1989

Toxic properties of d-limonene in insects and the earthworm Eisenia fetida

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Toxic properties of $d$-limonene in insects and the earthworm *Eisenia fetida*

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Iowa State University, 1989
Toxic properties of d-limonene in insects and the earthworm

*Eisenia fetida*

by

Laura L. Karr

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Entomology

Co-majors: Entomology
          Toxicology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa

1989
TABLE OF CONTENTS

INTRODUCTION AND LITERATURE REVIEW

| Introduction | 1 |
| Literature Review | 5 |
| Historical Background of Monoterpenoids | 5 |
| Monoterpenoid Biosynthesis | 8 |
| The Natural Occurrence of Monoterpenoids | 12 |
| The Biological Role and Consequences of the Monoterpenoids and Other Secondary Plant Compounds | 14 |
| d-Limonene Attributes and Uses | 16 |
| d-Limonene Toxicity | 18 |

REFERENCES CITED

20

PART I. INSECTICIDAL PROPERTIES OF D-LIMONENE

| ABSTRACT | 25 |
| INTRODUCTION | 26 |
| MATERIALS AND METHODS | 28 |
| Acute Topical Toxicity | 28 |
| Fumigant Activity | 28 |
| Oral Toxicity | 29 |
| Repellency | 31 |
| Residual Activity | 31 |
| Ovicidal and Larvicidal Properties | 32 |

RESULTS

| Acute Topical Toxicity | 34 |
Fumigant Activity 34
Oral Toxicity 34
Repellency 36
Residual Activity 36
Ovicidal and Larvicidal Properties 38

DISCUSSION 39
REFERENCES CITED 40

PART II. EFFECTS OF D-LIMONENE ON GROWTH AND REPRODUCTION IN THE GERMAN COCKROACH (ORTHOPTERA: BLATTELLIDAE) 41

ABSTRACT 42
INTRODUCTION 44
MATERIALS AND METHODS 46
Feeding Preferences 46
Growth Rate Effects 47
Oöthecal Treatment 47
Effects of "Lifetime-Feeding" of d-Limonene-Treated Diet on Fertility and Fecundity 48
Effects of Topical d-Limonene Treatment on Fertility and Fecundity 49
Effects of d-Limonene Vapor Exposure on Fertility and Fecundity 50

RESULTS 52
Feeding Preferences 52
Growth Rate Effects 53
Oöthecal Treatment 54
INTRODUCTION AND LITERATURE REVIEW

Introduction

Plants produce a myriad of chemicals known as secondary compounds or allelochemics. These materials are not required for plant growth and reproduction and appear to exist primarily for the protection they offer against insect herbivores. According to Hedin (1986), the chemical classes encompassed by the secondary compounds include: 1) phenolic compounds such as the flavonoids, tannins, and aromatic acids; 2) nitrogenous compounds such as alkaloids, amino acids, and amides; 3) proteinaceous materials including protease inhibitors, glycosidase inhibitors, and phytohemagglutinins; 4) lipids, including the fluorolipids, cyanolipids, and saponins; 5) many types of glycosides; and 5) the isoprenoids such as the sesquiterpene lactones and the monoterpenoids. The presence of secondary compounds in plants is conferred by specific genes, a fact that has been exploited for decades by plant breeders selecting for crop plants possessing traits deemed undesirable for insect pest establishment and survival.

Whereas plant breeders have strived to exploit insect-impairing secondary compounds within plants, chemical ecologists, natural product chemists, and insecticide toxicologists frequently have attempted to identify, isolate, and characterize the chemical factors responsible for resistance and either exploit the compounds directly as insecticides, or use them as models for new and more potent insecticides. Plant chemicals which have been used directly as insecticides are generally referred to as "botanicals." This group includes insecticides of significant his-
torical and economical importance: pyrethrum (from flowers of the composite genus Chrysanthemum), nicotine (from leaves of Nicotiana tabacum and Nicotiana rustica), rotenone (from the roots or resins of legumes in the Derris or Lonchocarpus genera) (Matsumura 1975), sabadilla (from the seeds of the lily Schoenocaulon officinale), and ryania (from roots, leaves and stems of South American plants in the genus Ranya) (Crosby 1971).

According to Hedin (1986), none of the botanicals have been as widely accepted and employed in practical agriculture as have the synthetic organic insecticides such as the organophosphates. This is because many of the structures are too complex to permit economical manufacture or because many of the compounds do not penetrate insect cuticle readily and require alternative routes of entry. A more complete understanding of the structures and modes of toxic action of such insect-impairing plant chemicals may, however, serve as a guide for the synthesis of analogous compounds that may be, under the appropriate circumstances, more effective, more stable, and more economical to manufacture than natural substances. The success that has been attained in producing a group of economical, useful, and potent agrochemicals based on the structure of a natural product is clearly exemplified by the synthetic pyrethroids, structural analogues of natural pyrethrum.

A group of secondary plant compounds for which insect-influencing properties have received growing attention in recent years is the monoterpenoids. These diverse ten-carbon hydrocarbons, alcohols, aldehydes, ketones, and carboxylic acids are dominant components of the essential
oils of plants, the volatile, odiferous chemical mixtures that contain many of the fragrances and flavors that characterize the plants from which they originate. Monoterpenoids are typically rather lipophilic compounds, and thus appear to have a high potential for toxic interference with the basic biochemical, physiological, and behavioral functions of insects. Indeed, many exhibit toxic, repellent, or attractant properties in various insect species (Brattsten 1983). Also, monoterpenoids are abundant in nature and represent a virtually untapped and unlimited source of potential insect-impairing chemicals. Additionally, monoterpenoids in general, and the monocyclic hydrocarbon d-limonene in particular, have frequently been touted as possible alternatives to conventional insecticides. A principal goal of my research was to evaluate d-limonene's performance and practical feasibility as an insecticide.

The evaluation of insecticidal properties of compounds such as d-limonene is timely from both practical and academic standpoints. As noted by Hedin (1986), "the exploitation of knowledge of the chemical bases of biological interactions (i.e., biorational approaches) may lead to the recognition and utilization (i.e., synthesis, chemically directed selections, gene insertions) of new molecules designed to act at a particular site or to block a key step in a biochemical process." As concerns deepen over the presence of, or potential for, synthetic pesticide residues in our foods, soils, and groundwater supplies, the development of such "biorational" approaches to managing insect populations becomes more pertinent and more desirable. Botanical insecticides, or
insecticides based on naturally occurring secondary plant compounds, may therefore represent an agreeable, sensible alternative to traditional synthetic pesticides. A thorough understanding of secondary compound roles in managing insect pests may also prove to be increasingly useful in the selection of crop plant varieties. As biotechnological techniques improve, permitting gene traits for secondary compounds to be transferred between crop plants, we may see biorational approaches aiding, or perhaps controlling, gene selection processes.

The mechanisms of plant and insect interactions have long been pondered by botanists, entomologists, biochemists, chemical ecologists, and evolutionary biologists. Plants and insects coevolve, and many of their coadaptations undoubtedly reflect the dynamic balance and interplay between factors such as the biosynthesis of defensive compounds by the plant, and the development of detoxification or other coping mechanisms by the insect. A clearer understanding of the complex interactions between plants, secondary compounds, and insects can only serve to advance our understanding of the intricacies of coadaptation and coevolution.

The purpose of my research was to evaluate the toxic properties of the monocyclic monoterpenoid d-limonene, p-mentha-1,8-diene, an allelo-chemic found in abundance in citrus oils. This project was designed to answer the following questions: what are the toxic properties of d-limonene, which insects and insect life stages does it affect, which routes of entry are most efficient, what sublethal effects are important, how feasible is d-limonene, in and of itself, as an insecticide, and what
are some possible modes of toxic action?

The specific objectives of the studies completed were:

1) To determine the spectrum of insecticidal properties of d-limonene in several common insects via several routes of entry and to evaluate the suitability and efficacy of the compound as a general-use insecticide.

2) To determine if d-limonene influences growth and reproduction in the German cockroach, *Blattella germanica*.

3) To assess the neurotoxic properties of d-limonene in a nontarget species, the earthworm *Eisenia fetida*, by employing non-invasive electrophysiological recording techniques.

Understanding the toxic properties and possible modes of action for d-limonene will advance our comprehension of chemical structure-activity relationships and plant and animal coevolution. Additionally, it may contribute to the biorational approaches to insect pest management that are so critical at this time.

**Literature Review**

**Historical Background of Monoterpenoids**

The flowers, fruits, leaves, and roots of plants often contain many volatile, odiferous chemicals which can be separated from the plant components by mild heating. Together, these principles are known as the "essential oils" and consist of a mixture of hydrocarbons, alcohols, aldehydes, ketones, acids, and esters.

According to de Mayo (1959), the essential oils were discovered centuries ago and the production and employment of the oils, primarily
for medicinal purposes, became significant in the sixteenth century. The exploitation of the oils of anise, clove, cinnamon, and others was rapid. Sixty oils are listed in the *Dispensatorium Valeri Cordi* of 1592. Pharmacists developed applications for the oils during the following two centuries, and chemists, such as Dumas, began to investigate the materials in the nineteenth century. Early chemists discovered that the more volatile fractions of the essential oils contained numerous hydrocarbons, all sharing the same molecular formula, \( C_{10}H_{16} \). The name "terpene" was applied to these compounds; it was derived from the German *terpentine* (turpentine). With the discovery of related substances containing many different functional groups (alcohols, ketones, etc.), the ending "-ene" of terpene, originally signifying an alkene, seemed inappropriate and the general term "terpenoid" has since been adopted as a generic name for this class of compounds. "Monoterpenoid" is currently used to refer to the 10-carbon terpenoids, "sesquiterpenoid" describes the 15-carbon terpenoids, and "diterpenoid", "triterpenoid", and "tetraterpenoid" refer to the 20-, 30- and 40-carbon terpenoids, respectively.

According to Dev et al. (1982), chemical investigations of the essential oils in the nineteenth century led to the isolation and purification of a number of monoterpenoids: borneol in 1840, linalool in 1853, limonene in 1870, geraniol in 1871, cineol in 1884, citral in 1888, and \( \alpha \)-pinene in 1890. The structures of most of these compounds also were elucidated by 1900. In spite of these early advances, further terpenoid research progressed rather slowly. This resulted, in part, from the fact that monoterpenoids, and terpenoids generally, usually
occur in nature as mixtures of closely related compounds including geometric and optical isomers. The methods of separation available to early workers simply were not effective in obtaining the pure samples necessary for many analyses and investigations.

The early difficulties encountered in terpenoid analysis were eventually overcome as more sophisticated separation and purification techniques evolved. Terpenoid researchers were then able to make some substantial contributions to organic chemistry, notably the detailed and exhaustive studies of bicyclic monoterpenoids which led to the 1899 discovery of Wagner rearrangement (Ingold 1953). This, in turn, has advanced our understanding of reaction mechanisms involving carbonium ions. Additionally, Wallach's work in the late 1880s, in which he proposed the "Isoprene Rule", has been a basic in the development of terpenoid chemistry in general (Dev et al. 1982). The "Isoprene Rule", in its most elementary form, states that the most likely structure for a terpenoid is that which permits its carbon skeleton to be divisible into iso-C₅ (or "isoprene") units (Linstromberg 1974). The rule has been helpful in deriving the structures not only of monoterpenoids, but also of a number of other natural products including the carotenoids and the steroids. The "Isoprene Rule" eventually was refined by Ruzicka (1953, 1959) into the "Biogenetic Isoprene Rule", a useful development in terpenoid chemistry that permitted terpenoid structures to be rationalized and deduced via accepted reaction mechanisms from hypothesized acyclic precursors. By 1977, 700 monoterpenoids, belonging to 38 skeletal types, had been described. These are conveniently grouped into three main categories:
acyclic, monocyclic, and bi- and tricyclic monoterpenoids.

