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Xenobiotic Detoxication Potential and Drug Induced Changes in Carbaryl Toxicity in the Alfalfa Leafcutter Bee, *Megachile rotundata* (Febricius)

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XENOBIOTIC DETOXICATION POTENTIAL AND DRUG INDUCED CHANGES IN

CARBARYL TOXICITY IN THE ALFALFA LEAFCUTTER BEE,

MEGACHILE ROTUNDATA (FEBRICIUS)

by

Richard M. Lee

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Zoology

(Entomology)

Approved:

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Major Professor   Committee Member

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Committee Member   Committee Member

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Logan, Utah

1972

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Richard M. Lee

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ABSTRACT

Xenobiotic Detoxication Potential and Drug Induced Changes

in Carbaryl Toxicity in the Alfalfa Leafcutter Bee,

Megachile rotundata (Fabricius)

by

Richard M. Lee

Utah State University, 1972

Major Professor: Dr. William A. Brindley
Department: Zoology

The alfalfa leafcutter bee, Megachile rotundata, one of the most important pollinators of alfalfa, generally became more susceptible to carbaryl as the adults aged. LD_{50} values for carbaryl toxicity were 240, 166, 109, and 51 μg carbaryl/gram for the 1, 2, 3, and 4 days-old adult male bees. For the female bees, these were 245, 551, 289, and 262 μg carbaryl/gram. Lipid content and microsomal enzyme activity (measured by EPN detoxication in vitro) decreased in both sexes as the bees aged.

Synergist ratios, defined as the ratio of the LD_{50} of carbaryl alone to the LD_{50} of carbaryl applied after the synergist piperonyl butoxide, were measured. These did not correlate with either decreasing LD_{50} values or decreasing EPN detoxication in the males being 12, 21, 42, and 53 for the 1, 2, 3, and 4-day-old bees. In female bees the synergist ratio corresponded more closely being 23, 49, 11, and 15 as the females

aged from 1 to 4 days. Published literature has suggested that such synergist ratios are usable as in vivo estimates of detoxication ability, higher values indicating more detoxication potential, but there may be difficulties in the proper estimation of the synergist ratio.

The use of enzyme-inducing drugs for protection of the bees from carbaryl poisoning was studied. Among the three drugs used, only chlorcyclizine increased the LD_{50} value (to about two fold) in the 4-day-old male. Aminopyrine increased the male's susceptibility to carbaryl while phenobarbital had no effect at all. None of the drugs significantly changed the female's susceptibility to carbaryl except aminopyrine which lowered the LD_{50} from about 262 to 75 $\mu g/gr$. Studies with in vitro EPN detoxication indicated that all three drugs caused increases in microsomal enzyme activity up to four or five fold and decreases in lipid content of about 20 to 30%.

(88 pages)

INTRODUCTION

Alfalfa is Utah's leading legume hay crop and the production of alfalfa seed is an important agricultural industry in the intermountain states. Its blossoms require tripping and cross pollination by insects for high yields of seed. An introduced wild bee, the alfalfa leafcutter bee, <u>Megachile</u> <u>rotundata</u> (Fabricius), has become one of the most important pollinators of alfalfa (Bohart, 1972) because of the low efficiency of the honey bee <u>Apis</u> <u>mellifera</u> in pollinating alfalfa. The alfalfa leafcutter bee is more valuable than many other wild pollinating insects because it is gregarious, will accept artificial nesting sites, and is therefore easily managed. It has been said that the alfalfa leafcutter bee is more susceptible to the commonly used insecticides than the honey bee (Johansen, Jaycox, and Hutt, 1963; Johansen and Eves, 1967). As a result, the protection of pollinators from poisoning continues to be a major concern of alfalfa seed producers.

This study was designed to make a laboratory test of the feasibility of developing a temporary insecticide resistance or tolerance in alfalfa leaf-cutter bees by means of enzyme induction through exposure of the bees to sublethal doses of drugs. This is not to be confused with the treatment of the insects with anticholinesterase antagonists such as atropine and 2-PAM--a technique found not to be effective with the honey bee (Barker, 1970).

An important part of the study was to determine the inherent sensitivity of the bees to an insecticide relative to age and sex. Many authors have shown that proper separating of insects by age and sex can show important differences in susceptibility (Schonbrod et al., 1965; Moradeshaghi, 1968; Benke and Wilkinson, 1971 a and b). With this information properly in hand, then the effects of the selected drugs upon the insecticide tolerance could be accurately determined.

In addition, several authors, including Ahmad and Brindley (1971) and Turnquist (1971) have shown that in vitro assays of microsomal detoxication of the insecticide EPN can be correlated with changing enzyme levels in insects and the effects of the drugs on insecticide tolerance and microsomal activity. An additional indicator of microsomal enzyme activity was measured by synergist ratios for carbaryl and piperonyl butoxide with a procedure modified from that suggested by Brattsten and Metcalf (1970). As the lipid and fat content of insects have also been implicated in relative differences in the penetration of and tolerance to lipophilic insecticides (Moore et al., 1967; Winteringham, 1969; Barnett and Berger, 1970) the lipid content of the leafcutter bee relative to age, sex, and drug exposure were also measured.

REVIEW OF LITERATURE

In recent years, studies of microsomal enzyme induction have
played a great role in the investigation of xenobiotic detoxication (Conney.
1967; Hodgson, 1968). There is a great deal of information concerning
various aspects of drug metabolism and microsomal enzyme induction
caused by xenobiotics (Brodie, Gillette, and LaDu, 1958; Conney and
Burns, 1962; Gillette, 1966; Conney, 1967; Hodgson, 1968; Kuntzman,
1969). It is believed that the microsomal enzyme system is multifunctional
and can catalyze various reactions including N-dealkylation, deamination,
aromatic hydroxylation, ether cleavage, nitro group reduction, azo cleav-
age, alkyl chain oxidation, O-dealkylation, desulfuration, epoxidation,
and the formation of glucuronides (Brodie, Gillette, and LaDu, 1958;
O'Brien, 1967). In most cases, these chemical reactions require oxygen
and reduced NADP both in vitro and in vivo (O'Brien, 1967).

The multifunctional oxidative enzymes responsible for the biotrans-
formation of xenobiotics are located in the smooth endoplasmic reticulum
fragment of the liver microsomes (Remmer and Merker, 1963; Gillette,
1966). It is also reported to be present in microsomes derived from
endoplasmic reticulum in insects (Cassidy, Smith and Hodgson, 1969;
Brindley and Dahm, 1970).

Enzyme induction in insects

As a result of extensive applications of insecticides, insects are constantly exposed to sublethal dosages of one or more insecticides during their life spans. Enzyme induction may then occur and the activities of detoxifying enzymes will be enhanced, hence augmenting the development of resistance. Therefore it is important to investigate the nature and the extent to which microsomal enzyme induction can occur in insects (Plapp and Casida, 1970).

Although there has been a great amount of study done on drug-induced insecticide and drug metabolism in mammals at the subcellular level (Terriere, 1968), fewer studies utilizing insects have been reported. Most of the early and detailed studies on microsomal enzyme induction in insects were conducted by Agosin and his co-workers at the University of Chile.

Agosin et al. , (1961) reported that DDT was metabolized to a kelthane [4,4'-dichloro-d-(trichloromethyl.)-benzhydrol] -like product by the microsomal enzyme system in the German cockroach, Blattella germanica L. This enzyme system essentially required NADPH (reduced nicotinamide-adenine dinucleotide), molecular oxygen and magnesium ions for activity. The same enzyme system also existed in several strains of the housefly, Musca domestica L. and the American cockroach, Periplaneta americana L.

Agosin et al. (1963) have studied the effect of DDT on the rate of ^{14}C-glucose metabolism and the levels of NADP in Triatoma infestans nymphs. The results indicated that DDT significantly increased the total

content of NADP and remarkably stimulated the pentose phosphate pathway.
The authors suggested that DDT was detoxified in $\underline{T}$. infestans, at least in
part, by a mechanism requiring NADPH.

Agosin, Ilivicky and Litvak (1967) also found that NAD kinase was
induced by DDT in $\underline{T}$. infestans. DDT was found to increase the incorporation
of ^{14}C-labeled leucine into the enzyme and the amount of enzyme protein.
NAD kinase induction was suggested to be related to a DDT-detoxication
mechanism of the insects by increasing the required cofactor supply of
NADP as found previously (Agosin et al., 1963).

In addition, Agosin and Dinamarca (1963) also noted that DDT
increased the total NADP content significantly in nymphs without chang-
ing the ratio of NADP/NADPH, while in adult males, neither the total NADP
level nor the ratio of NADP/NADPH was changed.

Later Ilivicky, Dinamarca and Agosin (1964) also studied the activity
of NAD-kinase of $\underline{T}$. infestans nymphs upon treatment with DDT and other
related compounds. DDT and some other analogs were found to increase
NAD-kinase activity while others were inhibitory. The authors claimed this
was an inductive effect rather than enzyme activation, since the intact organism
was required for increasing the enzyme level. They also speculated that DDT
might induce a special pattern of enzyme activity in $\underline{T}$. infestans nymphs. Such
an effect might change the physiological capacities of insects relative to DDT-
resistance.

To be consistant with the above interpretation, Agosin, Aravena and Neghme (1965) showed that DDT also stimulated protein synthesis in vivo by increasing the rate of DL-leucine-1-C^{14} incorporation into total protein. The results indicated that protein biosynthesis was stimulated by DDT, and increased only in nymphal T. infestans, which are very tolerant to DDT, and not in adult specimens, which are very susceptible. Similar results were obtained in previous work which demonstrated that DDT also stimulated an increase of NAD kinase in T. infestans nymphs.

Morello (1964) observed that T. infestans nymphs were more resistant to DDT with pretreatment of the microsomal enzyme inducer, 3-methylcholanthrene. More water soluble DDT metabolites were produced by the induced nymphs than by control nymphs or as by insects treated with microsomal enzyme inhibitors such as iproniazid and SKF 525-A.

Agosin et al. (1969), also found that DDT was metabolized to a Kelthane-like and phenolic type compound in vitro by microsomal preparations from T. infestans fifth-instar larval. The reactions required either NADPH or NADH. Pretreatment of phenobarbital resulted in an increase in both metabolites, but there were different induction kinetics for each. From the above observations, the authors suggested that possibly two microsomal enzyme systems were involved in DDT metabolism in T. infestans nymphs.

Tsukamoto, Shrivastava and Casida (1968) reported that the fifth chromosome in the housefly was active in conferring both high carbamate resistance and microsomal enzyme activity which is responsible for carbamate

detoxication by ring hydroxylation and attack on N-methyl and O-alkyl groups.

The third chromosome was secondary in importance. The second chromosome,

however, was also responsible for resistance but not related to the enzymic

detoxication activity. The investigators postulated that the 5th chromosome

gene(s) are controlling the microsome-NADPH system which is responsible

for carbamate detoxication. Thus these genes are important in the mechanism

of resistance to carbamate insecticides and may be also for other types of in-

secticides.

Plapp and Casida (1969) have studied nine insecticide chemicals which

were susceptible to metabolism by NADPH - dependent oxidative processes in

the housefly. These insecticides were found to be metabolized faster by re-

sistant strains than susceptible ones. The authors also observed that gene (s)

on autosome 2 in one resistant strain and on autosome 5 in another resistant

strain were controlling microsomal oxidase activities which were responsible

for detoxication of certain chlorinated hydrocarbon, pyrethroid, organophos-

phate, and methyl carbamate insecticides. They suggested that high ability to

oxidize insecticide chemicals is responsible for only a portion of the resist-

ance level found in the strains tested. The factor of absorption in addition to

oxidase activity, may play a role in resistance. This both establishes a

genetic basis, for the high oxidase activity in resistant housefly strains and

introduces other factors for consideration.

Schonbrod et al. (1968) measured two microsomal oxidative enzyme

activities by means of aldrin epoxidation and hydroxylation in fourteen strains

of houseflies selected for resistance with different insecticides. Their experiments were designed to investigate correlations between specific enzyme activities and resistance to specific insecticides or between a generalized enzyme increase and resistance to many insecticides. They concluded that there is no simple relation between insecticide resistance and microsomal enzyme activity. The lack of cross resistance between the carbamate resistant and naphthalene resistant strains indicated the involvement of more than one enzyme.

Gil et al. (1968) also observed that microsomal enzyme induction occurred in two strains of flies upon pretreatment with DDT. This enzyme induction by DDT was paralled by an increased rate of incorporation of the ^{14}C-labeled precursor, uracil, into total RNA. These authors suggested that cross resistance between DDT and pyrethrum may be related to microsomal enzyme induction.

