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Development of Resistance in Drosophila Melanogaster by Selective Pressure with Malathion (Organophosphate Insecticide)

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by

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Two strains of resistant Drosophila melanogaster were cultured by selective pressure with malathion. One group was exposed to a sublethal concentration for it's entire life cycle while the other group was exposed to an LC_{65} for twenty-four hours. The data obtained indicates that both strains developed equal degrees of resistance representing a four-fold increase in the LC_{50}. This appears to be the upper limit of resistance for these flies with respect to their genetic limitations. It is possible, however, that this represents a plateau of resistance and further selection might have caused an increase.

Upon obtaining resistance in both strains, population studies were conducted to determine the characteristics of the resistance. It was found that a cessation of treatment for five generations did not significantly reduce resistance. The cross breeding of wild flies with resistant flies resulted in offspring that had a mortality curve closely matching the projected mortality curve of flies assumed to have

resistance caused by a single dominant gene. There was no indication of resistance being sex linked. Finally, it was found that resistant flies are more susceptible to malathion when they are reared at a higher temperature (30°C. instead of 25°C.).

Introduction

Resistance to insecticides has become a perplexing problem in both the fields of agriculture and health. Since Melander first reported resistance in the San Jose Scale, there have been many instances of insect resistance (28). This poses serious problems to man, who must control insects in order to avoid famine and disease.

For many years man has attempted to circumvent the development of resistance in pest species by developing new and better insecticides. In the 1940's and early 1950's many new insecticides were discovered. They can be grouped into classes such as chlorinated hydrocarbons, organophosphates, carbamates and others. For each class of insecticide, resistant insects can be found (5, 15, 19, 23, 40). It has become apparent that insects are capable of developing multiple resistance. They even developed resistance to the "miracle insecticide" DDT (9, 24, 55). It was also found that many insects had developed cross-resistance to insecticides other than the ones with which they had been treated (24). To combat resistance, man must learn what causes it and what the mechanics of it are. First the mode of action of insecticides must be understood and then the genetics and physiology of resistance must be analyzed.

The intent of the experiments in this thesis was to help gain an insight into the mechanics and characteristics of insecticide resistance. Researchers have had varying degrees of success and failure in attempting to artificially induce resistance by means of selective pressure.

Two different methods of applying selective pressure on a wild colony of Drosophila melanogaster were used. One group was treated throughout the life cycle at a sublethal concentration of malathion. The other group was treated at a progressive and selective concentration of malathion at approximately the 50% lethal concentration (LC_{50}) for twenty-four hours.

Malathion was selected because it is representative of the organophosphate group of insecticides which have become very popular in recent years. The literature abounds with work done using DDT and the chlorinated hydrocarbons, but there is not enough on organophosphates and other promising insecticides.

The critical question of the thesis was whether or not resistance could actually be artificially induced with malathion. There does seem to be some question as to the difference between true resistance and a phenomenon known as "vigor tolerance". True resistance is usually based on genetic inheritance, while vigor tolerance is merely a non-genetic increase of biochemical tolerance.

There are two major theories as to how insects acquire resistance. The postadaptive theory of insect resistance holds that

resistance is a physiological tolerance of the insect to the poison. This produces insects with vigor tolerance rather than true resistance.

The preadaptive theory of insect resistance holds that resistance is acquired by the selection of genes that somehow favor a body mechanism that confers resistance to the insect. This could be by a number of mechanisms such as reduced absorption of insecticide through the insect cuticle, increased lipid content, or enhanced excretion of the poison. The usual cause of resistance, however, proves to be an enzymatic detoxicative mechanism (4).

Resistant genes are very often found in small numbers in an insect species and associated with an unfavorable survival characteristic. Intense selection causes the resistant factor to outweigh the unfavorable survival factor. The question in breeding resistance in Drosophila melanogaster is whether or not the species possesses the gene (s) for malathion resistance. These selected strains are difficult to analyze because they possess four-fold resistance, that is, the LC_{50} has increased four-fold, which could possibly be vigor tolerance. However, the characteristics of these flies compared with the literature indicates that they do possess true resistance. These studies are designed to prove artificial selection for resistance to malathion in D. melanogaster as well as to contribute to an understanding of the mechanism of resistance which is essential for long range control.

Review of Literature

Before becoming involved in a study of resistance, it is first necessary to become familiar with the mode of action of insecticides. Insecticides can be divided into common groups, such as organophosphates, chlorinated hydrocarbons, carbamates, pyrethrins and others. The compounds within a particular group may vary slightly in mode of action, but generally they follow similar metabolic pathways. In fact, insects resistant to a particular insecticide will often have some degree of resistance to other compounds within the same group but not to compounds of another group (3).

The majority of insecticides kill by acting either directly or indirectly upon the nervous system. The insect cannot tolerate even brief functional interruptions of the nervous system. Thus, the disruption of the sodium pump mechanism or interference with an enzyme at a synapse can cause death (32).

There are two modes of impulse transmission, axonic and synaptic. Axonic transmission is the carrying of an impulse along the axon to another cell. It is an electrical mechanism. Synaptic transmission is chemical and carries the impulse across the junction between cells (32).

If an insecticide can upset the balance of the sodium pump or prevent removal of acetylcholine from a synapse it can cause interference of nerve transmission.

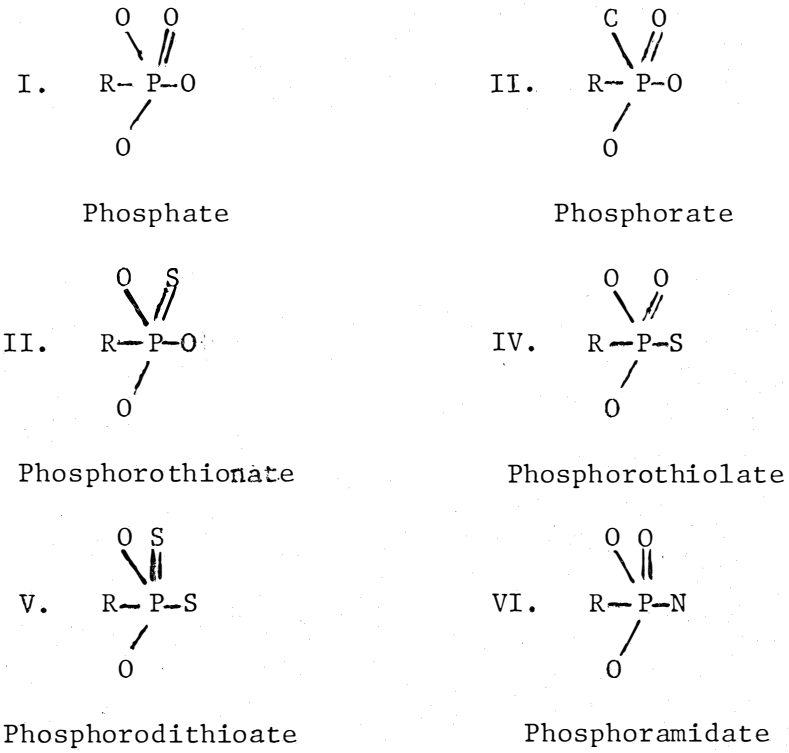
At cholinergic junctions, the enzyme cholinesterase hydrolyzes acetylcholine into acetate and choline. This prevents repeated impulses and clears the synapse for any following impulses.

Some poisons, such as DDT, interfere with axonic transmission while others, such as organophosphates and carbamates, interfere with synaptic transmission. Evidence suggests that the neuromuscular junction in insects is not cholinergic but that the central nervous system is. This means that the cholinesterase in the central nervous system is the target of organophosphates and carbamates (32).

Organophosphates constitute a large class of insecticides about which much is known at the molecular as well as the cellular level. The organophosphates were originally developed as nerve gases during World War II, but soon the insecticidal activity of TEPP and parathion were discovered and since then, thousands more have been formulated (33).

Organophosphate is used as a generic term to cover all toxic organic compounds containing phosphorus. Most of them can be thought of as esters of alcohols with a phosphorous acid or anhydrides of phosphorous acid with some other acid. Figure 1 gives the active areas of the major groups of organophosphates.

FIGURE 1: Active areas of six major groups of organophosphates.



It is widely accepted that the organophosphates kill animals by inhibiting cholinesterase. The resulting disruption of nervous activity is due to the accumulation of acetylcholine at nerve endings. This theory is based on several facts:

(1) Cholinesterase is a vital enzyme, judged by the fact that severe inhibition causes death.

(2) Symptoms of organophosphate poisoning (convulsions, excessive parasympathetic activity

[lachrymation, salivation]) indicate malfunction of the nervous system.

(3) Organophosphate products have been shown to be extremely good inhibitors of cholinesterase and poor inhibitors of other enzymes.

(4) Organophosphates which are good inhibitors of cholinesterase are toxic, those that are poor inhibitors are not. (33).

There has been some debate over whether it is cholinesterase or aliesterase inhibition that causes organophosphates to be lethal. However, several tests indicate cholinesterase inhibition as the major factor. One test showed that aliesterase can be inhibited with no ill effects (33). It has also been shown that acetylcholine levels in poisoned insects rises sharply (33). This would indicate the failure of cholinesterase to eliminate it.

