genes controlling resistance is of considerable importance to both the development and loss of resistance in insect populations.

The genetic basis of resistance may be determined by either single gene or polygenic mechanisms. Whether a monogenic or polygenic system will be selected by insecticide exposure depends on the amount of genetic variation available and also the extent and manner which this variation is acted upon by either artificial or natural selection (McKenzie, 1985). The adaptive response depends on whether the screening process selects for variation that is already present or for novel mutations. To date most of the insecticide resistance studies of laboratory populations of Drosophila have indicated polygenic resistance (Dapku and Merrel, 1977; King and Somme, 1958; Merrell and Underhill, 1956; Oshima, 1958; Singh and Morton, 1981). What is not certain though is whether the same polygenic inheritance occurs in nature. It has

been suggested (Roush and McKenzie, 1987) that monogenic inheritance of resistance is more likely in natural populations because the genetic variation in the insect populations of fields exposed to insecticides is unlimited. This results in selection of rare alleles causing major changes in the degree of resistance within the population. It is believed that pesticides exposure in the field selects for chance mutations by screening preferentially for variation outside the range of the initial phenotypic distribution, although to date no study has discovered such extreme variants. Even if these extreme genotypes exist, it is still not known if they will be fit enough to survive and become the predominant type. The genetic variation in a laboratory colony, on the other hand is limited and biological and environmental stress factors are minimized. This tends to promote the development of resistance originating from contributions by several different mechanisms (Brattsten et al, 1986) because selection in this case acts on variation within the distribution of naturally existing phenotypes (McKenzie et al, 1980). Similar responses producing polygenic inheritance may also occur in natural insect populations and are referred to as changes in "Vigour tolerance" of the population (Brown and Pal, 1971; and Georghiou, 1972).

## 1.8 The Number of Genes Influencing Quantitative Traits

Determining the number of genes for resistance segregating in natural populations of Drosophila melanogaster will help settle the

controversy of monogenic versus polygenic inheritance in natural populations. Estimates of the number of genes contributing to the variance of quantitative characters within and between populations are fundamental to the study of mechanisms of heredity and evolution. The multiple-factor theory of the inheritance and the evolution of quantitative characters (Wright, 1968) holds that heritable variance in quantitative traits within populations is caused by the segregation of multiple genetic factors, and that large evolutionary changes in quantitative characters generally occur through the Darwinian process of accumulation of numerous genetic factors with individually small effects.

A procedure for estimating the number of segregating factors was originally developed by Wright (in Castle, 1921; Wright, 1952 and 1968) for two inbred parental lines and later generalized by Lande (1981) to a method for heterogeneous parental populations. The procedure assumes that environmental variance is equal in all populations, that all loci have equal effects and act additively, and that there is no linkage, dominance or other genetic interactions. Two extreme parental populations are used in crosses to obtain F1, F2, B1 and B2 populations. The F2 and two backcross populations contain a component of variance, the segregation variance arising from genetic differences between the two parental populations. The observed segregation variance can be estimated from linear combinations of parental, F1, F2, B1 and B2 populations in four different ways (see section 2.9). These four ways are

equivalent when the environmental variance is the same for all populations and generations. The equation for estimating the minimum number of genes is based on the segregation variance and the difference between the means of the parental populations. Since the minimum number of genes estimates approximates the recombination index (the haploid number of chromosomes plus the mean number of recombination events per gamete) for most studies that have been done (Lande, 1981). We cannot reject the idea that actually large number of genes are involved in most quantitative traits in natural populations. This is consistent with the neo-Darwinian theory of evolution by gradual change. The effective number of genetic factors is also related to an upper bound on the magnitude of the factor of largest effect. If the effective number of factors is large, the proportion of the range between the two populations explained by the major factor must be small (Lande, 1981). Gene number estimates are considerably more reliable when environmental variance is small compared to genetic variance, particularly when gene number is large (Park, 1977). This is one of the reasons why inbred (isofemale) parental lines are used in the minimum number of genes estimates in this thesis.

## 1.9 Purpose of this Study

In spite of the fact that the biological, genetic and environmental factors operating in nature may be completely different from laboratory conditions, almost all studies on

insecticide resistance are carried out on laboratory colonies for obvious reasons.

The general purpose of this study was to compare natural and laboratory populations (both selected and susceptible) of Drosophila melanogaster for their resistance to an organophosphorus insecticide phosmet (Imidan®) and levels of mixed-function oxidase (MFO) activity [measured by 7-ethoxycoumarin-O-deethylase (7-ECD) specific activity]. The relationship between 7-ECD specific activity and levels of the terminal oxidase for MFO activity, cytochrome P-450, using an antiserum directed against cytochrome P-450 of a laboratory selected isochromosomal strain was also studied. It was hoped that this antiserum could be used to identify resistant genotypes in natural populations.

The number of genes controlling 7-ECD specific activity differences between two isofemale lines (both derived from a natural population of Drosophila melanogaster) was also estimated as part of the general effort to understand the rate of development and limits of resistance in nature. It was particularly hoped that estimating the number of genes will help settle the controversy over monogenic versus polygenic inheritance of resistance factors in natural populations.

# MATERIALS AND METHODS

## 2.1 Maintenance of Flies

Flies to be used in all experiments were fed banana medium : 10g malt powder, 20g sugar, 40g yeast powder, 1 banana, and 12g agar (Difco) per 1200ml $ddH_2O$, with 0.2% p-hydroxybenzoic acid (to inhibit fungal growth) and kept at $24^0C$ on a 12 hour day/night cycle in 16 dram plastic vials. However for longtime maintenance they were put on corn meal medium : 40g corn meal, 30g yeast powder, 12g agar, 30g sugar and 10g malt powder per 1400ml $ddH_2O$ with 0.2% p-hydroxybenzoic acid. The origin and nature of flies used in this study are given in Table 1.

## 2.2 Enzyme Assay

Crude fly extract for the mixed-function oxidase (MFO) assay was prepared by homogenizing 15 male flies per 350ul TKEG, pH 8.6 extraction buffer (50mM Tris, 50mM KCl, 1mM Sodium EDTA, 0.5mg/ml ovalbumin, 10% glycerol), then centrifuged at 15,000g for 10 minutes. The rate of deethylation of 7-ethoxycoumarin (7-ECD) activity was determined spectrofluorimetrically using a modified procedure from Prough et al (1978) as described by Houpt et al (1989) and Patil et al (1990). The reaction mixture contained 90ul of the extraction buffer described above with 33uM ethoxycoumarin, 0.5mM NADP, 2.5mM glucose-6-phosphate and 0.3

**TABLE I :** Strains of *Drosophila melanogaster* used in this study.

| Strain(s) | Origin | Nature |
|---|---|---|
| Canton S | Laboratory stock. | Susceptible. |
| IIID, IIIC | Hamilton, Ontario(1976). | Isochromosome III, Malathion resistant. |
| 11B, 52A, 5B, 56A and 109A | North Carolina. | Isochromosome III. |
| Dal-1, -2, ... and -14 | Hamilton, Ontario(1976). | Isofemale lines. |
| VC | Vineland, Ontario (1988). | Sampled from unsprayed plot. |
| VS | Vineland, Ontario(1988). | Sampled from sprayed plot. |
| VS-7, -11, -14 and -35 | Vineland, Ontario(1988). | Isofemale lines. |
| RS-VSS/14 | Vineland, Ontario(1988). | Selected on Imidan and maintained on medium with 14 µM of Imidan added. |
| FM7a | Laboratory stock. | Chromosome I balancer. |
| CyO/bw$^D$ | Laboratory stock. | Chromosome II balancer. |
| MKRS/TM3 | Laboratory stock. | Chromosome III balancer. |

units of yeast glucose-6-phosphate dehydrogenase.

For single fly enzyme assay, each male fly was extracted in 200ul of the extraction buffer and also centrifuged at 15,000g for 10 minutes. The reaction mixture was the same as described above. Forty microlitres of the fly extract in TKEG buffer was added to the reaction mixture to initiate the reaction which was carried out 30°C for 60 minutes, then terminated by adding 0.2vol of 1N HCl followed by 1vol of chloroform. The product, 7-hydroxycoumarin (7-HC), was extracted into 30vol of 0.5M Tris base (pH > 10) in order to maximize its fluorescence and then measured in a flourimeter with excitation at 380nm and fluorescence emission determined through a 400nm cut-off filter. Mock reaction mixtures containing known amounts of 7-hydroxycoumarin were used to calibrate the instrument. Specific activity has been expressed in units of picomoles of product formed per sec mg of protein.

## 2.3 Protein Quantification

Protein concentration for MFO specific activity measurements were by the method of Bradford (1976). Crude fly proteins were mixed with the reagent [0.0002% of Coomassie Blue (Serva Blue G catalog number 35050, Feinbiochemica) + 1.9% perchloric acid], vortexed and the absorbance measured 20 to 60 seconds later at 610nm on a Horizon Colorimeter. Bovine serum albumin (Boehringer-Mannhein 238 031) was used as a standard.

Protein concentration for the cytochrome P-450 antigenic response was determined by the method of Lowry et al (1951) with slight modifications. Samples containing 10ul of crude fly protein were mixed with 0.5ml water and 2.5ml reagent [49ml of 2% $Na_2CO_3$ + 1ml of (0.5% $CuSO_4(H_2O)_5$, 1.2% $Na_3$Citrate)] and incubated at room temperature for 10 minutes. Folin reagent (Fisher 50-P-24, 0.25ml of a 33% solution) was added and the absorbance at 660nm was measured after one hour. Bovine serum albumin was used as the standard.

### 2.4.1 Electrophoretic Analysis of Crude fly Proteins

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was used to separate crude fly proteins. The procedure followed was that of Laemmli (1970) as modified by Coulhart (1986). 1.5mm thick gels (16 X 14 cm) with a 10% resolution gel and a 4.8% stacking gel were used in all cases. All gels were run at $4^0$C. Gels were electrophoresed at 75V for 16 hours. All samples were mixed with an equal volume of 2X sample buffer [125mM Tris pH 6.8, 4% SDS, 10% B-mercapto ethanol, 0.01% bromophenol blue (BPB), 20% glycerol] and boiled for three minutes prior to loading. Flies were extracted in 5ul per fly of 0.05% CHAPS in TKEG pH 8.6, centrifuged a 15,000g for 10 minutes at $4^0$C and 10ul (sample + buffer) loaded per lane.

### 2.4.2 Western Blotting

SDS polyacrylamide gels were electro-blotted onto nitrocellulose membrane (Scheicher and Schuell, BA85) using a Hoefer Scientific Instruments transblot system. Gels were equilibrated in 25mM Tris, 60mM glycine buffer for 20 minutes at room temperature and together with the membrane sandwiched between Whatman 3mm filter paper as described by Towbin et al (1979). Transfer was carried out with a current of 0.25A for 6 hours with stirring at 4°C in transfer buffer (25mM Tris, 60mM glycine in 20% methanol). The blotted nitrocellulose was blocked with a 5% solution of skim milk powder in PBS ($NaHCO_3/Na_2CO_3$ buffer pH 7.2), air dried and stored between filter paper until immunostained.

### 2.4.3 Western Immunostaining

Rabbit antibodies (Ab1-B) used in the study was a gift from J. Pursey. For a description of how they were produced see Pursey (1989). Ab1-B is preferentially directed to strain IIID microsomal protein. This primary antibody diluted 1/1000 in 0.05% Tween in PBS pH 7.6, was applied to blocked-blotted nitrocellulose for 2 hours at room temperature with gentle agitation. Excess antibody was washed off three times with 0.05% Tween in PBS for 5 minutes each. The filters were then incubated with secondary antibody [alkaline phosphatase conjugated goat anti-rabbit IgG antibody (Inter Medico, TAGO Immunologicals 6530)], diluted 1/5000 in 0.05% Tween in PBS pH

7.6. Incubation and washing conditions were as described for the primary antibody. Alkaline phosphatase was developed using he nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate system (Blake et al, 1984). The substrate made up of [0.02ml 2M $MgCl_2$, 1ml 1mg/ml NBT (Sigma N6876) in 0.1M Tris-HCl pH 9.2, 0.1ml 5mg/ml BCIP (Sigma B8503) in N, N-dimethyl formamide, 9ml 0.1M Tris-HCl pH 9.2] was added and the filter was then incubated at $37^0$C until purple bands developed. This was followed by a brief wash in 0.05% Tween in PBS, pH 7.6 to remove traces of the substrate. The stained filter was stored after air drying.

### 2.4.4 Quantitation of Cytochrome P-450 (antigenic) Response

Two different models of densitometers were used in the quantitation of antigenic determinant response to the antibody Ab1-B. For data presented in Figures 6 and 12 a densitometer connected to a calibrator was used to detect the reflectance of each band revealed by immunodetection and to calculate the area under each reflectance peak. The area could then be used to calculate the relative amounts of antigenic determinant per mg of protein. The attenuation and speed were adjusted with respect to the least and most intense bands so as to acquire a satisfactory base line and peaks.

All other measurements of antigenic determinant response presented in the thesis were made with an LKB-UltroScan XL Enhanced

Laser Densitometer. The following parameters were used to measure the absorbance of the bands, A-axis normalized, Y-axis expansion factor 1, Y-step 1, X-step 250, Peak Width 2, 20 and 1, X-width 8 and Baseline 1. The absorbance measures were integrated to give the area of each band in absorbance units per millimetre. This was used to calculate the amount of antigenic determinant per mg protein applied to the original gel.

## 2.5 Immunoplate Assay for Detection of Resistant Genotypes

Single male flies were extracted in 200ul of TKEG pH 8.6 + 0.05% CHAPS and centrifuged at 15,000g for 10 minutes. Twenty microlitres of the fly extract + 180ul of coating buffer (100mM $Na_2CO_3/NaHCO_3$ pH 9.6) were loaded into each well and incubated at $4^0C$ for 16 hours. The wells were then washed three times with 0.05% Tween/PBS pH 7.6 and shaken upside down to drain. After this the wells were blocked with 5% skim milk powder in PBS for 1 hour and washed as described above. This was followed by incubation at room temperature for 2 hours with 200ul of the primary antibody, Ab1-B, diluted 1/1000 in 0.05% Tween/PBS. This was followed by the same washing conditions as above and then incubated at room temperature for 2 hours with the secondary antibody (alkaline phosphatase conjugate, Goat anti-rabbit IgG) and again washed as before. Alkaline phosphatase was assayed by incubation at $37^0C$ with 200ul of 1mg/ml of p-Nitrophenyl phosphate disodium (Sigma 104 phosphatase substrate) in diethylamine buffer (200ml of $ddH_2O$, 24.25ml of diethylamine, and

32.5ml of 1N HCl with the pH adjusted to 9.8 followed by 50mg of $NaN_3$ and 25mg of $MgCl_2 6H_2O$). The reaction in each well was stopped with 50ul of 2M NaOH and the content diluted in 0.75 ml of $ddH_2O$ to determine the absorption at 405nm.

## 2.6 Selection Experiment

Peach fruits infested with Drosophila larvae were collected from Vineland, Ontario on September 8, 1988. The fruits were placed in a population cage (45 X 30 X 30 cm) and sprayed on three consecutive days with 150uM of technical grade of phosmet (Imidan$^R$), an organophosphorus insecticide. Pupae that formed after this were collected into 8oz bottle containing banana medium and the larvae which subsequently developed from this collection were again sprayed with the same concentration of Imidan. The survivors were inter-mated in a population cage, eggs collected on banana medium in 8oz bottles and the flies maintained as VSS (selected from flies of a regularly sprayed plot at Vineland) in the middle of November, 1988.

The MFO specific activity measured for this line was 1.64 picomoles/sec mg as compared to 0.72 for Canton S. By the first week of April, 1989 the MFO specific activity for this line had dropped to 0.89 compared to 0.77 measured for Canton S.

The VSS was therefore reselected on corn meal medium containing increased doses (1, 2, 4, 8, 12 and 14 uM) of Imidan. The reselected line was then labelled RS-VSS/14 with MFO specific activity of 1.67 versus 0.69 for Canton S was maintained on corn medium containing 14uM of Imidan.

## 2.7 Resistance to Imidan

Resistance of flies to Imidan was measured either by the concentration (LC-50) required to kill 50% of the population over a 24-hour period of feeding on a medium containing the insecticide or by the percent of adults surviving 150uM of Imidan after 24 hours.

The required amount of Imidan dissolved in 2-propanol was added to a 1.5% agar, 1% sucrose solution at 45 - 50$^{0}$C and 2ml placed in a 17 X 100mm test tube which were then left to air-dry for 24 hours at 24$^{0}$C. Ten to twenty flies (5 - 8 days old) were added to the tube and kept 24$^{0}$C for 24 hours. The LC-50 was calculated by plotting probit survival versus log concentration and fitting points by linear regression.

## 2.8 Estimating the number of MFO genes

The procedure used for estimating the number of segregating factors influencing MFO activity was originally developed by Wright

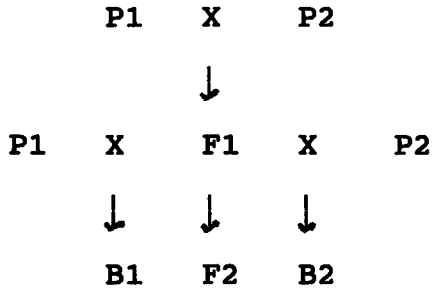
(in Castle, 1921; Wright, 1952 and 1968) for two homogeneous parental lines and later generalized by Lande (1981) to a method for heterogeneous parental populations. The procedure assumes that environmental variance is equal in all populations, all loci have equal effects and act additively, there is no linkage, dominance or other genetic interactions and that all favourable alleles have been fixed at both limits.

The minimum number of genes is estimated from the equation :

$$n_E = R^2 / 8 \sigma s^2 \qquad (1)$$

where $R^2$ is the square of the difference between the upper and lower selection limits and $\sigma s^2$ is the genetic variance segregating in a given backcross population or in the F2 (Figure 2).

Figure 2 : Hybrid crossings.




A representative isofemale line VS-7 with low 7-ECD specific activity and another VS-35 with high 7-ECD specific activity were used as parental lines (P1 and P2). The total variance corresponding to P1, P2, F1, F2, B1 and B2 populations can be described by the equations (Lande, 1981) :

$$\sigma_{P1}^2 = \sigma_{g1}^2 + \sigma_e^2 \quad (2)$$

$$\sigma_{P2}^2 = \sigma_{g2}^2 + \sigma_e^2 \quad (3)$$

$$\sigma_{F1}^2 = 0.5\,\sigma_{g1}^2 + 0.5\,\sigma_{g2}^2 + \sigma_e^2 \quad (4)$$

$$\sigma_{F2}^2 = 0.5\,\sigma_{g1}^2 + 0.5\,\sigma_{g2}^2 + \sigma_s^2 + \sigma_e^2 \quad (5)$$

$$\sigma_{B1}^2 = 0.75\,\sigma_{g1}^2 + 0.25\,\sigma_{g2}^2 + \sigma_s^2 + \sigma_e^2 \quad (6)$$

$$\sigma_{B2}^2 = 0.25\,\sigma_{g1}^2 + 0.75\,\sigma_{g2}^2 + \sigma_s^2 + \sigma_e^2 \quad (7)$$

where $\sigma_{P1}^2$, $\sigma_{P2}^2$, $\sigma_{F1}^2$, $\sigma_{F2}^2$, $\sigma_{B1}^2$ and $\sigma_{B2}^2$ refer to the total variance in the P1, P2, F1, F2, B1 and B2 populations respectively. $\sigma_{g1}^2$ and $\sigma_{g2}^2$ refer to the genetic component of the variance in the P1 and P2 populations respectively and

$\sigma_e^2$ is the non-heritable, environmental component of the variance. The F2, B1 and B2 variance components include $\sigma_s^2$, the segregation variance arising from genetic differences between the two parental lines.