Monoterpenoid Biosynthesis

Terpenoids are generated in living systems by the conversion of acetate (from fatty acid breakdown, carbohydrate metabolism, and certain amino acids) to a branched-chain intermediate, $\Delta^3$-isopentenyl pyrophosphate, the biological isoprene unit. According to Richards and Hendrickson (1964), the biosynthetic pathway (Figure 1) begins with condensation of two moles of acetyl coenzyme A to produce acetoacetyl coenzyme A. Acquisition of another acetyl residue at the central carbonyl group forms the branched, six-carbon hydroxymethylglutaryl coenzyme A. A stepwise reduction of the esterified carboxyl carbon of this substance yields mevalonate. A series of phosphorylations follows with the terminal hydroxyl function of the mevalonate activated as a pyrophosphate ester. Phosphorylation of the tertiary hydroxyl group activates the resulting molecule for decarboxylation concerted with the loss of phosphate and the generation of $\Delta^3$-isopentenylpyrophosphate.

Whereas the intermediates in the biosynthesis pathway prior to mevalonate may be converted to other substances, formation of mevalonate is essentially an irreversible process, and mevalonate, once formed, ultimately has one particular role: the production of isoprenoid substances including monoterpenoids, carotenoids, ubiquinones, plastoquinones, and steroids. The $\Delta^3$-isopentenyl pyrophosphate units generated from mevalonate join together via a series of reactions to produce 10-, 15-, 20-, 30-, 40-carbon, or larger unsaturated molecules. Further transformations may then give rise to the various skeletal types.
Polymerization of isopentenyl pyrophosphate units to form monoterpenoids begins with a reversible isomerization of $\Delta^3$- to $\Delta^2$-isopentenyl pyrophosphate (Figure 2). These two molecules undergo condensation to yield the ten-carbon precursors to the terpenoids, geranyl pyrophosphate and neryl pyrophosphate. The acyclic monoterpenoids arise via modification of the hydrolysis products of these pyrophosphates, geraniol, and its geometric isomer, nerol. Many biogenetic theories on the subsequent formation of other terpenoids from these precursors have been proposed. Specifically for monoterpenoids, Ruzicka (1953) proposed rationalization
of structures in terms of cyclization of a suitable acyclic precursor, such as geraniol, employing ionic or free radical intermediates. At present, according to Dev et al. (1982), most monoterpenoids can be accommodated within Ruzicka's system; however, there are some exceptions. It is important to realize that two general synthetic pathways exist for the formation of cyclic monoterpenoids: that described by Ruzicka, involving various cyclizations of acyclic precursors such as geraniol or nerol, and that which does not conform to the Biogenetic Isoprene Rule. The geraniol-based cyclic monoterpenoids may be grouped according to their carbon skeleton characteristics. These are indicative of the type of initial bond formation which occurred in the cyclization (Figure 3).
Figure 3. Cyclization possibilities for an acyclic precursor (after Dev et al. 1982)
The most dominant cyclization pathway encountered in nature is the 1,6 cyclization of the acyclic precursor, most probably nerol, to produce a cation which can lead to monocyclic substances including d-limonene, α-phellandrene, and α-terpinene. The cation may also partake in the formation of bicyclic monoterpenoids such as α-thujane, borneol, camphene, and α-pinene.

The Natural Occurrence of Monoterpenoids

Monoterpenoids are vastly dispersed in nature, and have been found in plants, animals, and bacteria, and in both terrestrial and marine environments. Monoterpenoids are abundant in the higher plants and may occur in any portion of a plant. Guenther (1948) recognized that the percentage and nature of the monoterpenoids in a specific plant may vary widely with the ontogeny of the plant. Additionally, the presence of these compounds in plants is under genetic control and may therefore be selected for both naturally and artificially. In plants, monoterpenoids exist in both free and combined states: hydrocarbons tend to occur in the free state, alcohols often occur in both the free state and as esters, and heavily oxygenated monoterpenoids typically are glycosylated. According to Nicholas (1973), α-pinene, cineole, and limonene are the most widely distributed and abundant of the monoterpenoids in plants.

Flower buds and flowers may contain an array of monoterpenoids; for instance, Minyard et al. (1969) found limonene, α-pinene, and β-caryophyllene in the essential oils of the buds of the cotton plant, *Gossypium hirsutum*. Fruits and seeds have also yielded large quantities of monoterpenoids: cherimoya, *Annona cherimolia*, was found to contain
linalool, α-terpineol, carvone, and 1,8-cineol (Idstein et al. 1984), and citrus fruits (Rutaceae) have long been known as a bounteous source of d-limonene. The oleoresins and resins secreted by many woody plants, especially in the Pinaceae and Burseraceae, are especially rich sources of some monoterpenoids as are the herbaceous plants in the families Labiatae, Lauraceae, Myrtaceae, Gramineae, and Umbelliferae. Whereas monoterpenoids have not been isolated from fungi (Turner 1971), they have been found in many marine plants and algae (Katayama 1962) and in at least one vertebrate, the alligator (Fester et al. 1937).

In recent years, many monoterpenoids have been identified in animals, especially insects. Some of these compounds have been implicated as sex and aggregation stimulants, and others appear to function as alarm and defensive substances. Young et al. (1973) identified linalool in volatiles produced by female Ips paraconfusus and in the frass of male Ips pini; linalool has also been identified as the main component of the mandibular gland secretion in several species of Colletes bees (Bergström and Tengö 1978). Various Andrena bees produce cephalic secretions containing neral, geranial, geraniol, and citronellol (Tengö and Bergström 1976).

A number of insects employ monoterpenoids in defensive secretions. For example, Nutting et al. (1974) identified α-pinene, myrcene, and limonene from frontal gland secretions of soldier termites, Tenuirostri-termes tenuirostris, and Brand et al. (1974) identified d-limonene, l-limonene, and several other monoterpenoids in the poison gland secretion of the ant Myrmicaria natalensis. The nymph of the tropical hemipteran
Hoea gambiae utilizes a mixture of mono- and sesquiterpenoids in a defensive secretion of the dorsal abdominal glands (Gough et al. 1985). Linalool was found to be a major component of the scent secreted by the metathoracic gland of another hemipteran, the cotton stainer, Dysdercus intermedius (Everton et al. 1979), and hemipterans in the Rhopalidae family have been found to release α-pinene, β-pinene, myrcene, limonene, and thymol from the metathoracic and dorsal abdominal glands (Aldrich et al. 1979). Coleopterans may also utilize monoterpenoids in defense secretions; larvae of the willow leaf beetle, Plagiodera versicolora, secrete methylcyclopentanoid monoterpenoids to deter feeding by conspecific adults and other willow herbivores (Raupp et al. 1986).

The Biological Roles and Consequences of the Monoterpenoids and Other Secondary Plant Compounds

Plant-produced monoterpenoids belong to a class of chemicals known as "secondary plant compounds." Such materials are produced by plants, often in significant quantities, but are not required for the plants' growth and development. In the 1930's, Muenscher (1939) summarized contemporary thought on the function of secondary plant compounds: "Many hypotheses have been advanced to explain the significance of toxic substances produced in plants, as, that they are developed to protect the plants against herbivorous animals, are waste products, are stages in the processes of metabolism. Of these suggestions the first appears the least acceptable, and the last the most plausible." Current thought on the matter is generally contrary to Muenscher. Because secondary plant compounds are often complex and costly molecules to build biochemically,
there is little evidence supporting the hypotheses that they are produced by plants as wastes or metabolic intermediates. There is, however, considerable evidence to support the theory that many secondary plant compounds were originally produced because of, and exist largely for, their anti-microbial and anti-herbivore (and, especially, anti-insect) functions. Whittaker (1971) has proposed replacing the term "secondary plant compound" with "allelopathic chemical" or simply "allelochemic", defining the latter as a non-nutritional chemical produced by one species for its effects on the growth, health, reproduction, and behavior of another species.

Plant allelochemics may negatively affect insects in a variety of ways. They may interfere with host selection, feeding, or oviposition. Allelochemics may also play integral roles in the plant resistance mechanism known as biochemical antibiosis. Allelochemics responsible for antibiosis may cause direct harm to insects by killing them outright, prolonging the length of the stadia, increasing the number of instars, inducing restlessness, decreasing fecundity, preventing the formation of proper food reserves, and hindering normal growth and weight gain (Painter 1951).

Antibiosis, however, is only one facet of the relationship between the allelochemic producer and receiver. Whereas many allelochemics do have adverse effects on insect herbivores, others have been successfully exploited by insects and are all-important compounds in the food-finding, feeding, and breeding success of herbivorous insects (Kingsbury 1978). Examples of allelochemic dependence and exploitation abound: Danaidae
butterflies sequester glycosides from milkweeds thereby becoming distasteful to predators; some *Dendroctonus* beetles synergize aggregation pheromones with host-provided monoterpenoids; and *Diabrotica* depend on host-produced tricyclic tetraterpenoids for host recognition. These relationships are not accidental nor novel but are the result of a long chronology of coevolution between plants and insects. Ehrlich and Raven (1964) have even suggested that the rapid and extensive diversification of the angiosperms, compared to other contemporary plant groups, occurred behind a biochemical shield of toxic secondary compounds that afforded them an unusual degree of protection from the phytophagous organisms of the time. Because the coevolution of angiosperms and insects has had more than 100 million years to operate, it is not surprising that some marvelous and intricate interactions have ensued between these two groups. Allelochemics, such as the monoterpenoids, are therefore perhaps best described as ecologically active participants in the complex and ever-changing interactions between components of ecosystems.

**d-Limonene Attributes and Uses**

*d*-Limonene (Figure 4), p-mentha-1,8-diene, is a monocyclic hydrocarbon monoterpenoid widely distributed in nature. The compound, which has also been known as cinene, cajeputene, and kautshln (Vindholz 1983), has the chemical formula \( \text{C}_{10}\text{H}_{16} \) and a molecular weight of 136.23. At room temperature, it exists as a liquid with a density of 0.84 g/ml and is pleasantly odiferous, emitting a strong, orange-like scent. *d*-Limonene evaporates from surfaces completely, leaving no residues. The material is found as its *d*- and *l*-forms in several essential oils including lemon,
orange, caraway, and bergamot (Pinder 1960). A racemic mixture, known as
dipentene, occurs in various turpentines. \( d \)-Limonene is practically

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\caption{\( d \)-Limonene (\( \text{p-mentha-1,8-diene} \))}
\end{figure}}
\]

insoluble in water but is readily miscible with alcohol, ethyl ether, and acetone.

\( d \)-Limonene has several minor industrial uses as a solvent and a
wetting and dispersing agent but it is frequently and abundantly employed
in the flavor and fragrance industry. FEMA (Flavoring Extract Manufac-
turers' Association) and the U.S. Food and Drug Administration gave the
compound GRAS (generally recognized as safe) status in 1965 (Opdyke
1979). The material also has been used clinically in a preparation for
dissolving cholesterol gallstones (Hisatsugu et al. 1972). Two more
novel uses of \( d \)-limonene include combining it with hydrogen disulfide for
use as a microencapsulated chemical marker in paraquat herbicide (Turner
et al. 1981) and using it as a detector of singlet oxygen in polluted
atmospheres through chromatographic quantitative identification of
optically active limonene oxidation products (Rawls and Estes 1978). In
recent years the compound has gained attention as the active ingredient
of several commercially available flea shampoos for pets.
d-Limonene Toxicity

*d*-Limonene has very low toxicity in mammalian species; the lowest published oral LD$_{50}$ value for rats is 4600 mg/kg (Duke 1977). The absorption, distribution, and excretion of the material has been thoroughly investigated in rats (Igimi et al. 1974), and its metabolism has been examined in rats (Regan and Bjeldanes 1976), rabbits (Kodama et al. 1974a), hamsters, dogs, guinea pigs, and humans (Kodama et al. 1974b). No data for inhalation toxicity in mammals are available.

A number of studies have addressed citrus oil and *d*-limonene toxicity toward insects. In their undistilled form, citrus oils have exhibited topical toxicity against the cowpea weevil (*Callosobruchus maculatus*), rice weevil (*Sitophilus oryzae*) (Su et al. 1972), house fly (*Musca domestica*), and red imported fire ant (*Solenopsis invicta*) (Sheppard 1984).