In addition Plapp and Casida (1970) demonstrated that a strain of housefly resistant to chlorinated hydrocarbons, which was fed high dietary levels of DDT or dieldrin, showed a significantly higher ability to metabolize the insecticides aldrin, allethrin, propoxur, DDT, and diazinon. The increases in metabolism ranged from 3 to 37% relative to the control values. The authors believed that the resultant rapid increase in the metabolism of the insecticides was an inducible phenomenon in the houseflies.

It was found in our laboratory that microsomal enzyme induction also occurs in the American cockroach, Periplaneta americana, by administration of sublethal doses of chlorcyclizine. Turnquist (1971) observed an

increase in the concentration of cytochrome P-450, and the activity of NADPH-neotretrazolium reductase and detoxication of EPN, and p-nitroanisole. Ahmad and Brindley (1969) demonstrated that chlorcyclizine and phenobarbital induced EPN detoxication, p-nitroanisole O-demethylation, and NADPH-neo-tetrazolium (NT) reductase activity in wax moth larvae.

Ahmad and Brindley (1969) have also studied modification of parathion toxicity to wax moth larvae by chlorcyclizine, aminopyrine, or phenobarbital. They found that pretreatment with sublethal doses of chlorcyclizine, amino-pyrine or phenobarbital in the larval medium reduced parathion toxicity. Chlorcyclizine decrease parathion toxicity to 32, 20, 4, and 10% mortality when fed for 2,4,8, and 10 days, respectively. Phenobarbital was somewhat more effective than aminopyrine and also offered protection against parathion if the time of exposure was increased. The authors suggested that the protective effect was neither immediate nor permanent and that it depended upon the dosage and length of drug pretreatment.

Moradeshaghi (1968) and Brindley (unpublished data) have treated alkali bees Nomia melanderi Cockerell, with chlorcyclizine and SKF 525-A prior to their treatment with parathion. Although chlorcyclizine consistently increased the parathion toxicity, SKF 525-A had variable effects.

Kuhr (1970) reported that in vitro metabolism of carbaryl in cabbage loopers, Trichoplusia ni (Hubner), was most rapid in the fat body and gut tissues, and these tissues were found to be most active in the last larval stage and prepupal forms. Homogenates of fat body and gut required oxygen and

NADPH as cofactors and the enzyme activity was inhibited by carbon monoxide and methylenedioxyphenyl synergists. The major metabolite formed by the fat body and gut was hydroxymethyl carbaryl.

In comparative studies of carbaryl metabolism among DDT-resistant, DDT-susceptible, and parathion resistant strains of the cabbage looper, Kuhr (1971) observed that a DDT-resistant strain had almost twice as much microsomal oxidative enzyme activity in both gut and fat body compared with those of a susceptible strain. An 8-fold increase of enzyme activity was discovered in the fat body and a 4-fold increase was discovered in the gut of parathion resistant strain as compared to a DDT-susceptible strain.

There were also differences in the levels of cytochrome P-450 in the gut and fat body from different strains of the looper. These differences closely paralled differences in enzyme activity from the same tissues (Kuhr, 1971).

As to sex differences, guts from male and female fifth instar cabbage looper larvae had almost the same ability to metabolize carbaryl. Fat body from females, however, had a higher ability than the males (Kuhr, 1971).

Nevertheless some investigators have failed to demonstrate induction of multifunction oxidative enzymes in insects.

Meksongsee, Yang and Guthrie (1967) reported that there was no significant decrease in the toxicity of carbamates in the housefly with pretreatment of microsomal enzyme inducers such as phenobarbital and DDT. However,

carbamate toxicity increased when phenobarbital was incorporated in the food of susceptible houseflies.

Similar results were obtained by Chakraborty and Smith (1967). The microsomal enzyme inducers, phenobarbitone (phenobarbital), and 3,4-benzopyrene failed to reduce the toxicity of carbamates to locusts and houseflies.

Synergism

Synergism is a particular type of joint action which produces a substantially more than additive toxic or pharmacolgical action of two substances used together. As related to insecticide action, synergism has been applied only when the synergist itself is not toxic at the dosage employed and when the mixture is appreciably more toxic than the other component alone.

In addition to the practical and economical importance of applications of synergist in insect control, basic investigations of synergism and synergist have also resulted in better understanding of detoxication mechanisms and biochemical processes of insect resistance to insecticide. Recent investigations have shown that virtually all examples of pronounced synergism are related to interference by the synergist in the in vivo metabolic detoxication of the insecticide (Metcalf, 1967).

Recently Brattsten and Metcalf (1970) have studied the distribution of mixed function oxidase detoxication activity in insects. The synergistic ratio of carbaryl with piperonyl butoxide (ratio of LD_{50} of carbaryl alone to

LD_{50} of carbaryl + synergist) was evaluated for fifty-four species of insects from eight orders and thirty-seven families. They claimed that the various values of carbaryl susceptibility and synergistic ratio were due to differences existing in the activities of microsomal oxidase detoxication which is blocked by the synergist piperonyl butoxide. Therefore a determination of the synergistic ratio offers a quantitative and relative measure of the activity of the multifunction oxidative detoxication in the insect body.

In studies of the relationship between the in vitro measurement of microsomal enzyme activity in the malpighian tubes of the cricket and in vivo microsomal detoxication capability, Benke and Wilkinson (1971b) made a comparison between the patterns of toxicity of carbaryl alone, synergistic ratios of carbaryl-piperonyl butoxide combinations, and a microsomal enzyme activity in crickets. The authors found that the toxicity of carbaryl and its synergism by piperonyl butoxide are correlated with in vitro microsomal expoxidase levels in the malpighian tubes of crickets. The conclusion reached was that microsomal enzyme activity was a realistic indicator of the cricket's in vivo microsomal detoxication capability and also reflected the susceptibility of the cricket to insecticides known to be metabolized by these enzymes. Therefore, the relationship obtained between microsomal activity and the degree of synergism of carbaryl with piperonyl butoxide supported Brattsten and Metcalf's (1970) suggestion.

In studies of a new type of carbamate synergists, phenyl 2-propynyl ethers, Barnes and Fellig (1969) found that the synergists potentiated 11 carbamate insecticides to a greater degree than the well known

methylene dioxyphenyl compounds. They also reported that the synergized

carbamates were able, in all cases, to overcome the resistance in a resist-

ant strain of flies. The synergistic ratios measured for the resistant strain

were greater than the synergistic ratios measured for the susceptible strain.

The authors suggested that it is very probable that the acquired resistance

involved an increase in the enzymes normally involved in the detoxication

process. It was also observed in this experiment that the synergist ratio

changed dramatically depending on the amount of piperonyl butoxide added.

Unfortunately, this limitation was not fully recognized by Brattsten and Metcalf

(1970) who pretreated all fifty-four species with a constant dose relative to

the carbaryl dose applied. At any given moment, the amount of mixed func-

tion oxidase enzymes will be a fixed quantity and therefore the dose of

synergist should also be fixed and not varied according to insecticide dose.

Effects of lipid on insecticide susceptibility

The effects of nutritional factors in insect resistance have been

thoroughly reviewed by Gordon (1961). Undoubtedly the diet could affect

the general vigor of insects. O'Brien (1967) has suggested the term "vigor

tolerance" to describe the possible development of general resistance in

insects to various insecticides and environmental pressures due simply to

physical well being.

Recently, many investigators have focused on the problem of fats

in various insects as related to susceptibility to insecticides. The studies

have been mostly concerned with total body fat with a few reports on some specific lipids.

Fast and Brown (1962) found that there was no significant difference between DDT-resistant and susceptible strains of Aedes aegypti larvae in total lipid or phospholipid content among 3 of 4 different stocks tested. The main fatty acid of the neutral lipids and phosphalipids found was palmitoleic acid while the main phospholipid moiety was cephaline.

Moore et al. (1967) treated boll weevils, Anthonomous grandis Boheman, which were either collected from fields or were laboratory reared, with toxaphene, toxaphene + DDT, carbaryl, and azinphosmethyl, topically or with a dry-film technique. Total lipids were extracted from weevils that survived or died from this treatment and the fatty acids in the extracts were determined.

Moore et al. (1967) found that dead weevils contained remarkably higher concentrations of stearic, linoleic, and lineolenic acids whereas the survivors had higher concentrations of palmitic and oleic acids. These results were more consistant in toxaphene or toxaphene + DDT treated weevils than carbaryl or azinphosmethyl treated weevils.

To study the qualitative composition of lipids in the bollworm, Heliothis zea (Boddie), as related to insecticide susceptibility, Barnett and Berger (1970) reared the bollworms on diets with different fatty acid compositions and treated the larvae topically with contact insecticides of 3 chemical classes (methyl parathion, endrin, and carbaryl). The LD_{50} of each

insecticide was determined for the five groups of experimental larvae. No differences were found in the LD_{50} of endrin and carbaryl. There was, however, no correlation between toxicity differences and variations of fatty acid composition. These authors suggested that high lipid content in insects might help by solubilizing more toxicant and increasing toxicant storage in various tissues, therefore reducing the criticial concentration at the target site.

Winteringham (1969) reviewed mechanisms of selective insecticidal action and discussed the possible roles of lipids in insect susceptibility to insecticides. He concluded that the chemical nature of the cuticular lipids might influence the penetration of toxicants into the insect integument. Lipoprotein in the hemolymph might affect the transport of the toxicant and also the rate of enzymatic activation or detoxication. Lipid content in the fat body might control the storage of fat soluble insecticides and determine the maximum internal dosage that the insect can tolerate. Lipids in the nerve tissues and surrounding sheath probably affect the penetration of insecticides to the target site and eventually their toxic actions. Similarly, lipids of cell membranes also play an important role in the subcellular activity of insecticides.

Effects of age and sex on enzyme induction

The effect of age and sex on detoxication potential and susceptibility to enzyme induction has been rather neglected.

Nakatsugawa and Dahm (1965) found that the fat body of adult American cockroaches; Periplanet a americana L.; had an increasing ability to activate

parathion up to an age of about three months. The reaction was belived to be catalyzed by microsomal mixed function oxidases. In addition, Schonbrod, Philleo and Terriere (1965) reported that naphthalene hydroxylation by house-fly microsomes varied with the insect's age. The production of metabolites increased about five times as the flies advanced from two to ten days of age. In general, there was good agreement between the susceptibility to the naphthalene vapors and napthalene metabolism except for two DDT-resistant strains of houseflies.

In our laboratory, Moradeshaghi (1968) observed that the percentage mortality due to parathion varied among different ages and sexes of alkali bees. The results showed that the mortality increased as the bees aged while the male bees were more susceptible to parathion than the female bees except for the 1-day-old bees. The difference in mortality due to parathion between one and four days after emergence was not highly signifi-cant for either sex. The difference between males four and seven days after emergence and between bees of both sexes at seven and ten days after emer-gence were highly significant (Moradeshaghi, 1968).

While studying the effect of age and induction on cockroach mixed function oxidase activity, Turnquist (1971) observed that female American cockroaches showed definite age-dependent changes in levels of activity of the microsomal mixed function oxidase chain. Cytochrome P-450 levels, EPN-detoxication, and p-nitroanisole O-demethylation activities were very low in young adult insects but increased steadily reaching a peak at about 100

days in fat body and at about 90 days in midgut and hindgut. The activities then declined rapidly reaching the levels of young insects at about 130 to 140 days of age. NADPH-NT-reductase activity was high in young insects and declined during the first few weeks of adult life. This activity also showed a peak at about 100 days.

Benke and Wilkinson (1971a and b) reported that levels of microsomal epoxidation and hydroxylation in the malpighian tubes of house crickets varied considerably with age and sex. The age-activity profile showed low levels of microsomal enzyme activity during the molts and was marked by a dramatic increase in activity during the first week of the adult stage, in the case of the female insect. Carbaryl toxicity and its synergism by piperonyl butoxide were also correlated with this enzyme activity in different ages and sexes of house crickets. The largest difference in each of the three parameters, carbaryl toxicity, synergist ratio and enzyme activity, occurred during the first week with the female adult insect. Male crickets, which consistently showed lower levels of microsomal epoxidase, were markedly more suscept-ible to carbaryl alone, although both sexes were equally susceptible to carbaryl-piperonyl butoxide combinations.