Malathion is a phosphorodithioate. Symptoms of poisoning take from several hours to as much as twenty-four hours to appear. The basis for the reaction is phosphorylation of cholinesterase whereby cholinesterase attaches to the organophosphate. Malathion is useful as an insecticide because it is highly toxic to insects but not to mammals. Most doubts as to whether or not cholinesterase inhibition is the true mode of action of malathion are based upon the fact that malathion itself is a poor cholinesterase inhibitor. It has been found, however, that the toxicity of malathion is closely related to it's conversion to malaoxon [0,0 dimethyl -S- (1,2 - bis-carboethoxy) ethyl phosphorothiolate] which is a more potent anticholinesterase by a factor of seven hundred to two thousand (22). E. Y. Spencer

and O'Brien conclude that it is the action of the oxidation products of organophosphates, such as malaoxon, as cholinesterase inhibitors that causes toxicity (46). The few exceptions involve action on ali-esterases.

In recent years there have been numerous cases of insect resistance, including several with severe economic and public health consequences. One of the first serious outbreaks was that of the California red scale which developed resistance to hydrocyanic acid (12). These were particularly troublesome because they exhibited cross resistance, that is, resistance to compounds other than the one used for treatment.

In 1953 resistance to DDT was suspected in Anopholes stephensi within a small area of Saudi Arabia (55). This was confirmed in 1955 and by 1957 it had spread throughout Iraq and Iran. This, of course, caused a serious malarial threat to the population. Replacement of DDT with dieldrin gave initial success but by 1960 the mosquitoes were resistant to dieldrin as well as DDT. As it turned out the resistance to DDT was of a low magnitude while the resistance to dieldrin was of a higher magnitude. It was becoming clear that resistance was complicating the control problem.

Organophosphate resistance was reported in the housefly in 1955, but by 1960 there were at least nineteen known species of insects found in the field with organophosphate resistance (5). Organophosphates very often give cross-resistance to other organophosphates.

Ten strains of red flour beetle, Tribolium castaneum, resistant to malathion were collected from storage facilities between 1963 and 1965 in which malathion had been used for various periods (45). Resistance was found to range up to 11.3 times for the LD_{50} compared to a susceptible strain reared in the laboratory.

Carter and Phillips were able to successfully induce resistance in a laboratory strain of the bollworm (10). Ten cycles of selection with parathion resulted in a progression through vigor tolerance to resistance. This is just one of many strains of insects that have developed resistance in the laboratory. Ribeiro and Mexia explain the theory of artificial selection for resistance (41). They state that resistance is generally due not to a mutation but to survival of the fittest and thus selection of existing genes. This applies to field populations as well as to laboratory colonies. The most important question to be asked when considering the possibilities for development of resistance is the homogeneity or heterogeneity of the population. A species exhibiting a homogeneic response to an insecticide, that is, a small range of variation in dose level between first kill and total kill, will not develop resistance easily. A species exhibiting a heterogeneic response, however, will quite likely develop resistance if a selective dose is applied.

It was found that the granary weevil, Sitophilus granarius, developed resistance when selected with Baygon and Fenthion pressure (23). They also found an increase in tolerance to other insec-

ticides. Forgash selected DDT resistant houseflies, Musca domestica, with WARF Anti-resistant and DDT (14). The flies were not resistant to the combination at first but soon became so, as well as becoming resistant to malathion, diazinon, ronnel and other insecticides. This is somewhat surprising because some of these insecticides, such as malathion, have different modes of action.

There have also been cases of failure of laboratory strains to develop resistance under selective pressure. Kalina attempted to increase DDT resistance in Drosophila melanogaster by selection (21). He noted that the treated flies showed a slightly slower life cycle, but development was normal until the later stages of pupal development when death occured. Apparently the DDT was being stored in fat tissues and when used for energy during pupation it was metabolized and caused death.

Treatment of fleas for more than forty generations on a substratum containing a sublethal concentration of DDT gave confusing results (48). The treatment speeded up the development of larvae, but only conferred 1.5 to two-fold resistance.

An attempt was made to induce resistance to malathion in body lice, Pediculus humanus (11). Three strains were treated every generation for from twenty-two to forty-four generations. None developed any resistance but two strains showed two-fold tolerance. The author selected at an LD_{50} level and attempted to increase the dose in later generations. It would appear that this strain did not have the genetic capability of developing resistance to malathion.

Since the resistant genes in a wild population are usually low in frequency before selection, it can be assumed that there are disadvantages associated with them, otherwise they would have been more common (12). When selection occurs, normal genes are replaced by resistant genes. When selection is discontinued, the population should return to normal depending on factors such as the dominance or susceptibility of the resistant gene(s). However, Crow's resistant D. melanogaster had still not lost resistance after three years (12). True resistance can last indefinitely if it becomes linked with a factor vital to life (19).

Several authors have given evidence supporting the preadaptive theory of selection (12, 19). Preadaptation is based upon selection of resistant genes that are already present in the insect population as opposed to mutation or non-genetic physiological tolerance (12). It was also noted that resistance increases more rapidly and to a higher degree if it is polygenic rather than monogenic. Increase in resistance is often slow at first but becomes more rapid as the resistant gene(s) become more common until a limit is reached for the insect and the insecticide. Progression of resistance is often geometrical, which explains the initial lag in onset of resistance and the subsequent rapid increase.

There have been several studies done on the inheritance and genetic basis of resistance. The genetical relationships of malathion and fenthion resistance in Culex pipiens were studied (47). The

author selected at the larval stage for ten generations before getting a strain with thirteen-fold resistance to malathion and fifty-fold resistance to fenthion. He found the resistance to be due to monofactorial inheritance of an incompletely dominant resistant gene.

An organophosphate locus in Tetramychus urticae has been suggested (36). Studying demeton -s- methyl in three strains, the authors found resistance due to a dominant gene. A test for allelism showed several major genes from this "organophosphate locus" which could be identical or allelic and give resistance to other organophosphates such as methyl parathion.

Busvine studied inheritance of DDT resistance in body lice (9). He found by studying hybrids from a cross between susceptible and resistant flies that since the resistance was only intermediate, the resistant gene was neither clearly dominant nor recessive. Nguy and Busvine, using similar techniques, also determined that resistance to parathion and malathion in the housefly is due to dominant genes (30). The genes are either allelic or closely paired.

It was determined that a resistant allele was causing quantitative increase in carboxyesterase production in malathion resistant Culex tarsalis (27). This monofactorial gene was giving thirteen-fold resistance. Herne and Brown also found a monofactorial dominant gene was responsible for thirty-fold resistance in a strain of T. urticae (18). They also give several cases of susceptible-resis-

tant crosses yielding resistant hybrids which upon back crossing with susceptible strains, give a 1:1 ratio of resistance, proving the resistant gene to be dominant.

There are more studies needed on the genetics of resistance. Each insecticide and each insect must be considered independently. It has been stated that many genes of the past exist in an insect population which can confer resistance but must be subjected to selection before they can exert their influence (2). The numbers of such genes vary from species to species. Insects can be found with recessive, intermediate, or dominant resistance, varying for different compounds. It is impossible to summarize all resistance for all insects, but it is possible to conduct definitive studies on major species and insecticides. Some of the more definitive studies of this nature have been conducted on the housefly, Musca domestica (20, 38, 39, 40). Enough data has been compiled to give a comprehensive summary of resistance in the housefly. Table 1 from Plapp gives an insight into the nature of resistant genes (38).

It was found that insecticide resistance in M. domestica falls into two classes; semi-dominant genes, which confer resistance by increasing detoxication mechanisms and recessive genes, whose mechanisms are not fully understood, but are extremely difficult to counteract.

Table 1: Nature of resistant genes in M. domestica.

Name of gene:	Mutant symbol:	Linkage groups:	Resistance spectrum:
DDT dehydrochlorinase	Deh	2	DDT, DDD
knockdown resistance	kdr kdr-o	3	Chlorinated hydrocarbons, pyrethroids.
DDT microsomal detoxication	DDT md	5	DDT, some OP's, carbamates.
dieldrin resistance	dld-r	4	cyclodienes, lindane.
altered ali-esterase	a	2	organophosphates.
oxidase	ox	2	carbamates, organophosphates.
organotin - r	tin	3	organotin, modifier of other genes.

The inheritance of resistant genes confers certain degrees of resistance upon insects. The biochemical mechanisms of resistance are as varied as the genetics. For each mode of action of an insecticide there is a mechanism for resistance to counteract it.

Many of the mechanisms of resistance are not understood well. The mechanism of cyclodiene resistance is obscure. It is known that cyclodienes act upon the central nervous system, but it is not known what the biochemical reaction involved in resistance is. Similarly, in the case of DDT, since the exact mode of insecticidal action is

not understood, it is extremely difficult to understand the function of DDT-ase, the enzyme known to be involved in many cases of DDT resistance (4).

The normal mechanism of resistance is enzymatic in nature. There are certain generalizations that can be made on the biochemical aspects of resistance. First, it has been noted that resistant and susceptible strains sometimes differ in the method of detoxication rather than in the rate. Changes in the regulation of enzyme synthesis are also important. Insect detoxicative enzymes may be allosteric proteins, which implies that the activity of their catalytic sites are strongly affected by the binding of small molecules to a different site on the protein (37).