Smith (1965) found that, of several ponderosa pine monoterpenoid vapors tested, limonene vapors were the most toxic to western pine beetles, *Dendroctonus brevicomis*. In similar studies, Coyne and Lott (1976) found topical applications of limonene to be toxic to the southern pine beetle, *Dendroctonus frontalis*. Taylor and Vickery (1974) noted that limonene exhibited contact toxicity in flies (Musca spp.), black flies (*Simulium damanosum*), mosquitoes (Culex and Anopheles spp.), and cowpea weevils (*Callosobruchus phaseoli*). Styer and Greany (1983) found limonene and several other citrus oil constituents to be toxic to larval and egg stage fruit flies (*Anastrepha suspensa*); similarly, Hink and Fee (1986) found topical applications and vapor exposures of the compound to
be most toxic to eggs, and decreasingly toxic to adults, larvae, and pupae of the cat flea, *Ctenocephalides felis*. Their findings suggested that vapors were the principle route by which the compound entered fleas. *d*-Limonene was also found to reduce fecundity in the cat flea (Collart and Hink 1986a) and was determined to be more toxic to malathion-resistant cat fleas than to malathion-susceptible cat fleas (Collart and Hink 1986b). Little is known regarding *d*-limonene's effects on terrestrial invertebrates other than insects.
REFERENCES CITED


Painter, R. H. 1951. Insect resistance in crop plants. Univ. Kansas Press, Lawrence, KS.


PART I. INSECTICIDAL PROPERTIES OF D-LIMONENE
d-Limonene (p-mentha-1,8-diene) is a monocyclic monoterpenoid found in citrus oils. The spectrum of insecticidal activity of d-limonene was examined by using the German cockroach, *Blattella germanica* (L.), house fly, *Musca domestica* L., rice weevil, *Sitophilus oryzae* (L.), and western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Bioassays were performed to determine topical, fumigant, oral, repellent, residual, ovicidal, and larvicidal activities. The material was slightly toxic when topically applied to German cockroaches and house flies and was synergized with piperonyl butoxide. High concentrations of vapor caused mortality in German cockroaches and rice weevils. Oral administration did not result in mortality to either adult or nymphal cockroaches but accelerated growth in nymphs. Repellent activity against German cockroaches was noted at high concentrations. No residual activity was observed on any of four surface types exposed to adult German cockroaches. d-Limonene inhibited western corn rootworm egg hatch at high concentrations and showed moderate toxicity in soil against third-instar western corn rootworm larvae. These findings indicate that the insecticidal properties of d-limonene are limited.
INTRODUCTION

Terpenoids are found in the essential oils of most higher plants. Monoterpenoids, ten-carbon compounds formed in nature via the condensation of isopentenyl pyrophosphate units, are among the industrially most important of the terpenoids. The monocyclic terpenoid d-limonene (p-mentha-1,8-diene) is a common monoterpenoid and is the main constituent of the terpenoid fractions of lemon and orange oils. It is purified from citrus peels via steam distillation.

Because the terpenoids are typically lipophilic, they possess a high potential for toxic interference with insect biochemical, physiological, and behavioral functions. In their undistilled form, citrus oils have exhibited toxicity against various insects including the cowpea weevil (Callosobruchus maculatus), rice weevil (Sitophilus oryzae) (Su et al. 1972), house fly (Musca domestica), and red imported fire ant (Solenopsis invicta) (Sheppard 1984). d-Limonene was found to be acutely toxic to Dendroctonus bark beetles (Coyne and Lott 1976, Smith 1965), eggs and larvae of the fruit fly Anastrepha suspensa (Styer and Greany 1983), and all stages of the cat flea (Ctenocephalides felis) (Hink and Fee 1986). It also was found to reduce fecundity in the cat flea (Collart and Hink 1986). d-Limonene is a common ingredient in soaps, perfumes, and food flavorings and has recently appeared as an active ingredient of some commercially available flea shampoos. d-Limonene exhibits low toxicity in mammals and has been given GRAS (generally recognized as safe) status by the United States Food and Drug Administration. It also has been used clinically in a preparation for dissolving cholesterol gallstones (His-
Because \textit{d-limonene} is a natural product with low mammalian toxicity, it has significant commercial appeal and has been touted as an alternative to synthetic insecticides. My study was done to evaluate the spectrum of activity of \textit{d-limonene} against some common insects in order to assess the suitability and efficacy of the compound as a general-use insecticide. A more complete understanding of the toxic effects that this plant-produced compound has on insects might also aid in our determination of its purpose and function in the plants producing it.
MATERIALS AND METHODS

Acute Topical Toxicity

Topical toxicity was examined by applying solutions of various concentrations of d-limonene (in acetone) to the abdominal venters of laboratory-reared, anesthetized adult (2 weeks post-molt) male German cockroaches, Blattella germanica (L.), and female house flies, Musca domestica L. (5 days post-eclosion, susceptible strain: Orlando regular), by using an electric microapplicator. Chlorpyrifos and pyrethrins were used as standards for comparison. The three chemicals were provided by Pet Chemical Co., Miami Springs, FL and were estimated to be at least 96% pure. Compounds were applied alone or with the synergist piperonyl butoxide (PB) in ratios of 1:1 and 1:5, insecticide to synergist. Three replicates of 20 insects each were treated with each of five concentrations of the chemicals (for d-limonene, treatments used were 8, 4, 2, 1, and 0.5 mg per cockroach or 2, 1, 0.5, 0.25, and 0.12 mg per house fly). Control treatments of acetone or PB also were administered. Mortality was recorded at 24 hr after treatment of insects. LD$_{50}$ values were estimated by using the Spearman-Karber method (Hamilton et al. 1977).

Fumigant Activity

Effects of d-limonene vapors were examined on German cockroach and rice weevil, Sitophilus oryzae (L.), adults. d-Limonene treatments were 0.01, 0.1, 1.0, 10, and 100 ppm. Dichlorvos, the standard for comparison, was obtained from Chem Service, Inc., Westchester PA; it was applied at rates of 0.001, 0.01, 0.1, 1.0, and 10 ppm. Control treatments also were administered, and treatments were replicated five times for each
chemical. For each concentration of each treatment, 25 rice weevils or 10 German cockroaches (5 females and 5 males) were placed in a small cage, which was then suspended via stainless-steel wire inside a 3-l jar. The cages were constructed from glass cylinders (6 X 2 cm diameter) with fine-mesh stainless-steel screens fastened to both ends of the cylinder with paraffin. Automatic micropipettes were used to dispense the various d-limonene or dichlorvos treatments into 1 dram vials also suspended with wire within the jars. Magnetic stir bars were placed in the jars, and the jars were sealed and placed on multiple magnetic stirrers to keep the volatilized toxins and air inside the jars well mixed. Mortality was assessed after 24 hr of exposure, and LC$_{50}$ values were estimated by the Spearman-Karber method.

Oral Toxicity

The effects of orally administered d-limonene were examined by using both adult and nymphal German cockroaches. Adult cockroaches (2 weeks post-molt), isolated in pint jars with screen lids, were fed a ground Purina$^\text{®}$ cat chow, corn-oil mixture (2:1), treated with d-limonene or, as a standard for comparison, chlordecone (obtained from Chem Services, Inc., Westchester, PA). Treated foods were offered in feeding stations constructed from 3-mm slices of 1-cm diameter Teflon$^\text{®}$ tubing to limit cuticular contact with food. Five male and five female adult cockroaches were used per treatment for each of three replications. d-Limonene treatments consisted of 0.1, 1.0, 10, and 25% active ingredient (AI) (by weight); chlordecone treatments consisted of 0.01, 0.1, and 1.0% AI. Control treatments consisting of untreated food and no food also
were included. Mortality was assessed after 24 hr, 48 hr, 7 days, and 14 days of exposure to treated foods.

Cockroach nymphs were subjected to long-term exposure to d-limonene-treated foods. Triflumuron insect growth regulator, provided by Mobay Chemical Corp., Kansas City, Mo. was used as a standard for comparison. Upon emerging from the egg cases of isolated females, single broods of cockroach nymphs were divided into three groups, each containing 10 individuals. Each group was allowed continuous access to food containing a different level of the test chemicals (0.1, 1.0, and 10% AI for d-limonene and 0.01, 0.1, and 1.0% AI for triflumuron). Control treatments of untreated food and no food also were administered. Foods were prepared as described for adult cockroach feeding. The experiment was replicated six times, and mortality and developmental aberrations were noted weekly for four weeks.

The effects of d-limonene-treated foods on German cockroach nymph weight and developmental rate were also studied. Single broods of newly-emerged cockroach nymphs were divided into 5 groups, each containing 6 individuals. Each groups was allowed continuous access to food containing a different level of d-limonene (0, 0.1, 1.0, 10, or 25% AI). Six broods were tested. Insects were held at 22°C under a light regime of 12 hr light, 12 hr darkness. Nymphs were weighed after six weeks of treatment and the number of days taken to reach the adult stage was determined. Analysis of variance was used to assess the treatment effects.
Repellency

A choice-box method was used for evaluating repellent properties of d-limonene. Pyrethrins were used as a standard for comparison. The choice box consisted of two clear, plastic chambers (9 X 8.5 X 2 cm) connected via 20 cm of 1-cm-diameter Tygon® tubing. Twenty-four hr before the start of the experiment, 10 adult cockroaches (2 weeks post-molt) were placed inside each of the choice boxes. Five replications of the experiment were performed each for female insects and male insects. Filter paper (5 cm, Whatman no. 5) treated with 0.001, 0.01, 0.1, 1.0, or 10 mg of d-limonene or pyrethrins was placed in one randomly-chosen chamber of a choice box. Untreated filter paper was placed in the other. Locations of the cockroaches with respect to the treated chamber were then noted at 4 hr and 48 hr after treatment. Analysis of variance and t tests were used to identify treatment differences.

Residual Activity

Short-term residual activity of d-limonene was examined by using four different surface types: painted wood, asbestos tile, glass, and galvanized steel. Chlorpyrifos was tested as a standard for comparison. The two chemicals were applied in ethanol to the surfaces. The ethanol was allowed to evaporate, and the surfaces were then placed into plastic boxes (9 X 8.5 X 2 cm) with adult cockroaches (5 male and 5 female, 2 weeks post-molt, per box). The spaces between the treated surfaces and the tops of the insides of the boxes were large enough to allow the insects to move around and small enough to prevent them from turning over. The insects were thus forced to maintain tarsal and abdominal
contact with the treated surfaces. *d*-Limonene doses were 0.001, 0.01, 0.1, 1.0, and 10 mg (AI)/cm²; chlorpyrifos doses were 0.01, 0.1, 1.0, 10, and 100 μg (AI)/cm². Control treatments of ethanol also were administered. Insects were forced to remain in contact with the treated surfaces for 72 hr, and mortality was then assessed.

**Ovicidal and Larvicidal Properties**

Ovicidal and larvicidal properties of *d*-limonene were examined by using eggs and larvae of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. Eggs from field-collected females were chilled for 20 weeks at 25°C and then were placed on moist (20%) seed-germination paper which had been pretreated with *d*-limonene at 0.001, 0.01, 0.1, 1.0, and 10% AI. Chlordimeform, obtained from Chem Service, Inc., Westchester, PA, was tested at 0.0001, 0.001, 0.01, 0.1, and 1.0% AI. The experiment was replicated five times with 60 eggs per concentration per replication. Eggs and treated papers were placed in glass petri plates (9 cm diameter) which were sealed with paraffin film. Eggs were allowed to remain in contact with the treated filter paper for 40 days. Percentage hatch was then determined by microscopic examination. LC50's were estimated with the Spearman-Karber method, based on the concentration of chemical required to inhibit 50% of the eggs from hatching.

Larvicidal activity of *d*-limonene was examined by exposing third-instar laboratory-reared western corn rootworms to treated soil. Chlorpyrifos was tested as a standard for comparison. Distilled water (10 ml) containing 1 ml acetone plus the chemical treatment was added to 50 g of sterile, oven-dried soil. The soil and the water mixture was blended
together with a wood applicator stick and was placed in a disposable petri plate (9 cm diameter). d-Limonene was added to the soil at concentrations of 1.0, 10, 100, and 1000 ppm; chlorpyrifos was tested at 0.1, 1.0, 10, and 100 ppm. The experiment was replicated four times. A sprouted corn seed, as a food source, and 10 larvae were placed on the treated soil for 48 hr. Mortality then was assessed, and \( \text{LD}_{50} \)'s were estimated by the Spearman-Karber method.
RESULTS

Acute Topical Toxicity

d-Limonene was only slightly toxic to German cockroaches and house flies (Tables 1 and 2). The compound exhibited quick knock-down activity. Symptoms of intoxication included twitching and convulsions, followed by paralysis. Dead insects exhibited considerable fluid loss. The Spearman-Karber analysis indicated that PB increased d-limonene toxicity up to 2.3-fold in cockroaches and up to ca. 17-fold in house flies. These synergistic ratios were similar to those observed for pyrethrins applied with PB. The synergistic action suggests cytochrome P-450 involvement in the detoxification or metabolism of d-limonene.