MATERIALS AND METHODS

Insect maintenance

Alfalfa leafcutter bee, <u>Megachile rotundata,</u> prepupae were purchased from commercial sources and stored at 2 C until incubation. About three weeks before the experiments began, the prepupae were incubated at 30C and 30-40% relative humidity, until the adults emerged. A photoperiod of 15 hours of light (from about 6 AM to 9 PM) and 9 hours of dark was provided by fluorescent bulbs. The adult bees were collected at 24-hour intervals as they emerged. Those bees collected were regarded as being one day old. They were collected usually between 1 and 2 PM in a darkened room where a light was located at one side of the rearing cage. About twenty bees of the same sex and size were kept in each plastic cage and fed with 25% sucrose solution applied to Cellucotton® strips. Cylindrical 50 dram plastic vials were used as the rearing cages. The measurement of the cage was 2 inches in diameter and 3 1/2 inches in height. Small holes were drilled in the lid and around the wall of the vial to provide ventilation. The sucrose solution was fed in a plastic chamber cemented to the bottom of the vial. Food was changed daily to prevent fermentation or contamination by fungus. The feedings were continued for various lengths of time, prior to bioassay tests. The bees could live more than 30 days under these conditions.

Chemicals

Carbaryl (1-naphthyl methylcarbamate), EPN (O-ethyl O-p-nitrophenyl phenylphosphonothioate), chlorcyclizine dihydrochloride [1-(4-chlorobenzhydryl)-4-methyl-piperazine] and aminopyrine (4-dimethylamino 1,5-dimethyl-2-phenyl-3-pyrazolone) were donated by the Union Carbide Corporation, the E.I. duPont de Nemours and Company, and the Abbott Laboratories, respectively. Phenobarbital (5-ethyl-5-phenyl barbituric acid), piperonyl butoxide, nicotinamide adenine dinucleotide phosphate, reduced sodium salt (NADPH) and all other chemicals were purchased from various commercial sources. The biochemicals were obtained from the Sigma Chemical Co.

Toxicity tests and drug pretreatment

The susceptibility of the bees to carbaryl was determined by topically applying $0.5\,\mu l$ of glass distilled acetone solution to the dorsal portion of the thorax with a microapplicator (Biotronics Incorporated, Brookings, South Dakota). Insects used as controls were similarly treated with $0.5\,\mu l$ of glass distilled acetone. The syringe delivery was calibrated with mercury. The bees were chilled with a block of ice before application. All bioassay experiments were conducted between 2 and 6 PM. Mortalities were recorded at 24 hours after the pesticide application and all moribund (slightly moving but uncoordinated) bees were considered dead. A similar procedure was followed to determine the LD_{50} of the response to carbaryl with pretreatment of piperonyl butoxide and thereafter determination of the synergistic ratio.

For statistical analysis of toxicity, the results were analyzed by probit analysis (Finney, 1964) using a computer (Sisson, unpublished procedure). Analyses of variance and least significant difference tests were used to test the significance of responses by the various age groups.

To test the effect of drugs on carbaryl toxicity, chlorcyclizine di-hydrochloride, aminopyrine, or phenobarbital were fed to the bees at their highest non-lethal concentration 72 hours before the application of carbaryl. The control bees were fed only 25% sucrose solution before and after carbaryl application and the results analyzed by an analysis of variance and least significant difference tests.

Dissections

The bees were weighed and dissected in a small petri dish partially filled with cold phosphate buffer (0. 1 M, pH 7. 4) which was set on a block of ice. The bees were decapitated and their posterior abdominal segments were removed. The entire guts were then pulled out and transferred to cold phosphate buffer in a 5 ml beaker. All tissues were homogenized immediately.

Preparation of microsomes

Seventy milligrams of whole gut tissue were transferred to a cold glass homogenizer tube containing 2 ml of ice cold 0. 2 M pH 7. 4 buffer solution. The homogenizing tubes were placed in a beaker filled with crushed ice. The whole gut tissue was homogenized with 20 up-and-down strokes using a precooled power driven Teflon® pestle. The gut homogenate was

transferred to a centrifuge tube and spun at 20,000 RPM for 2 hours at 4C

in a Sorvall RC-2 centrifuge. The supernatant was discarded and the micro-

somal pellet was resuspended in a glass tube with 0.5 ml of cold phosphate

buffer for biochemical assay.

Measurement of EPN detoxication activity

Resuspended microsomes of the whole gut homogenate were used

for EPN detoxication activity studies. The procedure of Kinoshita et al.

(1966) was followed with slight modifications as used by Turnquist (1971).

An EPN solution (7 mg/ml) in a mixture of 20% ethanol and 80% propylene

glycol was added immediately before use to phosphate buffer (0.1 M,

pH 7.8) in the amount of 0.25 ml per 2.4 ml of buffer. The incubation

mixture contained 0.5 ml of microsomal suspension, 0.1 ml NADPH solu-

tion (10 mg/ml glass distilled water), and 0.2 ml of EPN-buffer mixture in

a total volume of 0.8 ml. The mixture was incubated for 60 minutes at $37.5^{o}C$

with gentle shaking. For the control, the same mixture was boiled in water

for twenty minutes prior to incubation. The reaction was stopped by adding

1.8 ml of cold acetone and 0.8 ml of glycine-sodium hydroxide buffer (0.5 M,

pH 9.5) was added to intensify the color of the p-nitrophenol formed by EPN

degradation.

The mixture was centrifuged at 13,000 rpm for 20 minutes. The

amount of p-nitrophenol produced/70 mg wet weight tissue was measured

at 410 mμ with a Zeiss PMQ-II spectrophotometer. A standard curve for

p-nitrophenol was made by dissolving known amounts of the compound in 0.8 ml of the incubation mixture and then processed as described above. The resulting data were plotted on a graph and the activity was expressed as milliliters of p-nitrophenol produced per hour per milligram of fresh weight. Analyses of variance were used to measure the effect of drugs of the EPN-detoxication activity of whole gut homogenates.

Measurement of lipid content

Approximately two grams of adult male or female leafcutter bees were weighed to the nearest 0.1 mg. The lipids were extracted according to Leveille and Sauberlich (1963). The procedure involved homogenization in methanol, addition of chloroform, filtering through filter paper, and evaporating the sample to dryness with a gentle nitrogen stream. The lipids were then reconstituted in chloroform, dried with anhydrous sodium sulfate, and the solvent once again removed after transfer to tared vessels. The samples were then placed in a desiccator overnight until they reached constant weight. The results were expressed as percent lipid content of whole (fresh) body weight.

RESULTS

<u>Carbaryl susceptibility of the alfalfa leafcutter
bee as determined by means of LD_{50} and
synergistic ratio</u>

The analyses of variance of the quantal response for the dosage-

mortality data, unsynergized or synergized with piperonyl butoxide, are

shown in Tables 1 and 2. In all cases the F values at the 0.05 level were

highly significant. In other words, there was a highly significant regression

relationship between the independent variables (the dose of carbaryl applied)

and dependent variables (the response or mortality of the bees). Therefore

the change in the carbaryl dosage was highly associated with the change in

the mortality.

The mean responses, the population intercepts, and the re-

gression coefficients for all dose-mortality curves are shown in Table 3.

Regression equations, $Y = \bar{Y} + bx$, can be easily obtained from Table 3.

Computerized results of the probit analyses are shown in Figures

1 and 2. All regression lines from experiments including synergist shifted

to the left because of the block of microsomal detoxication enzyme systems

in the bees by pretreatment with the synergist. LD_{30}, LD_{50}, LD_{70} and LD_{90}

data and also the confidence intervals for those lethal dosages are indicated

in the graphs.

LD_{50} values with their 95% confidence intervals for different sexes and ages of the bees, with or without synergist, are summarized in Table 4. The results showed that the LD_{50} decreased as the bees aged with the exception of the 1-day-old females. However, the result of all possible comparisons by means of LSD (least significant difference) (Tables 12, 13 and 14) indicated that there were no differences between 1-day, 3-day, and 4-day-old groups of the bees in the female while all were significant in the male. The synergist ratios did not correlate well with decreasing microsomal detoxication in the male bees, nor were increasing syngergist ratios correlated with increasing tolerance to carbaryl. In the female, there was a less definite trend in synergist ratio. Although synergist ratios more closely relate to the carbaryl susceptibility of the females, there is no clear relation to the gut EPN detoxication.

Determination of the lipid content of the leafcutter bee

From Table 5, it is obvious that the lipid content of adult male and female leafcutter bees decreased as the bees aged. The males had a slightly greater percent lipid, but on an individual basis, the females, due to their large body contained more. Duncan's multiple range test was conducted to compare the difference of the lipid content in the different ages of the adult bees. The results indicated that there were significant differences for both sexes (Tables 12, 15, and 16).

Table 1. Analysis of variance for regression of the mortality of the alfalfa leafcutter bees due to carbaryl, without pretreatment of piperonyl butoxide (testing with hypothesis: β = o)

Sex	Age (days old)	Source of Variation	Degrees of Freedom	Mean Square	F test value[a]
Male	1	Regression	1	69.171	69.171**
		Residual of error term	∞	1.000	
	2	Regression	1	72.560	72.560**
		Residual of error term	∞	1.000	
	3	Regression	1	63.131	63.131**
		Residual of error term	∞	1.000	
	4	Regression	1	54.089	54.089**
		Residual of error term	∞	1.000	
Female	1	Regression	1	24.987	24.987**
		Residual of error term	∞	1.000	
	2	Regression	1	37.887	37.887**
		Residual of error term	∞	1.000	
	3	Regression	1	48.713	48.713**
		Residual of error term	∞	1.000	
	4	Regression	1	26.249	26.249**
		Residual of error term	∞	1.000	

[a] F (0.05) = 3.8399
**Significant at 0.05 level

Table 2. Analysis of variance for regression of the mortality of the alfalfa leafcutter bees due to carbaryl, with pretreatment of piperonyl butoxide (testing the hypothesis: β = o)

Sex	Age (days old)	Source of Variation	Degrees of Freedom	Mean Square	F test value[a]
Male	1	Regression	1	32.471	32.471**
		Residual of error term	∞	1.000	
	2	Regression	1	67.378	67.378**
		Residual of error term	∞	1.000	
	3	Regression	1	82.091	82.091**
		Residual of error term	∞	1.000	
	4	Regression	1	42.499	42.499**
		Residual of error term	∞	1.000	
Female	1	Regression	1	29.600	29.600**
		Residual of error term	∞	1.000	
	2	Regression	1	44.638	44.638**
		Residual of error term	∞	1.000	
	3	Regression	1	43.065	43.065**
		Residual of error term	∞	1.000	
	4	Regression	1	44.308	44.308**
		Residual of error term	∞	1.000	

[a] F (0.05) = 3.8399

**Significant at 0.05 level

Table 3. Intercept and slope values of the dose-response-regression line
for the alfalfa leafcutter bees treated topically with carbaryl,
with or without pretreatment of piperonyl butoxide