There is much literature on the mechanisms of resistance of insects to organophosphates. Most of it is concerned with enzymes, but some of it suggests other possible mechanisms. There has been some mention of slower absorption as a mechanism of resistance, but that is neither very common nor effective (39). Crow concludes that there are three types of organophosphate resistance (12). The type of resistance found in mites renders cholinesterase less vulnerable to inhibition. The type found in houseflies remoulds an unimportant enzyme originally inhibited by organophosphates into a breakdown enzyme which detoxifies the insecticide. The third type, found in Culex tarsalis, is a breakdown enzyme produced in greater quantities until there is enough to detoxify all of the

insecticide.

It was found that the thirteen-fold resistance found in Culex tarsalis produced more carboxyesterase but there was no qualitative change in it (27). The resistance was due solely to the production of a greater quantity of carboxyesterase.

The activity and reactions of cholinesterase from organophosphate resistant blue ticks, Boophilus decoloratus, were different from susceptible ticks (17). The cholinesterase of the resistant ticks possessed lower total activity and had two or three enzyme types which differed from the susceptible ticks in reaction to organophosphates. Oppenoorth discovered a higher detoxication rate in resistant strains (35). It is likely that detoxication is usually carried on by such enzymes as carboxyesterase or by hydrolysis by fat bodies.

The relationship between metabolism and differential toxicity of malathion in insects and mice has been investigated (22). In trying to account for the selective toxicity of malathion, the authors found degradation of malathion to be much more extensive in mice. Still, there was very little production of malaoxon in mice.

The steps involved in insecticide poisoning are penetration, metabolism, excretion or storage, attacking of target site and physiological consequences of attack on target (22). If a poison affects a susceptible strain but not a resistant one, there must be a difference in at least one of these steps. It has been shown

that penetration has but little effect. The major mechanisms of resistance are degradation of insecticide, substitution of enzymes or increased rate of metabolism.

The detection of resistance can be difficult and misleading. Resistance is not always the proper term for an unexpected lack of kill-effect of an insecticide (19). There are other factors which can exert an influence. Vigor tolerance is not resistance but can cause a change in dose-kill effect. Age of insect, sex, weight, stage of life cycle and method of application must also be considered. The first indication of resistance is an immunity to an expected lethal dose. To test for resistance, data must first be gathered on normal susceptible insects. Then there are sufficiently precise testing methods which can be used to detect resistance, such as, measurements of homogeneity or heterogeneity, or measurement of an increase in LD_{50} (or LC_{50}) (10, 19, 29). There are also methods of measuring the characteristics of a particular resistance. These include studies of factors affecting resistance such temperature, sex and other environmental factors. Biochemical analyses of enzymatic functions in the insect body help to discover mechanisms of resistance. One of the most common methods of studying the nature of resistance is the use of resistant-susceptible crosses and backcrosses. These can give vital genetic information on the causes of resistance. The studies done by Plapp and Hoyer on the housefly used the method of backcrossing to determine the nature of several

resistant genes (20, 38, 39, 40). Busvine used hybrids from crosses between resistant and susceptible strains to prove the dominance of one pair of genes resistant to malathion and incomplete dominance in another pair (7, 8). He also was able to use these methods to determine the number of genes involved and whether or not they were alleles or closely related pairs.

There has been much concern over the development of cross resistance among insects resistant to a particular insecticide. Usually cross resistance is conferred to insects against compounds similar to the original selective agent. A study of houseflies resistant to several organophosphates and carbamates has given a broad picture of cross resistance (3). Using seven strains of houseflies, the author uncovered two new types of resistance mechanisms and demonstrated the existence of at least three cross resistance mechanisms for carbamates.

It is logical that insecticides with similar modes of action might select for resistance mechanisms which would confer cross resistance to one another. Carbamates and organophosphates are both known cholinesterase inhibitors, so it is not surprising that there are several documented cases of cross resistance between them. However, it is surprising that insects selected by DDT would also show an increased tolerance to malathion and other organophosphates because DDT has an axonic effect while organophosphates are enzyme inhibitors (14). A possible explanation would be that the resistant

genes for both compounds are grouped in the same loci of a chromosome. It is also possible that resistant genes for both compounds had not exerted themselves under normal conditions but when selected by another compound the competition for survival becomes less favorable to susceptible flies and more favorable to flies with resistant genes. That is, the unfavorable characteristic with which the organophosphate resistant gene may have been associated now becomes less important under the new selective pressure. Thus, even though only DDT was being used to select the houseflies, organophosphate resistant genes could be inadvertantly selected too.

One means of combating resistance is the use of synergists. In some instances two insecticides or one insecticide and one appropriate compound can be combined to cause a toxic effect which is greater than the arithmetically expected toxicity of the combination.

Pyrethroids were the first group for which a set of synergists was developed. Now both organophosphates and carbamates can be effectively synergized. Some of the better known synergists are piperonyl butoxide, n-propyl isome, sesoxane and sesamine oil and it's derivatives.

The major draw back of synergists is that they also select populations of insects, making it possible for the insects to develop resistance to them. The houseflies selected by Forgash were also selected with WARF Anti-resistant combined with DDT (14). They were able to develop resistance to the combination.

A promising, but as yet not completely practical approach to control is "natural control". This involves attempting to control pest species of insects without the use of insecticides. Whitten mentions the possible use of chromosome rearrangements as a means of introducing lethal genes into a population (49). Another possible means of control would be the introduction of dominant susceptible genes to combat the inheritance of resistant genes. The problems encountered are detecting the resistant strain, analyzing the genes of the strain and developing a workable replacement strain. The proceedures are expensive and time consuming.

Materials and Methods

Drosophila melanogaster was used in this study because it is conveniently obtained and has a short life cycle. Two methods of selective breeding were used. One strain (F strain) was exposed continuously to a medium contaminated with a sublethal concentration of malathion. The other strain (RF strain) was developed by exposure to a medium with a concentration of malathion causing approximately 65 per cent mortality (LC_{65}) for twenty-four hours.

The medium used was Carolina Instant Drosophila Medium. The contaminating solution was 50% malathion (0,0 dimethyl phosphorodithioate) in xylene diluted to desired concentrations with a 90% water, 10% glycerine solution. Controls were tested with 10% glycerine in water and found to be unaffected. In all treatments and tests ten milliliters of solution were added to four grams of dry medium in half pint milk jars.

The constant exposure (F strain) was begun at concentrations of .00008%, .00005% and .00003% malathion. Three groups (A, B, C) were begun as a precaution against losing all flies due to an overdose of insecticide or a fungus attack. Fifteen flies were placed in each jar. Each group was treated in duplicate.

Thirty adults were transferred from each generation, fifteen per jar, within two to four days of emergence. After adults of each generation had produced substantial numbers of visible larvae,

the adults were discarded and the larvae allowed to mature. There was no transferral from one jar to another except when transferring adults to another breeding jar or testing adults for resistance. After transferral of adults to the breeding jars, the remaining adults were tested for resistance except for the F_2 generation. The treatments of the F strain were terminated after emergence of the F_9 generation while the RF treatments were terminated after emergence of the RF_9 generation.

Difficulty was encountered in obtaining survivors in the F_6 and F_7 generations. Therefore, the dose was lowered in the F_6 generation. The C group was combined with the B group after the F_8 generation because it was felt that no purpose was being served by maintaining three groups. The A and B groups were combined after the F_3 generation to provide a plentiful supply for the population studies which followed. Tests after the seventh and eighth generations showed both the A and B groups possessed the same degree of resistance.

The twenty-four hour exposure strain (RF) was developed more rapidly than the F strain, but only after initial problems. This was the first method attempted. Flies were placed in jars the same as in the F group, but the concentration of the solution was much higher, at approximately LC_{90} (.0008% malathion in H_2O). It proved very hard to obtain survivors and those that were obtained were sterile. So after first abandoning this method and beginning the F

strain, this method was tried again with a beginning solution of .0005% (approximate LC_{65}). This proved more successful. Adults were treated for twenty-four hours, then the survivors were transferred to untreated jars and allowed to breed. Adults were removed after a substantial number of larvae were observed. The concentration was increased to .001% for the RF_2 generation and kept at that level. Attempts at increasing the percent solution to .002 resulted in infertile survivors which died after three to four days.

The population studies were done by using different strains for different tests. This was necessary because of the large quantity of flies required. The F_9 strain was used for a test to determine if resistance is lost or diminished shortly after discontinuation of treatment. Two groups of F_9 adults were separated into jars containing untreated medium. Fifteen flies were placed in each jar. As with the selecting procedure, the adults were tested within two to four days of emergence. The adults transferred to breeding jars were removed when visible larvae developed. They were allowed to develop without treatment for five generations (F_9 - F_{14}). The flies of each generation were tested at what was thought to be the approximate LC_{50} (.002% solution). Data was also acquired with solutions of .001% and .00?% but the results were not used in statistical analysis because they are too low and too high respectively to give a good indication of a dose/mortality curve. It was felt that for the purposes of this test the LC_{50} would be an

adequate indicator of resistance.

The RF_6 strain was used to determine the influence of an introduction of wild flies into a resistant population. Twenty flies, ten males and ten virgin females were placed in each jar. Wild flies of each sex were contained at percentages of 0, 20, 40, 60, 80 and 100 and allowed to cross breed with the resistant flies. Tests were run in duplicate and were repeated twice. The adults were again removed after larvae were observed. Offspring were tested with .002% and .001% solutions. Mortality with respect to percentage of wild flies in the cross was measured.