The observed segregation variance can be esimated from linear combinations of the parental, F1, F2, B1 and B2 variances in the following four ways (Equations 8 - 11) :

$$\sigma_s^2 = \sigma_{F2}^2 - \sigma_{F1}^2 \quad (8)$$

$$\sigma_s^2 = \sigma_{F2}^2 - 0.5\,\sigma_{F1}^2 - 0.25\,\sigma_{P1}^2 - 0.25\,\sigma_{P2}^2 \quad (9)$$

$$\sigma_s^2 = 2\sigma_{F2}^2 - \sigma_{B1}^2 - \sigma_{B2}^2 \quad (10)$$

$$\sigma_s^2 = \sigma_{B1}^2 + \sigma_{B2}^2 - \sigma_{F1}^2 - 0.5\,\sigma_{P1}^2 - 0.5\,\sigma_{P2}^2 \quad (11)$$

These four ways are equivalent if the environmental variance is the same for all populations and generations as is evident from equations 2 to 7.

## 2.9 Extraction of Chromosomes

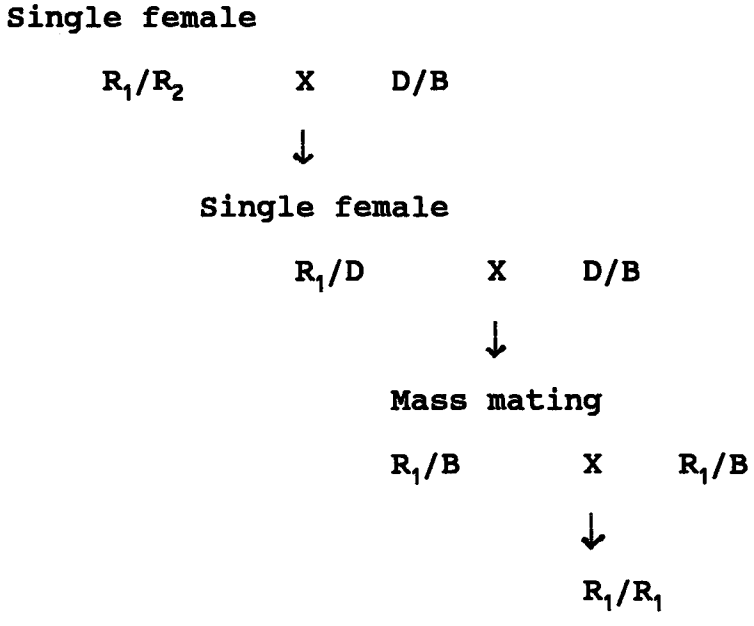
A number of isochromosomal strains that were homozygous for either first, second or third chromosomes were derived from VS-35 (an isofemale line with high 7-ECD specific activity) as well as from RS-VSS/14 (the laboratory-selected line which was maintained on banana medium containing 14uM of Imidan). Each isochromosomal strain sampled a different chromosome from one of these two populations. The extraction procedure made the sampled chromosomes homozygous within a susceptible background.

Individual females (one for each line ) from the populations were crossed to a balanced lethal strain carrying a chromosome and a dominant mutation to suppress recombination (B in Figure 3). Single females from the first cross carrying one of the two dominant mutations (D in Figure 3) were backcrossed to the balancer strain. The progeny from this cross carrying the same dominant mutation, as used in the backcross were then mated to obtain the desired isochromosomal strain (Figure 3).

The balanced chromosomes used were FM7a for the first chromosome, CyO for the second chromosome and TM3 for the third chromosome. For a description of mutations see Lindsley and Grell (1968). The relative viability of the chromosomes from VS-35 and RS-VSS/14 were determined as described by Halpern and Morton (1986). The progeny from the last cross (R/D X R/D) were scored and the observed

genotypic ratio, (R/R)/(R/R + R/D), was divided by the theoretical ratio (0.5 for chromosome I using only males, and 0.33 for chromosome II and III). The result was then taken as the relative viability of the VS-35 and RS-VSS/14 chromosome which had been extracted into the isochromosomal strain.

Figure 3 : Procedure for chromosome extraction.



# RESULTS

## PART ONE

### 3.1 Relationship between a Polypeptide Determinant (P2), 7-Ethoxycoumarin Deethylation Activity and Resistance.

The relationship between MFO activity, measured by 7-ethoxycoumarin deethylation activity (7-ECD) and a polypeptide determinant (P2) tentatively identified as belonging to cytochrome P-450 (Pursey, 1989) was investigated in isofemale and isochromosomal strains derived from natural populations. The correlation between 7-ECD specific activity and antigenic response, that is the amount of P2 as determined by immunostaining was examined.

Two Drosophila melanogaster populations were used in this study. The first consisted of several laboratory stock strains with low 7-ECD specific activity, a number of homozygous third chromosome strains derived from natural populations (a gift from Dr. C. F. Aquadro, Section of Genetics and Development, Cornell University) together with two malathion resistant, isochromosome three strains (IIIC and IIID) with high 7-ECD specific activity. The second group of flies were fourteen isofemale lines with variable but intermediate 7-ECD specific activity that were established from a collection at Dalewood Crescent, Hamilton, Ontario in 1976.

Crude fly extracts were used for 7-ECD activity measurements. The fly homogenates were also electrophoresed, electroblotted onto nitrocellulose and immunostained with the antiserum, Ab1-B (Pursey, 1989). Ab1-B was obtained by treating the serum Ab1 with Canton S (the susceptible strain) microsomal proteins. After this treatment Ab1-B remained reactive only to the proteins found in excess in IIID, the malathion resistant, isochromosome three strain used in the production of the antiserum Ab1.

Figure 4, courtesy of Jane Pursey shows P2, the gel isolated protein which is the main band examined in this study. Canton S and IIID strains microsomal proteins were stained for total protein or immunostained with preimmune serum, Ab1-B and Ab2-IgG, IgG fraction from a second antiserum. Fly extracts from strain IIID responded to both Ab1-B and Ab2-IgG more than those from Canton S, the susceptible strain (Figure 4). A densitometric analysis of the immunoblots was under taken to quantify the expression of P2 and three other proteins designated P1, P3 and P4 that were used as controls (Figure 5). More bands are seen in Figure 5 than Figure 4 because whole-fly extracts contained more proteins reacting with the antiserum than microsomal preparations. Increased amounts of P2 was associated with increased MFO-activity in the first group of flies, particularly

Figure 4 : Western immunoblot of Canton S and strain IIID microsomal proteins stained for total protein with amido black or immunostained with preimmune serum, Ab1-B or Ab2-IgG as indicated. Detection occurred with alkaline phosphatase linked second antibody and NBT/BCIP substrate. By courtesy of J. Pursey (1989).

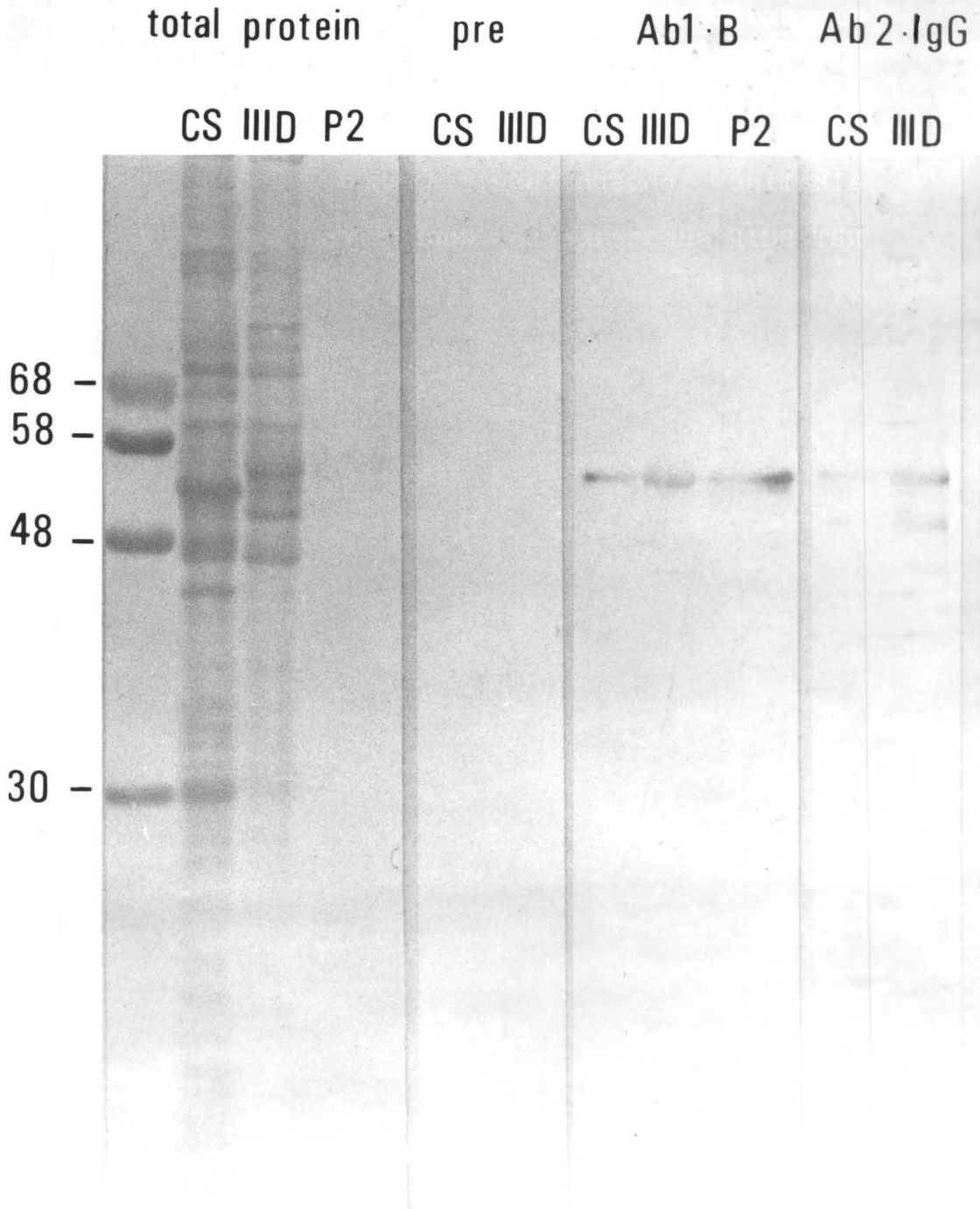
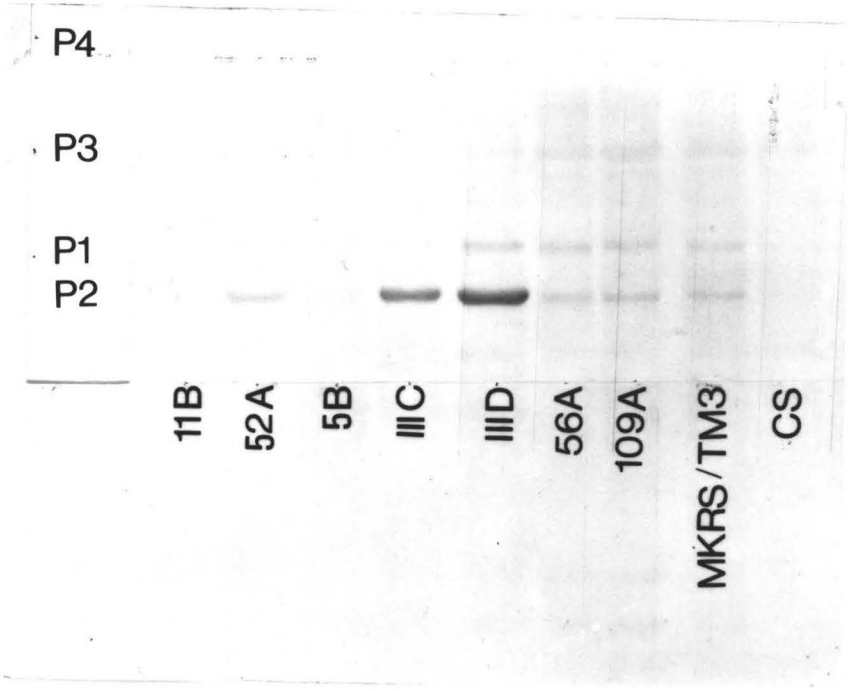

total protein
pre
Ab1·B
Ab2·IgG
CS IIID P2
CS IIID
CS IIID P2
CS IIID
68
58
48
30

Figure 5 : Western immunoblot of whole-fly extract of susceptible low MFO activity strains (CS and MKRS/TMS), selected resistant isochromosome III strains with elevated P-450 content (IIIC and IIID) and isochromosome III strains from North Carolina (11B, 52A, 56A and 109A) immunostained with Ab1-B antiserum. Blots were stained with alkaline phosphatase linked second antibody and NBT/BCIP substrate.


P4
P3
P1
P2
11B
52A
5B
IIIC
IIID
56A
109A
MKRS/TM3
CS

for strains IIIC and IIID which were selected by Halpern and Morton (1987). No such correlation was seen for the control bands P1, P3 or P4 (Figure 6). The correlation coefficient, r, obtained for antigenic response of P2 against 7-ECD specific activity is 0.939, $P < 0.001$. The correlation coefficients for P1, P3 and P4 against MFO specific activity were 0.277, 0.351 and 0.384. They were all shown to be non-significant. The correlation of the polypeptide determinant, P2 in the fly extracts detected by the antiserum, Ab1-B and MFO activity is consistent with its identification as cytochrome P-450 (Pursey, 1989), although it does not prove this identification. This is because the antiserum could detect some other proteins (eg glutathione transferase) which was also correlated with resistance.

The relationship between P2 and 7-ECD specific activity was also studied using isofemale lines from natural populations (collected from Dalewood Crescent). This set of strains have both 7-ECD specific activity and P2 antigenic response intermediate to the low activity laboratory stock strains and the selected lines used in the first study. Western immunoblot for these Dalewood lines are shown in Figure 7, two with intermediate activity and the other two with high activity within the group were used (see also Table 2). The P2 antigenic response for Dal-3 and Dal-10, although greater than the more susceptible strains were only one half that of the resistant, isochromosome three strains IIIC and IIID (Table 2). In spite of this, their 7-ECD activity were

Figure 6 : The relationship between antigenic response of polypeptide determinants from whole-fly extracts run on SDS ployacrylamide gels and 7-ethoxycoumarin specific activity for nine different Drosophila melanogasterstrains. Control P1 and P2 (Figure 5) polypeptides were compared. Canton S and strain IIID responses are indicated, the other points represent the remaining strains named in the legend of Figure 5.

Fig 6

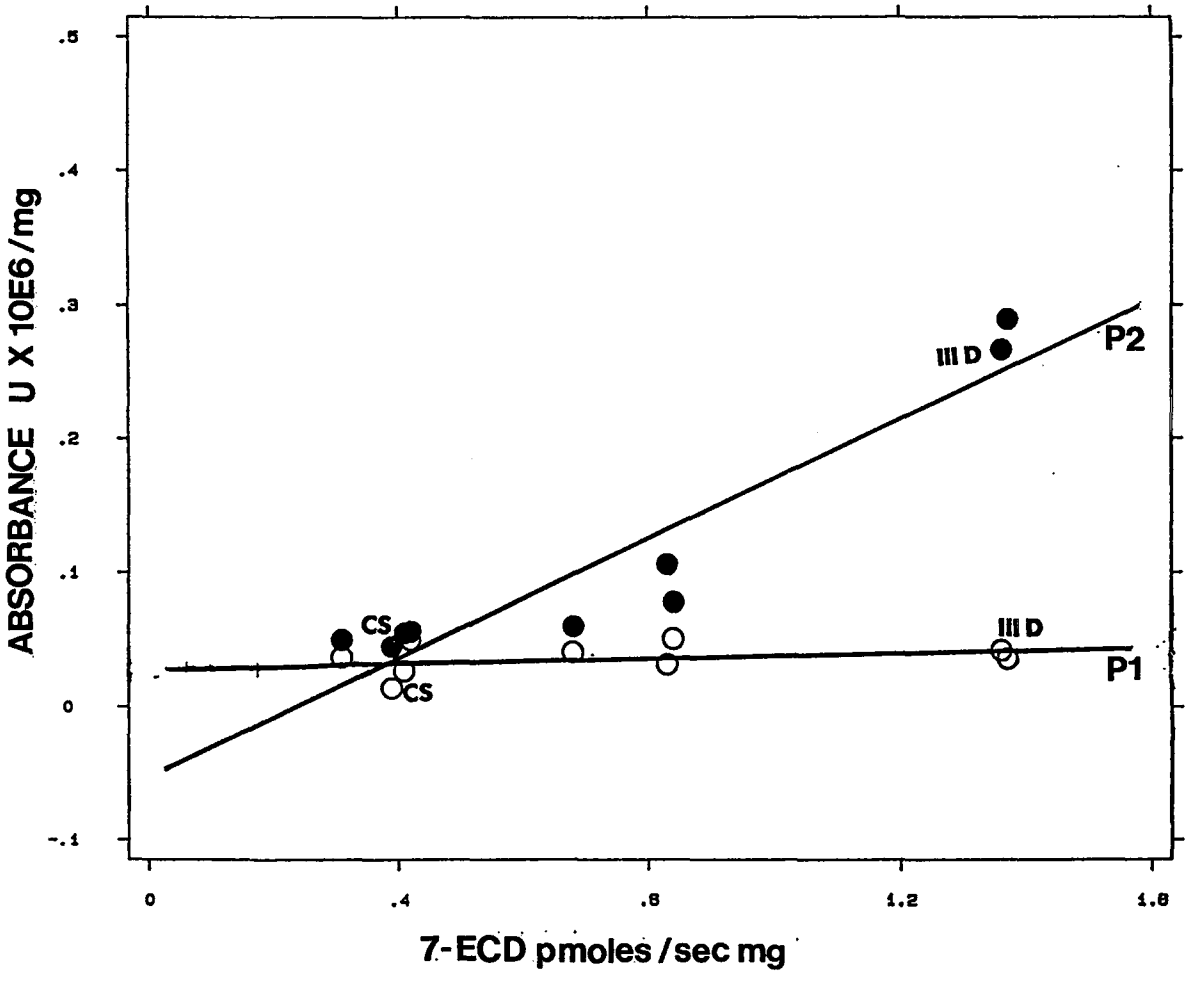

ABSORBANCE U X 10E6/mg
.5
.4
.3
.2
.1
0
-.1
0
.4
.8
1.2
1.6
7-ECD pmoles/sec mg
III D
P2
CS
CS
III D
P1

Figure 7 : Western immunoblot of whole-fly extracts run on SDS polyacrylamide gel of selected isochromosome III strains (IIID and IIIC) with high P-450 content, susceptible strain (CS) with low MFO activity and isofemale lines established from flies collected in Hamilton, Ontario (Dal-3, Dal-6, Dal-10 and Dal-13). Microsomal preparations from strains IIID was included in lane 1. Blots were stained with alkaline phosphatase linked second antibody and NBT/BCIP substrate.