Sex	Pretreatment of Synergist	Age (days old)	Mean Response ($\overline{Y}$)	Population Intercept (α)	Slope of Regression Coefficient (b)
Male	Without piperonyl butoxide	1	4.72	2.58	3.10
		2	4.88	3.24	2.74
		3	4.91	2.78	3.47
		4	5.42	4.15	3.42
	With piperonyl butoxide	1	4.88	6.36	4.36
		2	5.29	5.58	4.23
		3	5.03	7.14	1.81
		4	5.27	8.72	2.19
Female	Without piperonyl butoxide	1	5.41	4.19	4.81
		2	4.70	3.26	4.70
		3	5.31	3.39	3.83
		4	5.07	3.73	2.90
	With piperonyl butoxide	1	5.09	3.66	5.57
		2	5.04	3.59	4.31
		3	5.13	3.37	2.60
		4	5.34	3.40	3.37
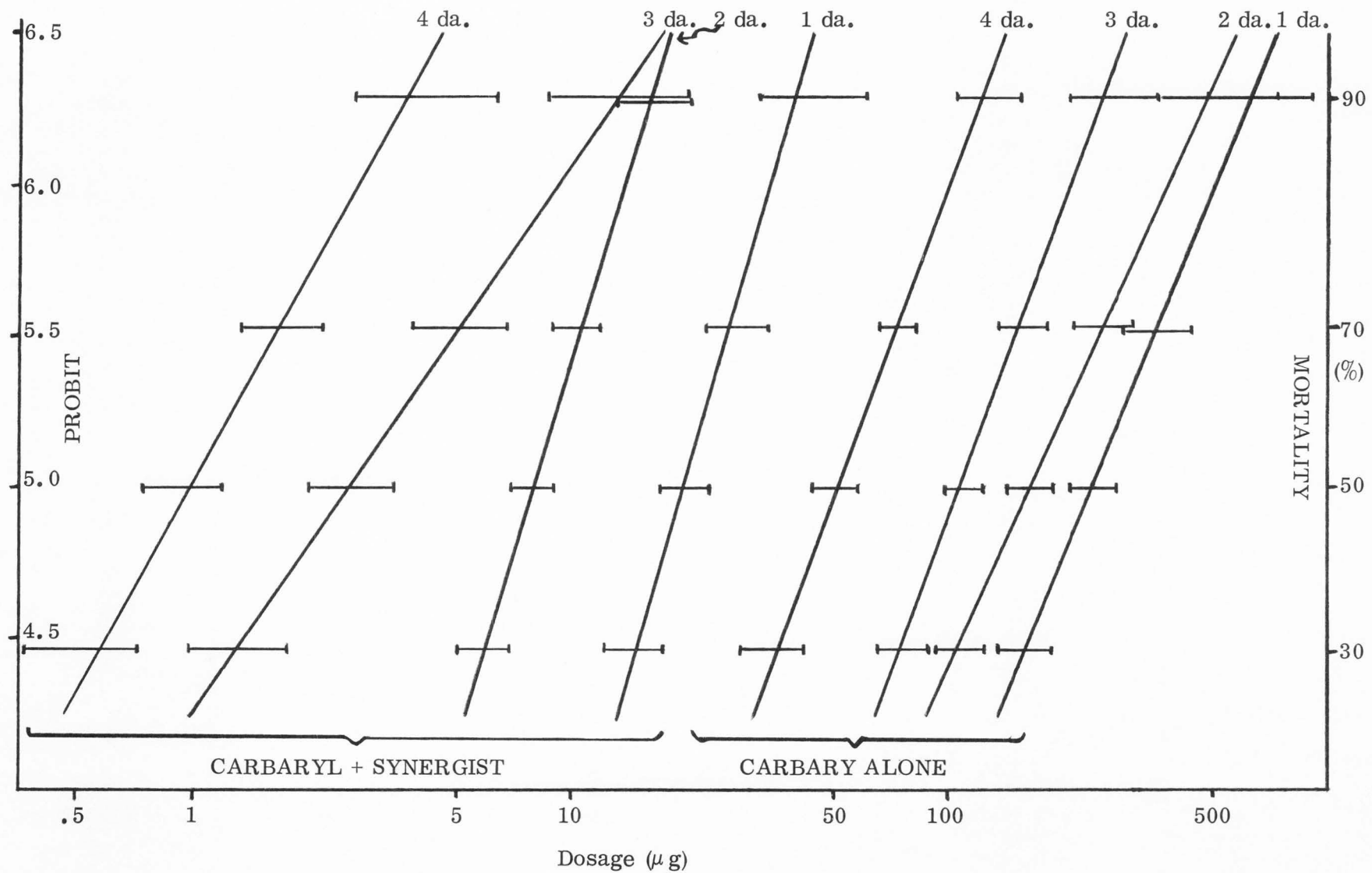
Figure 1. Regression lines for carbaryl toxicity with or without synergist in male leafcutter bees.

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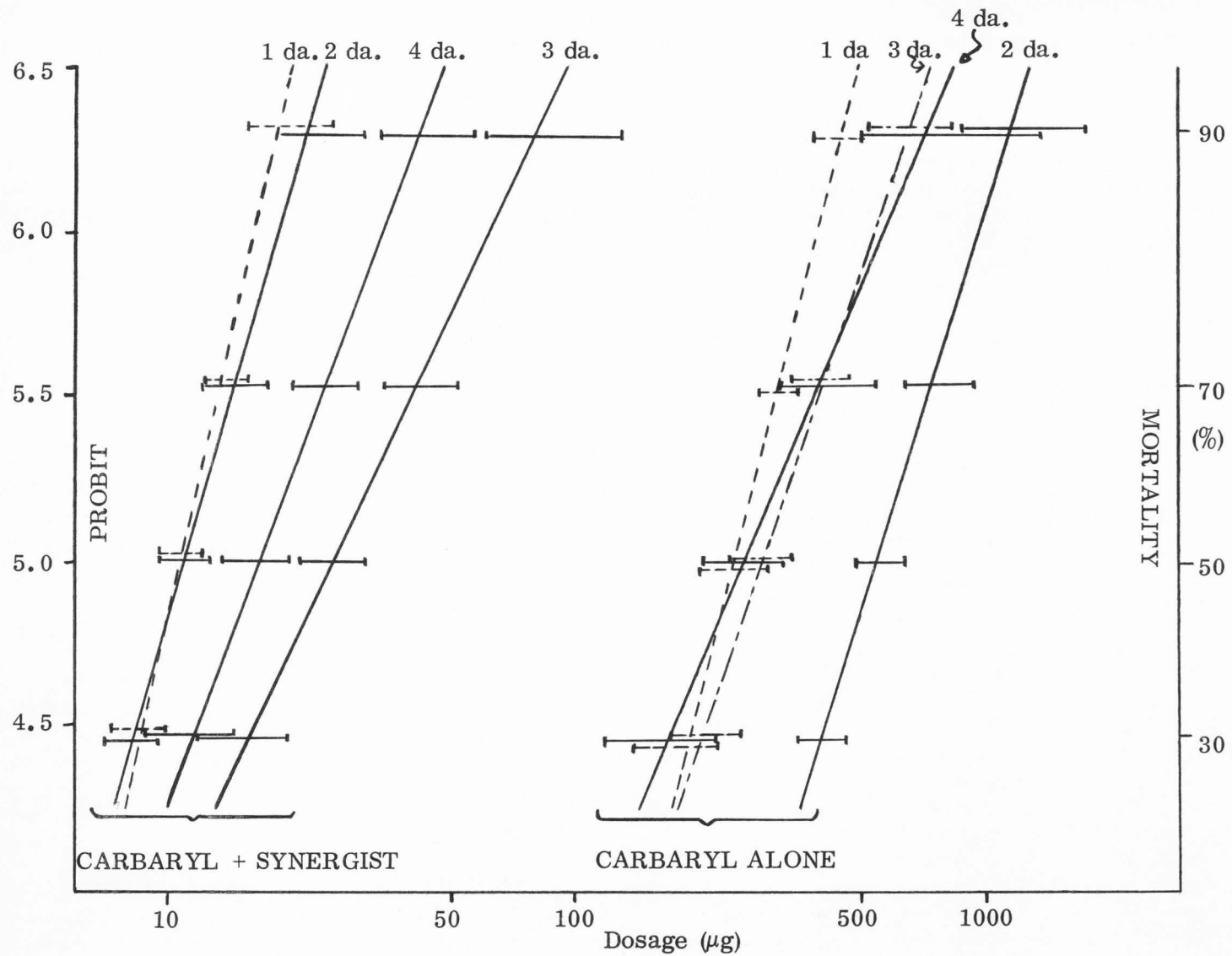
Figure 2. Regression lines for carbaryl toxicity with or without synergist in female leafcutter bees.

Table 4. LD$_{50}$ values for different sexes and ages of alfalfa leafcutter bees treated with carbaryl, with or without pretreatment of piperonyl butoxide

| Sex | Age (days old) | LD$_{50}$ | | | | Synergistic ratio[a] |
| | | Without piperonyl butoxide | | With piperonyl butoxide | | |
		μg carbaryl/gr bee	95% confidence limits	μg carbaryl/gr bee	95% confidence limits	
Male[b]	1	239.96	(206.96∼285.06)	20.03	(17.49∼23.36)	12
	2	166.49	(142.39∼196.96)	8.02	(6.96∼9.08)	21
	3	109.24	(97.35∼123.48)	2.62	(2.04∼3.38)	42
	4	51.30	(43.88∼57.41)	0.97	(0.73∼1.20)	53
Female[c]	1	245.48	(200.12∼280.38)	10.84	(9.62∼12.09)	23
	2	550.54	(486.16∼647.88)	11.27	(9.81∼12.90)	49
	3	289.39	(241.66∼335.59)	25.77	(21.16∼30.68)	11
	4	262.38	(207.29∼325.58)	16.99	(13.65∼20.23)	15

[a]S.R. = $\dfrac{\text{LD}_{50}\text{ of carbaryl}}{\text{LD}_{50}\text{ of carbaryl + synergist}}$

[b]Average body weight = 24 mg/bee

[c]Average body weight = 33 mg/bee

Determination of enzyme activity of the
whole gut homogenates of the alfalfa
leafcutter bee

Enzyme activity was expressed as micrograms of p-nitrophenol

formed from EPN per 70 mg fresh weight gut homogenate after one hour of

incubation. For preparing every 70 mg gut sample, 1.7 and 1.4 gms of

bees were required for the male and the female, respectively. Figure 3

is the standard p-nitrophenol curve used to determine the concentration

of p-nitrophenol produced per 70 mg fresh weight gut homogenate per hour.

Levels of EPN-detoxication activity from one day to four days in both males

and females are shown in Table 6. There was a similar pattern of decline

in the enzyme activity as in the lipid content. It was found that the more the

bees aged, the more the enzyme activity decreased. The changes were com-

parable in each sex.

Statistical analyses also showed that there was a significant dif-

ference among the enzyme activities of different age groups of bees (Tables

12, 17, and 18).

The levels of EPN-detoxication activity observed from the different

age groups of the bees may help to explain the bee's decreasing tolerance to

carbaryl (Tables 4 and 6). Although carbaryl and EPN are very different

insecticides, EPN detoxication measures the overall activity of the micro-

somal electron transport chain which also would provide significant detoxica-

tion of carbaryl (Wilkinson, 1968).

Table 5. The lipid content of whole body homogenates of the alfalfa
leafcutter bee

Sex	Age (days old)	Lipid Content[a] (%)	Percent of Control	Percent Decrease
Male	1 (control)	9.93± 0.34 (5)[b]	100	--
	2	7.06± 0.16 (4)	71	29
	3	6.07 ± 0.09 (4)	61	39
	4	5.98 ± 0.14 (4)	60	40
Female	1 (control)	7.69 ± 0.34 (6)	100	--
	2	6.21 ± 0.35 (5)	81	19
	3	4.79 ± 0.16 (5)	62	38
	4	4.55 ± 0.22 (5)	59	41

[a]Lipid content is expressed as percentage of lipid weight to the total
whole body weight $\pm\, t._{95(n-1)}S_{\bar{x}}$.
[b]Number in parentheses shows the number of determinations made.
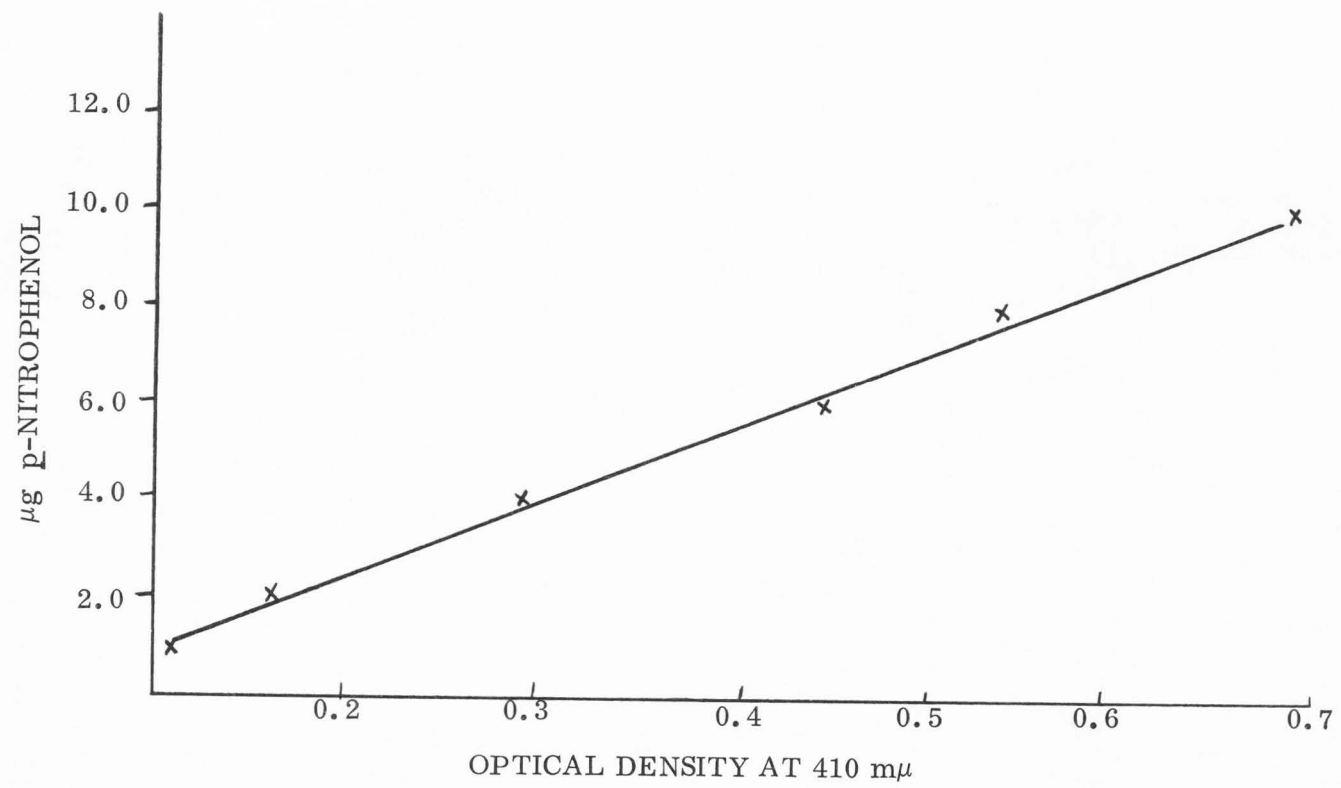
Figure 3.   Standard curve used in the determination of p-nitrophenol concentration from optical density