Both the RF_6 and F_9 strains were combined for the test to determine differences in susceptibility between male and female flies. Both wild (control) and resistant flies were tested. Ten males and ten females were placed in each jar and tested for resistance. The resistant flies were treated at .001% and .002%. The wild control flies were treated at .0004% and .0007%. Tests were run in duplicate and repeated twice.

The test for a difference in susceptibility with change of temperature was investigated using F_{12}, F_{13}, and F_{14} flies. The results were pooled. There were ten trials of flies at room temperature (approximately 25°C.) and eleven trials at 30°C. Fifteen flies were placed in each jar and tested for resistance with .002% solution.

DATA

Table 2: Differences in tolerance among wild, F_9 and RF_6 flies.

Approximate LC_{50} for each trial (% malathion)

Trial	F_9	RF_6	Wild
1.	.0016	.0018	.00045
2.	.0018	.0018	.00043
3.	.0022	.0019	.00038
4.	.0025	.0020	.00059
5.	.0019	.0020	.00029
6.	.0018	.0017	.00044
7.	.0020	.0023	.00048
8.	-	-	.00038
9.	-	-	.00061
10.	-	-	.00041
Approx. LC_{50}	.0020%	.0019%	.00045%

The results of t tests indicate the following:

Wild:F_9 was highly significant (0.001).

Wild:RF_6 was highly significant (0.001).

F_9:RF_6 was not significant.

Table 3: Differences in susceptibility between male and female flies.

Number dead / 10

	Resistant flies				Wild flies			
	Male		Female		Male		Female	
% malathion:	.001%	.002%	.001%	.002%	.0004%	.0007%	.0004%	.0007%
	3	7	3	8	4	8	5	9
	3	7	2	7	7	9	6	9
	2	4	2	5	5	8	6	8
	1	8	0	3	4	6	7	8
Total:	9	26	7	23	20	31	24	34

The results of t tests indicate the following:

Wild-Male:Female was not significant.
Resistant-Male:Female was not significant.

Table 4: Comparison of susceptibility at 25°C. and 30°C. at .002% malathion in F_{12}, F_{13}, and F_{14}.

Number dead / 15

Trial	25° C.	30° C.
1.	9	15
2.	7	12
3.	10	14
4.	7	15
5.	4	15
6.	7	14
7.	5	15
8.	9	15
9.	6	15
10.	6	15
11.	-	15
Total:	70	160

The results of a t test indicate:

25° C.:30° C. was significant (0.05).

Table 5: Susceptibility of F_{10} - F_{14} at .002% solution of malathion.

Number dead / 10

Trial	F_{10}	F_{11}	F_{12}	F_{13}	F_{14}
1.	4	2	8	9	6
2.	3	3	3	7	4
3.	3	5	6	6	5
4.	6	2	5	5	3
5.	-	-	4	3	-
6.	-	-	3	4	-
7.	-	-	-	4	-
Total:	16	12	29	38	18

Analysis of variance gave an F value of 1.36 which is not significant.

Table 6: Susceptibility of crosses between wild and resistant flies to .002% malathion.

Number dead / 20

Trial	0% Wild	20% Wild	40% Wild	60% Wild	80% Wild	100% Wild
1	8	7	8	11	18	20
2	9	11	11	17	16	20
3	12	15	9	10	12	20
4	7	12	14	17	20	-
5	-	10	13	13	17	-
6	-	-	10	-	-	-
Total	36	55	65	68	83	60

A one way analysis of variance gave an F value of 9.35 which is highly significant (0.001).

Figure 2: Progressive Tolerance of F and RF Strains at .001% Malathion

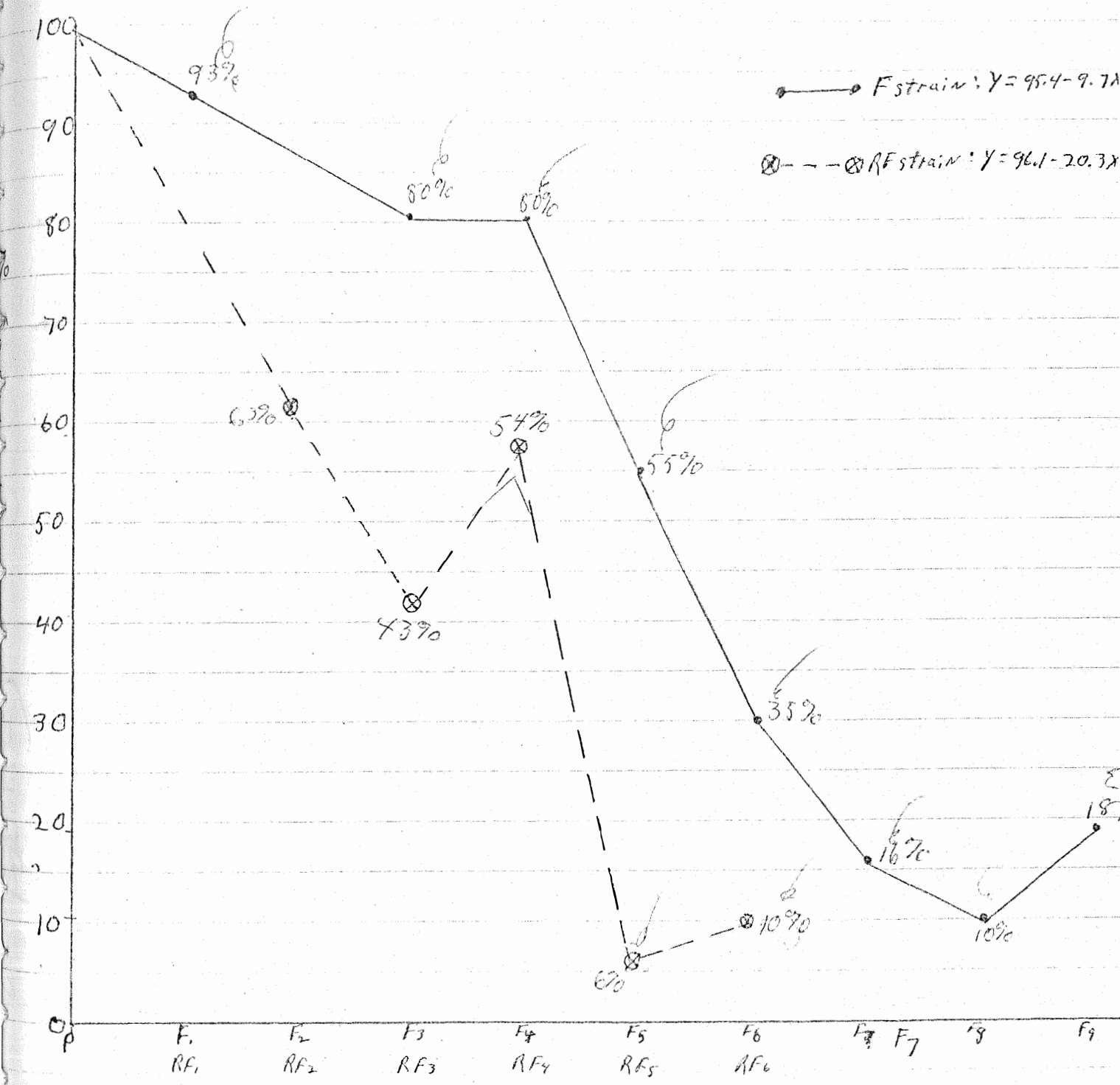
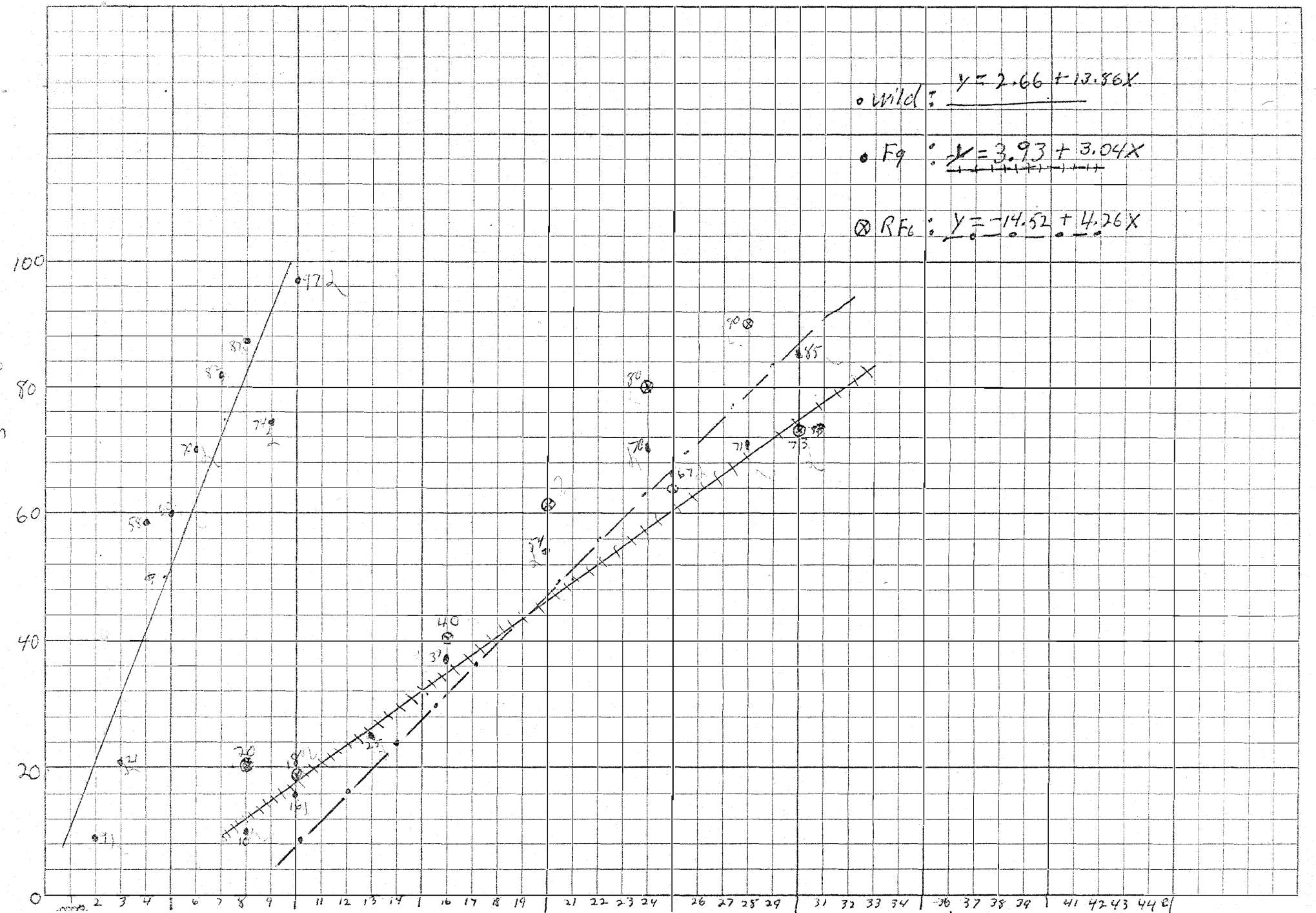