Fumigant Activity

High concentrations of d-limonene (ca. 20 ppm) were toxic to both cockroaches and rice weevils (Table 3). Dichlorvos exhibited much greater toxicity, with LC\textsubscript{50}'s of 0.01 ppm and 0.007 ppm for cockroaches and rice weevils, respectively. Dead insects in the d-limonene treatments exhibited fluid loss like that observed under topical exposure, and the cuticles were slightly softened. In addition, moisture condensed on the insides of the jars containing intoxicated insects; this was not observed for dichlorvos treatments.

Oral Toxicity

No significant mortality was observed under any of the d-limonene treatments for either adult or nymphal cockroaches (Table 3). Chlordecone, however, was lethal to adults, exhibiting an LC\textsubscript{50} of <0.01% AI for 14 days of exposure. Triflumuron at 0.1% AI caused 50% mortality
Table 1. Acute topical toxicity of d-limonene applied with and without piperonyl butoxide to adult German cockroaches

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of insects tested</th>
<th>24 hr LD$_{50}$ (μg/insect)$^a$,$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-limonene</td>
<td>300</td>
<td>700 (610 - 810)</td>
</tr>
<tr>
<td>1 d-limonene : 1 PB</td>
<td>300</td>
<td>360 (200 - 480)</td>
</tr>
<tr>
<td>1 d-limonene : 5 PB</td>
<td>300</td>
<td>300 (180 - 560)</td>
</tr>
<tr>
<td>chlorpyrifos</td>
<td>296</td>
<td>0.18 (0.13 - 0.25)</td>
</tr>
<tr>
<td>pyrethrins</td>
<td>300</td>
<td>0.89 (0.52 - 1.51)</td>
</tr>
<tr>
<td>1 pyrethrins : 1 PB</td>
<td>300</td>
<td>0.46 (0.28 - 0.77)</td>
</tr>
<tr>
<td>1 pyrethrins : 5 PB</td>
<td>300</td>
<td>0.59 (0.34 - 1.05)</td>
</tr>
</tbody>
</table>

$^a$Control mortality: acetone = 1.6%. PB = 0%.

$^b$95% confidence limits indicated.

Table 2. Acute topical toxicity of d-limonene applied with and without piperonyl butoxide to female house flies

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of insects tested</th>
<th>24 hr LD$_{50}$ (μg/insect)$^a$,$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-limonene</td>
<td>300</td>
<td>90 (70 - 130)</td>
</tr>
<tr>
<td>1 d-limonene : 1 PB</td>
<td>300</td>
<td>25 (18 - 35)</td>
</tr>
<tr>
<td>1 d-limonene : 5 PB</td>
<td>298</td>
<td>5.2 (3.7 - 7.5)</td>
</tr>
<tr>
<td>pyrethrins</td>
<td>300</td>
<td>0.60 (0.35 - 1.02)</td>
</tr>
<tr>
<td>1 pyrethrins : 1 PB</td>
<td>300</td>
<td>0.07 (0.01 - 0.99)</td>
</tr>
<tr>
<td>1 pyrethrins : 5 PB</td>
<td>300</td>
<td>0.03 (0.01 - 0.94)</td>
</tr>
</tbody>
</table>

$^a$Control mortality: acetone = 3.3%. PB = 1.6%.

$^b$95% confidence limits indicated.
after 3 weeks exposure to nymphs.

Although d-limonene caused no significant mortality nor visible morphological aberrations in nymphs, it accelerated nymph growth significantly (Table 4). Analysis of variance indicated that insect weight at 6 weeks following emergence and days to the adult stage were both significantly influenced by d-limonene treatment ($F = 47.09; \text{df} = 4, 170; P = 0.01$ and $F = 3.02; \text{df} = 4, 170; P = 0.05$, respectively). Higher d-limonene doses resulted in greater weights and faster developmental rates than did lower doses or control treatments.

**Repellency**

Analysis of variance indicated that, at 4 hr post treatment, there were no significant d-limonene dose differences. By 48 hr, significant dose differences were observed ($F = 5.7; \text{df} = 5, 45; P < 0.01$) with higher d-limonene concentrations repelling significantly more insects than lower concentrations (Table 5). Although d-limonene had significant repellency at higher levels, it repelled significantly fewer insects than did equivalent concentrations of pyrethrins. d-Limonene also seemed to exhibit attractive properties at low concentrations (0.001 mg). No significant differences were observed between the responses of male and female cockroaches.

**Residual Activity**

After 72 hr of contact with surfaces treated with d-limonene, no significant mortality was observed on any surface tested (Table 3). Chlorpyrifos, however, was quite toxic; concentrations of 0.2$\mu$g/cm$^2$ on galvanized steel and 3.7$\mu$g/cm$^2$ on painted wood killed 50% of the cock-
Table 3. Toxicity of d-limonene via various methods of exposure to three species of insects

| Insect                  | Total number tested & control mortality | Route of exposure                        | LD$_{50}$ or LC$_{50}$
|-------------------------|-----------------------------------------|------------------------------------------|--------------------------
| German cockroach        | 250 (2%)                                | fumigation, adult                        | 23.3 ppm (17.5-31.0)    |
| rice weevil             | 625 (3%)                                | fumigation, adult                        | 19.0 ppm (13.2-27.3)    |
| German cockroach        | 120 (0%)                                | oral, adult                              | no mortality            |
| German cockroach        | 180 (0.5%)                              | oral, nymph                              | no mortality            |
| German cockroach        | 250 (2%)                                | contact with treated surface, adult      | no mortality            |
| western corn rootworm   | 1500 (6%)                               | contact with treated substrate, egg      | 1.8% AI (0.8%-2.9%)     |
| western corn rootworm   | 160 (7%)                                | contact with treated soil, third instar  | 12.2 ppm (4.5-32.6)     |

*95% confidence limits indicated.

Table 4. Influence of d-limonene on nymphal German cockroach weight and rate of development

<table>
<thead>
<tr>
<th>Treatment (% AI)</th>
<th>Total number of insects tested</th>
<th>Mean (and standard deviation) insect weight (mg) at six weeks of age</th>
<th>Mean (and standard deviation) days to the adult stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 %</td>
<td>36</td>
<td>19.3 (1.1)</td>
<td>113.7 (22.7)</td>
</tr>
<tr>
<td>10</td>
<td>36</td>
<td>12.2 (4.1)</td>
<td>129.0 (10.3)</td>
</tr>
<tr>
<td>1</td>
<td>36</td>
<td>3.7 (1.4)</td>
<td>140.8 (6.0)</td>
</tr>
<tr>
<td>0.1</td>
<td>36</td>
<td>4.0 (2.0)</td>
<td>177.0 (35.4)</td>
</tr>
<tr>
<td>0</td>
<td>36</td>
<td>3.6 (1.7)</td>
<td>169.0 (47.9)</td>
</tr>
</tbody>
</table>
Table 5. Repellency of d-limonene and pyrethrins toward adult German cockroaches

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Dose (mg)</th>
<th>% Repellency</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-limonene</td>
<td>10.0</td>
<td>87% a</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>67 b</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>61 bc</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>52 bc</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>24 d</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>44 c</td>
</tr>
<tr>
<td>pyrethrins</td>
<td>10.0</td>
<td>95% a</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>96 a</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>69 b</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>61 bc</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>68 b</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>56 c</td>
</tr>
</tbody>
</table>

*Treatments sharing a common letter within a chemical do not differ significantly (*t* = 2.101; df = 18; *P* < 0.05).

roaches. In may be concluded that d-limonene, unlike chlorpyrifos, exhibits no residual toxicity on either porous or nonporous surfaces.

Ovicidal and Larvicidal Properties

d-Limonene exhibited a slight degree of toxicity against western corn rootworm eggs (Table 3) but was not nearly as toxic as chlordimeform (*LC*₅₀ = 0.001% AI). Against third-instar western corn rootworms (Table 3), d-limonene was only about 10 times less toxic than chlorpyrifos (*LC*₅₀ = 0.95 ppm). The appearance of dead larvae after d-limonene exposure was quite unusual: larvae were very much darkened and nearly liquified, with a highly-softened cuticle. Chlorpyrifos-intoxicated insects did not exhibit such an appearance.
DISCUSSION

*d*-Limonene was not found to be a uniformly potent toxin against the insects tested. Because of its lack of residual activity and low toxicity via other routes of exposure it must be judged, overall, to be a poor candidate for general employment as an insecticide. Yet, it exhibited some unusual properties that should be investigated further: in particular, the synergism of the compound with piperonyl butoxide, the effects observed when orally administered to immatures, the repellent-attractant effects on behavior, and the toxicity toward soil-dwelling larvae.

Better understanding of the spectrum of insecticidal activity of *d*-limonene and other terpenoids could result in practical applications. Additionally, knowledge of structure-activity relationships and symptomatology also could help elucidate toxic modes of action of naturally-occurring terpenoids in insects.
REFERENCES CITED


PART II. EFFECTS OF D-LIMONENE ON GROWTH AND REPRODUCTION IN THE GERMAN COCKROACH (ORTHOPTERA: BLATTELLIDAE)
ABSTRACT

d-Limonene (p-mentha-1,8-diene), a monocyclic monoterpenoid, possesses numerous toxic, repellent, and attractive properties in various insects and may interfere with aspects of insect growth and reproduction. Studies were carried out in the German cockroach, Blattella germanica, to evaluate d-limonene's attractiveness in cockroach diet, to quantify d-limonene-induced growth stimulation, to examine embryotoxic properties of the compound, and to examine its effects on fertility and fecundity when administered via the oral, topical, and respiratory routes of entry. Untreated diet was significantly preferred over diet treated with high levels (25 and 10% active ingredient) of d-limonene. The threshold for acceptance was between 1 and 10% d-limonene. d-Limonene was found to significantly influence the days required by nymphs to reach the adult stage; insects fed diet treated with 25, 10, 1, and 0.1% d-limonene reached the adult stage in significantly fewer days than did insects receiving no d-limonene. Application of high doses of d-limonene to the oothecae of gravid female cockroaches significantly decreased the chances of young emerging from them but did not affect female mortality. The feeding of d-limonene-treated diet during nymphal development and through the early, pre-mating period of the adult stage did not influence the number of broods produced per pair, the number of offspring per brood, the days required to produce a brood, the total offspring produced by a pair, or the female lifespan. A pre-mating topical, near-lethal application of d-limonene to the female had some deleterious effects on the reproductive parameters of: occurrence of reproduction, number of broods
produced, total offspring produced by a pair, the number of days required to produce a brood, and the female lifespan. A pre-mating single exposure to sublethal levels of d-limonene vapors did not have a significant influence on any of the reproductive parameters examined.
INTRODUCTION

Monoterpenoids are abundant and diverse ten-carbon components of the essential oils of many higher plants. They are typically rather lipophilic compounds and thus have a high potential for toxic interference with the basic biochemical, physiological, and behavioral functions of insects. Indeed, many monoterpenoids have been shown to exhibit various toxic, repellent, and attractive properties in numerous insect species (Brattsten 1983, Osborne and Boyd 1974, Siegfried 1987) including the German cockroach (Inazuka 1983, 1982). In addition, a number of the essential oils, the more complex terpenoids, and terpenoid derivatives have been shown to reduce the productivity of various stored product insects (Amos et al. 1982, Singh et al. 1989), and negatively impact growth, development, and reproduction in a number of herbaceous insects (Rogers et al. 1987, Brattsten 1983).