Table 6. Levels of EPN-detoxication activity of whole gut homogenates
of the alfalfa leafcutter bee

Sex	Age (days old)	EPN-detoxication[a] activity	Percent of control	Percent decrease
Male	1 (control)	2.06 ± 0.08 (3)[b]	100	--
	2	1.49 ± 0.08 (3)	72	28
	3	0.46 ± 0.00 (3)	22	78
	4	0.30 ± 0.01 (3)	15	85
Female	1 (control)	1.99 ± 0.17 (3)	100	--
	2	1.23 ± 0.34 (3)	62	38
	3	0.62 ± 0.14 (8)	31	69
	4	0.24 ± 0.13 (4)	12	88

[a]Enzyme activity is expressed as micrograms of p-nitrophenol formed from EPN per 70 mg fresh weight gut homogenate per hour $\pm t._{95,(n-1)} S_{\bar{x}}$

[b]Number in parentheses shows the number of determinations made.

Modification of carbaryl toxicity to the
alfalfa leafcutter bee by drugs

The analyses of variance of the dosage-mortality data after drug

exposure are shown in Table 7. F values at the .05% level were also highly

significant for a regression relationship between the dose of carbaryl applied

and the mortality of the bees after pretreatment with different drugs. The

various parameters of the regression equation are listed in Table 8.

Computerized results of the probit analyses were also plotted on

the graphs with the coordinates of dosage in milligrams and the mortality

in probits. The LD_{30}, LD_{50}, LD_{70} and LD_{90} values are indicated and the

95% confidence intervals for each lethal dosage are also shown in the graphs

(Figures 4 and 5).

LD_{50} values with their 95% confidence intervals for different sexes

of the bees pretreated with the drugs are also summerized in Table 9.

Chlorcyclizine-fed male bees decreased in carbaryl susceptibility by almost

two fold, but in females chlorcyclizine did not significantly change the response.

Aminopyrine remarkably enhanced the susceptibility of both sexes to carbaryl.

There was no modification of carbaryl toxicity observed in bees of either sex

pretreated with phenobarbital. Statistical analyses indicated that there were

no significant carbaryl toxicity differences between control and phenobarbital-

treated, and among control, phenobarbital-treated and chlorcyclizine-treated

male and female bees respectively (Tables 12, 19, and 20).

Table 7. Analysis of variance for regression of carbaryl toxicity of the alfalfa leafcutter bees pretreated with chlorcyclizine, aminopyrine or phenobarbital (testing the hypothesis: β = o)

Sex	Drug fed	Source of variation	Degree of freedom	Mean square	F test value[a]
Male	Chlorcyclizine (0.75%)	Regression	1	36.074**	36.07**
		Residual of error term	∞	1.000	
	Aminopyrine (0.75%)	Regression	1	80.845**	80.45**
		Residual of error term	∞	1.000	
	Phenobarbital (0.375%)	Regression	1	11.751	11.751**
		Residual of error term	∞	1.000	
Female	Chlorchyclizine (0.2%)	Regression	1	41.486	41.486**
		Residual of error term	∞	1.000	
	Aminopyrine (0.75%)	Regression	1	21.162	21.162**
		Residual of error term	∞	1.000	
	Phenobarbital (0.375%)	Regression	1	13.947	13.947**
		Residual of error term	∞	1.000	

[a] F(0.05) = 3.8399
**Significant at 0.05 level

Table 8. Intercept and slope values of the dose-regression lines for the alfalfa leafcutter bees treated topically with carbaryl, with pre-treatment of chlorcyclizine, aminopyrine, or phenobarbital

Sex	Drugs fed	Mean response $(\overline{Y})$	Population intercept (α)	Slope or regression coefficient (b)
Male	Chlorcyclizine (0.75%)	5.12	3.73	2.62
	Aminopyrine (0.75%)	5.49	3.81	1.57
	Phenobarbital (0.375%)	4.96	4.34	1.73
Female	Chlorcyclizine (0.2%)	5.05	3.76	4.13
	Aminopyrine (0.75%)	5.47	4.52	3.20
	Phenobarbital (0.375%)	4.92	4.29	0.95
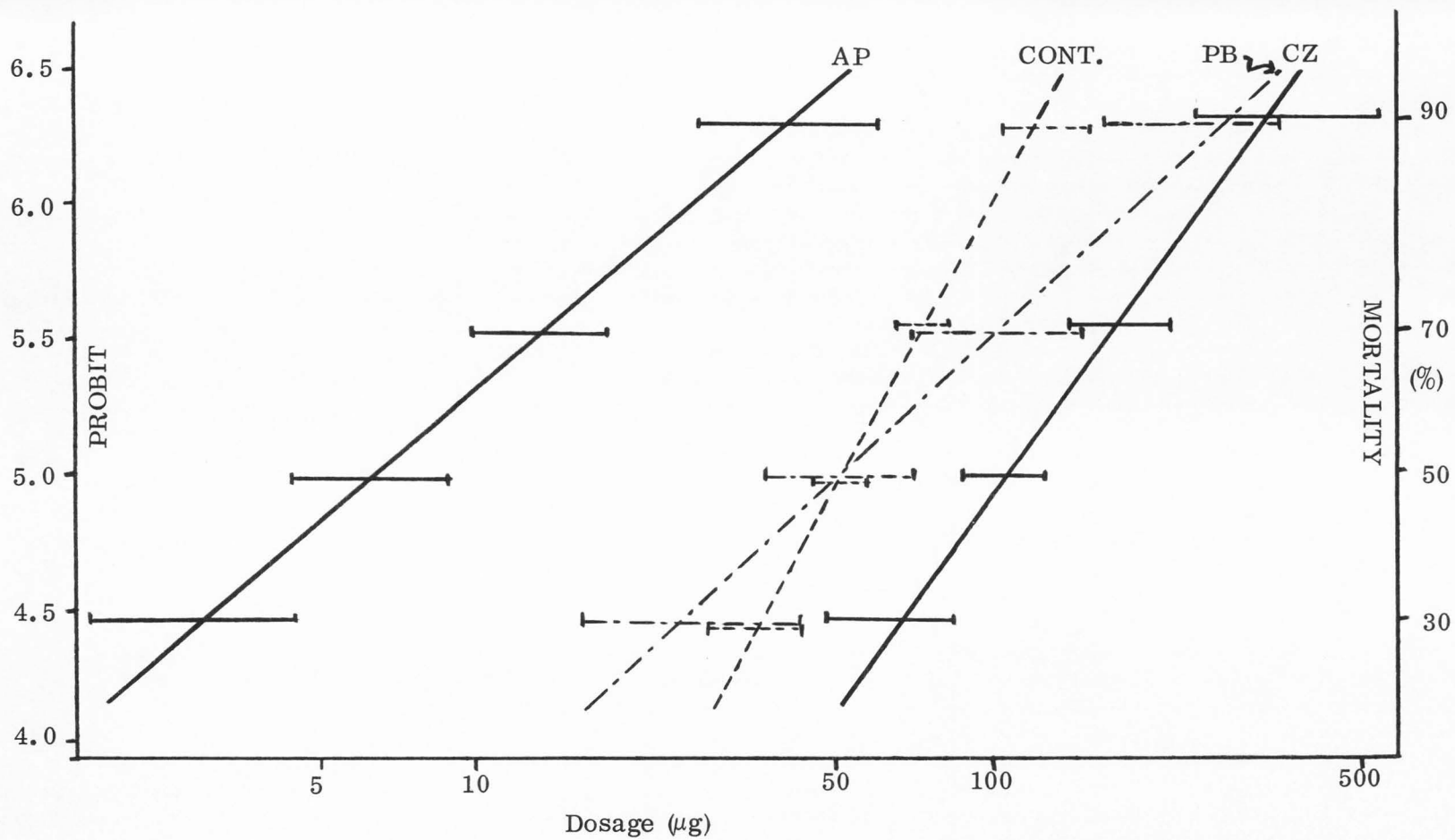
Figure 4. Regression lines showing effects of drugs on carbaryl toxicity to male leafcutter bees.
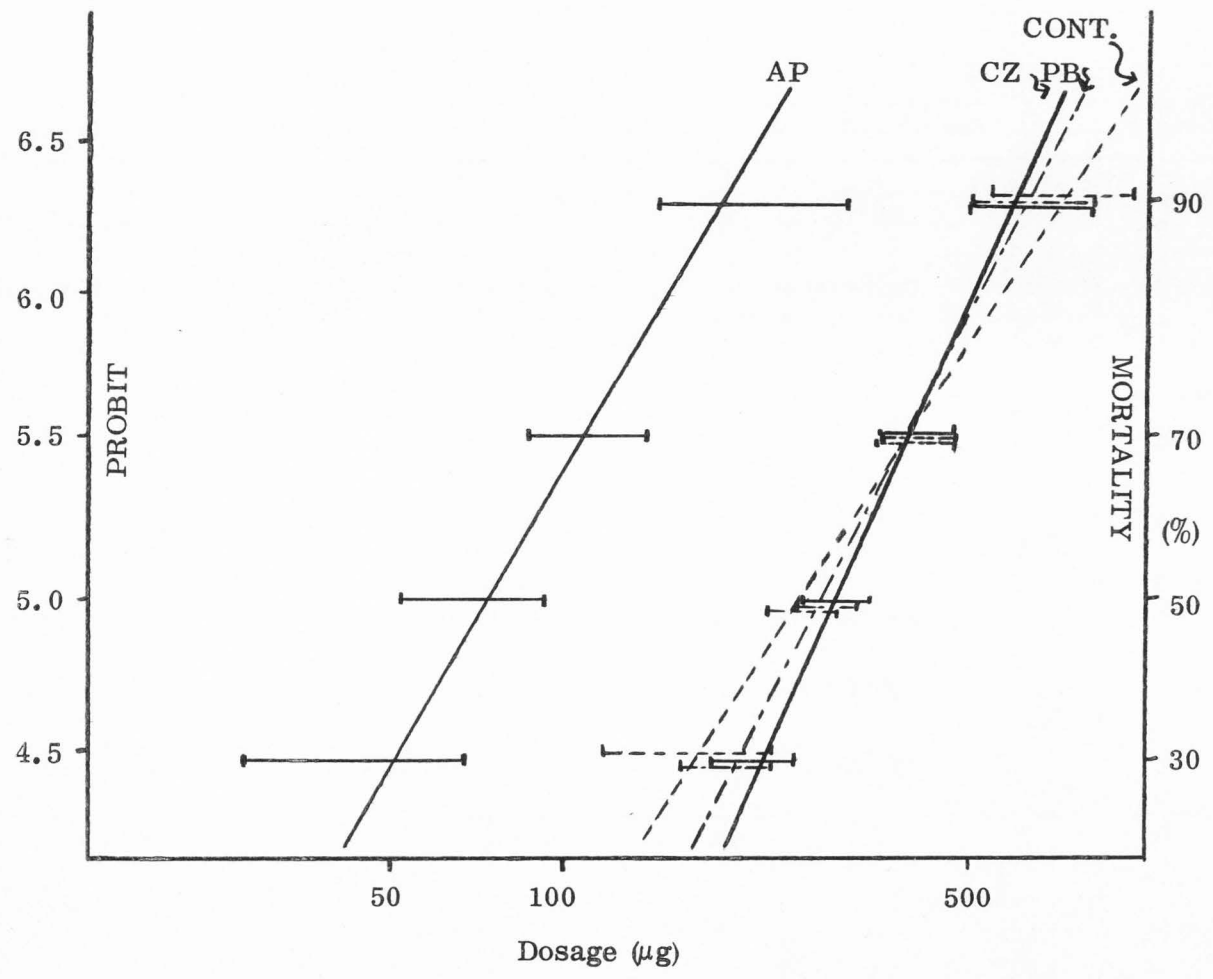
Figure 5. Regression lines showing effects of drugs on carbaryl toxicity to female leafcutter bees.