Figure 3/4: Dose/Mortality Regression Lines for F_9, RF_6 and wild flies



Curves

Figure 4: Dose/mortality ~~Regression lines~~ for F_{10}-F_{14}

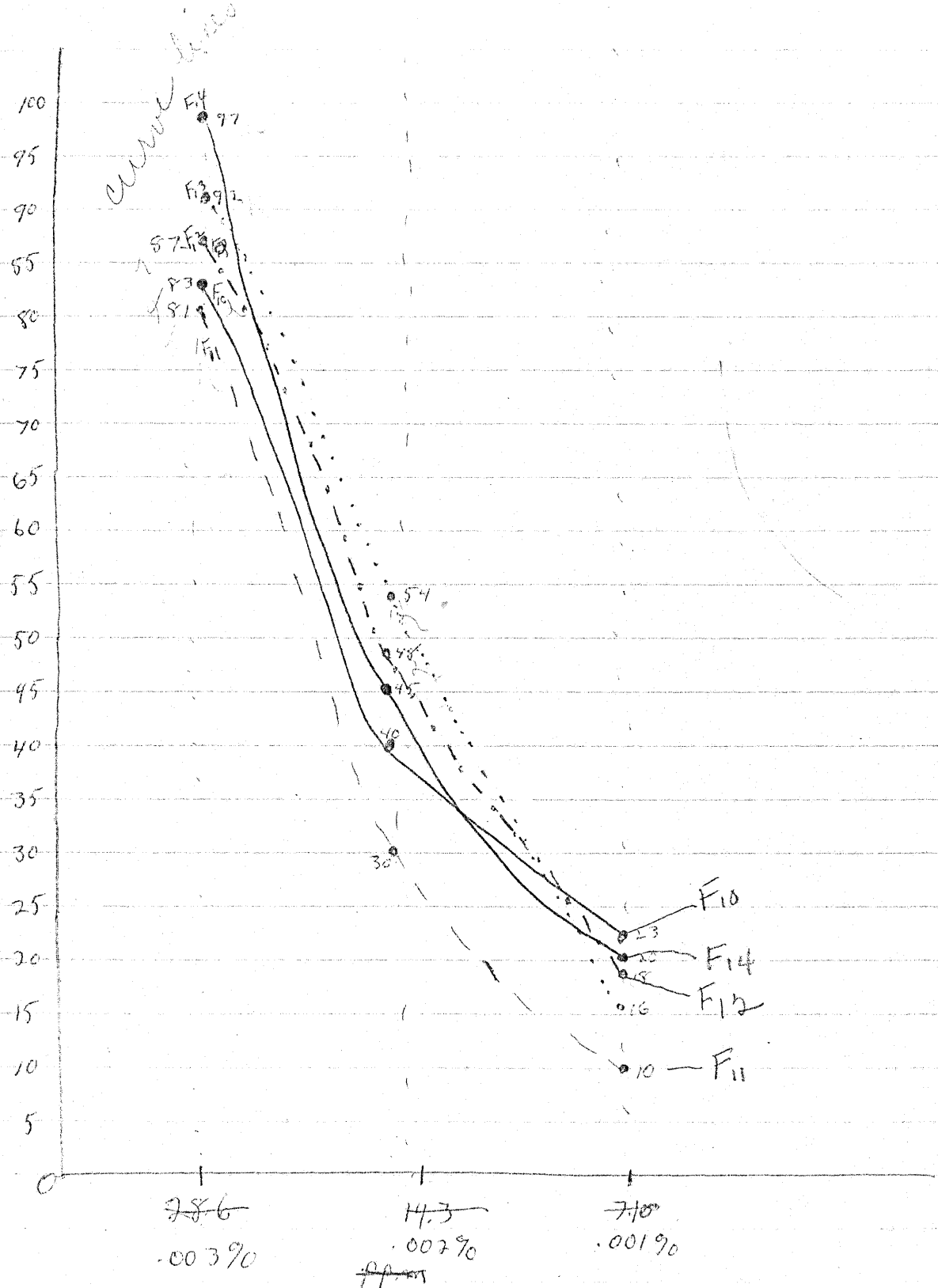
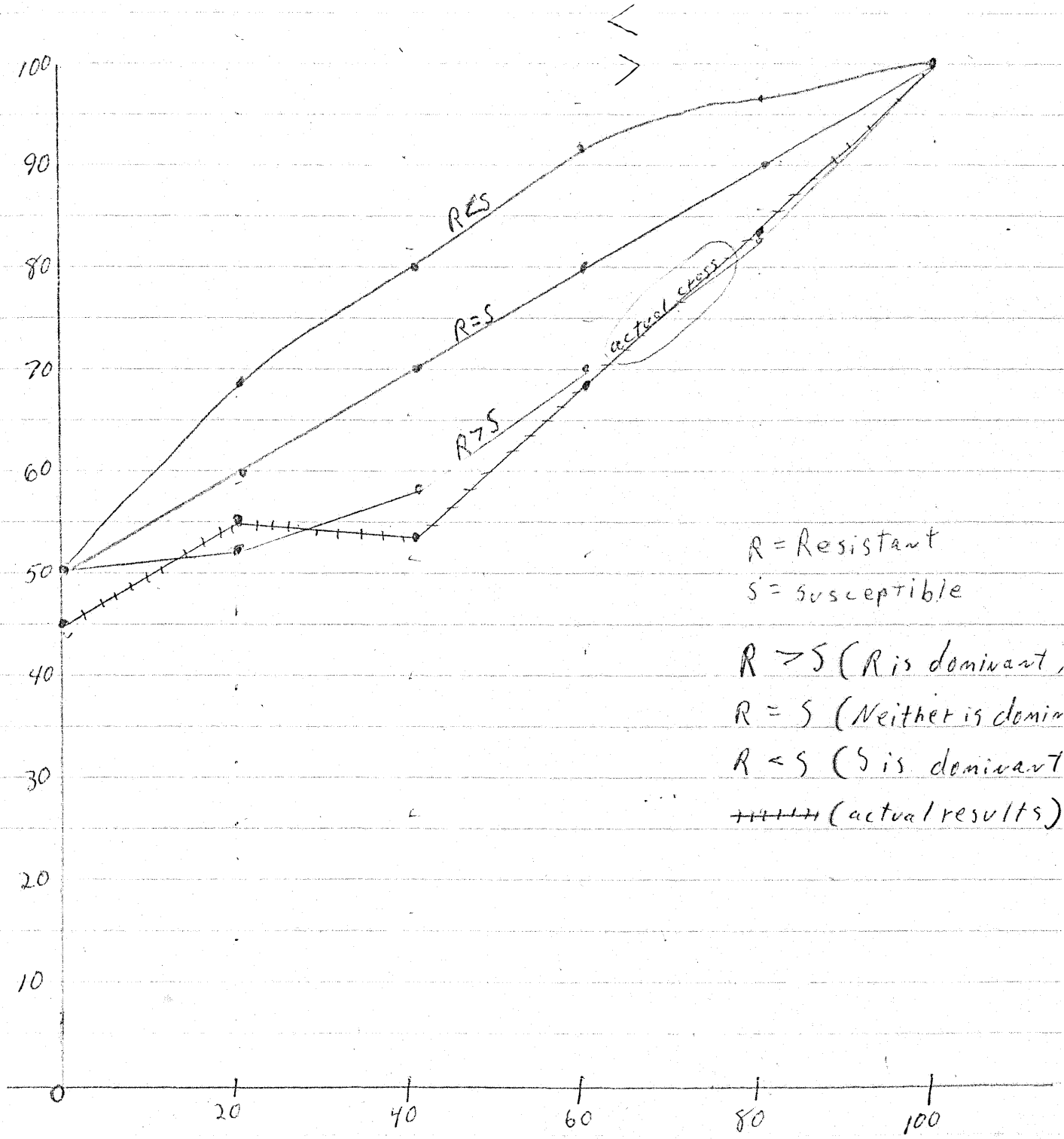


Figure 5:

~~Table~~ Possible and Actual Dose/Mortality Regression Lines for Wild x Resistant

at .002%



Discussion

The flies of both the F_9 and RF_6 strains appear to possess almost equal degrees of resistance. The LC_{50} for the F_9 strain was .002% and for the RF_6 strain .0019%. This compares to an LC_{50} of .00045% for the wild strain representing an increase of 4.4-fold and 4.2-fold respectively. There was no significant difference between the F_9 and RF_6 strains when analyzed by a t test, but there was a significant difference at .001 level of probability between the resistant strains and the wild strain (Table 2).