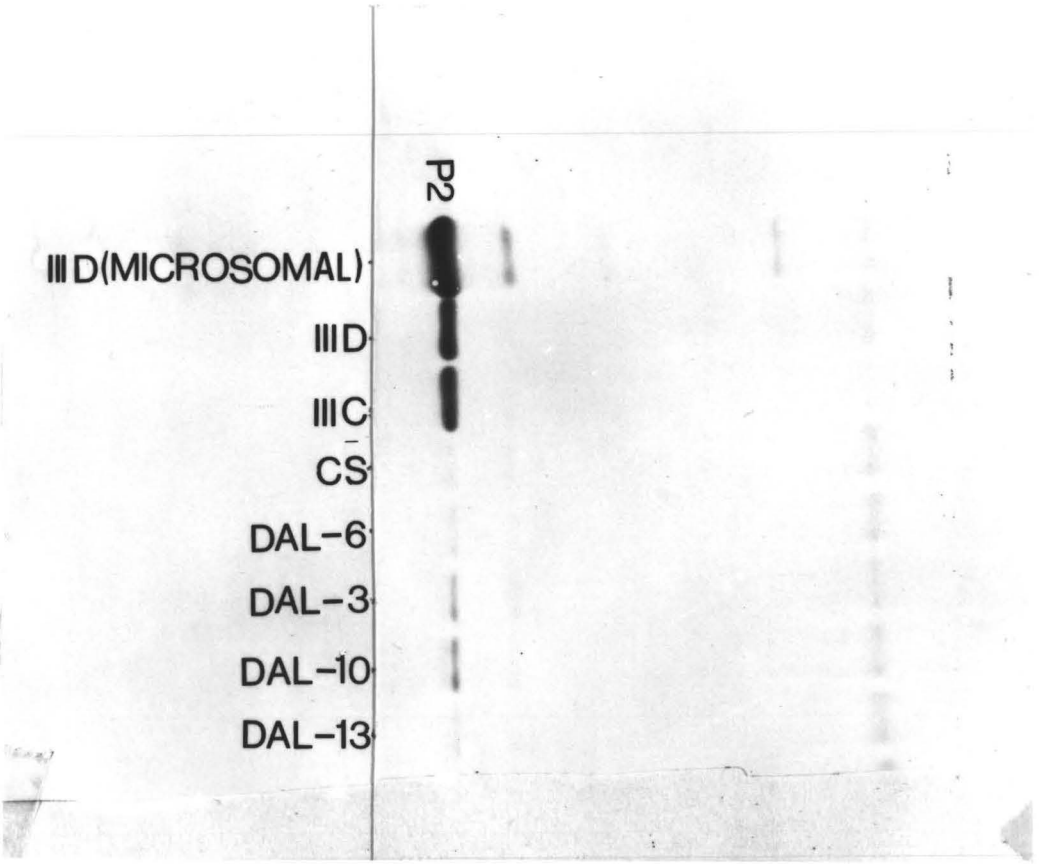

P2
III D(MICROSOMAL)
III D
III C
CS
DAL-6
DAL-3
DAL-10
DAL-13

**TABLE2:** P2 antigenic response and 7-ECD specific activity of laboratory stock, isofemale lines from nature and laboratory-selected isochromosomal strains. Numbers in brackets are standard errors.

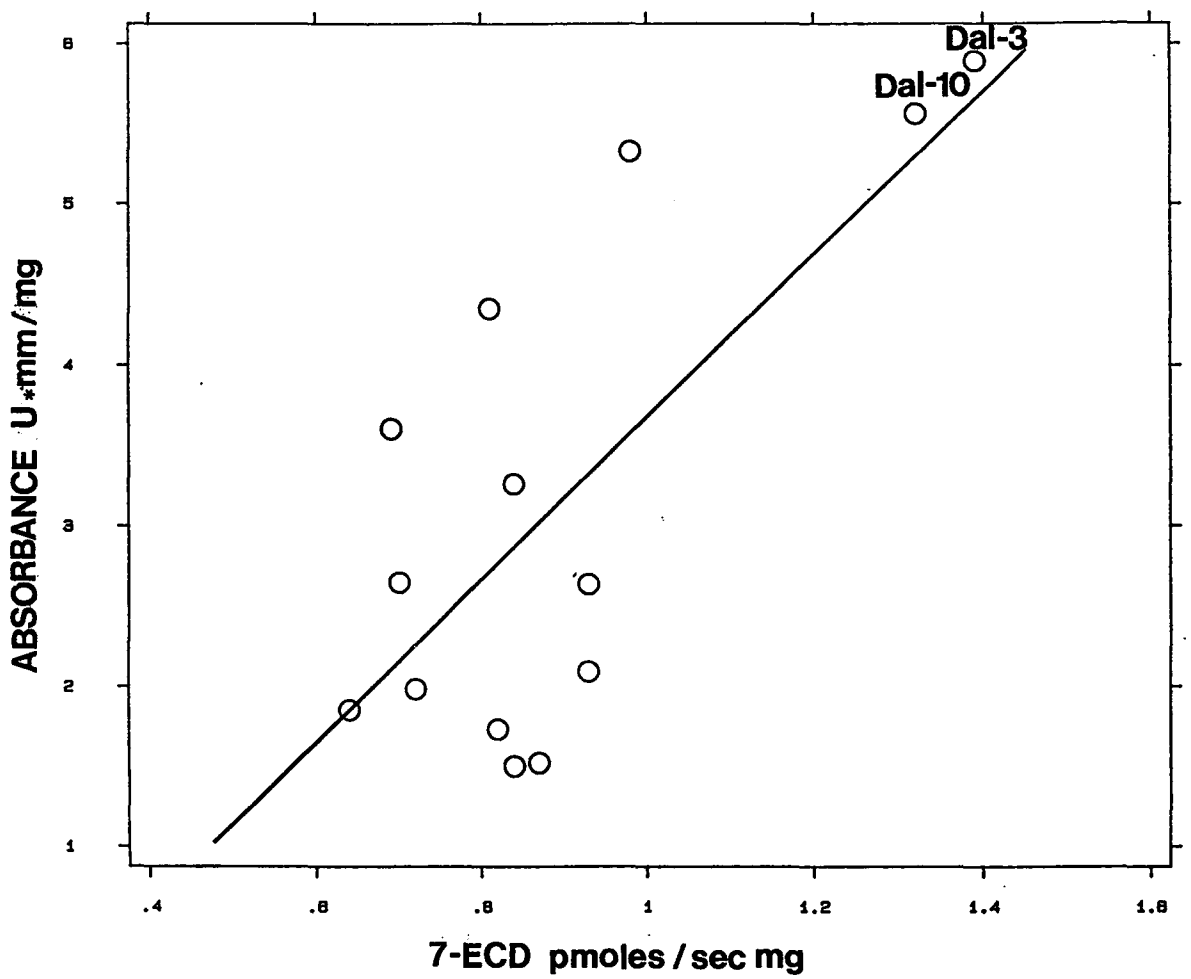
| LINE | Antigenic Response AU.mm/mg | 7-ECD S.A. pmoles/sec mg |
|---|---|---|
| CS | 2.80 (0.51) | 0.80(0.03) |
| DAL-13 | 4.53(0.96) | 0.89(0.04) |
| DAL-10 | 6.83(1.22) | 1.35(0.12) |
| DAL-3 | 6.21(0.98) | 1.50(0.08) |
| DAL-6 | 4.12(0.47) | 0.90(0.05) |
| IIIC | 15.81(2.67) | 1.74(0.13) |
| IIID | 16.02(1.09) | 1.86(0.14) |
| IIID(microsomes) | 28.42(2.03) | ND |

ND = Not Determined.

nearly as great. This suggests that other proteins besides P2 could be involved with increased MFO activity. The antigenic response of P2 from a microsomal preparation of strain IIID (kindly provided by J. Pursey) is also included in Figure 7 to compare with the crude fly extracts from the same strain (see also Table 2). The P2 response in the microsomal preparation is almost twice that of the crude fly extract, consistent with the presence of this polypeptide in the microsomal fraction. The association between P2 antigenic response and 7-ECD specific activity among the entire group of 14 Dalewood isofemale lines is shown in Figure 8. There is a significant positive correlation between P2 expression and MFO activity, $r = 0.7107$, $P < 0.01$, however no significant correlation was obtained when Dal-10 and Dal-3 are removed. These two strains appear to constitute a high activity group distinct from the other strains.

The MFO activity also correlated positively with Imidan resistance among the Dalewood isofemale lines ($r = 0.7426$, $P < 0.01$). Similarly, the antigenic response of P2 correlated with strain LC-50 (resistance) ($r = 0.8559$, $P < 0.001$). These results suggest an involvement of the 50K polypeptide determinant (P2) in insecticide resistance and MFO specific activity. However as stated earlier P2 may be a protein not directly involved in MFO activity, although this is unlikely because of the presence of heme activity in this band (Pursey, 1989).

**Figure 8 : The relationship between the antigenic response of polypeptide determinant (P2) from whole-fly extract proteins and 7-ethoxycoumarin specific activity for fourteen isofemale lines established from flies collected in Hamilton, Ontario. Only Dal-3 and Dal-10 strains are labelled to show how they appear to belong to a distinct high activity group.**


Dal-3
Dal-10
ABSORBANCE U*mm/mg
7-ECD pmoles / sec mg
6
5
4
3
2
1
.4
.6
.8
1
1.2
1.4
1.6

## 3.2 Sex differences in the levels of the Polypeptide Determinant (P2).

An association between the polypeptide determinant, P2 and 7-ECD activity was demonstrated by results presented. Patil et al (1990) found that the 7-ECD specific activities of male flies were 90 - 93% that of females. The levels of this insecticide resistance-related polypeptide determinant was examined in males and females of several Drosophila melanogaster lines by immunostaining with Ab1-B. This was done to determine if levels of P2 correlates with MFO activity in the two groups. It was found that the levels of the P2 antigen was lower in females than in males, in all cases. The antigenic response of P2 per mg of protein to the antiserum Ab1-B ranged from about 40% lower in females of the selected strains to 64% in females from the isofemale strains having low 7-ECD specific activity (Figures 9 and 10). Two resistant isochromosome three strains, a selected population and two isofemale strains from natural populations, one with low 7-ECD activity and the other with high activity were used in this study. Since a correlation of increased MFO activity and increased amounts of antigen P2 was observed among strains, it was surprising that smaller amounts of P2 were found in females than in males. These differences were also examined at single fly level. In single flies the antigenic response of P2 in females was only 21% that of the males compared to 64% when flies were pooled together (Figure 11 and Tables 3a and b).

Figure 9 : Western immunoblot of male (M) and female (F) whole-fly extracts run on SDS polyacrylamide gel of selected isochromosome III strains (IIIC and IIID) with high P-450 content, a laboratory-selected population (RS-VSS/14) and isofemale lines collected from Vineland, Ontario. Blots were stained with alkaline phosphatase linked second antibody and NBT/BCIP substrate.

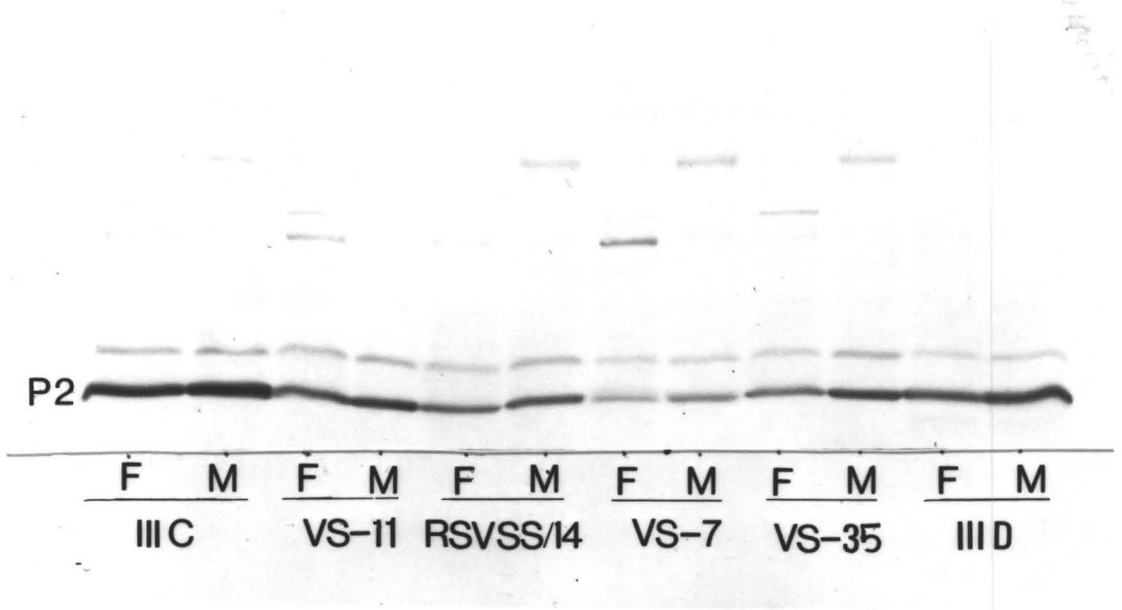

P2
F M F M F M F M F M F M
III C
VS-11
RSVSS/14
VS-7
VS-35
III D

Figure 10 : Male and female differences in polypeptide determinant P2 response from whole-fly extracts. Strains represented are selected isochromosome III (IIID and IIIC) with high P-450 content, a laboratory-selected population (RS-VSS/14) and isofemale lines collected from Vineland, Ontario.

Fig 10

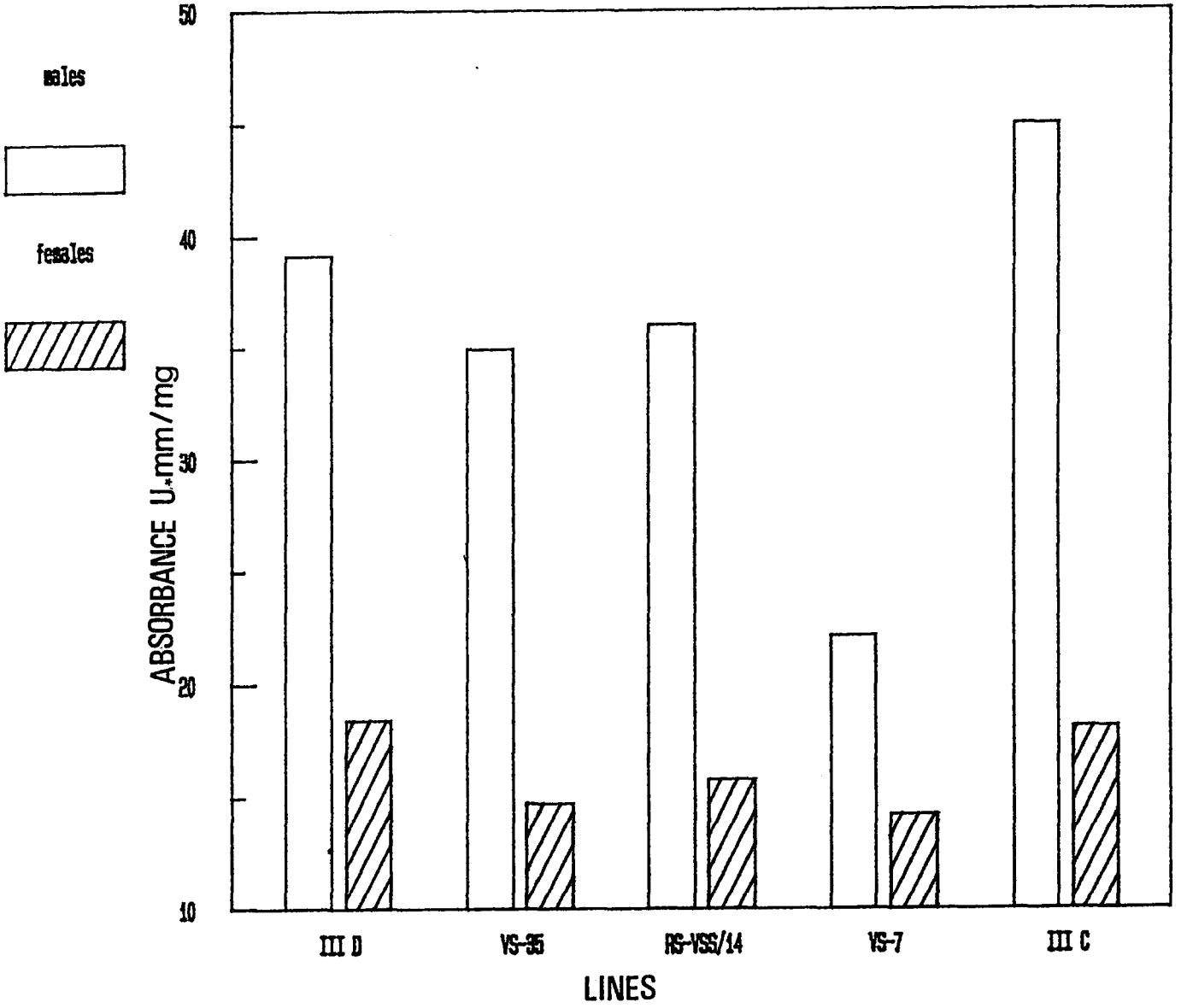
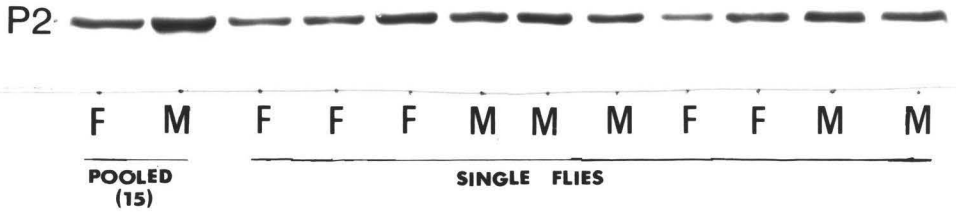

males
females
50
40
30
20
10
ABSORBANCE U.mm/mg
III D
VS-35
RS-VSS/14
VS-7
III C
LINES

Figure 11 : Western immunoblot of single and pooled male (M) and female (F) whole-fly extracts run on SDS polyacrylamide gel of a selected isochromosome III strain (IIID) with high P-450 content. Blots were stained with alkaline phosphatase linked second antibody and NBT/BCIP substrate.


P2
F M F F F M M M F F M M
POOLED (15)
SINGLE FLIES

TABLES 3a and b : Sex differences in P2 polypeptide determinant for strain IIID.

(a) Single Flies.

| Sex | N | Mean (AU*mm/mg) | Variance | Standard error. |
|---|---|---|---|---|
| Male | 5 | 148.3 (100%) | 506 | 10 |
| Female | 5 | 31.3 (21%) | 101 | 4.5 |

(b) Pooled Flies.

| Sex | N | Mean (AU*mm/mg) | Variance | Standard error. |
|---|---|---|---|---|
| Male (15) | 1 | 39.0 (100%) | NA | NA |
| Female (15) | 1 | 19.4 (64%) | NA | NA |

NA = Not Applicable.