Table 9. Effect of drugs on carbaryl toxicity to alfalfa leafcutter bees

| Sex | Drug fed | LD$_{50}$ | | Percent of control |
		µg carbaryl/gr bee	95% confidence limits	
Male	None (control)	51.30	43.88~57.41	100
	Chlorcyclizine	106.09	88.26~124.47	207
	Aminopyrine	6.28	4.46~8.82	12
	Phenobarbital	51.38	35.72~77.40	100
Female	None (control)	262.38	207.29~325.58	100
	Chlorcyclizine	293.18	263.96~323.69	112
	Aminopyrine	75.29	52.14~93.46	29
	Phenobarbital	282.34	154.28~595.76	108

Effects of drugs on enzyme activity of the whole gut homogenate of alfalfa leafcutter bees

Treatment with chlorcyclizine, aminopyrine, or phenobarbital caused rapid depletion of lipid as shown in Table 10. Statistical analyses showed that there were significant differences in lipid content between drug treated and non-treated (control) bees. However, there were no differences among drug-treated bees themselves (Tables 12, 21, and 22). It is evident that lipid levels do not provide an explanation for drug-induced changes in susceptibility. Neither do they adequately explain normal age-susceptibility relationships.

Effects of drugs on enzyme activity of the whole gut homogenate of alfalfa leaf-cutter bees

Pretreatment of the bees with either chlorcyclizine, aminopyrine, or phenobarbital dramatically stimulated EPN detoxication in both male and female leafcutter bees (Table 11). Statistical analyses also supported the fact that there were significant differences in enzyme activity between the drug-treated bees and non-treated (control) bees (Tables 12, 23, and 24).

Table 10. Effects of drugs on the lipid content of whole body homogenates
of alfalfa leafcutter bees

Sex	Drug fed	Lipid content[a] (%)	Percent of control	Percent decrease
Male	None (control)	5.98 ± 0.14 (4)[b]	100	--
	Chlorcyclizine	4.40 ± 0.40 (3)	74	26
	Aminopyrine	4.84 ± 0.35 (4)	81	19
	Phenobarbital	4.08 ± 0.37 (4)	68	32
Female	None (control)	4.55 ± 0.08 (4)	100	--
	Chlorcyclizine	3.74 ± 0.23 (4)	82	18
	Aminopyrine	3.81 ± 0.34 (4)	84	16
	Phenobarbital	3.82 ± 0.23 (4)	84	16

[a] Lipid content expressed as percentage of mg lipid weight to mg total whole
body weight $\pm\, t._{95,\,(n-1)} S_{\overline{x}}$.

[b] Number in parentheses shows the number of determinations made.

Table 11. Effects of drugs on the rates of EPN-detoxication activity of whole gut homogenates of 4-day-old alfalfa leafcutter bees

Sex	Drug fed	EPN-detoxication[a] activity	Percent of control
Male	None (control)	0.28 ± 0.16 (4)[b]	100
	Chlorcyclizine	1.52 ± 0.13 (4)	543
	Aminopyrine	1.17 ± 0.16 (4)	418
	Phenobarbital	1.40 ± 0.14 (4)	500
Female	None (control)	0.35 ± 0.08 (4)	100
	Chlorcyclizine	1.46 ± 0.06 (4)	417
	Aminopyrine	1.45 ± 0.26 (4)	414
	Phenobarbital	1.66 ± 0.20 (4)	474

[a]Enzyme activity is expressed as micrograms of p-nitrophenol formed from EPN per 70 mg fresh weight gut homogenate per hour $\pm t._{95, (n-1)} S_{\bar{x}}$.

[b]Number in parentheses shows the number of determinations made.

Table 12. Summarized results of multiple range tests of comparisons of carbaryl susceptibility, lipid content, and gut enzyme activity in alfalfa leafcutter bees relative to age and drug exposed[a]

	Male				Female			
A. Among different age groups								
Carbaryl toxicity (LD_{50})	$\overline{Y}_{4\,day}$	$\overline{Y}_{3\,day}$	$\overline{Y}_{2\,day}$	$\overline{Y}_{1\,day}$	$\overline{Y}_{1\,day}$	$\overline{Y}_{4\,day}$	$\overline{Y}_{3\,day}$	$\overline{Y}_{2\,day}$
	51.30	109.24	166.49	239.96	245.48	262.38	289.39	550.54
Lipid content	$\overline{Y}_{4\,day}$	$\overline{Y}_{3\,day}$	$\overline{Y}_{2\,day}$	$\overline{Y}_{1\,day}$	$\overline{Y}_{4\,day}$	$\overline{Y}_{3\,day}$	$\overline{Y}_{2\,day}$	$\overline{Y}_{1\,day}$
	5.98	6.07	7.09	9.93	4.55	4.79	6.21	7.69
Enzyme level	$\overline{Y}_{4\,day}$	$\overline{Y}_{3\,day}$	$\overline{Y}_{2\,day}$	$\overline{Y}_{1\,day}$	$\overline{Y}_{4\,day}$	$\overline{Y}_{3\,day}$	$\overline{Y}_{2\,day}$	$\overline{Y}_{1\,day}$
	0.30	0.46	1.49	2.06	0.24	0.62	1.23	1.99
B. Among effect of different drugs (AP: aminopyrine; Cont: not treated; PB: phenobarbital; CZ: chlorcyclizine)								
Carbaryl toxicity (LD_{50})	$\overline{Y}_{AP}$	$\overline{Y}_{Cont}$	$\overline{Y}_{PB}$	$\overline{Y}_{CZ}$	$\overline{Y}_{AP}$	$\overline{Y}_{Cont}$	$\overline{Y}_{PB}$	$\overline{Y}_{CZ}$
	6.28	51.30	51.38	106.09	75.29	262.18	282.34	293.18
Lipid content	$\overline{Y}_{PB}$	$\overline{Y}_{CZ}$	$\overline{Y}_{AP}$	$\overline{Y}_{Cont}$	$\overline{Y}_{CZ}$	$\overline{Y}_{AP}$	$\overline{Y}_{PB}$	$\overline{Y}_{Cont}$
	4.08	4.40	4.84	5.98	3.74	3.81	3.82	4.55
Enzyme level	$\overline{Y}_{Cont}$	$\overline{Y}_{AP}$	$\overline{Y}_{PB}$	$\overline{Y}_{CZ}$	$\overline{Y}_{Cont}$	$\overline{Y}_{AP}$	$\overline{Y}_{CZ}$	$\overline{Y}_{PB}$
	0.28	1.17	1.40	1.52	0.35	1.45	1.46	1.66

[a]Underlining between treatments mean that there are no significant differences between those treatments. A lack of underlining between treatments indicates that there are significant differences between those treatments.

DISCUSSION

The alfalfa leafcutter bees were more tolerant to carbaryl than honey bees. The LD_{50} for carbaryl to the leafcutter bees by topical application was as high as 52 $\mu g/g$ for the male and 262 $\mu g/g$ for the female. Similarly, several other carbamate insecticides such as landrin and lannate are not very toxic to either larval or adult leafcutter bees (Guirguis, unpublished data). The apparent insensitivity of the leafcutter bee to carbamates contrasts with the susceptibility of the honey bee, Apis mellifera, for which Brattsten and Metcalf (1970) estimated the carbaryl LD_{50} to be 2.3 $\mu g/g$. The carbaryl synergist ratio for the honey bee was 2.9.

Alfalfa leafcutter bees are relatively more tolerant to carbaryl than to organophosphate insecticides for which the LD_{50} values are similar to those for the honey bee (Guirguis and Brindley, unpublished data). A similar result was found in Waller's work (1967) in which he also found carbaryl was the safest insecticide as compared with azinphosmethyl, malathion, DDT and parathion.

Since carbaryl is toxic to honey bees but comparatively safe for leaf-cutter bees, we have a case of selective toxicity in the traditional sense. This is usually interpreted as an interspecific phenomenon. In the case of the house-fly, the carbaryl LD_{50} was estimated at more than 900 $\mu g/g$ with a synergist ratio of more than 72 (Brattsten and Metcalf, 1970). Hence, the alfalfa

leafcutter bee is between the honey bee and housefly in carbaryl suscepti-
bility.

Selective toxicity, however, might also be interpreted in an intra-
specific fashion by comparing different ages of insects, or different sexes
within the same species. The female leafcutter bee is more tolerant of
carbaryl than the male, especially after the first day. Although the carbaryl
sensitivity of the female is relatively similar for 1, 3, and 4 days of age, in
the male there is a clear trend toward a loss of tolerance as the bee ages.
If we view these toxicity differences as being no different, in principle, than
differences noted between species, then it may be useful to apply the principles
of selective insecticidal action to an interpretation of the results and the design
of experiments.

Winteringham (1969) has proposed an analysis of the mechanisms of
selective insecticidal action. Intoxication is achieved through successful
travel of toxic molecules down to the site of action. This, in turn, depends
on survival of the molecule through a series of competing processes and rate
limiting barriers. Dissolution of the toxicant in the epicuticular wax, inert
storage in indifferent tissues (tissues not containing toxicant-sensitive sites
of action), or excretion or detoxication processes may be competing factors.
While there are some other factors such as external integument, rate of
facilitated diffusion, internal structural barriers, competition of normal enzyme
substrates for enzymes inhibitable by the toxicant, or varying degrees of

sensitivity of the site of action, my study has focused on only some of the important factors relating to selective toxicity.

The experiments with the alfalfa leafcutter bee was designed to estimate metabolism (as estimated by the synergist ratios), detoxication enzyme activity (as measured by EPN detoxication in vitro), and lipid content (as measured by whole body extracts).

Previous studies with enzyme induction in insects in our laboratory (Ahmad and Brindley, 1969; Moradeshaghi, 1968) have utilized the organophosphate parathion. Although enzymatic metabolism of insecticides usually involves detoxication, certain reactions are well established as activations, for example the oxidation of phosphorothionates to phosphates (as in parathion to paraoxon). Carbaryl was selected as the insecticide for this study as most of its microsomal oxidations are clearly detoxications. Hydroxymethyl carbaryl is slightly activated with respect to carbaryl and their abilities to inhibit acetylcholinesterase (Dorough, 1970). However, hydroxymethyl carbaryl is an unstable metabolite so the overall detoxication is still served and the activation is not nearly as striking as the comparison between phosphorothionates and phosphates (O'Brien, 1967). Kuhr (1970, 1971) has found hydroxymethyl carbaryl to be the major metabolite in susceptible and resistant cabbage loopers.

The tissue of the leafcutter bees selected to measure in vitro EPN detoxication was the gut. The gut is easily dissected from the insects and was, indeed, the only tissue sufficiently abundant to be sampled in both

sexes. Several studies have shown that homogenization of whole insects can lead to the release of inhibitors of microsomal enzymes which make reasonable activity measurements difficult or impossible (Terriere, 1968). The use of gut tissue only avoided difficulty.

The insecticide susceptibility of insects is related to their microsomal enzyme activity which, in turn, is partially age and sex dependent. Similar results for parathion susceptibility and age in the alkali bee were observed by Moradeshaghi (1968). The susceptibility of the insects increased with age as shown for carbaryl toxicity with the leafcutter bees in this study.

In the case of American cockroaches, however, levels of microsomal enzyme activity increased as the cockroaches aged, reaching a peak at about the age of 3 months (Turnquist, 1971). Enzyme activities in the female American cockroach reached their highest levels at 100 and 90 days in fat body and gut, respectively. Parathion activation had also been observed to increase up to three months of age in adult American cockroaches (Nakatsugawa and Dahm, 1965). An analogous pattern was also found in houseflies (Schonbrod et al., 1965) in the microsomal hydroxylation of naphthalene. The metabolites of naphthalene were reported to increase five times as the flies aged from two to seven days.