Hoskins and Gordon state that for a population of insects to be susceptible to selection, they must possess a certain degree of heterogeneity (19). Heterogeneity can be determined by finding the slope of the dose/mortality regression line. A population possessing homogeneity as opposed to heterogeneity will have a very steep slope. That is, there will be a small range of increase in dose level between first kill and total kill. A heterogenous population, having a flatter slope, will have a wider dose range between first and total kill and can, therefore, be selected more easily. Selection for resistant genes creates a tendency toward heterogeneity. Thus, if the insect population is acquiring resistance, the slope of the line will flatten out. Flies not possessing the proper resistant genes will become more homogenous upon selection with a resultant increase in slope. These insects may also increase their LC_{50} or (LD_{50}) but

this increase will be limited and will represent vigor tolerance rather than true resistance.

A comparison (Figure 3) of the slopes of the dose/mortality regression lines for the strains used in these experiments shows that the selected flies are exhibiting an increase in heterogeneity along with the greater than four-fold increase in the LC_{50}.

Examination of Figure 2 shows that in the F strain, increased resistance to malathion came about suddenly while in the RF strain the increase was steady. The F strain had a lag of five generations before there was a significant increase in tolerance, but then the increase was rapid until it leveled off at the same level that the RF strain did. This pattern is not unusual (12). The initial lag indicates the slow accumulation of resistant genes. The build up is slow up to a critical point and then the frequency of the resistant gene increases geometrically, resulting in a rapid increase in the level of resistance. The pressure on the RF strain is great enough to select a sufficient number of resistant genes in the first generation for resistance to show immediately. It is worth noting, however, that the original wild flies were homogenic enough to cause difficulty in selection, but not enough to prevent it. The first attempt at selecting with an LC_{90} was a failure because the survivors had been weakened enough to prevent viability. When the concentration was lowered to an approximate LC_{65}, selection was successful.

The leveling off of resistance at 4.4-fold and 4.2-fold may represent either the upper limit of resistance or a plateau of resistance. Since both strains leveled off at the same point, it appears quite possible that they have reached the upper limit of resistance to malathion. This is a comparatively low level of resistance which indicates the action of only one gene (40). Most species with more than one gene influencing the level of resistance have a larger degree of resistance. The fact that all attempts at increasing the pressure on both strains met with failure, either death or sterility, indicates that this is not a plateau of resistance and there is only one gene causing resistance. It is possible, however, that exposure to another chemical could activate another resistance mechanism and in turn cause an increase in resistance to malathion.

Another factor suggesting resistance rather than vigor tolerance is that after five generations without treatment there was no significant decrease in the level of resistance of the F strain (Table 5 and Figure 4). The graph (Figure 4) illustrates quite well the fact that there is very little difference in the dose/mortality lines between F_{10} and F_{14}. Insects exhibiting vigor tolerance would be expected to lose their tolerance shortly after cessation of exposure to the poison because their tolerance would be physiological rather than genetic. Since true resistance is genetic it would be expected that it would be passed from generation to generation in-

definitely. The only factor that would reduce resistance would be a reduction in frequency of the resistant gene in the population. This would only happen if the gene was either recessive or associated with some characteristic unfavorable for survival. The latter factor might be more in evidence in the field where environmental conditions are more extreme, but in the laboratory where conditions are relatively uniform and competition is not a major factor it apparently has little effect (12). This indicates that the gene for resistance to malathion is not recessive.

It is also unlikely that the resistant gene is a mutation. Very few cases of resistance due to selection are a result of mutations. The F strain did produce one apparent mutation. One group from the F_3 generation was slow in emerging and when they did emerge they had dark coloring. However, tests indicated they did not possess any degree of resistance whatever. Apparently the mutation was a response to some other environmental factor such as lack of moisture. Of course, it is possible that rather than a mutation it was the result of selection of a gene already present, much the same as selection for the resistant gene. Whatever the case, the trait disappeared immediately with the next generation and was not reproducible.

While attempting to select flies at the LC_{90} level, it was noticed that often of the two or three survivors there would be no females. So it was decided to test for a difference in susceptibility between male and female flies. T tests were run on both wild and

resistant flies. The results indicate that there was no significant difference in susceptibility between either the wild or resistant males and females (Table 3). This eliminates the theory that the initial difficulties in starting the twenty-four hour selection series was due to greater susceptibility of female flies. It also indicates that resistance is not sex-linked as is the case with several species (16, 49).

There have been varying reports of differences in susceptibility at different temperatures (16, 27). Flies treated at .002% solution of malathion at room temperature (approximately 25°C.) and at 30°C. were analyzed by a t test (Table 4). The results clearly indicate a large increase in susceptibility with an increase in temperature. At 30°C. there was almost total kill after twenty-four hours while at room temperature there was less than 50% kill. Assuming that cholinesterase inhibition is the mode of action of malathion in these flies, as has been shown to be the case in other insects, it is possible that resistance is due to either an increase in cholinesterase production or, more likely, the production of another enzyme such as carboxyesterase which can either take the place of cholinesterase, detoxify malathion or inhibit the metabolism of malathion to malaoxon (34). Matsumara and Brown found that carboxyesterase production in Culex tarsalis was more heat labile than the carboxyesterase of the susceptible strain (27). If a mechanism similar to this was the cause of resistance in the F and RF strains of