The selected isochromose three strain, IIID was used for this study. Fifteen flies pooled together for each of the two sexes were included to compare with the single flies. The differences in pooled and single flies could be due to the fact that for the single fly experiment each fly was extracted in 200ulof TKEG buffer while for the pooled flies experiment 15 flies were extracted in 75ul of buffer. Sex differences in P2 implies other factors between sexes compensate to give the realized MFO activity levels. There was no significant differences in variability of P2 expression between the two sexes ($F = 5.0$, $P < 0.05$) (Table 3a).

## 3.3 Microplate Assay for the Identification of Resistant Genotypes.

The possibility of developing a less expensive (compared to immunoblotting) microplate assay was examined to determine if antiserum Ab1-B could be used to identify resistant genotypes in field populations of Drosophila melanogaster. Early detection of resistant genotypes is essential to any control programme. Since other proteins reacting with the antiserum (eg P1, P3 and P4 in Figure 5) could interfere with the microplate assay it was necessary to determine if significant differences between resistant and susceptible genotypes could be detected. As a preliminary test to see if such a difference remains even if all polypeptide bands reacting with Ab1-B were included, the bands P1, P2, P3 and P4 (Figure 5) were summed and plotted against

7-ECD activity for nine strains (Figure 12). A significant correlation was found for this sum ($r = 0.957$), $P < 0.001$) in spite of the fact that, individually, only P2 gave a significant positive correlation. This was partly due to the fact that the correlations of P1, P3 and P4 with 7-ECD activity were all positive although not significant (see section 3.1).

The positive association of MFO activity with summed band intensity following western blotting suggested that the total proteins in crude fly extracts could be used as a measure of MFO activity. This was explored using single male flies from the susceptible Canton S and resistant IIID strains. Individual flies were extracted in TKEG + 0.05% CHAPS buffer and the total fly protein plus coating buffer absorbed to the wells of the microplate which were then reacted with primary and secondary antibodies. Tests of reaction time and dilution were made in order to determine the conditions for best resolution between resistant and non-resistant variants. The optimum resolution was observed after 30 minutes of incubation and when the flies were extracted in 200ul of the TKEG and CHAPS buffer (Figure 13).

The following factors were also investigated to increase the difference between the two variants. 1) Blocking time of well, 2) Substrate concentration, 3) Dilution of primary antiserum and secondary antibody and 4) Reaction time. The

**Figure 12 : The relationship between sum of all detectable polypeptide determinants (P1, P2, P3 and P4) (Figure 5) and 7-ethoxycoumarin specific activity of nine different strains of Drosophila melanogaster. Canton S and strain IIID responses are indicated, the other points represent the other strains named in the legend of Figure 5.**

Fig 12

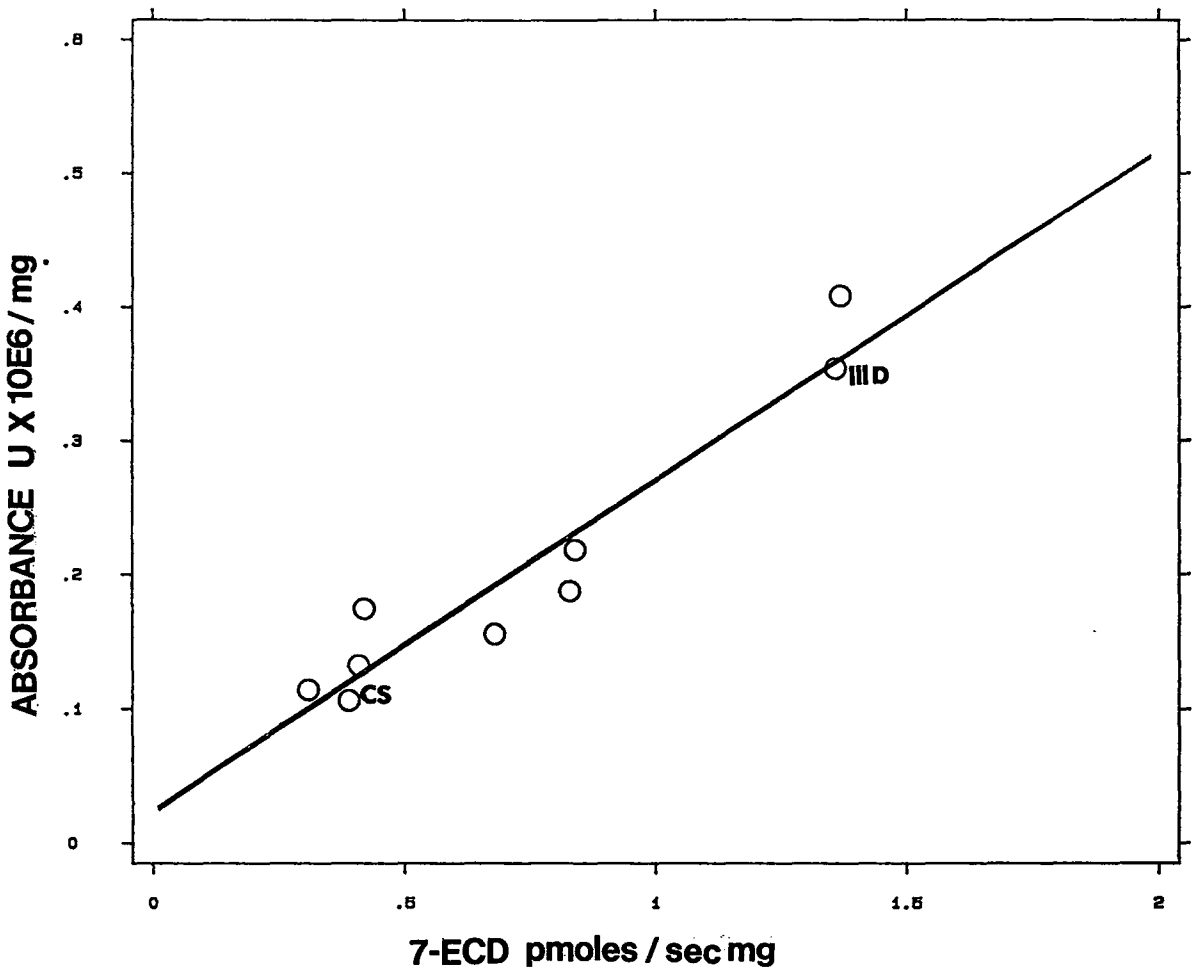
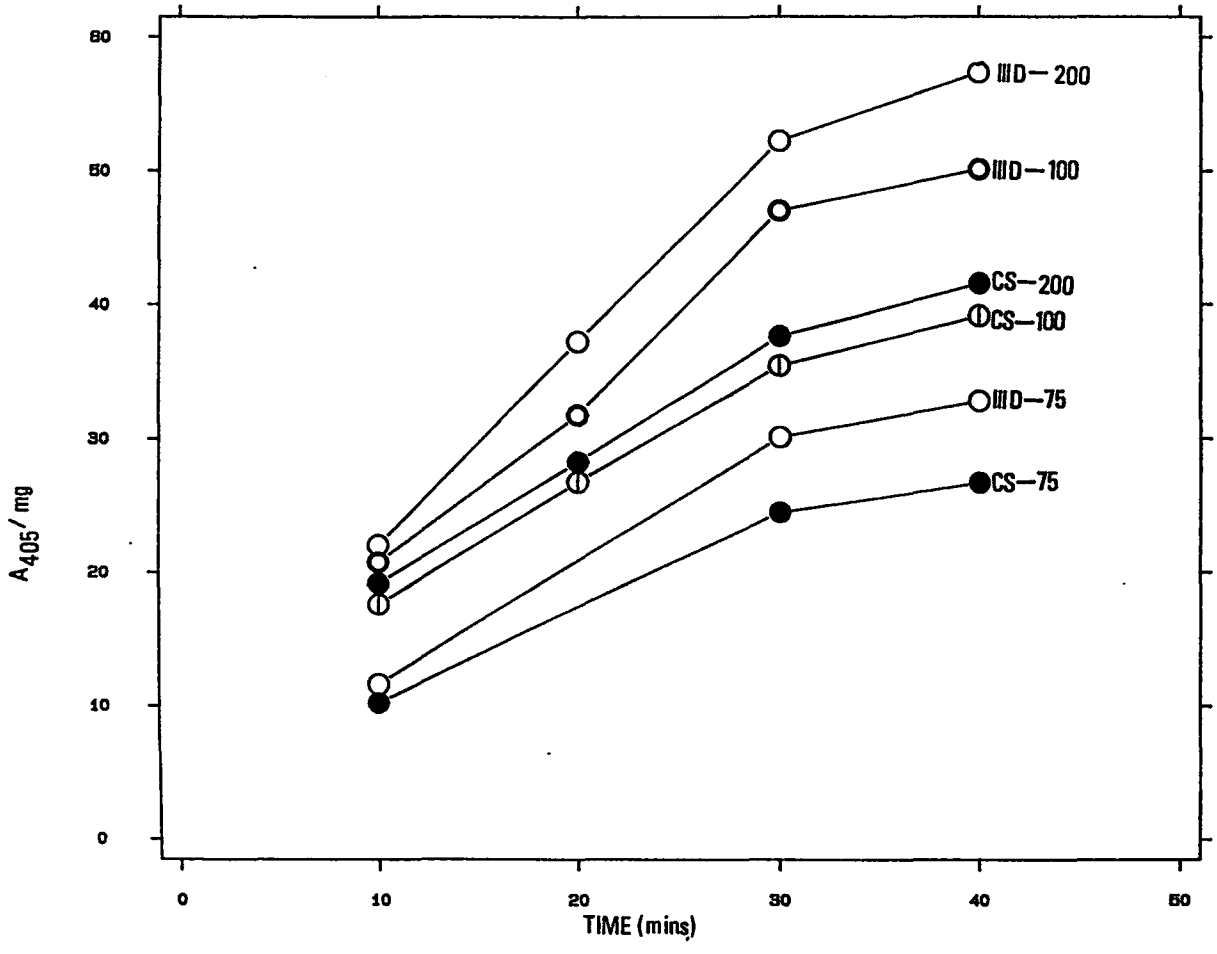

ABSORBANCE U X 10E6 / mg
.8
.5
.4
.3
.2
.1
0
0
.5
1
1.5
2
7-ECD pmoles / sec mg
IIID
CS

Figure 13 : Antigenic response of whole-fly proteins of susceptible (CS) and resistant IIID strains. Response with time and three different amounts of extraction buffer (75, 100 and 200 ul) are presented. Response was determined in a microplate assay using p-nitrophenyl phosphate disodium substrate in diethylamine buffer as substrate for secondary antibody. Each well contains proteins from one fly.

Fig 13


60
50
40
30
20
10
0
0
10
20
30
40
50
TIME (mins)
$A_{405}$/mg
IIID—200
IIID—100
CS—200
CS—100
IIID—75
CS—75

results are summarized in Table 4. Blocking wells for 1 or 2 hours (columns B and C) instead of 30 minutes (column, A the basic method) produced no change. For substrate concentrations, 1mg/2ml (column D) and 1mg/4ml (column E) produced lower absorbance in both strains compared to 1mg/ml (column A) but the resolution between the strains was not significantly different. Changing the dilution of the primary antiserum to 1/100 instead of 1/1000 and the secondary antibody to 1/500 instead of 1/5000 (column F) produced about 2.5-fold increase in absorbance for both strains but a lower resolution compared to the basic method. Decreasing the dilution of the primary antiserum to 1/100 and maintaining the concentration of the secondary antibody at 1/5000 (column G) also gave higher absorbance values in both strains, however maintaining the concentration of the primary antiserum at
1/1000 and decreasing the dilution of the secondary antibody to 1/500 (column H) did not have any effect on either the absorbance and the resolution between the two strains. Reacting both the antiserum and the secondary antibody at 37°C (column I) instead of room temperature (column A) produced no change.

At best the microplate assay gave only 50% greater response per mg of fly extract for the resistant strain (IIID) relative to the susceptible strain (Canton S). The small resolution between the two strains compares poorly to the 5.82-fold increase obtained for the polypeptide determinant P2 from the immunoblots

Table 4 : Microplate assay for whole-fly antigenic response (Absorbance at 405 nm per mg of protein).

| | A | B | C | D | E | F | G | H | I |
|---|---|---|---|---|---|---|---|---|---|
| CS | 37.7 | 41.4 | 42.7 | 28.8 | 23.4 | 126.5 | 109.5 | 41.9 | 45.1 |
| IIID | 52.3 | 58.3 | 53.2 | 41.6 | 35.0 | 147.6 | 136.5 | 53.4 | 59.4 |
| Ratio | 1.39 | 1.41 | 1.25 | 1.44 | 1.50 | 1.17 | 1.25 | 1.27 | 1.32 |

A = Basic method presented in the methods sections.

B = Wells were blocked for 1 hour (vs 30 minutes).

C = Wells were blocked for 2 hours (vs 30 minutes).

D = Concentration of substrate in buffer is 1mg/2ml (vs 1mg/ml).

E = Concentration of substrate in buffer is 1mg/4ml (vs 1mg/1ml).

F = (1) Dilution of antiserum used at room temperature is 1/100.

(2) Dilution of secondary antibody used at room temperature is 1/500.

G = Dilution f antiserum used at room temperature is 1/100.

H = Dilution of secondary antibody used at room temperature is 1/500.

I = Both antibodies were reacted at $37^{0}$C.

(Figure 6) and the 3.34-fold increase obtained for the sum of all detectable polypeptides (Figure 12). The poor resolution observed in the microplate assay could mean that only very little amounts of the antigens were absorbed in the wells or that many more polypeptides other than the four detectable ones following SDS polyacrylamide electrophoresis and immunoblotting were responding to the antiserum when used with immunoplates.

**PART TWO**

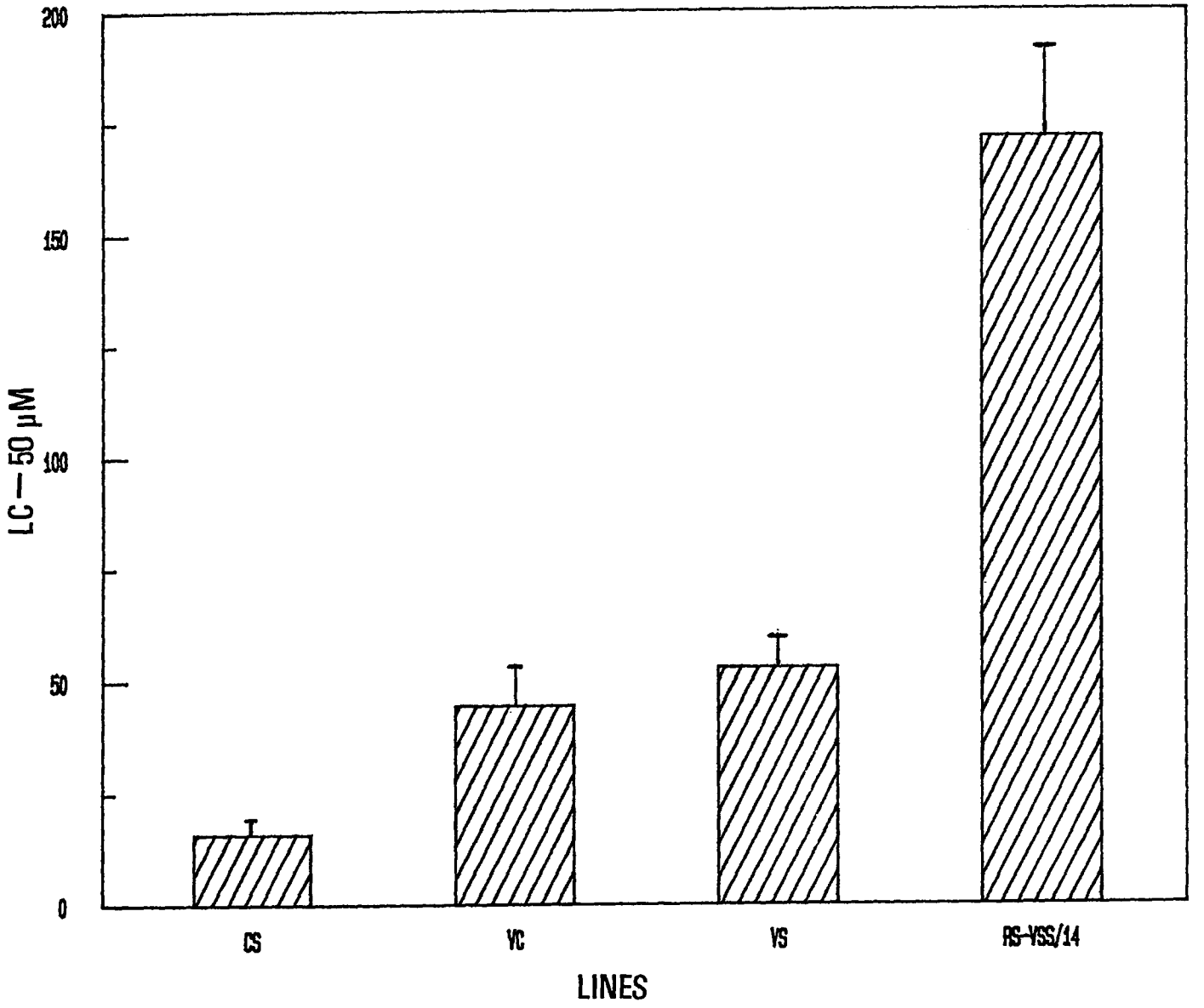
### 3.4.1 Comparison of Resistance in Natural and Laboratory Populations.

Experiments in this section were performed to study the differences between the resistance of two natural populations and to compare them to a susceptible laboratory strain (Canton S) and a line selected from infested peach fruits. The flies for this study were collected from Vineland, Ontario. Two collections were made, one from a plot on a peach farm which is regularly sprayed (VS) with Imidan, an organophosphorus insecticide, and the second from an unsprayed plot (VC) about 100 metres away from the first plot.

The LC-50 of Imidan for the VS sample was not very different from the VC sample, however both populations were significantly more resistant to Imidan than Canton S (Figure 14). The possibility that flies collected from the peach orchard were segregating resistance genes was tested by selecting a population by exposure to Imidan (see section 2.6). A line, RS-VSS/14 was obtained from peach fruits infested with Drosophila larvae by spraying with Imidan followed by selecting with increasing amounts of Imidan in banana medium. This produced a 3-fold increase relative to the population it was collected from, the regularly sprayed plot (VS) (Figure 14) suggesting that all

Figure 14 : Comparison of resistance to Imidan of a Laboratory stock (CS), unsprayed (VC), regularly sprayed (VS) and laboratory-selected (RS-VSS/14) populations. VC and VS are flies collected from peaches in the orchard at Vineland, Ontario while CS and RS-VSS/14 are flies raised in the laboratory on banana medium. Resistance was measured by LC-50, the concentration of Imidan that will kill approximately 50% of the population.

Fig 14



resistance genes were not homogenously fixed in the natural population.