There is also a dramatic example of the relationships of age with house crickets (Benke and Wilkinson, 1971b). Microsomal epoxidation and hydroxylation in the malphigian tubes of house crickets increased dramatically during the first week and then leveled off. From this and the foregoing discussion, there seems to be good agreement that as microsomal enzyme

activity increases, insects tend to become more resistant to insecticides.

Of course, as in the alfalfa leafcutter bee increasing microsomal activity

may not be correlated in the increasing age as in the housefly (Schonbrod,

Philleo, and Terriere, 1965). Hence, every insect species must be studied

as a separate case. In this study, the comparative data has been collected

only for one to four days old leafcutter bees. The bees were fed on sucrose

solution only, and further aged bees were not considered for experiments

because of possible complications with nutritional factors.

For the drug effects study, leafcutter bees were treated with carbaryl

as four days old, because of the three days of drug feeding which preceeded

carbaryl application. Similar schedules were used by Moradeshaghi (1968)

for alkali bees.

That the alfalfa leafcutter bee was not more resistant to carbaryl

with increasing age, might be due to the physiological status of the bees

because of their diapause as a prepupal stage. It was also evident that the

lipid content also plays a role in the case of the leafcutter bee (Figure 6)

as there is a close correlation between lipid content and enzyme activity.

A very useful indicator of detoxication as an in vivo biological

response is obtained by the use of synergists. It is now well accepted that

many microsomal oxidative detoxications may be blocked by synergists in

vivo, which themselves have no effect on the biological response or on the

site of action. Thus, if differential toxicity between insects and mammals

or other beneficial insect is eliminated by the presence of suitable synergists,
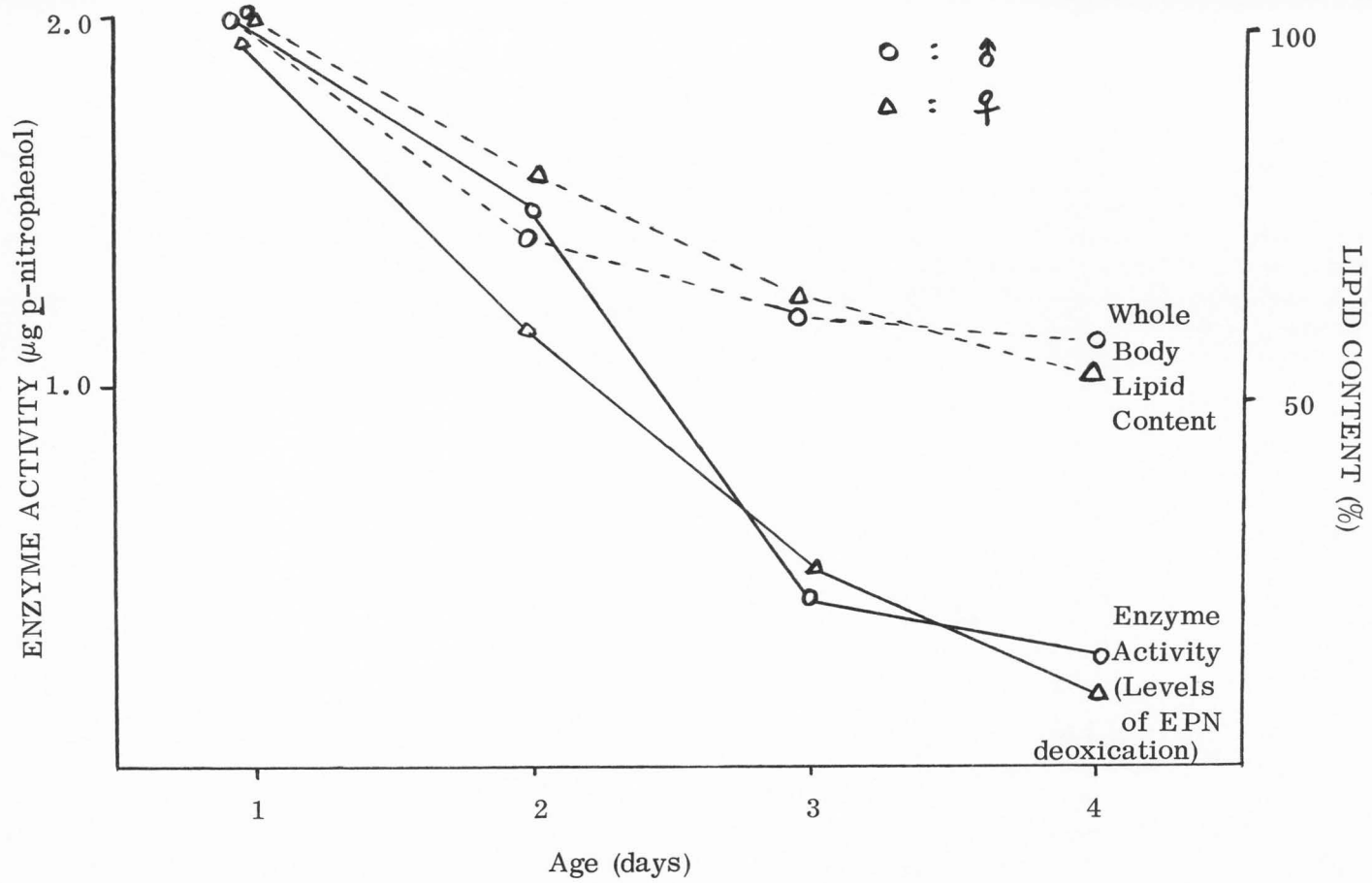
Figure 6. Relation between lipid content and enzyme activity.

such selectivity is very likely to be due to differences in oxidative metabolism and therefore the metabolic detoxication potential may be estimated (Winteringham, 1969; Brattsten and Metcalf, 1970).

Recently Brattsten and Metcalf (1970) have evaluated the synergistic ratio of carbaryl with piperonyl butoxide in relation to carbaryl LD_{50} values with 54 species of insects from eight orders and 37 families. They concluded that there was generally a good correlation between wide ranges of suscepti- bility to carbaryl and the synergist ratio, and interpreted this as due to dif- ferences in the activities of microsomal enzyme activity which is inhibited by piperonyl butoxide. Similar results were reported by Benke and Wilkinson (1971b). They also found a good agreement between the toxicity of carbaryl with its synergism by piperonyl butoxide and in vitro microsomal epoxidase levels in the malpighian tubes of house crickets of different ages and sex. In this study, however, the synergist ratio failed to correlate with either the toxicological or biochemical data determined for the leafcutter bee.

In addition, I found that there was something impractical about the procedure of the synergist ratio. As a rule, after bioassay, statistical analyses for inspection of dose mortality curves must be conducted for deter- mination of the LD_{50} and fitting of a regression line for the quantal response. To evaluate the synergist ratio, two regression lines are required for com- parison; one for carbaryl alone and one for carbaryl with synergist. If these two regression lines are not parallel, the synergist ratio will change dramatically depending on the dosage chosen for reference. But, obviously,

the activities of detoxication enzymes do not depend upon the synergist dose. For example, the synergist ratio for 3-day-old male bees (Figure 1) will be 20, 30, or 60, depending on whether the LD_{90}, the LD_{70}, or the LD_{30} is chosen for comparison.

Furthermore, the validity of the synergist ratio is also doubtful from a statistical point of view. LD_{50} is a point estimate; therefore the synergist ratio is a quotient of two variables. Hence, theoretically this figure will change drastically depending on the values chosen for comparison and their own standard deviations.

Next, an additional precaution is also necessary regarding the determination of the amount of piperonyl butoxide required. As Barnes and Fellig (1969) pointed out, the synergist ratio changed drastically depending on the amount of synergist applied.

Nutrition may have an effect on the resistance of insects to insecticides. At least, insects can develop modest tolerance to insecticidal toxicity and environmental stress due simply to physical well being (O'Brien, 1967). Lipid storage may be a function of nutrition and may also affect insecticide susceptibility. Recently, the effects of lipids in insects in regard to resistance to insecticides have been investigated. The roles of lipid in insects, relevant to selective toxicity is multifunctional. Lipids play many important roles such as controlling the penetration rate of the toxicant at integument and nerve tissue, or inert storage of lipophilic molecules. They also may affect the transport of the toxicant as lipoprotein (Winteringham, 1969).

Moore et al. (1962) found qualitative differences in lipids occurring in bollweevils treated with toxaphene, toxaphene + DDT, carbaryl, or azinphosmethyl. Significantly higher levels of steric, linoleic, and linolenic acids were present in dead weevils whereas higher levels of palmitic and oleic acids were found in the survivors. Barnett and Berger (1970) also reported that different lipid components in diets affect the mortalities of bollworms due to endrin and carbaryl. Fast and Brown (1962), however, found no significant difference in total lipids or phospholipid content between DDT-resistant and susceptible strains of mosquito larvae.

In this study, there was a close correlation between lipid content and carbaryl toxicity among different age groups of leafcutter bees. The younger bees, which had higher lipid content, also had a higher LD_{50} value.

The practical goal of protecting the alfalfa leafcutter bee from insecticides by an enzyme induction technique has motivated this study.

The most commonly used microsomal enzyme inducers have been chlorcyclizine, aminopyrine, phenobarbital and 3-methylcholanthrene (Sher, 1971). In some cases, DDT and dieldrin have been successfully used to modify toxicant storage in mammals (Street, 1968).

Pretreatment with sublethal doses of chlorcyclizine, aminopyrine or phenobarbital in the larval medium are reported to reduce the mortality of wax moth 6th instar larvae due to parathion (Ahmad and Brindley, 1969). The effects of different lengths of drug exposure were also tested. The authors suggested that the protective effect is neither immediate nor permanent

and that it depends upon the dosage and length of drug pretreatment. In connection with this work, Ahmad and Brindley (1971) have also studied effects of chlorcyclizine or phenobarbital on in vitro detoxication activity by larval wax moth gut homogenates. Chlorcyclizine or phenobarbital incorporated into the media of 6th or 7th instar wax moth larvae increased the rates of EPN detoxication, p-nitroanisole O-demethylation, and NADPH-neotetrazolium (NT)-reductase activity up to about two or three-fold. Turnquist (1971) also used chlorcyclizine to increase levels of cytochrome P-450, EPN-detoxication, p-nitroanisole O-demethylation and NADPH-NT-reductase activities in young American cockroaches. Chlorcyclizine was also found to make alkali bees more susceptible to parathion toxicity whereas SKF 525-A either had no effect or protected one sex without protecting the other. Because of variable results with SKF 525-A and the low level of tolerance induced, Moradeshaghi and Brindley (unpublished manuscript) suggested that it is unlikely to be a practical method of protecting bees from parathion poisoning in the field.

In my study, all three of the drugs used enhanced the microsomal enzyme activity by increasing EPN-detoxication up to four or five fold in alfalfa leafcutter bees. These drugs all decreased lipid content 20 to 30%. In vivo chlorcyclizine exposure to males, increased the LD_{50} up to 207%. Conversely, aminopyrine increased the toxicity of carbaryl dramatically in both sexes of the bees. On the other hand, phenobarbital had no measurable effects.

It was surprising to find, although the drugs had similar effects upon EPN detoxication and lipid content, that there were greatly different effects upon carbaryl susceptibility of the alfalfa leafcutter bees. These effects on toxicity of carbaryl were related to sex, the females being unaffected by either phenobarbital or chlorcyclizine. The males were also unaffected by phenobarbital but were protected from carbaryl by chlorcyclizine. Aminopyrine made both sexes more susceptible to the insecticide. I have done no experiments to clarify these points but some possibilities might be mentioned. Several workers, including Conney (1967) have suggested that the different effects of enzyme inducing drugs in mammals may be due to the influence of sex hormones. The general effect of the drug and its age-response relationship might also be affected. Hence, there is no reason to suspect that male and female leafcutter bees must be equally affected. Also, there are known to be several categories of enzyme inducing drugs which may have different effects. There are also two major patterns of carbaryl metabolism and the influence of one pattern relative to the other might provide an explanation for the effects of one drug on stimulating a tolerance and another drug in decreasing tolerance. That is, carbaryl, if first de-carbamylated to naphthol, is immediately detoxified. If, however, the N-methyl group is first hydroxylated, despite the fact that the N-hydroxy-methyl carbaryl may be eventually metabolized to naphthol, the presence of toxicant is prolonged and detoxication is delayed. Further and more detailed experiments would be required to explain these variations.