d-Limonene (p-mentha-1,8-diene) is a monocyclic hydrocarbon monoterpenoid and is the principal terpenoid constituent of citrus oils. At room temperature it exists as an oily liquid with a density of 0.84 g/ml and emits a strong but pleasant orange-like scent. Although d-limonene was shown to be acutely toxic to Dendroctonus bark beetles a number of years ago (Smith 1965, Coyne and Lott 1976), it has again gained attention as a potential insecticide; in fact, it is currently commercially available as the active ingredient of several flea shampoos for pets. The material exhibits topical toxicity to all life stages of the cat flea (Ctenocephalides felis) (Hink and Fee 1986) and is known to reduce its fecundity (Collart and Hink 1986). Whereas d-limonene has some pro-
nounced benefits as a management tool for ectoparasites on animals (in particular, low mammalian toxicity), its potential for employment as a general-use insecticide is limited. In earlier studies (Karr and Coats 1988), it was demonstrated that the chemical had low topical toxicity toward the German cockroach, *Blattella germanica*, and house fly, *Musca domestica*. Additionally, d-limonene exhibited no residual activity on surfaces and was not toxic when administered orally via the diet, even at levels of up to 25% active ingredient (AI). When the compound was orally administered to German cockroach nymphs, however, it was shown to cause a significant stimulation in weight gain and developmental rate. Further investigations into the effects of d-limonene on parameters of German cockroach growth and reproduction are reported here. The objectives of these studies were: to compare attractiveness of diet treated with d-limonene to that of untreated diet, to quantify d-limonene growth-stimulation, to examine the embryotoxic properties of d-limonene, and to examine the effects of d-limonene on fertility and fecundity when administered via three routes of entry.
MATERIALS AND METHODS

Feeding Preferences

In an earlier study (Karr and Coats 1988) nymphal German cockroaches fed high levels of d-limonene treated diet gained weight and reached adulthood more rapidly than did cohorts fed untreated diet or diet treated with low levels of d-limonene. To determine if the apparent growth stimulation was simply due to insects consuming more of the treated food, a choice experiment was initiated employing adult male German cockroaches (two weeks post-molt, wild strain maintained in laboratory for five years). Treatments consisted of paired comparisons of the amount of treated diet consumed vs. the amount of untreated diet consumed by groups of insects over 14 days. Experimental units were wide-mouth 40 ml jars containing five male cockroaches, a small cardboard shelter, and two feeding stations: one containing the d-limonene-treated diet and one containing untreated diet. d-Limonene was obtained from Pet Chemicals, Inc., Miami Springs, FL, and was estimated to be 96%-98% pure. Each jar was topped with a piece of nylon hosiery, held in place by a rubber band. Insects were held at 22°C in a light regime of 12 hr dark, 12 hr light and were watered daily by lightly misting the nylon jar covers with distilled water.

Treated diets consisted of 0, 1, 10, and 25 % AI (by weight) d-limonene thoroughly blended into a paste of two parts Purina® cat chow and one part corn oil. Diets were administered via feeding stations constructed from 3 mm slices of 10 mm diameter Teflon® tubing. Feeding stations were used to help minimize cuticular contact with treated food.
Five replications of each of the four treatment levels were used. Feeding stations containing the diets were weighed at the start of the experiment and were weighed after 14 days with the insects. *T*-tests were used to determine the significance of the differences between the amounts of untreated diet consumed and amounts of treated diet consumed.

**Growth Rate Effects**

To quantify further the growth acceleration noted in an earlier experiment (Karr and Coats 1988), sibling groups of freshly-eclosed German cockroach nymphs were divided into five subgroups, each containing four to six nymphs. Each of the five subgroups received a different level of *d*-limonene in their diet; treatments were 0, 0.1, 1, 10, and 25 % AI (by weight) in the cat chow/corn oil paste described above in "Feeding Preferences." The experiment was replicated four times, using a different sibling group for each replicate. Insects were held at 20° C under 12 hr light, 12 hr dark. Each nymph was housed individually and was allowed continuous access to the treated diet. The diet was replaced every three days to ensure consistency in the level of *d*-limonene in the diet. Insects were held in jars described earlier under "Feeding Preferences." Analysis of variance (ANOVA) and Duncan's multiple range test (1955) were used to evaluate treatment effects.

**Oöthecal Treatment**

Hink and Fee (1986) indicated that *d*-limonene inhibited embryological development in eggs of the cat flea. The German cockroach presented an opportunity to examine potential embryological interference in an insect which does not simply drop its eggs in its environment, but which
carries them in the more protected confines of the oôtheca until im-
mediately prior to hatching. d-Limonene was applied directly to the 
venter of oôthecae protruding from the bodies of gravid female German 
cockroaches by using an electric microapplicator calibrated to deliver 
one-microliter aliquots. Gravid females employed were within seven days 
of copulation and exhibited oôthecae which protruded from the abdomen and 
were light-brown to chestnut in color. Applications were made to females 
anesthesized with a combination of CO₂ gas and chilling. Treatments con-
sisted of 0, 210, 420, 840, and 1680 micrograms of d-limonene per oô-
theca, and there were three replicates, each consisting of ten gravid 
females. The 840 and 1680 microgram doses represent one and two micro-
liters of undiluted d-limonene; the control treatment consisted of one 
microliter of acetone, and the remaining treatments were one microliter 
applications of d-limonene diluted with acetone. Treated insects were 
contained under the conditions described earlier under "Feeding Pref-
erences." Gravid females were held at 20°C under a 12 hr light, 12 hr 
dark and were observed for 21 days following treatment. Two variables 
were monitored: 1) the number of oôthecae yielding live young and 2) the 
number of females dying. Analysis of variance and Duncan’s multiple 
range test were used to evaluate treatment effects.

Effects of "Lifetime-Feeding" of d-Limonene-Treated Diet on 
Fertility and Fecundity

Upon emerging from oôthecae, sibling groups of German cockroaches 
were fed a cat chow/corn oil paste, described earlier in "Feeding Prefer-
ences", treated with 0, 1, 10, or 25% d-limonene by weight. The treated
diet was offered in the Teflon® tubing feeding stations described earlier, and the feeding stations and treated diet were replaced weekly until the cockroaches reached adulthood. Upon reaching adulthood, the cockroaches were separated and housed individually for one week. They were still offered treated diet during this isolation. Then, a male and a female cockroach pair were united in a 40 ml jar (described earlier) and were offered untreated diet. For each treatment level, pairing combinations consisted of: a male and a female receiving treated diet, a male receiving treated diet with a female receiving untreated diet, a male receiving untreated diet with a female receiving treated diet, or a male and a female both receiving untreated diet. Initially, at least five cockroach pairs were assigned to each pairing combination within each treatment level. Pairs were held at 20°C under 12 hr light, 12 hr dark. The insects were monitored daily for offspring production until the female cockroach died. Analysis of variance and Duncan's multiple range test were used to evaluate treatment effects on total number of offspring produced, number of broods produced, number of nymphs per brood, days required to produce a brood, and female lifespan.

Effects of Topical d-Limonene Treatment on Fertility and Fecundity

Male and female cockroaches were isolated from one another immediately upon reaching the adult stage. Individuals were housed separately for three to seven days and then were treated topically with a single near-lethal or sublethal dose of d-limonene or, as a control treatment, acetone. Treatments were applied to the abdominal venters of cockroaches anesthetized with a combination of cold treatment and CO₂ gas by using an
electric microapplicator calibrated to deliver one-microliter aliquots. Treatments consisted of 840 micrograms d-limonene (one microliter, undiluted), 420 micrograms d-limonene (diluted in acetone to one microliter), or one microliter of acetone. Because 840 micrograms was greater than the LD$_{50}$ value of 700 micrograms/insect (Karr and Coats 1988), sufficient pairs were treated to ensure that at least 10 pairs survived for further reproductive evaluation. Pairs of male and female cockroaches were united in 40 ml jars (described earlier) following treatment. For each treatment level, pairing combinations consisted of: a male and a female both receiving d-limonene, a male receiving d-limonene with a female receiving acetone, a male receiving acetone with a female receiving d-limonene, or a male and a female both receiving acetone. Ten pairs were assigned to each pairing combination within each treatment level. The insects were held at 22°C under 12 hr light, 12 hr dark. Pairs were monitored daily for offspring production until the female died. Analysis of variance and Duncan's multiple range test were used to evaluate treatment effects on total number of offspring produced, number of broods produced, number of nymphs per brood, days required to produce a brood, and female lifespan.

**Effects of d-Limonene Vapor Exposure on Fertility and Fecundity**

Male and female cockroaches were isolated from one another immediately upon reaching the adult stage. Individuals were housed separately for three to seven days and then were exposed to sublethal concentrations of d-limonene vapors for a single 24 hr period. For each vapor exposure concentration, cages of insects were suspended via stain-
less-steel wire inside a 3-l jar. The cages were constructed from glass cylinders (6 X 2 cm diameter) with fine-mesh stainless-steel screens fastened to both ends of the cylinder with paraffin. Automatic micro-pipettes were used to dispense d-limonene treatments into 1 dram vials also suspended with wire in the jars. Magnetic stir bars were placed in the jars, and the jars were sealed and placed on multiple magnetic stirrers to keep the volatilized d-limonene and the air in the jars well mixed. Control insects were placed in the exposure apparatus for 24 hr but received no d-limonene exposure.

D-Limonene treatments consisted of nominal vapor concentrations of 0, 0.5, 1, or 5 ppm. Pairs of male and female cockroaches were united in 40 ml jars (described earlier) following treatment. For each vapor concentration, pairing combinations consisted of: a male and a female both receiving d-limonene, a male receiving d-limonene with a control female, a control male with a female receiving d-limonene, or a control male with a control female. A total of fifteen pairs were assigned to each pairing combination within each vapor concentration. Insects were held at 22°C under 12 hr light, 12 hr dark. Pairs were monitored daily for offspring production until the female cockroach died. Analysis of variance procedures and Duncan's multiple range test were used to evaluate treatment effects on total number of offspring produced, number of broods produced, number of nymphs per brood, days required to produce a brood, and female lifespan.
RESULTS

Feeding Preferences

When offered a choice between untreated diet and diet treated with d-limonene, male German cockroaches chose untreated diet significantly over diet treated with 10 or 25% d-limonene ($t = 5.03; \text{df} = 4, \ P = 0.007$ and $t = 2.8; \text{df} = 4; \ P = 0.05$) (Table 1). Whereas high d-limonene concentrations were not very acceptable to the insects, the lower 1% AI concentration was accepted: the difference between 1%-treated diet consumed and untreated diet consumed was not significant at the 0.05 level ($t = -0.88; \text{df} = 4, \ P = 0.43$). The threshold for either discrimination or acceptance lies therefore between 1 and 10% AI. This finding indicates that d-limonene possesses no attractive qualities or feeding arrestant or stimulant attributes and suggests that the compound is actually somewhat repellent in the diet. In a previous study it was found that d-limonene-treated filter paper also was repellent to German cockroaches (Karr and Coats 1988).

Table 1. German cockroach food preferences: amount of untreated diet consumed vs. amount of diet treated with d-limonene consumed

<table>
<thead>
<tr>
<th>Dose (% AI)</th>
<th>Mean (± SE) difference: mg untreated - mg treated diet consumed</th>
<th>Significance of the difference ($P &gt; t$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.14 ± 4.29</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>1</td>
<td>-7.34 ± 8.32</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>10</td>
<td>33.10 ± 6.58</td>
<td>0.007</td>
</tr>
<tr>
<td>25</td>
<td>57.68 ± 20.98</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Growth Rate Effects

As was suggested in earlier studies (Karr and Coats 1988), d-limonene administration via the diet was found to significantly influence the time required to reach the adult stage \((F = 12.5, df = 4, P < 0.01)\). It took significantly fewer days to reach adulthood when d-limonene was present in the diet than when it was absent (Table 2). The dose-response relationship between days to the adult stage and percent d-limonene in the diet (Figure 1) was logarithmic and had a high coefficient of determination \((r^2 = 0.994)\). Possible explanations for this phenomenon may include hormonal interference (e.g., juvenile hormone inhibition or antagonism), antibiotic effects, or enzyme induction. Further research is needed to elucidate the mechanism of monoterpenoid-induced growth stimulation.