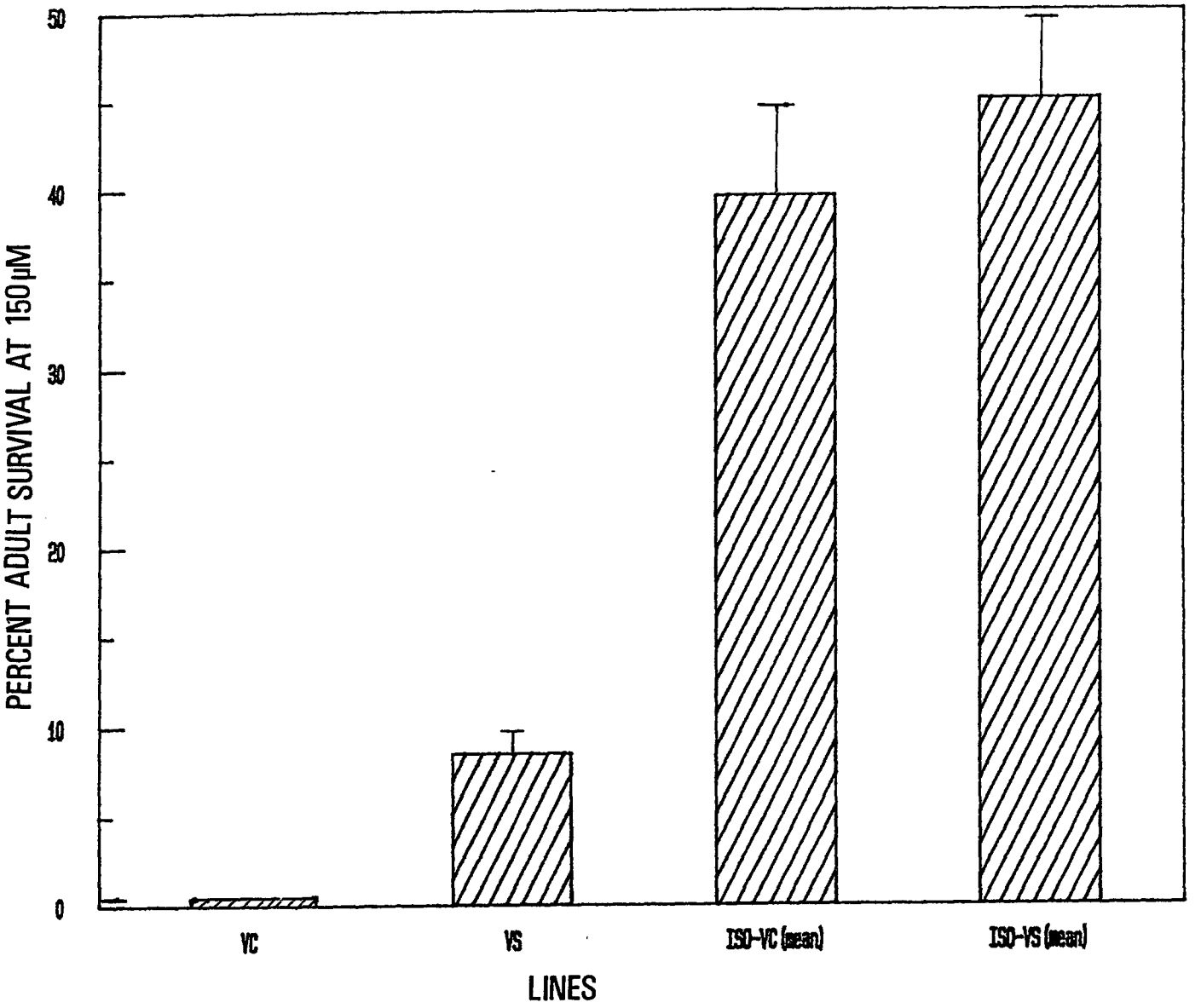
### 3.4.2 Comparison of mean Resistance level of Isofemale lines to that of the sample from which they were established and a selected population.

Resistance conferring genes from the collected flies were sampled by establishing 15 isofemale lines, from the unsprayed plot (VC-1 to VC-15) and 24 from the sprayed plot (VS-1 to VS-24). If resistance factors were segregating in the original population and fixed in these isofemale lines, their variability will give some idea about the original genetic variability for resistance.

The resistance levels were measured by feeding adult flies 5 to 8 days old agar and sucrose medium containing 150uM of Imidan over 24 hours and determining percent survival. None of the flies collected from the unsprayed (VC) plot survived at this dose, while 8.5% of the flies collected from the plot regularly sprayed (VS) survived. The isofemale lines on the average were more resistant than the population from which they were established (Figure 15). On the other hand, isofemale lines from sprayed and unsprayed plots were not significantly different (Figure 15). The isofemale lines were therefore not a representative sample of the original populations. This may be

Figure 15 : Comparison of resistance to Imidan at 150uM of flies collected from unsprayed (VC) and regularly sprayed plots at Vineland, Ontario and the mean of isofemale lines established from the two samples, ISO-VC and ISO-VS respectively.

Fig 15


PERCENT ADULT SURVIVAL AT 150µM
0
10
20
30
40
50
VC
VS
ISO-VC (mean)
ISO-VS (mean)
LINES

the result of a better physiological state achieved by growth on laboratory food and consequently less severe environmental conditions or the fact that resistant flies were more likely to establish isofemale lines.

Selection in the laboratory produced a 2.1-fold increase over the mean of the isofemale lines from the natural population. On the other hand only a 1.5-fold increase in 7-ECD activity was produced (Table 5).

### 3.4.3 Distribution of 7-ECD Specific Activity and Resistance levels of a Natural Population.

The variability of 7-ECD specific activity and its relationship to resistance in a natural population was investigated by the association between 7-ECD specific activity and Imidan resistance. The isofemale lines established from the unsprayed plot and the plot regularly sprayed with Imidan were used in this study.

No major differences were found in the variability or the mean 7-ECD specific activity between isofemale lines from sprayed and unsprayed plots. Mean and variance for the unsprayed population are 1.15 pmoles/sec mg and 0.0586 respectively and 1.17 pmloes/sec mg and 0.074 for the plot sprayed regularly with Imidan. The distribution of activity among isofemale lines was also similar (compare Figures 16 and 17).

**TABLE 5 :** Comparison of 7-ECD specific activity and resistance (percent adult survival at 150μM Imidan) of natural isofemale lines and a laboratory selected line (RS-VSS/14).

| | Mean Resistance of Isofemale lines. % | Resistance of selected line. % | Mean 7-ECD specific activity of isofemale lines. pmoles/sec mg | 7-ECD specific activity of selected line. pmoles/sec mg |
|---|---|---|---|---|
| | 42.3 | 88.0 | 1.16 | 1.67 |
| Ratio | 2.1 | | 1.45 | |

**Figure 16 : Frequency distribution of 7-ethoxycoumarin specific activity among isofemale lines established from the unsprayed plot (VC) collection of *Drosophila melanogaster*.**

Fig 16

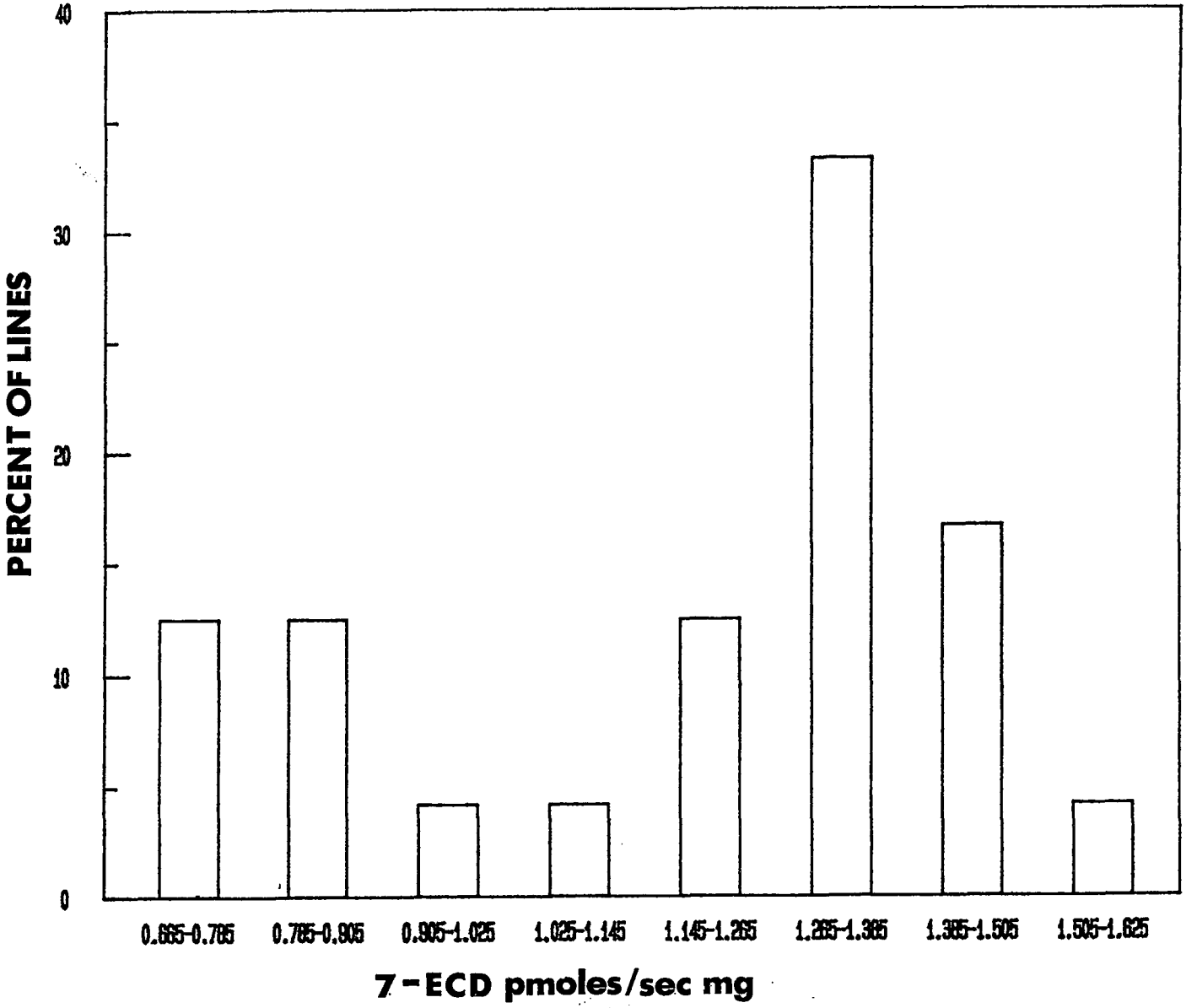
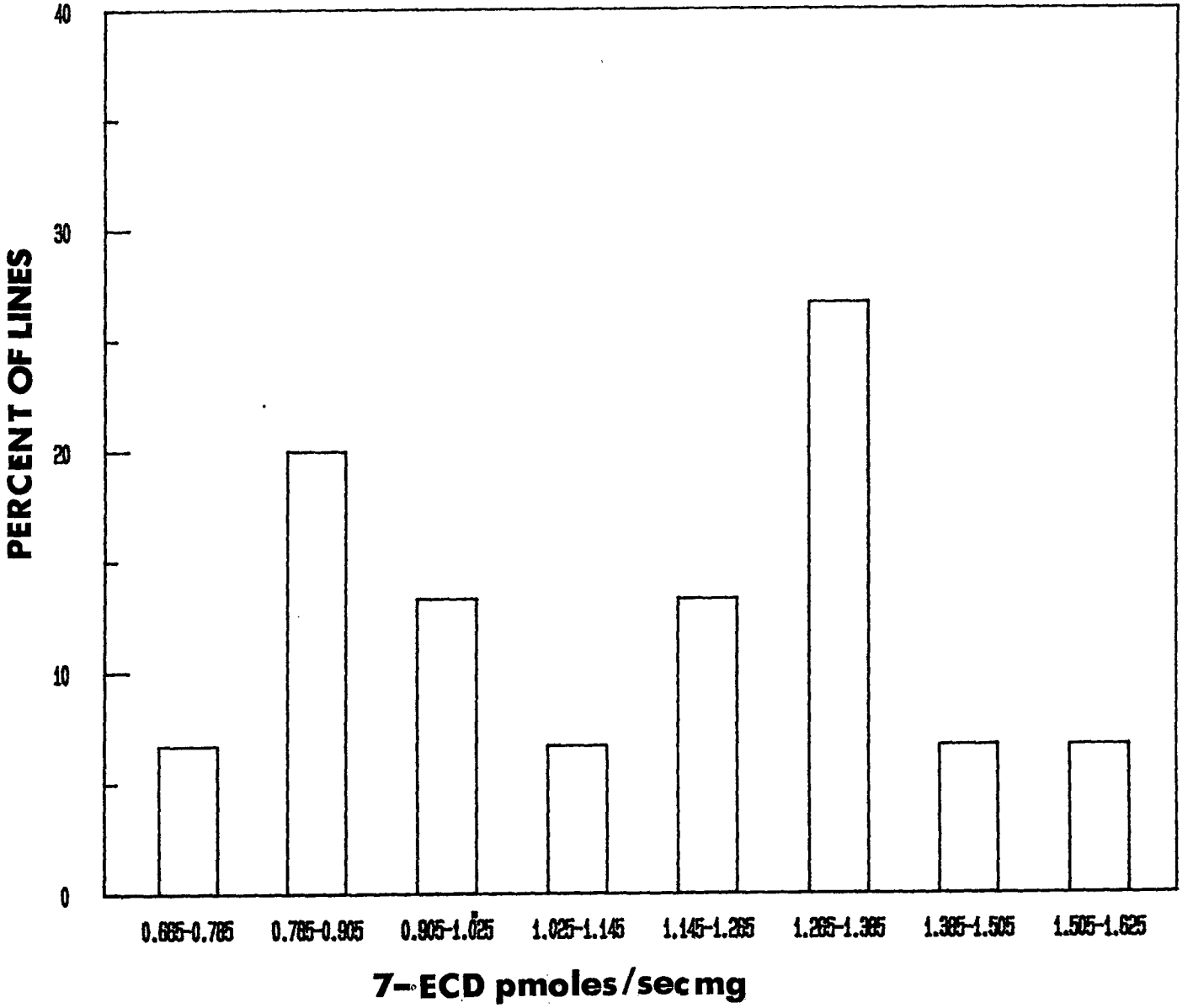

PERCENT OF LINES
40
30
20
10
0
0.665-0.785
0.785-0.905
0.905-1.025
1.025-1.145
1.145-1.265
1.265-1.385
1.385-1.505
1.505-1.625
7-ECD pmoles/sec mg

Figure 17 : Frequency distribution of 7-ethoxycoumarin specific activity among isofemale lines established from a regularly sprayed plot (VS) collection of Drosophila melanogaster.


PERCENT OF LINES
40
30
20
10
0
0.665-0.785
0.785-0.905
0.905-1.025
1.025-1.145
1.145-1.265
1.265-1.385
1.385-1.505
1.505-1.625
7-ECD pmoles/sec mg

There were no major differences between the distribution of resistance levels of the sprayed and the unsprayed populations (Figures 18 and 19). The variances for the sprayed and unsprayed populations are 280 and 339 respectively.

The sprayed and the unsprayed populations have been pooled together for subsequent analysis because there was no significant differences in the 7-ECD specific activity and resistance levels. This pooled group of isofemale lines showed a bimodal distribution of 7-ECD specific activity. However there was no corresponding bimodal distribution of resistance (Figures 20 and 21). Therefore resistance differences are not perfectly correlated with activity differences. Despite the differences in the distribution of 7-ECD specific activity and resistance levels of the pooled group of isofemale lines, there was a significant positive correlation between them ($r = 0.7569$, $P < 0.01$) (Figure 22). This result confirms the importance of 7-ECD activity in insecticide resistance of natural populations of Drosophila melanogaster. The bimodal distribution of 7-ECD activity suggests two alleles of the same gene may be affecting 7-ECD specific activity. Resistance levels may be affected by other genes besides this one.

Figure 18 : Frequency distribution of resistance to Imidan. Isofemale Drosophila melanogaster lines established from flies collected at the unsprayed plot at Vineland, Ontario exposed to 150uM of Imidan for 24 hours.

Fig 18

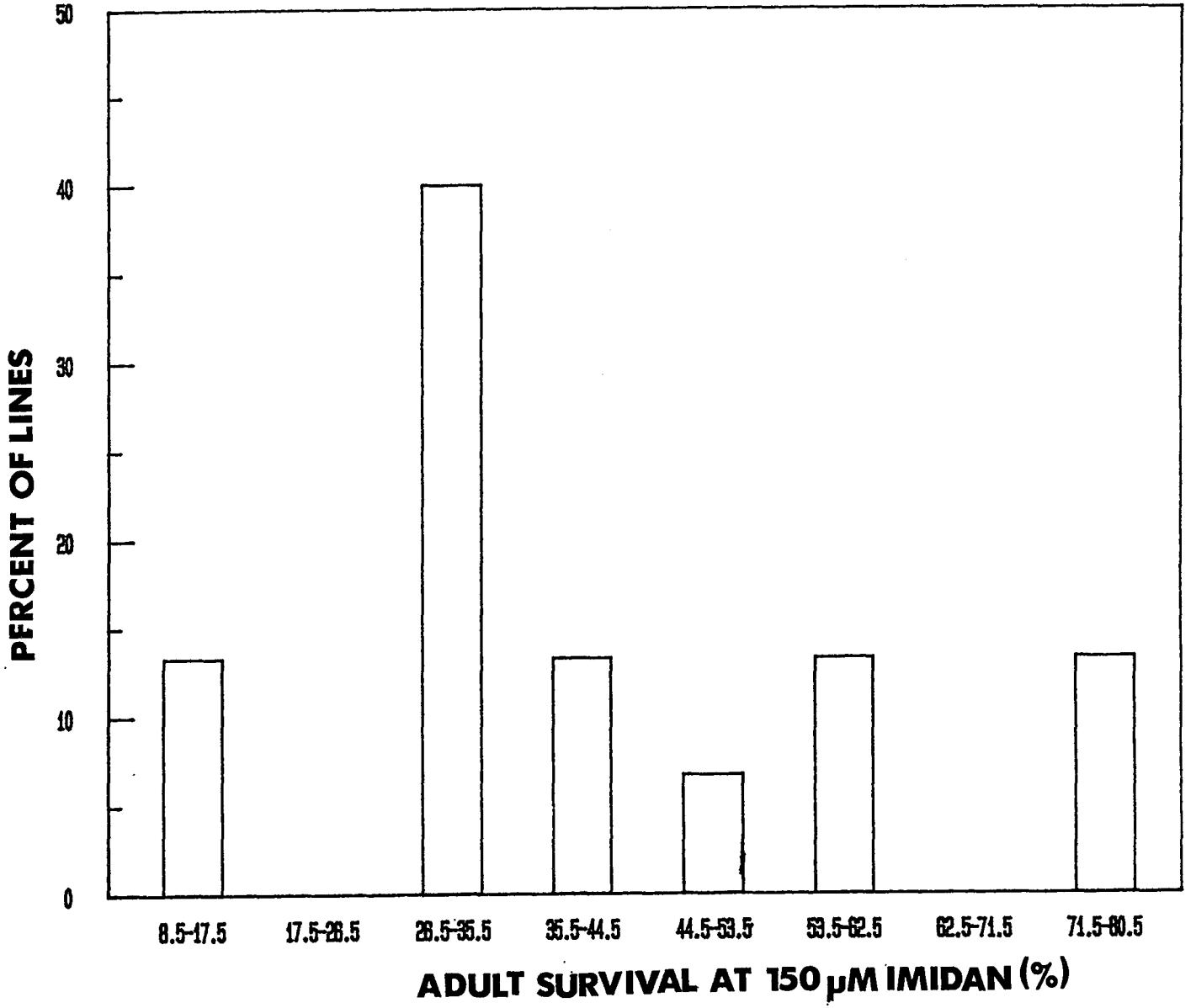

PERCENT OF LINES
50
40
30
20
10
0
8.5-17.5
17.5-26.5
26.5-35.5
35.5-44.5
44.5-53.5
53.5-62.5
62.5-71.5
71.5-80.5
ADULT SURVIVAL AT 150 µM IMIDAN (%)

Figure 19 : Frequency distribution of resistance to Imidan. Isofemale Drosophila melanogaster lines established from flies collected at the regularly sprayed plot at Vineland, Ontario exposed to 150uM of Imidan for 24 hours.