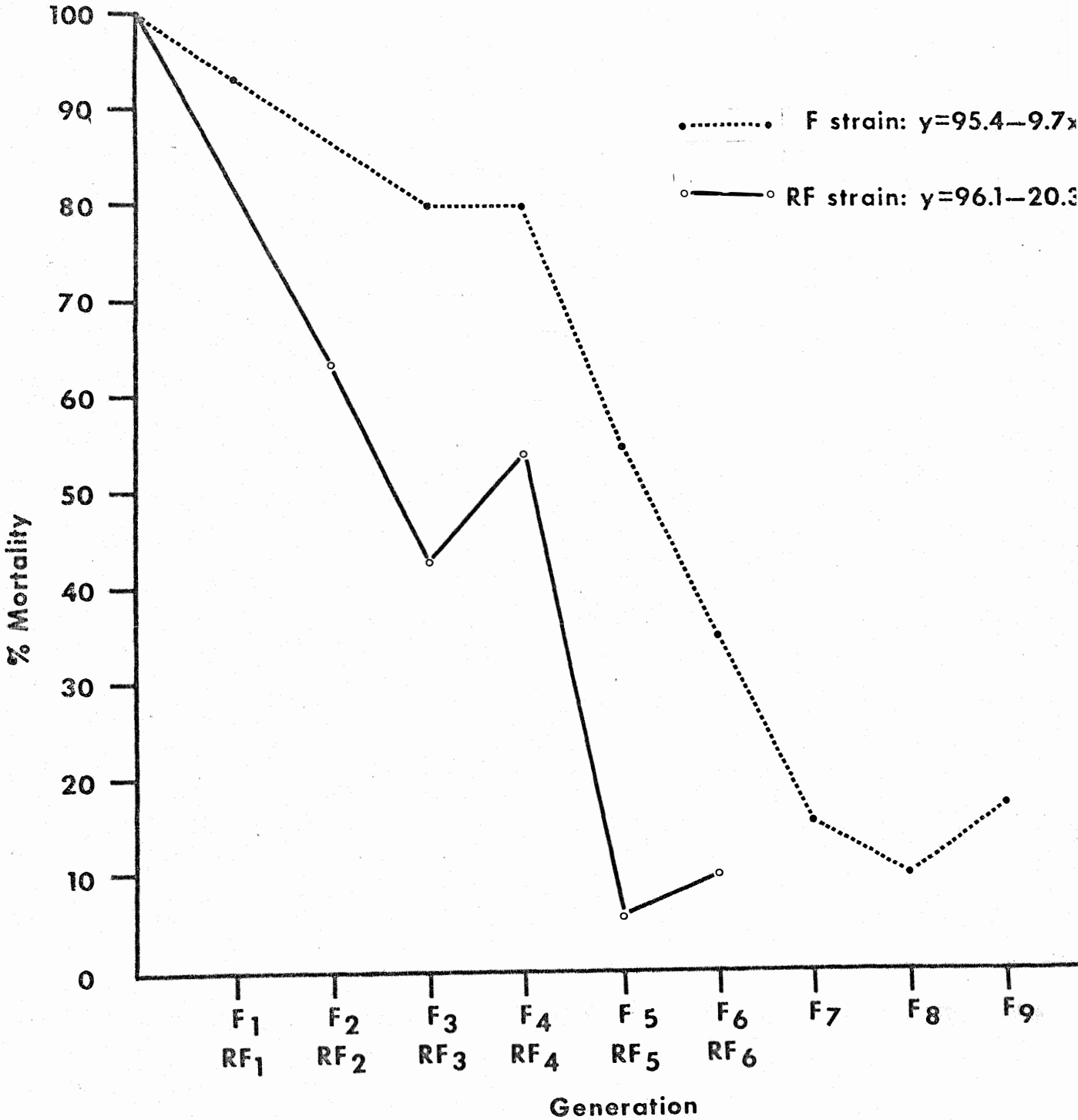


Figure 2: Progressive tolerance of F and RF strains at .001% Malathio

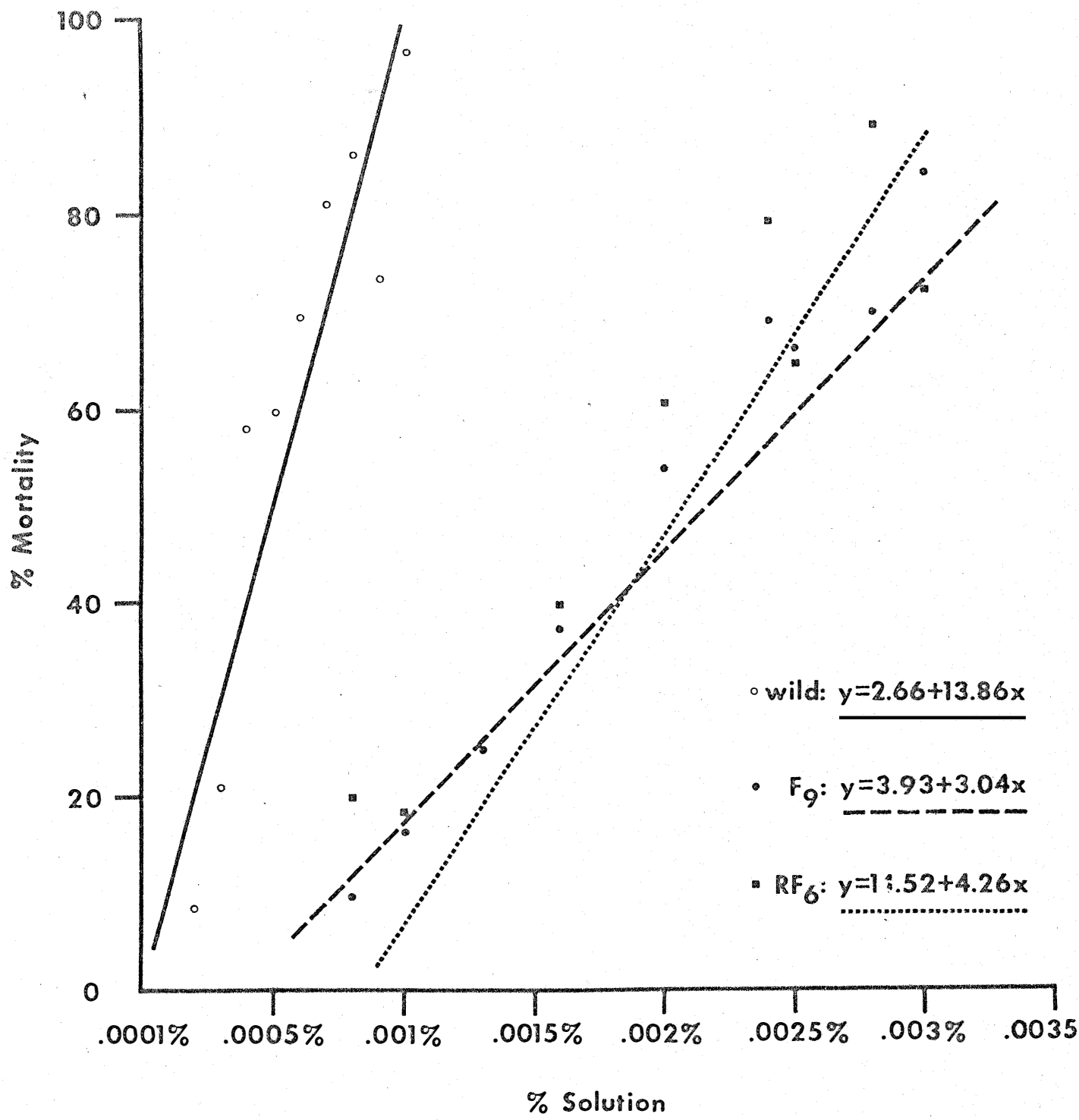


Figure 3: Dose/mortality regression lines for F_9, RF_6 and wild flies

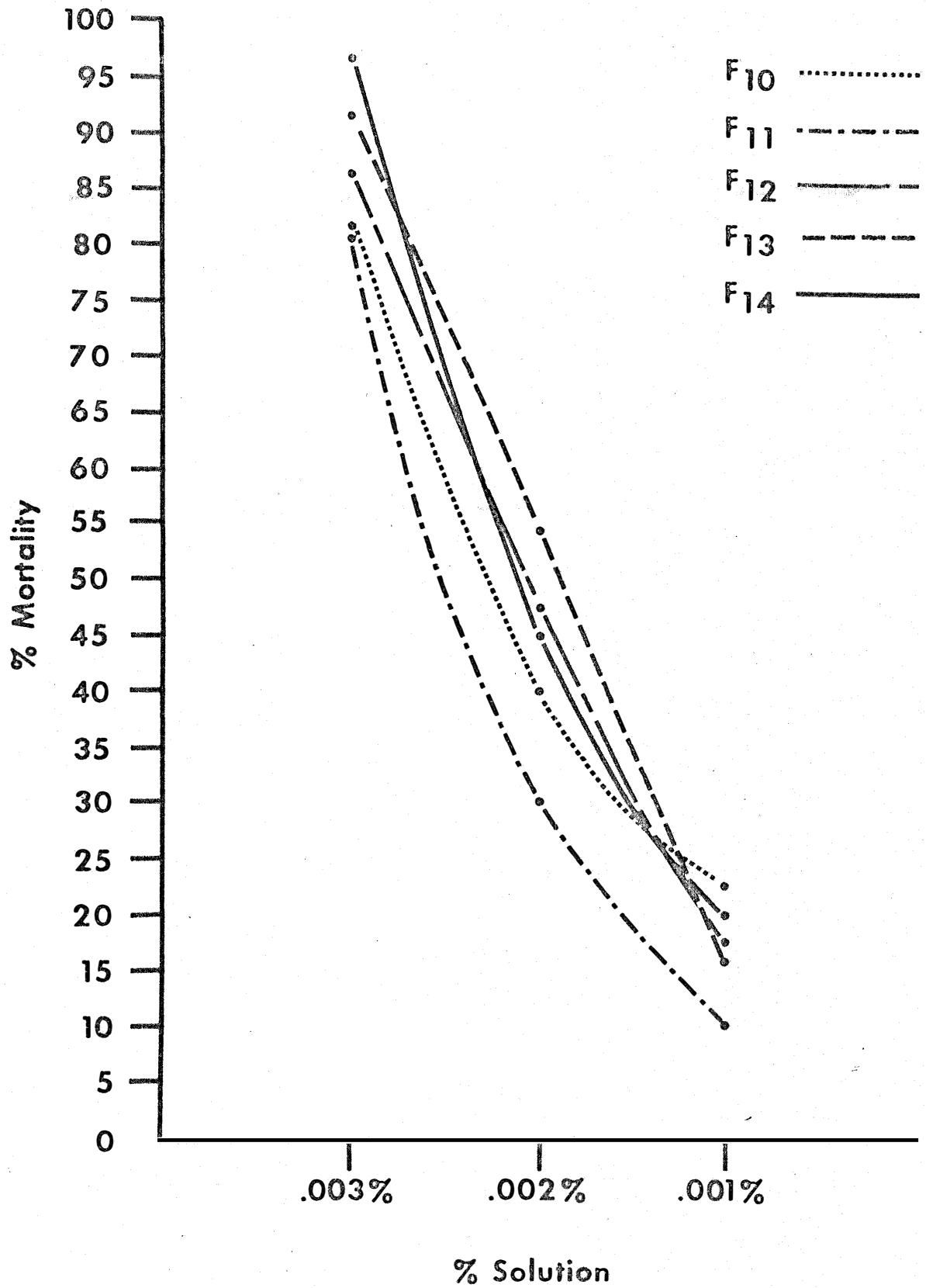


Figure 4: Dose/mortality curves for F_{10}–F_{14}

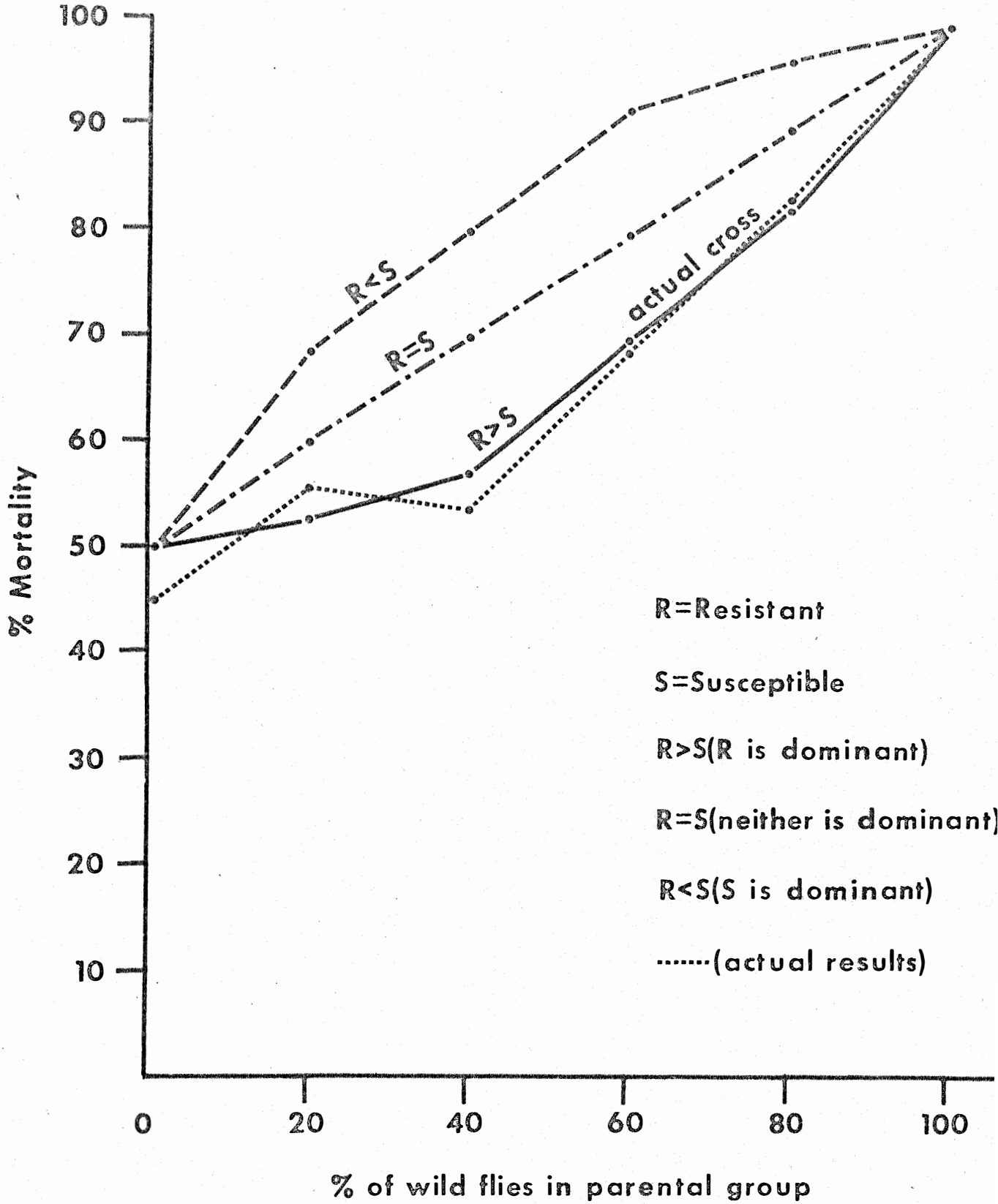


Figure 5: Possible and actual dose/mortality regression lin
for wild x resistant cross at .002%

D. melanogaster used in these experiments it would follow that an increase in temperature would destroy the defense mechanism.

Table 6 shows the results of an analysis of variance of the susceptibility to .002% solution of malathion (approximate LC_{50}) of the F_1 offspring from a cross between wild and RF$_6$ flies. The flies showed a loss of tolerance not directly proportional to the percentage of wild flies used in the cross. This indicates that there is not equal transmission of resistance and susceptibility Nguy and Busvine found that malathion resistance in the housefly, Musca domestica, is due to a single dominant gene (30). Busvine also found that hybrids between susceptible and resistant body lice were intermediate in resistance, thus not displaying either dominance or recessiveness (9). Figure 5 shows the mortality of the F_1 hybrids plotted against the percentage of wild flies used in the cross. It also shows the projected mortalities plotted against percentage of wild flies under assumed conditions of one dominant resistant gene, one recessive resistant gene and intermediate resistance and susceptibility. The LC_{50} is assumed to be .002% malathion. The graph shows that the actual results match very closely the projected results of a monofactorially dominant resistant gene. Dominance of the resistant gene would also explain the persistence of resistance in the absence of treatment and unfavorable environmental conditions.

Conclusions

It can be seen that the homogeneity of a population of insects has a great influence on the ability of such a population to develop resistance. This, of course, influences the ability of man to control them. A highly homogenous group of insects most likely will not develop resistance because a selective dose will be difficult to achieve and therefore will not pose any long range control problems. However, a heterogenous population of insects will develop resistance quite readily and continue to develop resistance to each new insecticide as it is applied.

Researchers have had difficulty in selecting for resistance (11, 21). Kalina used DDT to select D. melanogaster and although he obtained some survivors, they were unable to produce viable offspring (21). He stated that he felt the DDT was being stored in the fat of the larvae and then being consumed by the pupae, thus killing them. Cole treated body lice with malathion for several generations and found no significant increase in resistance (11). In light of the difficulty encountered in this study in first attempting to select D. melanogaster at an LC it would appear that one of the problems in artificial selection for resistance is the determination of the proper dose level. An analysis of the dose/mortality curve of a wild, susceptible population will show that the dose must be neither too selective nor underselective. The difficulty in select-

ing at the LC_{90} was a matter of overselecting while at the LC_{65} selection was effective. Care must be taken not to reduce the genetic variation too greatly. The initial lag in onset of resistance in the F strain may have been due to either initial underselection or the normal lag due to a slow buildup of resistant genes in the population. Figure 2 shows that resistance did not significantly increase in the F strain until the higher doses were used. At the lower doses acclimatization was occuring as indicated by the fact that untreated wild flies could not have survived for one entire life cycle at such high concentrations. During this acclimatization there was a gradual selection of resistant genes which progressed geometrically with selection by the higher doses.