Utilization of enzyme induction has been a challenging issue. From this study, however, the prospect of using enzyme induction to protect pollinators does not appear to be very promising. In some cases, effects of inducers are not always readily predictable and also not very great (Moradeshaghi and Brindley, unpublished data). In addition, only maximum lethal dosages of the drugs were used in this study. Therefore the potential of the technique value is doubtful. This view is also shared by Plapp and Casida (1970). In their report, they conclude that the laboratory conditions used to demonstrate induction may not be comparable to actual field conditions of use of either DDT or dieldrin. Such condition of long period and constant exposure to high concentrations of insecticides or drugs seems unlikely to exist under field conditions.

In conclusion, the only way to test the hypothesis further, whether enzyme induction can protect pollinators, is to actually do the tedious bioassay for numerous drugs with different concentrations and using insects of various physiological states.

SUMMARY AND CONCLUSIONS

The alfalfa leafcutter bee, <u>Megachile</u> <u>rotundata</u>, generally became more susceptible to carbaryl as the adults aged. LD_{50} values for carbaryl toxicity were 240, 166, 109, and 51 μg carbaryl/gram for the 1, 2, 3, and 4-day-old adult male bees. For the female bees, these were 245, 551, 289, and 262 μg carbaryl/gram. Lipid content and microsomal enzyme activity also decreased in both sexes as the bees aged.

Synergist ratios failed to correlate with either decreasing LD_{50} values or decreasing EPN detoxication in the males, being 12, 21, 42, and 53 for the 1, 2, 3, and 4-day-old bees. In female bees the synergist ratio corresponded more closely, being 23, 49, 11, and 15 as the females aged from 1 to 4 days.

The use of enzyme-inducing drugs for protection of the bees from carbaryl poisoning were studied. Among the three drugs used, only chlor-cyclizine increased the LD_{50} values about two fold in the 4-day-old male. Aminopyrine increased the male's susceptibility to carbaryl while phenobarbital had no effect at all. All three drugs, however, caused an increase in microsomal enzyme activity up to four or five fold and a decrease in lipid content about of 20 to 30%.

From this study, the prospect of using enzyme induction to protect pollinators appears not very promising. The effects are neither predictable

nor potentially great for practical use. Therefore the only way to test the

hypothesis further, whether enzyme induction can protect pollinators, is to

actually do the tedious bioassay for numerous drugs with different concentra-

tions and insects of various physiological states.

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APPENDIXES

Table 13. Least significant difference among different ages of the male leafcutter bee

	$LD_{50}(X_i$ day$)-LD_{50}$ (4 day)	$LD_{50}(X_i$ day$)-LD_{50}$ (3 day)	$LD_{50}(X_i$ day$)-LD_{50}$ (2 day)
LD_{50} (1 day) = 239.96	188.66**	130.72*	73.47*
LD_{50} (2 day) = 166.49	115.19*	57.25*	
LD_{50} (3 day) = 109.24	57.94*		
LD_{50} (4 day) = 51.30			

$$(LD_{50_i} - LD_{50_i'}) \geqslant t \sqrt{V(LD_{50_i}) + V(LD_{50_i'})}$$

*Significant (α = 0.05)

Table 14. Least significant difference among different ages of the female leafcutter bee

	$LD_{50}(X_i \text{ day}) - LD_{50}(1 \text{ day})$	$LD_{50}(X_i \text{ day}) - LD_{50}(4 \text{ day})$	$LD_{50}(X_i \text{ day}) - LD_{50}(3 \text{ day})$
LD_{50} (2 day) = 550.54	305.06*	288.16*	261.15*
LD_{50} (3 day) = 289.39	43.91	27.01	
LD_{50} (4 day) = 262.38	16.90		
LD_{50} (1 day) = 245.48			

$$(LD_{50_i} - LD_{50_{i'}}) \geq t \sqrt{V(LD_{50_i}) + V(LD_{50_{i'}})}$$

*Significant ($\alpha = 0.05$)

Table 15. Analysis of variance and Duncan's multiple range test for lipid content in different ages of the male leafcutter bees

SV	DF	SS	MS	F
Total	17	983.8101		
Treatment	3	47.6680	15.8893	
				328.97**
Error	13	0.6285	0.0483	

Duncan's critical values for different n's

$P_{\sqrt{}} = 13$	2	3	4
$\sqrt{p}$	4.260	4.442	4.560
Rp $(n_1=5, n_2=4)$	0.443	0.462	0.474
$R'p$ $(n_1=n_2=4)$	0.469	0.489	0.502

$\overline{Y}_{4 \text{ day}}$	$\overline{Y}_{3 \text{ day}}$	$\overline{Y}_{2 \text{ day}}$	$\overline{Y}_{1 \text{ day}}$
5.98	6.07	7.06	9.93

F(.01, 3, 13) = 5.74
**Highly significant ($\alpha = 0.01$)

Table 16. Analysis of variance and Duncan's multiple range test for lipid content in different ages of the female leafcutter bees

SV	DF	SS	MS	F
Total	21	767.0839		
Treatment	3	34.9473	11.6491	
				115.34**
Error	17	1.7165	0.1010	

Duncan's critical values for different n's

$P_{\sqrt{}} = 17$	2	3	4
$\sqrt{p}$	4.099	4.275	4.391
$Rp(n_1=6, n_2=5)$	0.557	0.581	0.597
$R'p(n_1=n_2=5)$	0.582	0.607	0.624
$\overline{Y}_{4 \text{ day}}$	$\overline{Y}_{3 \text{ day}}$	$\overline{Y}_{2 \text{ day}}$	$\overline{Y}_{1 \text{ day}}$
4.55	4.79	6.21	7.69

$F(.01, 3, 17) = 5.19$
**Highly significant ($\alpha = 0.01$)

Table 17. Analysis of variance and Duncan's multiple range test for enzyme levels in different ages of the male leafcutter bees

SV	DF	SS	MS	F
Total	12	20.2927		
Treatment	3	6.3401	2.1134	
				8.13**
Error	8	0.0205	0.0026	

Duncan's critical values for different n's

$P_{\nu} = 8$	2	3	4
ν_p	4.746	4.939	5.057
$Rp(n_1=n_2=3)$	0.142	0.148	0.152
$\bar{Y}_{4\ day}$	$\bar{Y}_{3\ day}$	$\bar{Y}_{2\ day}$	$\bar{Y}_{1\ day}$
0.30	0.46	1.49	2.06

$F(.01, 3, 8) = 7.59$
**Highly significant ($\alpha = 0.01$)

Table 18. Analysis of variance and Duncan's multiple range test for enzyme levels in different ages of the female leafcutter bees

SV	DF	SS	MS	F
Total	18	20.1504		
Treatment	3	6.2689	2.0896	
				67.844**
Error	14	0.4307	0.0308	

Duncan's critical values for different n's

$P_{\nu} = 14$	2	3	4
$\sqrt{p}$	4.210	4.391	4.508
$Rp(n_1=n_2=3)$	0.425	0.443	0.455
$Rp'(n_1=3, n_2=4)$	0.400	0.417	0.428
$Rp''(n_1=3, n_2=8)$	0.354	0.369	0.379
$Rp'''(n_1=4, n_2=8)$	0.320	0.334	0.343
$\overline{Y}_{4\ day}$	$\overline{Y}_{3\ day}$	$\overline{Y}_{2\ day}$	$\overline{Y}_{1\ day}$
0.24	0.62	1.23	1.99

$F(.01,\ 3,\ 14) = 5.56$
**Highly significant ($\alpha = 0.01$)

Table 19. Least significant difference among effects of the different drugs on carbaryl toxicity to the male leaf-cutter bees (cont.: not treated, CZ. chlorcyclizine, AP: aminopyrine, PB: phenobarbital).

	$LD_{50}(Drug_i) - LD_{50}(AP)$	$LD_{50}(Drug_i) - LD_{50}(Cont.)$	$LD_{50}(Drug_i) - LD_{50}(PB)$
$LD_{50}(CZ) = 106.09$	99.81*	54.79*	54.71*
$LD_{50}(PB) = 51.38$	45.10*	0.08	
$LD_{50}(cont) = 51.30$	45.02*		
$LD_{50}(AP) = 6.28$			

$$(LD_{50_i} - LD_{50_i}') \geq t\sqrt{V(LD_{50_i}) + V(LD_{50_i}')}$$

*Significant ($\alpha = 0.05$)

Table 20. Least significant difference among effects of the different drugs on carbaryl toxicity to the female leafcutter bees. (Cont.: not treated; CZ: chlorcyclizine; AP: aminopyrine; PB: phenobarbital.)

	$LD_{50}(Drug_i) - LD_{50}(AP)$	$LD_{50}(Drug_i) - LD_{50}(Cont.)$	$LD_{50}(Drug_i) - LD_{50}(PB)$
$LD_{50}(CZ) = 293.18$	217.89**	30.80	10.84
$LD_{50}(PB) = 282.34$	207.05**	19.96	
$LD_{50}(Cont.) = 262.18$	187.09**		
$LD_{50}(AP) = 75.29$			

$$(LD_{50_i} - LD_{50_i'}) \geqslant t\sqrt{V(LD_{50_i}) + V(LD_{50_i'})}$$

*Significant ($\alpha = 0.05$)

Table 21. Analysis of variance and Duncan's multiple range test for effects of drugs on lipid content of the male leafcutter bees. (Cont.: not treated; CZ: chlorcyclizine; AP: aminopyrine; PB: phenobarbital.)

SV	DF	SS	MS	F
Total	15	362.1881		
Treatment	3	8.0552	2.6851	
				41.44**
Error	11	0.7132	0.0648	

Duncan's critical values for different n's

$P_\gamma = 11$	2	3	4
γ_p	4.392	4.579	4.697
$Rp(n_1=4, n_2=3)$	0.602	0.627	0.643
$Rp'(n_1=n_2=4)$	0.558	0.582	0.597

$\bar{Y}_{PB}$	$\bar{Y}_{CZ}$	$\bar{Y}_{AP}$	$\bar{Y}_{Cont.}$
4.08	4.40	4.84	5.98

F(.01, 3, 11) = 6.22
**Highly significant ($\alpha = 0.01$)

Table 22.   Analysis of variance and Duncan's multiple range test for effects of drugs on lipid content of the female leafcutter bees.   (Cont.: not treated; CZ: chlorcyclizine; PB: phenobarbital; AP: amino-pyrine)

SV	DF	SS	MS	F
Total	16	255.4465		
Treatment	3	1.7434	0.5811	
				14.07**
Error	12	0.4954	0.0413	

Duncan's critical values for different n's

P_γ	2	3	4
γ_p	4.320	4.504	4.622
$Rp(n_1=n_2=4$	0.436	0.455	0.467

$\overline{Y}_{CZ}$	$\overline{Y}_{AP}$	$\overline{Y}_{PB}$	$\overline{Y}_{Cont.}$
3.74	3.81	3.82	4.55

$F(.01, 3, 12) = 5.95$
**Highly significant ($\alpha = 0.01$)

Table 23. Analysis of variance and Duncan's multiple range test for effects of drugs on enzyme activity of the male leafcutter bees. (Cont.: not treated; CZ: chlorcyclizine; AP: aminopyrine; PB: phenobarbital)

SV	DF	SS	MS	F
Total	16	22.9993		
Treatment	3	3.7406	1.2469	
				81.50**
Error	12	0.1836	0.0153	

Duncan's critical values for different n's

P_{γ}	2	3	4
$\sqrt{P}$	4.320	4.504	4.622
$Rp(n_1=n_2=4)$	0.268	0.279	0.287

$\overline{Y}_{Cont.}$	$\overline{Y}_{AP}$	$\overline{Y}_{PB}$	$\overline{Y}_{CZ}$
0.28	1.17	1.40	1.52

$F(.01, 3, 12) = 5.95$
**Highly significant ($\alpha = 0.01$)

Table 24. Analysis of variance and Duncan's multiple range test for effects
of drugs on enzyme activity of the female leafcutter bees. (Cont.:
not treated; CZ: chlorcyclizine; PB: phenobarbital; AP: amino-
pyrine)

SV	DF	SS	MS	F
Total	16	28.5744		
Treatment	3	4.2161	1.4054	
				67.244**
Error	12	0.2502	0.0209	

Duncan's critical values for different n's

$P_{\nu} = 12$	2	3	4
ν_p	4.320	4.504	4.622
$Rp(n_1=n_2=4)$	0.311	0.324	0.333
$\overline{Y}_{Cont.}$	$\overline{Y}_{AP}$	$\overline{Y}_{CZ}$	$\overline{Y}_{PB}$
0.35	1.45	1.46	1.66

$F(.01, 3, 12) = 5.95$
**Highly significant ($\alpha = 0.01$)

VITA

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Doctor of Philosophy

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