Fig 14

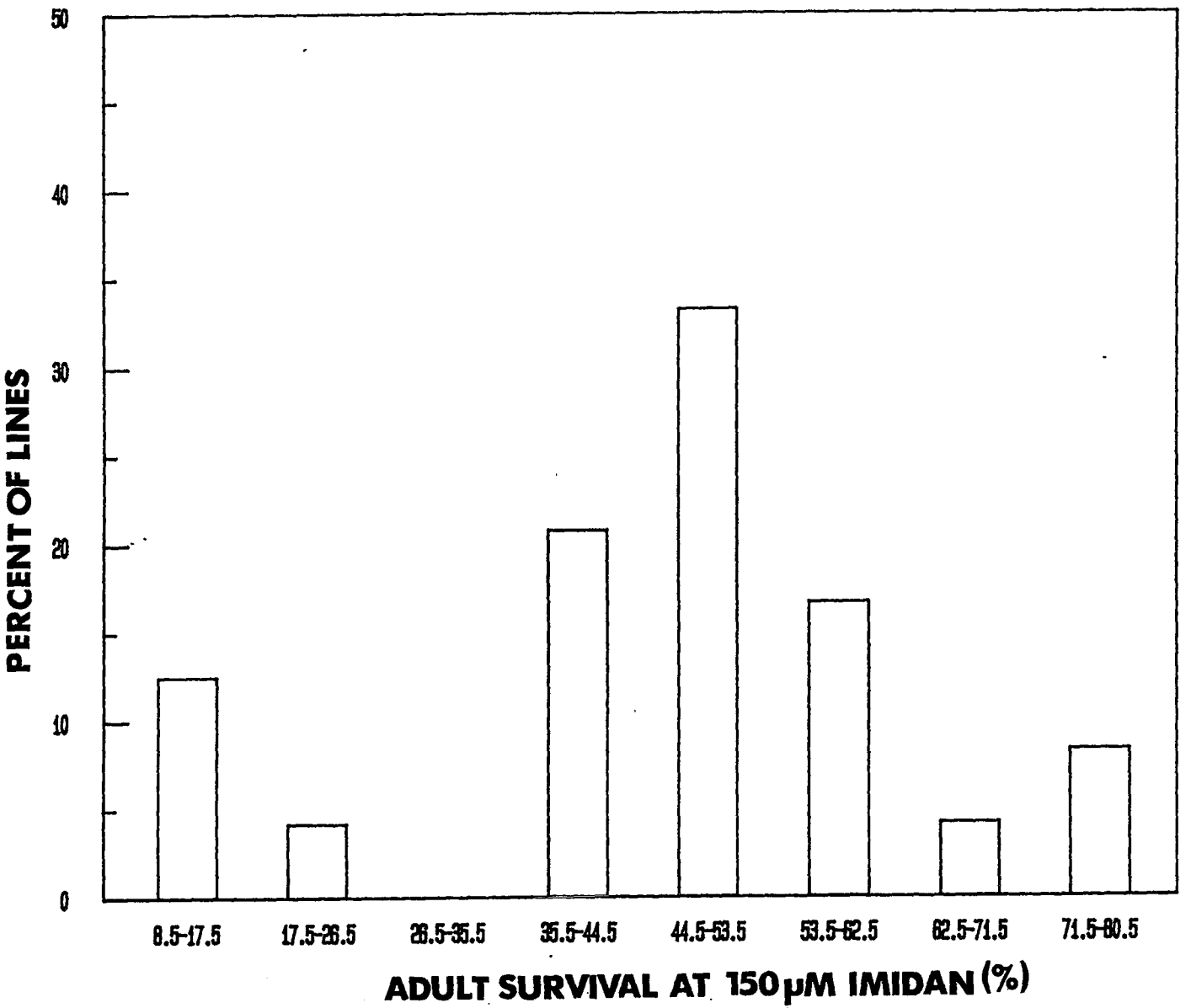

PERCENT OF LINES
50
40
30
20
10
0
8.5-17.5
17.5-26.5
26.5-35.5
35.5-44.5
44.5-53.5
53.5-62.5
62.5-71.5
71.5-80.5
ADULT SURVIVAL AT 150 µM IMIDAN (%)

Figure 20 : Frequency distribution of 7-ethoxycoumarin specific activity. Isofemale Drosophila melanogaster lines from unsprayed and regularly sprayed plots at Vineland, Ontario were pooled together.

Fig 20

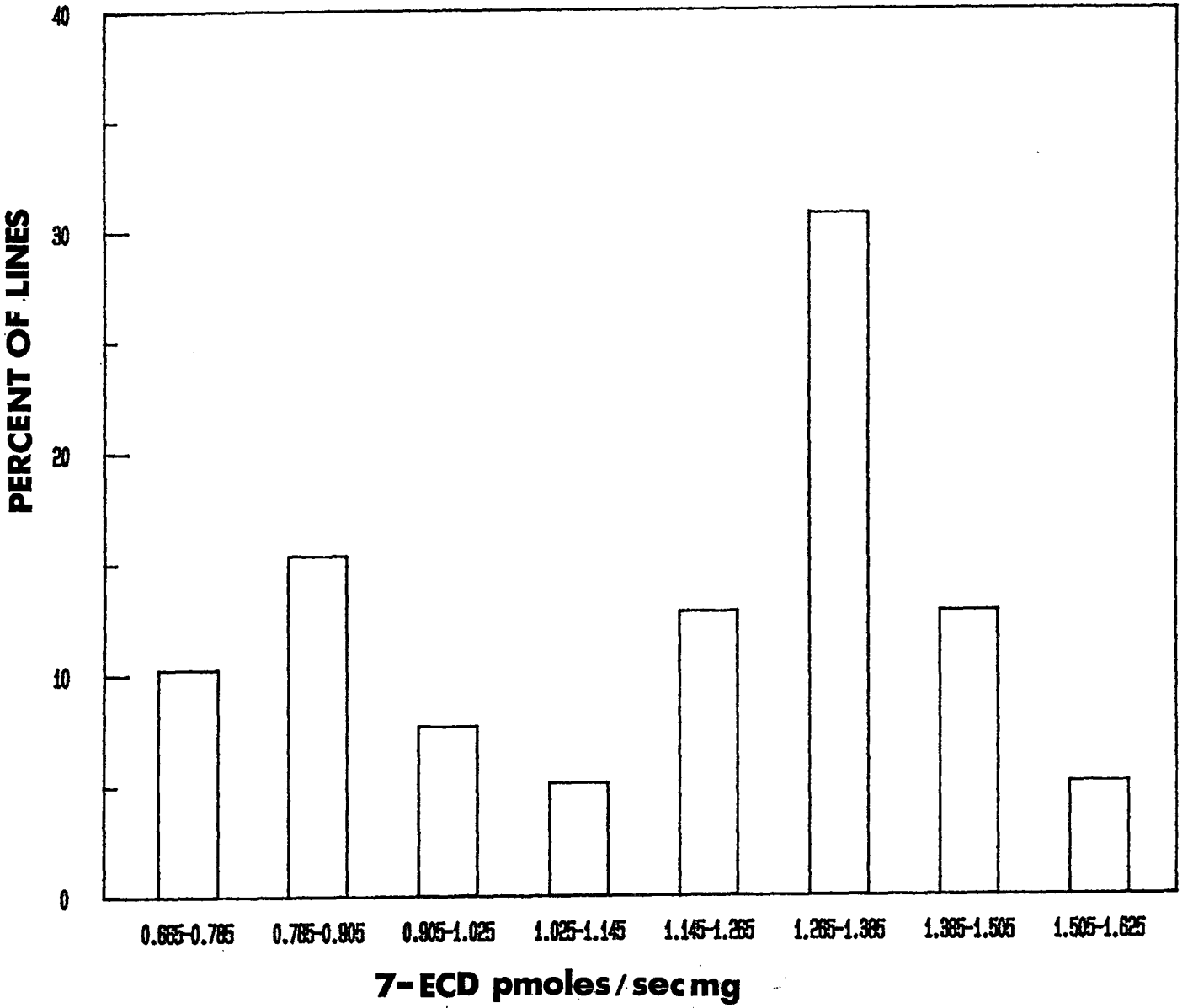

PERCENT OF LINES
40
30
20
10
0
0.665-0.785
0.785-0.905
0.905-1.025
1.025-1.145
1.145-1.265
1.265-1.385
1.385-1.505
1.505-1.625
7-ECD pmoles/sec mg

Figure 21 : Frequency distribution of resistance to Imidan. The pooled group of Drosophila melanogaster isofemale lines from unsprayed and regularly sprayed plots at Vineland, Ontario were exposed to 150uM of Imidan for 24 hours.

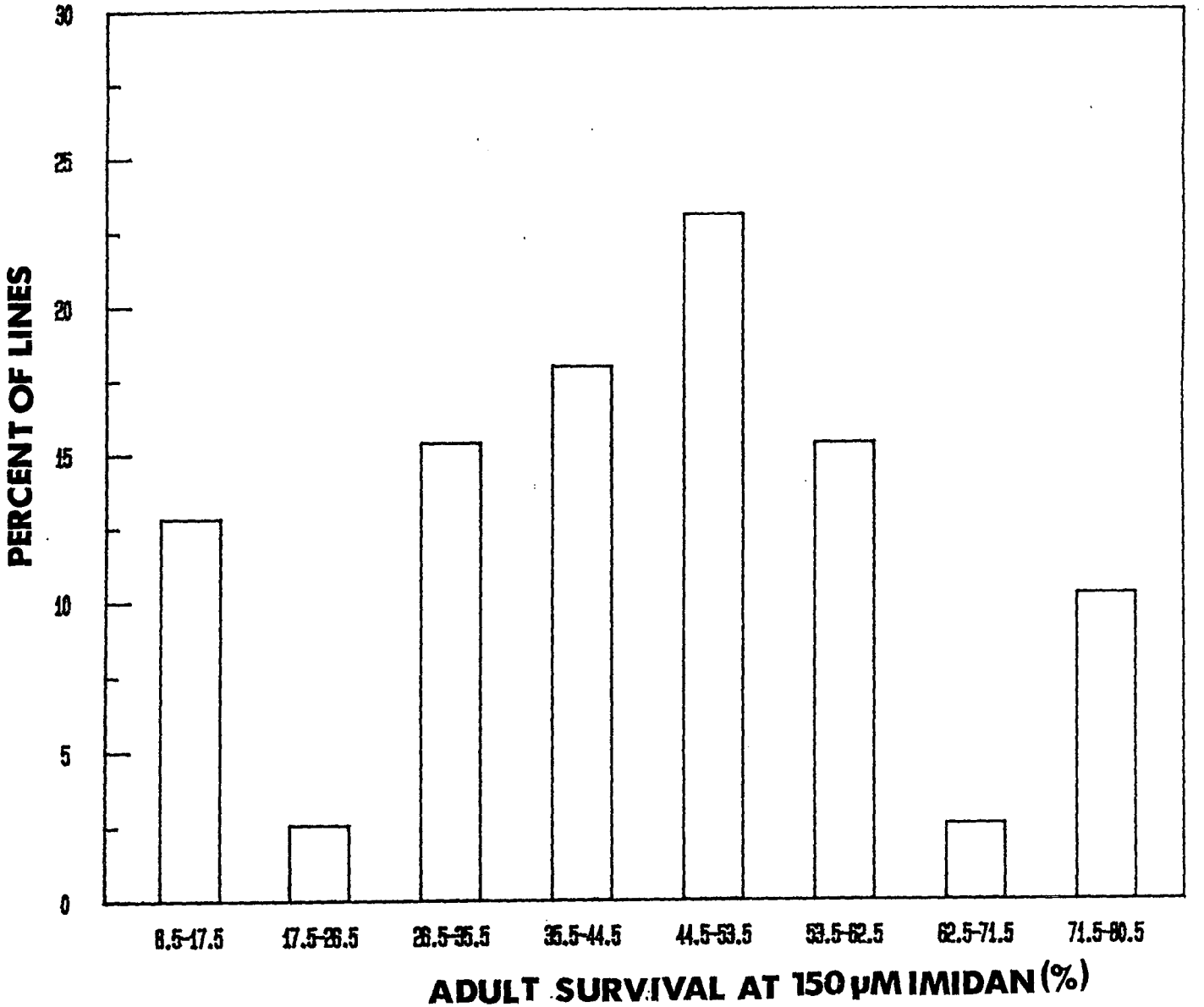
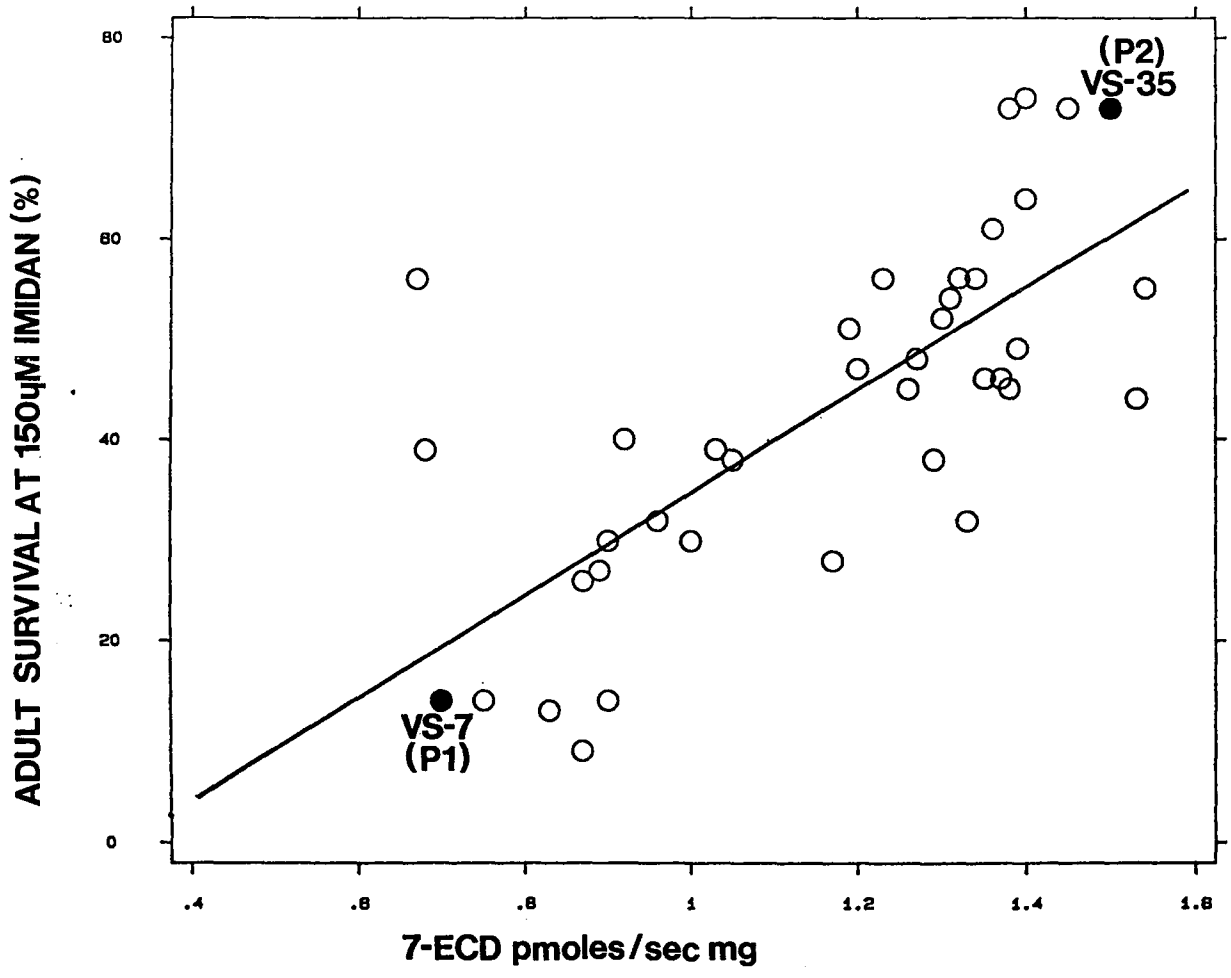

PERCENT OF LINES
30
25
20
15
10
5
0
8.5-17.5
17.5-26.5
26.5-35.5
35.5-44.5
44.5-53.5
53.5-62.5
62.5-71.5
71.5-80.5
ADULT SURVIVAL AT 150 µM IMIDAN (%)

Figure 22 : The relationship between resistance to Imidan and 7-ethoxycoumarin specific activity. The pooled group of isofemale lines from unsprayed and regularly sprayed plots were used. Resistance to Imidan was determined by percent adult survival at 150uM after 24 hours. The isofemale strains (P1 and P2) used in determining the number of genes affecting MFO activity (section 3.5.4) are indicated by filled circles.