Malathion is an organophosphate insecticide. As such, it is mainly a cholinesterase inhibitor. The results of the experiments done in this study indicate resistance of D. melanogaster to malathion is due to monofactorial inheritance of a dominant resistant gene which enables the insect to produce a heat susceptible enzyme which counteracts the inhibiting effects of malathion. Neither resistance nor susceptibility to malathion is sex linked in D. melanogaster. The exact mechanism of this resistance is not known, but it seems likely that it is similar to the mechanism of other malathion resistant insects (7, 10, 20, 27).

Summary

The critical question of this research was whether or not Drosophila melanogaster possessed the genetic mechanism to enable it to develop resistance to malathion by selective pressure. Two methods of selection were used to develop separately, two resistant strains. One method was continuous exposure of a group of flies to a sublethal, but increasing concentration of malathion. The malathion was mixed with water and added to dry instant Drosophila media. The second method of selection was treatment for twenty-four hours with a lethal concentration that killed 65% of the flies (LC_{65}). This concentration was used for one generation then doubled to what would have been an LC_{95} for an untreated group of flies and kept at that level for the remainder of treatment.

Both strains showed similar dose/mortality regression curves and almost identical LC_{50} values. It was determined that they exhibited true resistance rather than vigor tolerance. The dose mortality curves indicate slightly more heterogeneity than the normal susceptible strain, while vigor tolerance would have shown more homogeneity in the form of a steep dose/mortality curve.

The failure of the resistant strain to lose resistance after cessation of treatment for five generations also indicates true resistance. True resistance is genetically passed from generation to generation. The only way for the flies to maintain resistance

to the insecticide is to have the genes passed on from parents to offspring. Without resistant genes present the resistance would fade away shortly after cessation of treatment.

It is necessary to have a susceptible population with a certain amount of heterogeneity in order to select for resistance. If the dose/mortality curve is too steep it would be impossible to select the stronger flies from the weaker flies. This is what caused difficulty in beginning the twenty-four hour treatment strain. However, it was possible to select by using a lower dose.

After an initial lag in the F strain resistance was acquired rapidly in both strains. After the resistance progressed to an almost identical degree in both strains, however, it leveled off. It was not possible to increase the resistance further by increasing the dose. This indicates the flies have reached the uppermost limit of their resistance. Since it is a comparatively low degree of resistance (four to five-fold), it is probable that it is produced by the influence of only one gene.

Based on the assumption that there was, in fact, only one resistant gene and that it was dominant, a projected mortality curve was established for a cross of wild and resistant flies at differing percentages. This projected curve fit very closely with the values actually obtained indicating the existence of monofactorial dominant resistance.

A study done to detect a difference in susceptibility between

male and female flies showed no significant difference between the males and females of either resistant or wild flies. This indicates that resistance is not sex linked as is the case with some other insects.

The drastic difference in susceptibility of the F strain upon the raising of the temperature 5° C. indicates the mechanism of this resistance is dependent upon the production of an enzyme that is sensitive to increased temperature.

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