Table 2. The effect of d-limonene on growth rate of German cockroach nymphs

<table>
<thead>
<tr>
<th>Dose (% AI)</th>
<th>Mean days (±SE) to the adult stage(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>107.75 ± 6.22 a</td>
</tr>
<tr>
<td>10</td>
<td>113.11 ± 6.13 ab</td>
</tr>
<tr>
<td>1</td>
<td>123.00 ± 5.37 bc</td>
</tr>
<tr>
<td>0.1</td>
<td>131.55 ± 3.31 c</td>
</tr>
<tr>
<td>0</td>
<td>143.95 ± 6.90 d</td>
</tr>
</tbody>
</table>

\(^a\)Means followed by the same letter are not significantly different \((P = 0.05; \text{Duncan's [1955] multiple range test})\).
Oöthecal Treatment

Application of d-limonene directly to the oöthecae of gravid female German cockroaches significantly decreased the chances of young emerging from them. ANOVA revealed that d-limonene dose was a highly significant factor influencing the proportion of oöthecae yielding young \((F = 10.20; \ df = 4, 14; P < 0.01)\) but it did not significantly influence female mortality (Table 3). The highest d-limonene doses (1680 and 840 micrograms) did not cause significantly more mortality than the lower or control doses despite the fact that the reported \(LD_{50}\) value for d-limonene in the German cockroach is 700 micrograms per insect (Karr and Coats 1988). This indicates that the oötheca is a significant barrier that can hinder the passage of toxins from the oötheca to the female.
The relationship between d-limonene dose and reduction of the production of young was a logarithmic one (Figure 2) and had a high coefficient of determination \( r^2 = 0.82 \).

Table 3. The effect of d-limonene when applied to the oothecae of gravid German cockroaches

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>Mean female % mortality (±SE)</th>
<th>Mean % of oothecae producing offspring (±SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.3 ± 3.3</td>
<td>90.0 ± 3.3 a</td>
</tr>
<tr>
<td>210</td>
<td>3.3 ± 3.3</td>
<td>73.3 ± 17.6 b</td>
</tr>
<tr>
<td>420</td>
<td>3.3 ± 3.3</td>
<td>70.0 ± 5.8 bc</td>
</tr>
<tr>
<td>840</td>
<td>3.3 ± 0</td>
<td>16.7 ± 3.3 d</td>
</tr>
<tr>
<td>1680</td>
<td>6.6 ± 3.3</td>
<td>16.7 ± 8.8 d</td>
</tr>
</tbody>
</table>

*Means followed by the same letter are not significantly different \( P = 0.05; \) Duncan's [1955] multiple range test.

\[
Y = 249.74 - 32.19 \ln X \\
r^2 = 0.82
\]

Figure 2. Influence of topical d-limonene application to oothecae on the production of young
Effects of "Lifetime-Feeding" of d-Limonene-Treated Diet on Fertility and Fecundity

ANOVA results indicated that lifetime feeding of d-limonene-treated diet to German cockroach male and/or female nymphs did not significantly influence the mean number of broods per pair \( (F = 0.34; \ df = 9, \ 83; \ P > 0.05) \), mean offspring per brood \( (F = 1.51; \ df = 9, \ 83; \ P > 0.05) \), mean days to produce a brood \( (F = 0.95; \ df = 9, \ 83; \ P > 0.05) \), mean total offspring per pair \( (F = 0.43; \ df = 9, \ 83; \ P > 0.05) \), or the female lifespan \( (F = 1.62; \ df = 9, \ 83; \ P > 0.05) \). Duncan's multiple range test (Table 4) failed to detect differences between treatments or any consistent trends related to treatment level, indicating that oral administration of d-limonene-treated diet during the nymphal stage did not significantly influence reproductive capacity of adult cockroaches. It would be valuable to examine this topic further with a larger sample of cockroach pairs; in particular, the effects of treated diet when administered to adult cockroaches after mating and during the embryological development of the offspring should be studied, especially in light of the fact that d-limonene topical treatment of oothecae has been shown to hinder production of young.

Effects of Topical d-Limonene Treatment on Fertility and Fecundity

ANOVA results indicated that pre-mating topical treatment of male and/or female German cockroaches with d-limonene did not significantly influence the mean number of broods per pair \( (F = 2.21; \ df = 6, \ 69; \ P > 0.05) \), mean offspring per brood \( (F = 1.13; \ df = 6, \ 69; \ P > 0.05) \), mean days to produce a brood \( (F = 1.94; \ df = 6, \ 69; \ P > 0.05) \), mean total
Table 4. The effects of "lifetime-feeding" of d-limonene-treated food on reproductive parameters in the German cockroach

<table>
<thead>
<tr>
<th>% in diet</th>
<th>Male</th>
<th>Female</th>
<th>n</th>
<th>Mean number of broods per pair&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean offspring per brood&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean days to produce a brood&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean total offspring per pair&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean days female lifespan&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>25</td>
<td>10</td>
<td>10</td>
<td>3.3 a</td>
<td>26.6 ab</td>
<td>42.7 a</td>
<td>86.5 a</td>
<td>251.1 a</td>
</tr>
<tr>
<td>0</td>
<td>25</td>
<td>5</td>
<td>5</td>
<td>3.7 a</td>
<td>22.4 b</td>
<td>39.9 a</td>
<td>81.0 a</td>
<td>160.0 b</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>2.7 a</td>
<td>31.7 ab</td>
<td>46.8 a</td>
<td>89.7 a</td>
<td>158.7 b</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>12</td>
<td>12</td>
<td>3.5 a</td>
<td>23.0 b</td>
<td>43.0 a</td>
<td>83.7 a</td>
<td>180.1 ab</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>7</td>
<td>7</td>
<td>3.4 a</td>
<td>25.5 ab</td>
<td>58.1 a</td>
<td>91.3 a</td>
<td>190.6 ab</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>3.0 a</td>
<td>25.8 ab</td>
<td>69.9 a</td>
<td>84.3 a</td>
<td>212.0 ab</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>9</td>
<td>9</td>
<td>3.7 a</td>
<td>25.5 ab</td>
<td>41.4 a</td>
<td>93.8 a</td>
<td>180.0 ab</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>10</td>
<td>10</td>
<td>3.5 a</td>
<td>33.6 a</td>
<td>40.9 a</td>
<td>115.0 a</td>
<td>194.5 ab</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>4.0 a</td>
<td>33.4 a</td>
<td>34.9 a</td>
<td>133.0 a</td>
<td>169.7 b</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>3.7 a</td>
<td>25.2 ab</td>
<td>35.9 a</td>
<td>91.3 a</td>
<td>158.0 b</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means followed by the same letter are not significantly different (<i>P</i> = 0.05; Duncan's [1955] multiple range test).
offspring per pair \((F = 1.48; \text{df} = 6, 69; P > 0.05)\), or the female lifespan \((F = 1.42; \text{df} = 6, 69; P > 0.05)\). Duncan's multiple range test did, however, detect some individual treatment differences and several interesting trends (Table 5). Treatment of both the male and female of a pair with 840 \(\mu\text{g}\) of \(d\)-limonene, for example, prevented reproduction altogether and decreased the female lifespan significantly compared to the control treatment. Treatment of the female only with 840 \(\mu\text{g}\) reduced reproduction to 60% of the control, and significantly decreased number of broods per pair, the mean total offspring per pair, and the female lifespan compared to the control. Additionally, this treatment significantly increased the days required to produce a brood compared to the control. Overall, treatment of males appeared to have little if any effect on reproduction, and treatment of females with the lower 420 \(\mu\text{g}\) dose impacted only the proportion of pairs reproducing while not affecting the reproductive capacity of pairs that did reproduce. These findings suggest that, whereas \(d\)-limonene may have a negative impact on reproduction, this impact is minor overall, accounting for only a small proportion of the variability in reproductive parameters. It is also important to note that the threshold dose required to elicit even this minor reproductive disruption was very high, exceeding the normal \(LD_{50}\) value.

Effects of \(d\)-Limonene Vapor Exposure on Fertility and Fecundity

ANOVA results indicated that pre-mating vapor exposure of male and/or female German cockroaches with \(d\)-limonene did not significantly influence the mean number of broods per pair \((F = 0.44; \text{df} = 9, 149; P > 0.05)\), mean offspring per brood \((F = 0.15; \text{df} = 9, 149; P > 0.05)\), mean
Table 5. The effects of topical d-limonene treatment on German cockroach reproductive parameters

<table>
<thead>
<tr>
<th>Micrograms applied</th>
<th>Male</th>
<th>Female</th>
<th>% Pairs not reproducing</th>
<th>Mean number of broods per pair</th>
<th>Mean offspring per brood</th>
<th>Mean days to produce a brood</th>
<th>Mean total offspring per pair</th>
<th>Mean days female lifespan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>840</td>
<td>840</td>
<td>10</td>
<td>100</td>
<td>194.6 b</td>
<td>0.0</td>
<td>55.0 b</td>
<td>164.0 b</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>840</td>
<td>10</td>
<td>80</td>
<td>2.0 b</td>
<td>80.7 a</td>
<td>94.4 ab</td>
<td>212.8 ab</td>
</tr>
<tr>
<td></td>
<td>840</td>
<td>0</td>
<td>10</td>
<td>50</td>
<td>3.4 ab</td>
<td>27.7 a</td>
<td>49.5 b</td>
<td>122.7 a</td>
</tr>
<tr>
<td></td>
<td>420</td>
<td>420</td>
<td>10</td>
<td>70</td>
<td>4.0 a</td>
<td>31.2 a</td>
<td>44.9 b</td>
<td>123.1 a</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>420</td>
<td>10</td>
<td>20</td>
<td>3.1 ab</td>
<td>33.4 a</td>
<td>53.5 b</td>
<td>107.0 ab</td>
</tr>
<tr>
<td></td>
<td>420</td>
<td>0</td>
<td>10</td>
<td>40</td>
<td>3.7 a</td>
<td>33.2 a</td>
<td>45.5 b</td>
<td>120.5 a</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>20</td>
<td>4.4 a</td>
<td>28.4 a</td>
<td>42.8 b</td>
<td>125.4 a</td>
</tr>
</tbody>
</table>

*Means followed by the same letter are not significantly different (P = 0.05; Duncan's [1955] multiple range test).
days to produce a brood ($F = 1.09; \text{df} = 9, 149; P > 0.05$), mean total offspring per pair ($F = 0.84; \text{df} = 9, 149; P > 0.05$), or the female lifespan ($F = 1.11; \text{df} = 9, 149; P > 0.05$). Duncan's multiple range test (Table 6) did not detect any dose- or sex-related differences between treatments, nor did it suggest the existence of trends related to treatment. These findings indicate that exposing cockroach adults to a single treatment of a low concentration of d-limonene vapors prior to mating has no effect on their subsequent reproductive capacity. In light of the finding that topical d-limonene treatment negatively impacts reproduction only at doses exceeding the LD$_{50}$ value, it would be valuable to repeat vapor exposure at concentrations nearing or exceeding the reported LC$_{50}$ value of ca. 20 ppm (Karr and Coats 1988).
### Table 6. The effects of d-limonene vapor exposure on German cockroach reproductive parameters

| Vapor concentration (ppm) | Male | Female | n  | Mean number of broods per pair<sup>a</sup> | Mean offspring per brood<sup>a</sup> | Mean days to produce a brood<sup>a</sup> | Mean total offspring per pair<sup>a</sup> | Mean days female lifespan<sup>a</sup> |
|----------------------------|------|--------|----|------------------------------------------|---------------------------------|--------------------------------|---------------------------------|--------------------------------|--------------------------|
| 5                          | 5    | 5      | 15 | 1.3 a                                    | 10.7 a                          | 33.9 ab                        | 82.0 a                          | 169.2 ab                      |
| 0                          | 5    | 5      | 15 | 2.0 a                                    | 11.7 a                          | 23.6 ab                        | 111.1 a                         | 193.1 ab                      |
| 5                          | 0    | 0      | 15 | 1.2 a                                    | 10.4 a                          | 23.1 ab                        | 59.1 a                          | 164.9 b                        |
| 1                          | 1    | 1      | 15 | 1.5 a                                    | 13.8 a                          | 52.1 a                         | 62.7 a                          | 190.7 ab                      |
| 0                          | 1    | 1      | 15 | 1.3 a                                    | 10.8 a                          | 19.8 b                         | 94.3 a                          | 201.2 ab                      |
| 1                          | 0    | 0      | 15 | 1.2 a                                    | 13.6 a                          | 24.2 ab                        | 71.0 a                          | 201.6 ab                      |
| 0.5                        | 0.5  | 0      | 15 | 1.8 a                                    | 13.9 a                          | 29.3 ab                        | 92.4 a                          | 233.8 a                       |
| 0                          | 0.5  | 0      | 15 | 1.7 a                                    | 12.7 a                          | 31.9 ab                        | 74.5 a                          | 188.2 ab                      |
| 0.5                        | 0    | 0      | 15 | 1.6 a                                    | 11.8 a                          | 25.5 ab                        | 89.9 a                          | 217.6 ab                      |
| 0                          | 0    | 0      | 15 | 2.0 a                                    | 13.0 a                          | 38.8 ab                        | 80.6 a                          | 213.0 ab                      |