ADULT SURVIVAL AT 150μM IMIDAN (%)
80
60
40
20
0
.4
.6
.8
1
1.2
1.4
1.6
7-ECD pmoles/sec mg
(P2)
VS-35
VS-7
(P1)

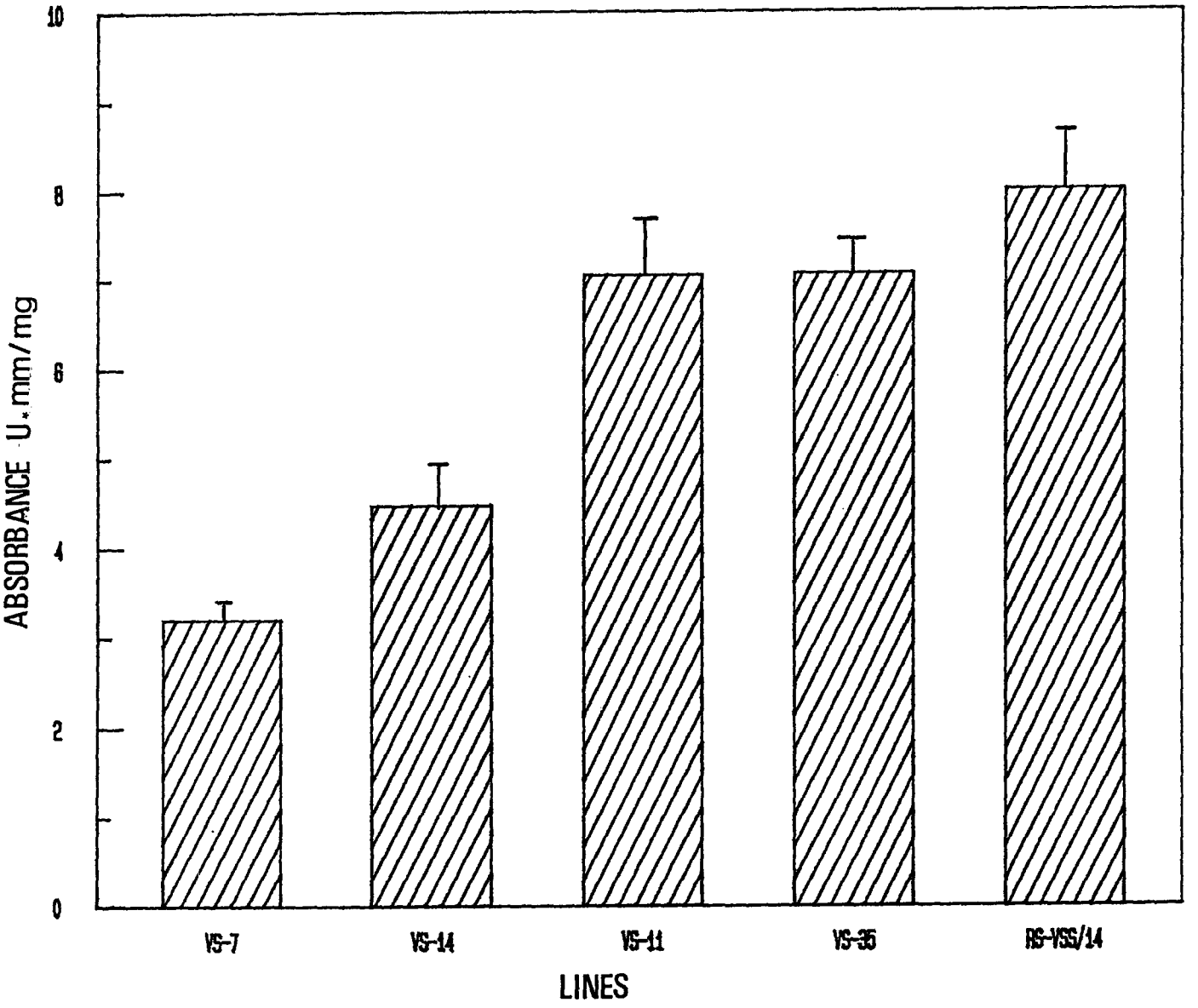
### 3.4.4 "P2" Antigenic levels in Natural Populations and a Laboratory Selected Line.

The purpose of experiments reported in this section was to determine the levels of P2, the polypeptide determinant which had been associated with insecticide resistance in section 3.1 in flies collected from Vineland, Ontario. It was also to determine if the genetic factors controlling the amount of P2 had been selected in this population or could be selected by further exposure of flies from it to Imidan. Levels of P2 antigenic response of these flies were also compared to a laboratory population selected from flies also collected from Vineland. Six isofemale strains were used in this study, two (VS-7 and VS-14) with low 7-ECD activity, one (VS-4) with intermediate activity and two (VS-11 and VS-35) with high 7-ECD activity and a laboratory-selected line (RS-VSS/14). The latter was selected by spraying infested peach fruits and using increasing amounts of Imidan in banana medium and then maintained on medium containing 14uM of Imidan.

The same antiserum, Ab1-B used in sections 3.1 and 3.3 was used in this study. Crude fly homogenates were electrophoresed on 10% SDS polyacrylamide gels, electroblotted onto nitrocellulose and then immunostained. Significant differences were found in the levels of the P2 polypeptide between strains with low, intermediate and high 7-ECD specific activity. However the difference between the strains showing high 7-ECD specific activity and the selected

strain, RS-VSS/14 was not significantly different (Figure 23). Selection in the laboratory did not increase significantly either 7-ECD specific activity of P2 polypeptide determinant levels beyond the highest values obtained for isofemale lines established from natural populations, although Imidan resistance did increase.

Figure 23 : Comparison of P2 antigenic response of whole-fly extracts run on SDS polyacrylamide gels of laboratory-selected (RS-VSS/14) and isofemale lines from nature (Vineland, Ontario) with different levels of 7-ECD activity.


10
8
6
4
2
0
ABSORBANCE U*mm/mg
VS-7
VS-14
VS-11
VS-35
RS-VSS/14
LINES

**PART THREE**

## 3.5 The number of genes controlling 7-ECD Specific Activity.

The number of genes is one of the genetic factors which influence the development of resistance (Georghiou and Taylor, 1976). The number of genes controlling resistance will determine the limits of progress from recurrent selection with pesticide. The role of 7-ECD activity in insecticide resistance was confirmed in the previous sections. It is therefore important to know the number of genetic factors controlling the activity of this enzyme.

Wright (1968) suggested selecting up and down a phenotypic scale to obtain the two populations to be used in gene number estimates. However in this study two isofemale lines with extreme 7-ECD specific activity were used. Time limitations prevented initiation of new selection experiment. As well it was necessary to investigate the genetic basis of the difference between two 7-ECD specific activity groups which were identified in section 3.4.3 (see Figures 20 and 22). These isofemale lines had been maintained in laboratory population size for almost 2 years and therefore should be fixed for alternate alleles. Wright's method could therefore be applied to determine the number of genes controlling the activity difference between the extreme isofemale lines.

### 3.5.1 Distribution of 7-ECD Specific Activity in Populations used for determination of number of genes.

MFO activity distributions of male flies of parental [VS-7(P1) and VS-35(P2), see Figure 22] and hybrid populations (F1, F2, B1 and B2) are presented in this section. MFO activities were measured on single flies and the distribution of these populations were analyzed to determined variation within and between populations.

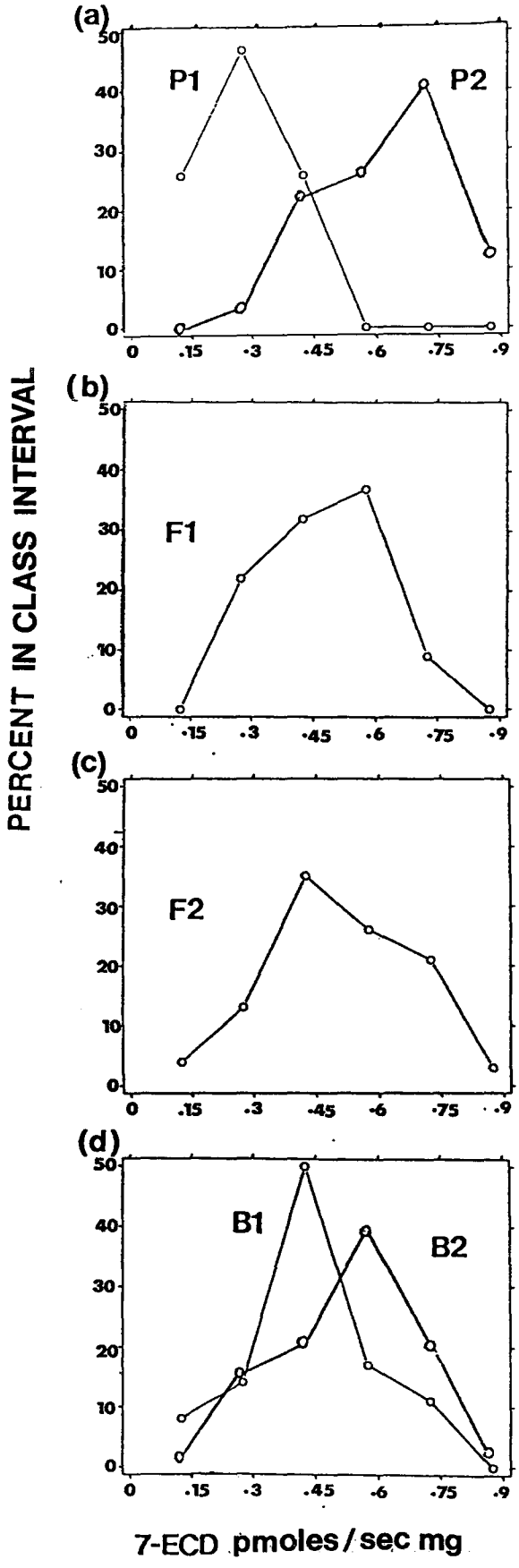
The variances of MFO activity in the two parental lines (P1, = 0.0097 and P2, = 0.0219) were found to be significantly different ($F = 2.26$, $P < 0.05$) (Table 6). The MFO activity distribution shows continuous variation in both parental populations with about 25% of individual activities overlapping (Figure 24a). The variance of the distribution of F1 generation was found to be significantly different from P1 ($F = 1.84$, $P < 0.05$), although it was not different from P2 ($F = 1.22$, $P < 0.05$). The variance of the distributions of P1, P2 and F1 were significantly less than the F2 population. However the variance of the two backcross populations (B1 and B2) were not significantly different from the F2 (Table 6 and Figures 24a - d), although as expected both were greater than the P1, P2 and F1 populations. The variation in the F2 and the backcross generations are due to both environmental and segregating genetic variances and therefore are expected to be higher than variances

**TABLE 6 :** Statistics for 7-ECD specific activity for all populations. Numbers in brackets are standard errors.

| Population | N | Mean, $\bar{X}$ pmoles/sec mg | Variance, $\sigma^2$ |
|---|---|---|---|
| P1 | 66 | 0.2791(0.012) | 0.0097 |
| B1 | 65 | 0.4349(0.019) | 0.0231 |
| F1 | 65 | 0.4758(0.017) | 0.0179 |
| F2 | 140 | 0.4989(0.015) | 0.0302 |
| B2 | 65 | 0.5300(0.019) | 0.0241 |
| P2 | 65 | 0.6279(0.018) | 0.0219 |

Figure 24 (a - d) : Frequency distribution of 7-ethoxycoumarin (7-ECD) specific activity in low 7-ECD parental (P1), high 7-ECD parental (P2), F1, F2, backcross to P1 (B1) and backcross to P2 (B2) populations. Points on the curves are percent in class interval and are plotted at the centre of the class intervals. A class interval equals 0.15.

Fig 24


(a)
P1
P2
(b)
F1
(c)
F2
(d)
B1
B2
PERCENT IN CLASS INTERVAL
0
10
20
30
40
50
.15
.3
.45
.6
.75
.9
7-ECD pmoles / sec mg

of P1, P2 and F1 which are solely due to environmental causes. The continuous variation observed in all the populations suggest some strong environmental and/or segregating genetic factors controlling 7-ECD specific activity.

The mean 7-ECD specific activity determined for B2, F1 and F2 hybrid populations were not different from the expected values (Table 6). Theoretically F1 and F2 supposed to be the mid-value between P1 and P2 values while B2 is the third-quarter value. The mean for backcross one (B1) was however unusually high and not significantly different from F1, F2 or B2.

### 3.5.2 Transformation of Original Measurements

The fact that the variances of P1 and P2 were significantly different suggested the need for transforming the original measurements to correct for a possible scale effect.

The original measurements were transformed on a scale of $X_T = \log (X + ab)$ where X is the original measurement and $X_T$ its transformed value, a the intercept and b is the regression coefficient in the regression equation relating the standard deviations and the means of the P1, P2 and F1 populations. This transformation is expected to make their variances equal (Wright, 1968. p232). Table 7 gives the statistics for the populations based on transformed data. The variances of P1, P2 and F1 were

**TABLE 7 :** Statistics for 7-ECD specific activity for all populations (original measurements transformed on a scale of $\log(X + 0.4672)$).

| Population | N | Mean, $\bar{X}_T$ | Variance, $\nabla_T^2$ |
|---|---|---|---|
| P1 | 66 | -0.1309 | 0.0034 |
| B1 | 65 | -0.0471 | 0.0056 |
| F1 | 65 | -0.0299 | 0.0039 |
| F2 | 140 | -0.0223 | 0.0065 |
| B2 | 65 | -0.0117 | 0.0061 |
| P2 | 65 | 0.0356 | 0.0035 |

not significantly different from each other after transformation.

### 3.5.3 Gene Action.

Population means for 7-ECD specific activity were analyzed using untransformed data to determine their fit to an additive model. Data are presented as plots of specific activity against the proportion of genome derived from the P2 strain containing high 7-ECD specific activity alleles (Figures 25). The means of P1, F2 and P2 have been joined by a line. In theory if the genes are acting additively, then the means of the two backcross populations, B1 and B2 should also lie on this line. They both deviated from this line although in opposite directions. However if the line is drawn through F1 (F1 and F2 are supposed to be equal) B1 shows a major deviation. The mean of F1 did not deviate significantly from the expected position of this line (Figure 25).

The mean phenotypes if they are additive should also give a triangular pattern when the variances are plotted against the population means on a transformed scale (Lande, 1981). The F1 and backcross generations are expected to be at the mid-points of the edges connecting the parental and F2 populations. The triangular plot (Figure 26) revealed B1 and F1 generations showing slight deviations from the expected positions. The F1 population also shows some deviation from the midpoint of the two parental

**Figure 25 : The relationship between 7-ethoxycoumarin specific activity and the proportion of parental population (P2) genome.**

Fig 25

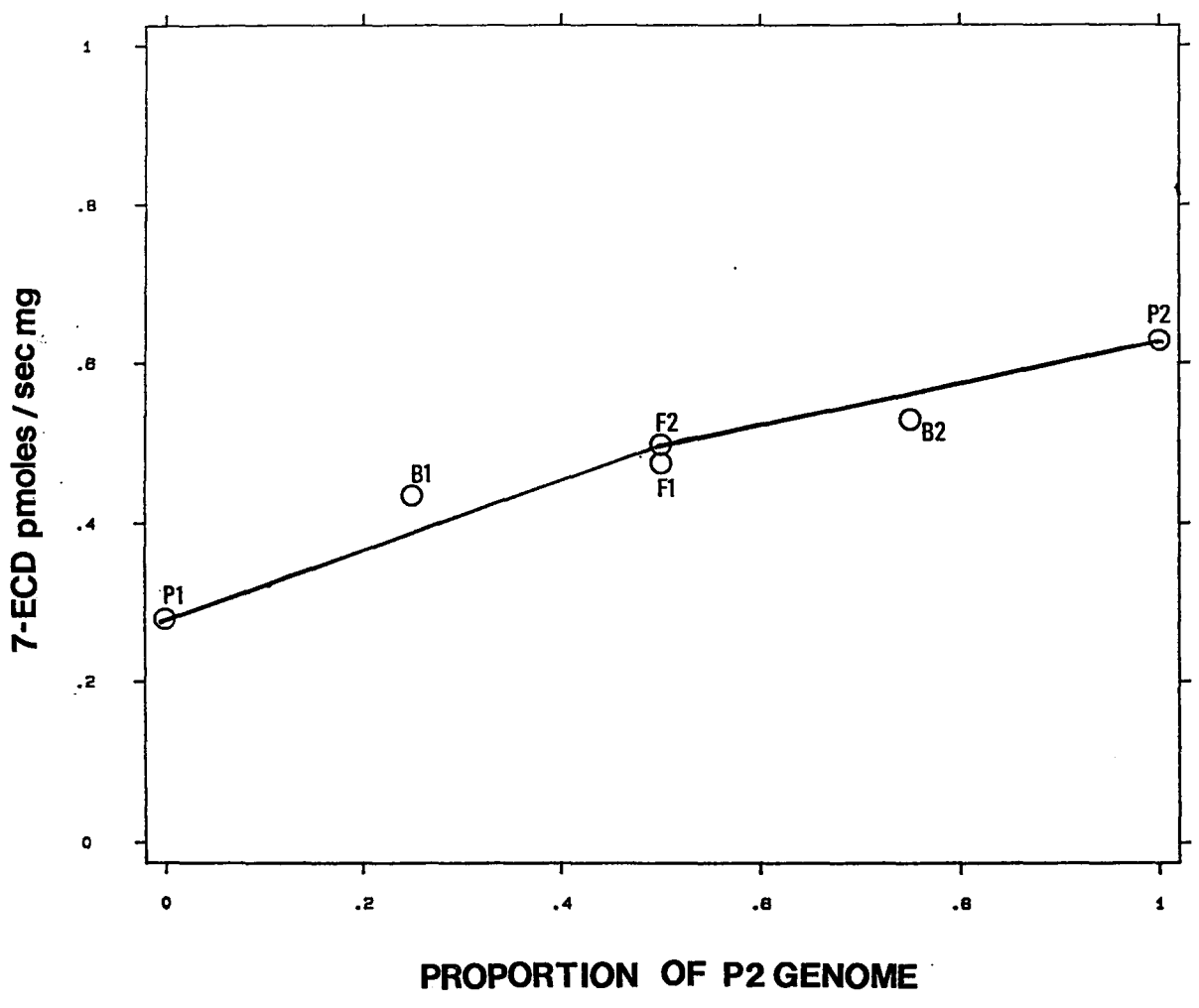
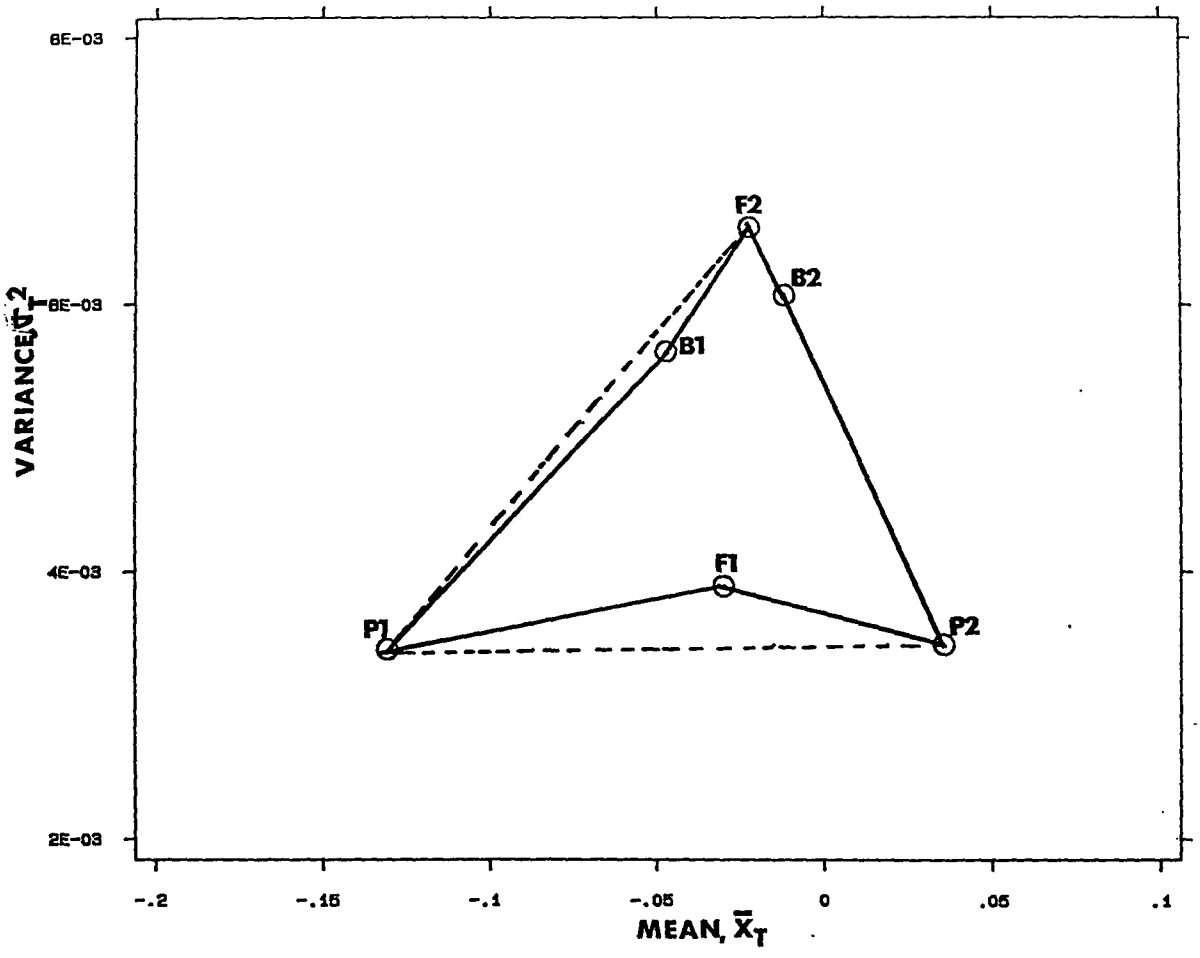

7-ECD pmoles / sec mg
1
.8
.6
.4
.2
0
P1
B1
F2
F1
B2
P2
0
.2
.4
.6
.8
1
PROPORTION OF P2 GENOME

Figure 26 : A triangular plot of transformed means and variances of parental and hybrid populations.