<sup>a</sup>Means followed by the same letter are not significantly different (P = 0.05; Duncan's [1955] multiple range test).
DISCUSSION

d-Limonene was found to have some interesting, but subtle effects on parameters of insect growth and reproduction. Because diet treated with high concentrations of the material was not preferred over untreated diet, the growth stimulation apparent when the material was fed to German cockroach nymphs can be assumed to have been due to some factor other than feeding stimulation or arrestment. One feasible explanation for d-limonene-induced growth stimulation might be inhibition or antagonization of juvenile hormone effects. Such juvenile hormone involvement should be reflected in reproductive interference as well since the hormone is known to play a role in vitellogenesis. The data obtained from the three reproductive analyses carried out, however, indicated that, at best, d-limonene had only minor negative effects on reproductive factors. Additionally, these effects (decreasing the proportion of pairs reproducing, the number of broods per pair, the total offspring per pair, and the female lifespan) were observed only when the female insect received very high topical applications (in excess of the normal LD<sub>50</sub> value). Perhaps the same negative effects would have been observed under vapor exposure had treatment levels nearer to the LC<sub>50</sub> value been chosen. The fact that reproductive interference is a potential consequence of a near-lethal d-limonene exposure suggests that, when the compound, or its conceivable analogues, are used as insecticides, insects not killed outright may be rendered less fit for survival and future reproduction. This notion is reinforced by the data obtained from the treatment of the oöthecae of gravid female German cockroaches. Whereas exposing the
oötheca alone to high levels of d-limonene did not harm the female insect, the developing offspring were destroyed. It is likely, therefore, that a thorough application of a lethal d-limonene dose to the body of a gravid female insect (as might occur in the treatment of ectoparasitic insects on animals) would be expected to compromise survival not only of the female, but of the offspring as well. These findings indicate that, as natural or synthetic analogues of d-limonene are investigated as potential insecticides, it would be wise, in assessing efficacy of control, to look not only at direct topical toxicity, but to assess negative impacts on biotic potential as well.
REFERENCES CITED


PART III. TOXIC EFFECTS OF D-LIMONENE IN THE EARTHWORM EISENIA FETIDA
ABSTRACT

d-Limonene, a monocyclic monoterpenoid with known insecticidal properties, was assayed using a standard method of cutaneous exposure for general lethality effects as well as neurotoxicity effects on escape reflex pathways in earthworms, *Eisenia fetida*. Neurotoxicity effects were assessed by non-invasive electrophysiological techniques and involved: (a) quantification of the impacts of chronic and acute sublethal exposures on impulse conduction in the worms' medial (MGF) and lateral (LGF) giant nerve fiber pathways, (b) determination of whether such effects were generalized or localized within various body regions, and (c) determination of the reversibility of neurotoxicity effects. The LD$_{50}$ value for d-limonene alone was 6.0 ppm and the LT$_{50}$ value for exposure to 12.6 ppm was 4.9 hr. Effects on lethality were not synergized significantly by either piperonyl butoxide or sesame oil. Generally, chronic and acute intoxication involved a rapid and predictable cascade of behavioral and morphological symptoms including increased mucus secretion, writhing, clitoral swelling, and elongation of the body. In addition, chronic d-limonene exposures induced significant weight loss, but there was no effect on MGF and LGF conduction velocities even though abnormal rebounding of MGF impulses and spontaneous LGF spiking were often evident. Acute exposures, however, induced significant decreases in conduction velocity in both the MGF and LGF but the effects were regionally specific; for example, LGF velocities were significantly reduced in the posterior one-half of the body but not in the anterior half. The magnitude of conduction velocity decreases was directly
related to both concentration and duration of exposure. Other neurotoxic manifestations of acute exposures included occasional blocking of LGF impulse propagation and reduced amplitude in the muscle potential which normally accompanies each MGF spike. Decreases in conduction velocities following acute exposures were reversed once d-limonene exposure ceased. The time course of this reversibility was related to both exposure concentration and duration.
INTRODUCTION

d-Limonene (p-mentha-1,8-diene) is a monocyclic monoterpenoid abundant in the essential oils of citrus and currently used as an insecticide in some formulations of flea shampoos for pets. The general insecticidal properties of undistilled citrus oils and d-limonene have been noted in a number of species including the cowpea weevil (Callosobruchus maculatus), rice weevil (Sitophilus oryzae) (Su et al. 1972), house fly (Musca domestica), red imported fire ant (Solenopsis invicta) (Sheppard 1984), Dendroctonus bark beetles (Coyne and Lott 1976, Smith 1965), cat flea (Ctenocephalides felis) (Hink and Fee 1986), and western corn rootworm (Diabrotica virgifera virgifera) (Karr and Coats 1988).

In view of these insecticidal properties, there is now an obvious need for developing a broader base of toxicological information for this compound, especially information related to possible mode(s) of action as well as effects on non-target invertebrate groups. Therefore, a non-target species, the earthworm Eisenia fetida, was selected and assayed for possible neurotoxicity effects related to d-limonene exposure.

Earthworms have been extensively used as representative soil invertebrates for a wide variety of toxicity screening tests (Ruppel and Laughlin 1977, Goats and Edwards 1981, Roberts and Dorough 1984). Whereas Eisenia fetida is not necessarily the most sensitive of the earthworm species available (Dean-Ross 1983), it is a hardy and dependable model well-suited for bioassays requiring a large number of subjects and low mortality rates in control animals. Additionally, it may be easily and rapidly reared under laboratory conditions.
The principal advantage of using earthworms for assessing neurotoxicity is that electrical impulse activity can be easily monitored from either of two giant nerve fiber pathways, known as the medial and lateral giant fiber systems (MGF and LGF, respectively), by using completely intact worms that may be tested before, during, and after exposure to toxicants (Drewes et al. 1984, Drewes and Vining 1984, and Drewes et al. 1987). This is accomplished by placing worms on printed circuit board recording grids (O'Gara et al. 1982) and obtaining transcutaneous, multi-channel recordings of giant fiber impulse traffic along the worm. This approach permits spatial and temporal resolution of toxin-induced disruptions in central as well as peripheral nervous system function along the entire animal. Furthermore, because intact organisms rather than in vitro nerve preparations are used, it is also feasible to assess whether neurotoxicity requires metabolic activation.

The specific objectives of these studies were to: 1) determine the LC_{50} and LT_{50} for d-limonene alone and in combination with two metabolic inhibitors in Eisenia fetida by using a standard method of cutaneous exposure; 2) describe the sequence of behavioral and morphological symptoms associated with toxicity; 3) characterize and quantify the impacts of acute and chronic sublethal exposures on impulse conduction in the worms' giant fiber pathways; 4) determine if neurotoxicity effects were generalized or localized within various body regions; and 5) determine if neurotoxicity effects were reversible.
MATERIALS AND METHODS

Animal Maintenance and Chemical Exposure

Laboratory cultures of *Eisenia fetida* were reared in the dark on a mixture of soil and horse manure at 24-26°C. Only clitellate specimens were employed in lethality and electrophysiology bioassays; animals weighed approximately 0.5 g and ranged from 6 to 9 cm in length. All tests were carried out at room temperature (21-22°C). All animals were starved for 24 to 48 hr prior to testing; this was sufficient time for animals to void gut contents thus ensuring that little or no fecal production occurred during exposure, weighing, or testing.

Exposure to d-limonene was achieved via a vapor/contact exposure method modified from Drewes et al. (1984). Exposure occurred in 95 ml capped glass vials containing a lining of qualitative-grade filter paper (total surface area of paper per vial=115 cm$^2$) saturated with a 2 ml aliquot of distilled water. A microsyringe was used to deliver d-limonene (Pet Chemical Co., Miami Springs, FL; approximately 96% pure) into the vial, but because d-limonene is a volatile compound, care was taken to minimize the opening of a vial once the material was added. Nominal d-limonene concentrations were expressed as parts per million based on the micrograms of the material added per unit volume of the vial (e.g., 0.8 mg per 95 ml vial=8.4 ppm).

$L_{C50}$ and $L_{T50}$ trials were conducted by exposing earthworms to d-limonene alone or in combination with the cytochrome P-450 mixed-function oxidase-inhibiting synergists piperonyl butoxide (PBO) and sesame oil (Pet Chemical Co., Miami Springs, FL). PBO was applied at 1 part PBO to
2 parts d-limonene, and sesame oil was applied at a one-to-one ratio with d-limonene. The filter paper in the exposure vials was pretreated with synergist by dissolving in 2 ml of acetone carrier. This volume saturated the filter paper and the acetone was then evaporated leaving a relatively uniform distribution of synergist on the paper. After evaporation, two ml of distilled water and one worm were added to each vial. Worms were exposed to the synergist alone for four hours. d-Limonene was then added to each vial and worms were subsequently monitored for symptoms of intoxication and mortality at 0.25 hr, 1 hr, 3 hr, 6 hr, 10 hr, 24 hr, and 48 hr of exposure. It seldom was necessary to open the vials for monitoring behavioral effects because the vials could be held up to a bright light permitting the animal to be viewed through the filter paper. Ten animals were exposed to each of six treatments: 0, 4.2, 6.3, 8.4, 10.0, and 12.6 ppm of d-limonene alone, or in combination with PBO or sesame oil. LC$_{50}$ and LT$_{50}$ values were calculated according to the trimmed Spearman-Karber method (Hamilton et al. 1977). Mortality was defined as failure to produce any body movement upon tactile stimulation. Dead worms invariably showed signs of rapid and extensive decomposition.

The same exposure protocol was used for worms subjected to electrophysiological testing except that no synergists were employed and d-limonene was delivered into the vials 0.5 hr before the worms were added. Animals designated for chronic d-limonene treatment were exposed to concentrations of 0, 1.0, 2.1, 4.2, 6.3, and 8.4 ppm for a 48 hr period (10 animals per concentration). Treatment was interrupted once at 24 hr to permit weighing and electrophysiological testing. Animals designated
for assessment of the localization of neurotoxicity (n=8) were exposed to 42.1 ppm d-limonene for 0.5 hr. Worms designated for acute d-limonene exposure received concentrations of 0, 2.6, 10.0, or 42.1 ppm for exposures of either 0.25 hr, 0.5 hr, or 1 hr. A group of ten animals was used in each of the 12 concentration/exposure combinations. Reversibility of neurotoxicity effects was studied in these same groups by placing the worms in clean vials containing only untreated filter paper and 2 ml of distilled water. Subsequently, the groups were electrophysiologically tested at 3, 6, 12, 24, and 48 hours after transfer to the clean vials.

Electrophysiological Methods

Methods for non-invasive detection of giant nerve fiber activity in earthworms were described previously (O’Gara et al. 1982, Drewes 1984). Briefly, before and immediately following exposure, worms were placed ventral-side down on a moistened printed circuit board recording grid, composed of pairs of parallel electrodes (2 mm spacing between electrodes in a pair; 10 mm between adjacent electrode pairs). Care was taken to ensure that worms were motionless and in relatively relaxed and normal states of elongation when electrophysiological data were taken. To evoke all-or-none MGF or LGF spikes, the head or tail end, respectively, of the worm was gently touched with the tip of a fine bristle attached to a hand-held probe. Resultant signals detected by each electrode pair were fed into differential recording amplifiers, filtered, and displayed as two separate digitized traces by using a Tektronix 5113 storage oscilloscope and 5D10 waveform digitizer. The peak-to-peak time interval
(measured to within 0.1 milliseconds) between corresponding spikes in the two displayed traces was measured on-line from the oscilloscope screen. Calculations of absolute velocities (m/s) were made by dividing conduction distance between electrode pairs (1 cm) by the conduction time indicated on the oscilloscope. Mean absolute MGF and LGF conduction velocities were obtained from six replicated conduction velocity measurements per animal. Five to ten minutes were required to obtain these measurements from each worm. Paired t tests were used to evaluate the differences between conduction velocity means (measured in m/s) taken before and after d-limonene exposure. For graphic presentation conduction velocity values were expressed as relative conduction velocities, relative conduction velocity being defined as the ratio of the mean absolute velocity following d-limonene exposure to mean velocity prior to exposure. Permanent records of impulse conduction were made by directly photographing the oscilloscope screen with a Polaroid® camera or by digital-to-analog conversion of the recording and transference from the oscilloscope to a chart recorder.