8E-03
6E-03
4E-03
2E-03
VARIANCE, $\sigma_T^2$
F2
B2
B1
F1
P1
P2
-.2
-.15
-.1
-.05
0
.05
.1
MEAN, $\bar{X}_T$

populations P1 and P2. This is because the F1 variance was significantly different from the parental populations. The point for B2 does not lie at the expected mid-point position between P2 and F2. This is an indication of the non-significant differences found between F2 and B2 variances. The results from both the transformed and untransformed data suggest that the genes influencing the specific activity of 7-ECD act nearly additively.

### 3.5.4 The minimum number of genes controlling 7-ECD Specific Activity and the contribution of a major factor.

The minimum number of genes controlling the variation between specific activity of 7-ECD of two isofemale lines can be estimated in four ways using equations which are stated again below (Lande, 1981).

1). $\sigma_s^2 = \sigma_{F2}^2 - \sigma_{F1}^2$

2). $\sigma_s^2 = \sigma_{F2}^2 - 0.5\,\sigma_{F1}^2 - 0.25\,\sigma_{P1}^2 - 0.25\,\sigma_{P1}^2$

3). $\sigma_s^2 = 2\,\sigma_{F2}^2 - \sigma_{B1}^2 - \sigma_{B2}^2$

4). $\sigma_s^2 = \sigma_{B1}^2 + \sigma_{B2}^2 - \sigma_{F1}^2 - 0.5\,\sigma_{P1}^2 - 0.5\,\sigma_{P2}^2$

The minimum number of genes is estimated from the equation :

$$n_E = R^2 / 8\sigma_s^2,$$

where $R^2$ is the square of the difference between parental means and $\sigma_s^2$ is the genetic segregation variance determined from one of the above equations (Wrights, 1968 and Lande, 1981).

Three of the estimates were consistently near 1 and not significantly different (Table 8). The third estimate gave a value

**TABLE 8 :** Segregation variance, minimum number of genes with standard error, and contribution of major factor estimates for 7-ECD specific activity for all four ways.

| Method | $\sigma_s^2$ | $n_E$ | S.E. | P |
|---|---|---|---|---|
| 1 | 0.0027 | 1.3 | 0.2 | 0.88 |
| 2 | 0.0029 | 1.1 | 0.1 | 0.95 |
| 3 | 0.0014 | 2.4 | 0.3 | 0.65 |
| 4 | 0.0043 | 0.8 | 0.2 | 1.12 |

of 2.40 with a standard error estimate of 2.28. The large standard error in this particular estimate is the result of the deviations reported in Figures 25 and 26. The unusually high variances of B1 and B2 resulted in either under or over estimation of the segregation variance from equations 3 and 4 (see above). However the segregation variance estimate from equation three was affected more resulting in significantly lower variance (Table 8) and consequently a higher number of genes and standard error estimates.

The maximum proportion of the difference between the parental populations (p) due to the major factor is estimated by the following formula (Lande, 1981) :

$$p = 1 / n_E$$

The p values from this estimate using the four minimum number of gene estimates reported above ranged from 0.65 to 1.11 (Table 8). These results indicate a single gene or only a few genes could be controlling the difference in variability of 7-ECD specific activity between the two isofemale Drosophila melanogaster lines.

## 3.6 Extraction of Chromosomes from VS-35 and Laboratory Selected Populations

Work in this section was carried out to determine which chromosomes from the isofemale line VS-35 used as P2 (high 7-ECD specific activity population) in the previous section were

responsible for MFO activity and Imidan resistance. Chromosomes from this population were also compared to chromosomes from the laboratory selected line, RS-VSS/14. Isochromosome strains homozygous for first, second and third chromosomes extracted from these two populations were also examined for their relative chromosomal viability and resistance to Imidan (LC-50).

First chromosomes from both resistant populations were slightly more viable in isochromosomal strains than their corresponding balancer chromosomes, FM7a. Relative viabilities ranged form 103 to 157% (Table 9). Viability of second chromosomes from the selected line, RS-VSS/14 although variable, were not different on the average from the balancer chromosome, CyO. On the other hand second chromosomes from the isofemale line VS-35 were more viable (mean = 124%) relative to the balancer chromosome, CyO. Third chromosomes from both populations were consistently less viable than the balancer chromosome, TM3. These results suggest that in both the laboratory selected line and the isofemale line established from nature, only the third chromosome had a detrimental effect on the viability of Drosophila melanogaster. This is due to greater frequency of semi-lethals on chromosome three and not to resistance genes.

All chromosomes from the resistant populations have an effect on Imidan resistance when compared to their corresponding balancer line (which contain other genes substituted in isochromosomal

**TABLE 9:** 7-ECD specific activity, imidan resistance and viability of *Drosophila melanogaster* strains. Numbers in brackets are standard errors.

| LINES | 7-ECD Specific Activity. mean(s.e) pmoles/sec mg | LC-50 µM* | Relative chromosomal viability. %* |
|---|---|---|---|
| FM7a | 0.52(0.04) | 15 | NA |
| MKRS/TM3 | 0.59(0.03) | 14 | NA |
| $C_yO/bw^D$ | 0.48(0.03) | 11 | NA |
| VS-35 | 1.53(0.06) | 91 | NA |
| RS-VSS/14 | 1.64(0.06) | 184 | NA |
| Isochromosomal I / VS-35 | 0.49(0.03) | 25-46<br>35 | 103-157<br>123 |
| RS-VSS/14 | 0.52(0.03) | 32-59<br>43 | 113-124<br>117 |
| Isochromosomal II / VS-35 | 0.51(0.03) | 37-56<br>46 | 93-159<br>124 |
| RS-VSS/14 | 0.68(0.04) | 47-70<br>58 | 78-136<br>104 |
| Isochromosomal III / VS-35 | 1.33(0.05) | 70-87<br>80 | 73-84<br>79 |
| RS-VSS/14 | 1.13(0.05) | 104-169<br>139 | 81-96<br>86 |

NA : Not applicable.

* : Range and mean (below).

strains). However the effect is greatest for chromosome three. Isochromosome three strains from the two populations had LC-50s comparable to their respective original populations (Table 9).

Third chromosomes also had major effects on the 7-ECD specific activity in both populations. A minor effect of the second chromosomes on 7-ECD specific activity in the laboratory selected population (RS-VSS/14) was also observed.

Taken with the estimates of number genes controlling 7-ECD specific activity, the results suggest a major genetic factor affecting both resistance and 7-ECD activity together with a group of minor factors affecting each separately.

# DISCUSSION

The factors influencing the potentiality of a population for development of resistance and the rate, stability and regression of such resistance are questions of primary interest in applied entomology (Rozeboom, 1963). It is in the light of this statement that studies presented in this thesis were carried out.

The study has been divided into three sections. The first section dealt with the relationship between 7-ECD specific activity and a polypeptide determinant, P2, which had been tentatively identified as cytochrome P-450 (Pursey, 1989). Sex differences in the expression of P2 were also studied under this section. The second section involved studies on variability in 7-ethoxycoumarin (7-ECD) specific activity and resistance levels in two natural samples of Drosophila melanogaster. The specific activity of 7-ECD, resistance levels and expression of P2 in the two samples were also compared to a laboratory selected line. This line was selected from peach fruits infested with larvae of Drosophila melanogaster. The fruits were collected from a plot regularly sprayed with Imidan and is the same plot where one of the two natural samples was collected. The final section of the thesis deals with the number of genes and chromosomes controlling 7-ECD specific activity and resistance to Imidan in a natural population of D. melanogaster.

## 4.1 7-ECD Specific Activity versus P2, a Polypeptide Determinant and Sex differences in P2

The role of MFO activity and cytochrome P-450 in insecticide resistance in D. melanogaster has been established by various studies, Morton and Holwerda (1985) and Sundseth et al (1990) among others. High MFO activity found in resistant strains could be due to either an increased production of a type of cytochrome P-450, normally (or also) found in susceptible strains or to a unique form or molecule with higher activity selected as a result of exposure to insecticide. A significant positive correlation was found in 7-ECD specific activity and expression of P2, determined by immunoblots for two groups of flies (Figures 6 and 8). Together these cover all levels of 7-ECD specific activity, that is low, intermediate and high. They also included both laboratory selected and natural populations. This results implies that the increased production of P2 causes increased 7-ECD specific activity in all strains of D. melanogaster. Using monoclonal antibodies the levels of a 56K cytochrome P-450 was also correlated with N-nitrosodimethylamine demethylase activity in different strains of Drosophila (Sundseth et al, 1989). Increased expression of P2 could be the result of duplication of structural genes or as has been found in the mouse system due to a regulatory gene coding for an inducer protein (Karenlampi et al, 1983).

The 60% greater levels of P2 found in male fly extracts relative to female fly extracts (Figures 9, 10 and 11 and Table 3) may be inconsistent with their slightly lower 7-ECD activity. This was reported by Patil et al (1990) to be 90 - 93% that of females. The difference in the two studies is only possible if females have higher levels of other factors such as G6P, G6PD and NADP which are important for the 7-ECD activity. Developmental regulation of P-450 genes was first suggested by Conney et al (1969) in which the level of hydroxylated testosterone metabolites generated from rat liver microsomes was shown to vary as a function of age and sex. Since then some sex-specific P-450 proteins have also been characterised (Nebert and Gonzalez, 1987). However, these differences are all hormonally regulated in mammals and a different mechanism may be responsible for the differences seen in insects. In a study with three housefly strains (one susceptible and two resistant) cytochrome P-450 levels per mg of protein in females were found to be only 46 - 65% that of males (Matthews and Casida, 1970).

The results of the microplate assay were rather disappointing. It had been hoped that this assay could be used to detect resistant genotypes in the field. Although there was large differences between resistant (IIID) and susceptible (Canton S) fly extracts using immunoblots following SDS electrophoresis (Figures 5, 6 and 12), the microplate assay could produce at best only a 45% difference. It could be that many more non-microsomal antigens in the whole-fly extract other than the four

detectable microsomal proteins (Figure 5) were responding to the non-specific antiserum and collectively masking the difference in P2 between the two strains. This study could be tried again with microsomal proteins and monoclonal antibodies. However, the use of microsomal proteins will negate the easy of a microplate assay. Sundseth et al (1989) using monoclonal antibodies and microsomal proteins were able to obtain substantial differences between strains on microplates.

## 4.2 7-ECD Specific Activity and Resistance Levels in Natural and Laboratory Selected Populations of Drosophila melanogaster

Part two of the study characterized the variability in resistance and 7-ECD specific activity in two natural samples of D. melanogaster. Isofemale lines established from the two samples were compared to a laboratory selected line derived from the same population.

Very little difference was found between MFO activity and resistance of flies sampled from the sprayed (VS) and unsprayed (VC) plots. The similarity of the two populations was seen in both sampled and isofemale lines (Figures 14, 15, 16, 17, 18 and 19). This may be due to the close proximity of the two sites which will permit constant migration between the two populations. Despite the similarity between the two populations in both sampled and isofemale flies, the isofemale lines had higher mean percent

survival on Imidan than the samples from which they were established (Figure 15). This difference between the populations and isofemale lines is probably due to the better nutrition the isofemale lines had in the laboratory. Flies used for resistance measurements of the two samples were fed banana medium for only three days. All 39 females from the two samples successfully established isofemale lines. This was an indication of a good sample of major alleles. The isofemale lines because of inbreeding were as a group expected to have lower fitness and therefore less resistant to Imidan. This results suggested the existence of very few or no semi-lethals or sterile genes in the population.

Laboratory selection resulted in a line having both higher MFO activity and resistance than the mean value of the isofemale lines. At 150uM of Imidan, the selected line had a resistance level of 88% (survival) compared to the mean of 42.3% for the pooled population of isofemale lines (Table 5), a 2.1-fold increase. The level of 7-ECD activity however increased 1.45 times only (Table 5). Disproportionate increases in resistance and microsomal oxidase activity has also been observed in several studies on housefly (Plapp, 1976; Perry et al, 1971; Terriere and Yu, 1974 and Chang and Hodgson, 1975). These findings could mean laboratory selection produced other resistance mechanisms aside from increased 7-ECD activity.

The difference in resistance levels and 7-ECD specific activity

between the selected line and the isofemale lines indicates that genes were still polymorphic in the population before selection. The existence of polymorphism in the natural population is also indicated by the variability seen among the isofemale lines. The significant positive correlation observed between resistance levels and 7-ECD specific activity among the isofemale lines (Figure 22) is an indication of the significance of the MFO system in organophosphorus resistance in natural Drosophila populations. However, the more continuous distribution of resistance (Figure 21) compared to the bimodal distribution of 7-ECD activity (Figure 20) implicates other factors aside from those responsible for increased 7-ECD specific activity in the control of resistance. Resistance mechanisms such as those involving an altered acetylcholinesterase or increased glutathione-s-transferase activity are known to be involved in organophosphorus resistance in Drosophila melanogaster (Brattsten et al, 1986).

Many studies have suggested a polygenic system may control insecticide resistance in laboratory-selected populations (Dapkus and Merrell, 1977; King and Somme, 1958; Merrell and Underhill, 1956; Oshima, 1958 and Singh and Morton, 1981). What is not clear though is the number of genes controlling resistance in nature. The distribution of resistance levels and 7-ECD specific activity and the correlation between them among the isofemale lines established from nature suggested that two alleles of a major gene control both resistance levels and 7-ECD specific activity with extra minor

genes also influencing resistance levels (Figures 20, 21 and 22). The two 7-ECD groups (Figure 20) were found to be related to P2 expression (Figure 23). VS-7 and VS-14 from the lower activity group contained less P2 antigen than VS-35 and VS-11 from the higher activity group. The independently selected line, RS-VSS/14 with high MFO activity also had a higher P2 expression (Figure 23). Therefore the same polygenic system uncovered in laboratory selected populations may be involved in the overall resistance of Drosophila melanogaster to insecticide in nature.

Laboratory selection increased the level of P2, the polypeptide determinant by only about 1.47-fold compared to the mean obtained for four of the isofemale lines (Figures 23). This compares well with the 1.45-fold increase obtained for 7-ECD specific activity. These results again demonstrates the importance of P2 in 7-ECD specific activity.

## 4.3 The minimum Number of Genes and Chromosomes controlling 7-ECD Specific Activity.

Work in part three of the study is on (1) the distribution of 7-ECD specific activity in isofemale parental populations (P1 and P2) together with F1, F2, B1 and B2 hybrid populations, (2) the number of genes and (3) the chromosomes controlling 7-ECD specific activity and resistance to Imidan.

A continuous distribution of MFO activity was found in all populations. There was also overlapping between parental populations (P1 and P2) MFO activity distribution as well as between B1 and B2 hybrid populations (Figures 24a - d). These results may be due to either a strong environmental effect on high 7-ECD specific activity alleles or unfixed genetic differences between isofemale lines. The minimum number of genes was estimated to be about 1. This depending on the magnitudes of environmental and genetic variance could produce a discontinuous variation in the F2 population. This however was not observed, although a discontinuous variation (bimodal) was observed in the original population of isofemale lines (Figure 20). The environmental and/ or unfixed genetic effects obscured the expected bimodal distribution in the F2. Continuous variation has also been observed in the specific activity of alcohol dehydrogenase. Variants of this enzyme that differ in kinetic properties can be identified in

Drosophila population (Laurie-Ahlberg, 1985). The genes controlling alcohol dehydrogenase were believed to be modulated by genes on several chromosomes.

Epistatic interaction and other genetic effects such as dominance seem to contribute very little to 7-ECD specific activity variation in nature, since transformed and untransformed data (Figures 25 and 26) both closely fit an additive model. This is consistent with the large additive components found for other traits representing responses to stress such as exposure to ethanol (Parsons, 1983 and Cohan et al, 1989). It, however, contradicts results for artificial selection on insecticide resistance in the housefly, in which different selected factors including increased detoxification and reduced penetration contribute synergistically to selected characters (Georghiou, 1972).

The minimum number of genes estimated for 7-ECD specific activity of about one obtained for the major difference between the two activity groups of Drosophila melanogaster is consistent with the extracted chromosome experiments. Only the third chromosome of the isofemale population VS-35 (the parental population or P2 having high 7-ECD activity) had a significant effect on 7-ECD specific activity (Table 9). The 7-ECD activity in the other isochromosomal lines was similar to the strains from which the balancers were derived. What is not certain though is the actual number of genes on chromosome three that affect 7-ECD activity. Houpt et al (1989)

reported at least two genes on chromosome three (near 3-58) controlling increased mixed-function oxidase in a laboratory selected strain. Hallstrom (1985) found that 7-ethoxycoumarin-O-deethylation in the Hikone R strain is semidominantly inherited and determined by one or two genes on the third chromosome.

Using the balancer strains as reference, both the laboratory-selected population (RS-VSS/14) and the isofemale line (VS-35) from nature revealed that additional genes on chromosomes one and two besides MFO genes on chromosome three make significant contribution to resistance of D. melanogaster (Table 9). Adult resistance to DDT in Drosophila was also found to be under a polygenic system of inheritance involving all three major chromosomes (Crow, 1954 and Dapkus and Merrel, 1977).

## 4.4 Further Research

Work presented in this thesis involved variability and the number of genes controlling the MFO system in a natural population of Drosophila melanogaster. It is hoped that in future more diverged natural populations, selected up and down a 7-ECD activity scale to fixation could be used to estimate the number of MFO genes. This may remove some of the overlap in 7-ethoxycoumarin specific activity observed in the parental and hybrid populations. It should also be interesting to estimate the number of genes controlling resistance itself. It is also hoped that in future it would be

possible to determine the actual number of genes on chromosome three involved with the mixed-function oxidase system in natural populations of D. melanogaster possibly through molecular techniques.

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