MGF and LGF electrophysiological data for chronic exposure (24 to 48 hr) was collected from the geometric centers of worms, over a 1-cm conduction distance centered at approximately body segment 50. To determine if neurotoxicity was localized within specific body areas, MGF and LGF conduction velocities were measured at four separate axial regions, or sectors, along the body. Each sector was ca. 25 segments in length. Sector 1 was anterior to the clittellum and included the head segments, sector 2 included the clittellum and the region immediately posterior to
it, sector 3 was posterior to 2, and sector 4 was posterior to sector 3 and included the pygidial segments. In a third group of animals, used for studying acute d-limonene exposure and recovery from neurotoxic effects, all conduction velocity data were collected from sector 3.
RESULTS

Mortality and Symptoms of Intoxication

Lethality effects of d-limonene are summarized in Table 1 and Figure 1. Pretreatment with the mixed-function oxidase (MFO) inhibitor sesame oil did not significantly alter either the 48 hr LC₅₀ or the LT₅₀ values for d-limonene. Although PBO pretreatment induced a very slight reduction in the LC₅₀ value, this occurred with a concomitant increase in the LT₅₀ value. PBO treatment also resulted in a slight flattening of the dose-response curve (Figure 1). Taken together, these data suggest that a cytochrome P-450-containing MFO system is probably not important in either detoxifying or activating d-limonene in the earthworm.

The LT₅₀ value calculated for the 12.6 ppm dose indicated that d-limonene acted relatively rapidly; only 6% of all mortalities occurred after 24 hr of exposure. Likewise, intoxication commenced rapidly upon exposure and involved a stereotyped cascade of symptoms. Within 0.5 hr of exposure, worms receiving d-limonene doses in the LC₅₀ range produced

Table 1. Toxicity of d-limonene with and without synergists to Eisenia fetida

<table>
<thead>
<tr>
<th>Treatment</th>
<th>48 hr LC₅₀ (ppm)ᵃ</th>
<th>LT₅₀ (hr)ᵇ⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-Limonene</td>
<td>6.0 (5.1-7.1)</td>
<td>4.9 (3.5-6.8)</td>
</tr>
<tr>
<td>d-Limonene + PBO</td>
<td>4.8 (4.2-5.5)</td>
<td>9.2 (6.7-12.6)</td>
</tr>
<tr>
<td>d-Limonene + sesame oil</td>
<td>6.4 (5.0-8.0)</td>
<td>6.6 (5.1-8.5)</td>
</tr>
</tbody>
</table>

ᵃ95% confidence limits indicated. All control mortalities = 0.

ᵇLT₅₀ data based on exposure to 12.6 ppm only.
Figure 1. Dose response curve for d-limonene when applied alone and with the synergist piperonyl butoxide.

Writhing movements (particularly in the posterior one-third), increased mucus secretion and coelomic fluid discharge, significant swelling of the clitellum (Figure 2), and general elongation of the body. Despite continuous exposure, clitellar swelling began to subside in most animals by 3 hr of exposure and ceased in all surviving animals by 48 hr. Elongation and writhing movements persisted in surviving worms through 24 hr but ceased by 48 hr. Animals receiving sublethal doses sometimes exhibited a series of intersegmental constrictions in the posterior one-half of the body at 3 to 6 hr of exposure. These were generally followed, between 24 to 48 hr, by autotomy at the constrictions. Up to one-half of the posterior of the body was sometimes lost in this manner but autotomy was seldom followed by death of the surviving head section. In worms receiving lethal d-limonene exposures, death was preceded by
ataxia, limpness of the body, and eruptive lesions in the body wall, especially in the clitellar region.

Animals exposed to higher d-limonene concentrations showed some additional symptoms. For instance, at 42.1 ppm or more, spastic twisting, particularly in the posterior end, occurred within the first 5 minutes of exposure, often resulting in highly contorted movements (Figure 2); this was followed by severe ataxia and a general rigidity within 15-20 minutes of exposure. Worms receiving 100 ppm or more became ataxic and showed a general pulsating or trembling of the body within 10 minutes. Death occurred within 2 to 6 hr at 42.1 ppm and within 1-2 hr at 100 ppm.

Figure 2. Silhouettes, photographed from videotape recordings, of normal, untreated worm (left), worm at 30 min post-exposure, 6.3 ppm (middle), and worm at 5 minutes post-exposure, 100 ppm (right)
Effects of Chronic Exposure

Sublethal concentrations of d-limonene had a significant influence on worm weight loss after either 24 or 48 hr exposures. There was no significant difference in mean weight loss between these two groups. The relationship between weight loss and d-limonene dose (Figure 3) was best described by a linear model and had a significant coefficient of determination \( r^2 = 0.88 \). Weight loss was probably related to the combined effects of increased mucus secretion and coelomic fluid discharge through dorsal pores which occur as general responses to irritation (Edwards and Lofty 1977). Comparable weight losses were observed when earthworms were exposed to dieldrin (Drewes and Vining 1984).

Whereas weight loss was induced by chronic d-limonene exposure, MGF and LGF conduction velocities were not affected. Paired t tests detected
no differences between mean conduction velocity measurements taken prior to d-limonene exposure and after 24 or 48 hr exposure (Figure 4). Also depicted in Figure 4 is a mortality curve based on deaths that occurred during chronic exposure. Exposure concentrations were well within the LC$_{50}$ range determined earlier (Figure 1); nevertheless, no sublethal effects on conduction velocity were noted, even at concentrations at which mortality soon followed. The mortality values determined here for chronic exposure were somewhat below the values expected based on LC$_{50}$ data (Table 1); this was probably because the vials were opened and the worms removed for 5-15 min periods at 24 hr for electrophysiological

![Graph](image)

**Figure 4.** Toxic effects of d-limonene following 48 hr chronic exposure. Mortality for chronically exposed animals is shown on the right coordinate with each triangle representing % mortality for a group of 10 worms. Relative conduction velocities for the same animals are shown on the left coordinate, each circle representing the mean relative conduction velocity for 10 worms except at 6.3 ppm where n=8 and 8.4 where n=4 (due to mortality during exposure)
monitoring thereby permitting some dissipation of \textit{d}-limonene vapors.

Despite the absence of chronic exposure effects on conduction velocity, other effects involving both giant fiber waveform and excitability were evident. The typical MGF and LGF waveforms in normal, untreated worms are as shown in Figures 5. In response to tactile stimulation of the head (Figure 5A), MGF spikes were initiated anteriorly and propagated posteriorly. Each was typically followed, after 1.5-2.0 ms, by a giant motor neuron spike and then by a muscle potential (Drewes 1984). LGF spikes, in contrast, were normally initiated by posterior touch and propagated anteriorly. Muscle potentials rarely accompany a single LGF spike or two widely spread spikes (Figure 5B). Departures from these normal conditions were observed at 24 hr exposure in half of the animals receiving 4.2 ppm, five of eight animals receiving 6.3 ppm, and four of five animals receiving 8.4 ppm where abnormal "rebound" spiking was prevalent in the MGF pathway. Such spikes often occurred following initiation of a single touch-evoked MGF spike. The spike, after normal propagation into posterior segments, was followed, after 10 to 20 ms, by initiation of one or more spikes propagated back anteriorly, as indicated by the inverted spike waveform and reversed timing sequence (Figure 6A).

To determine the site of rebound initiation along the body, recording sites were moved progressively posterior (Figure 6B and 6C) until rebound spiking occurred nearly synchronously (but with opposite spike polarity) at the two sites. This indicated that the site of initiation was somewhere between the two recording electrodes. Invariably, the site(s) of initiation for rebound spikes were in the posterior one-half
Figure 5. MGF and LGF spikes in a normal earthworm. A) A typical recording of a pair of MGF spikes. The spikes, evoked by anterior touch, were conducted posteriorly and recorded at two sites, 1.0 cm apart, in the posterior half of the worm. Each MGF spike (open circle) was accompanied by a giant motor neuron spike potential (dot) and a muscle potential (triangle). B) A pair of widely spaced LGF spikes, initiated by touching the tail end. Recording sites are the same as in A.
Figure 6. Rebound spiking in worms exposed to 6.3 ppm d-limonene for 24 hr. The initial MGF spike in each record was evoked by a light tactile stimulus to the anterior end. A) The reversed timing and inverted waveform of the second MGF spike pair indicate that the site of initiation (star) for the anteriorly conducted rebound spike was posterior to recording site 2. Note the reduced amplitude of giant motor neuron spike potential and lack of muscle potentials (large and small triangles, respectively) B) A second MGF spike, detected at site 2, failed to conduct to site 3. A third MGF spike (rebound spike) was initiated between sites 2 and 3 (star). C) A posteriorly conducted MGF spike was followed by three rebound spikes, with initiation sites sometimes anterior or posterior to recording site 3.
of the worm, at approximately two-thirds to three-fourths of the distance between the head and tail. This position was solely within the LGF sensory field and approximately 10 to 20 segments posterior to known limits of the MGF sensory field in normal worms (Vining and Drewes 1985). During any series of rebound spiking, and in the absence of any overt body movement, it was also evident that the exact locus of spike initiation was shifted to slightly more anterior or posterior sites (Figure 6C). The tendency for rebounding was decreased strikingly by 48 hr of exposure, occurring in only one of ten animals at 4.2 ppm, in one of eight animals at 6.3 ppm, and none of four surviving animals at 8.42 ppm.

Another effect of chronic d-limonene exposure was the occurrence of apparently "spontaneously" generated, often rhythmic bursts of LGF spikes. Such spikes appeared in the absence of any externally applied stimulus. The loci for initiation of these spikes were variable but were generally similar to those of MGF rebound spiking. After 24 hr of exposure, spontaneous spiking was observed in one of ten animals exposed to 2.1 ppm d-limonene, five of ten animals in the 4.2 ppm treatment, four of the eight animals surviving the 6.3 ppm treatment, and in three of the five animals surviving 8.4 ppm. Spontaneous spiking was never seen in control animals, or those receiving 1.0 ppm. Like the MGF rebounding effects, spontaneous LGF spiking was transitory; by 48 hr it was observed in only one animal exposed to the 6.3 ppm treatment.

A third effect of chronic d-limonene exposure, with concentrations of 4.2 ppm or greater, was marked decreases in the amplitude of giant motor neuron spikes and muscle potentials in the MGF pathway (Figure 6)
in comparison to untreated control animals (Figure 5A).

Effects of Acute Exposure

Because the abnormal spike initiation observed during chronic exposure occurred only in posterior segments of the worms, special attention was given to possible region-specific effects during acute exposure. This was done by comparing conduction velocity effects in four equally spaced sectors along the length of eight worms. Such data could not always be collected from posterior sectors of some worms due to blocking of giant fiber spikes in these sectors.

A single 0.5 hr exposure of 42.1 ppm caused statistically significant decreases in the mean conduction velocities of impulses in both the

![](chart.png)

Figure 7. MGF conduction velocity in four body regions. Each sector represents one-fourth (ca. 25 segments) of the length of the worm. Sector 1 is the most anterior. For sectors 1-3 n=8, for sector 4 n=6

*,** indicate significant differences at $P = 0.05$ and $P = 0.01$ levels, respectively
MGF and LGF. Additionally, the severity of these decreases was closely linked to the body sector examined. In the MGF (Figure 7), the effects on conduction velocity were greatest in the third (ca. segments 50-75) sector but statistically significant slowing was also observed in the other sectors. Regional differences were also observed for effects on LGF conduction velocity (Figure 8). Statistically significant differences between pre- and post-treatment velocities were observed only in the third and fourth sectors. Because the third sector appeared to be especially sensitive to the effects of d-limonene on both the MGF and LGF pathways, all conduction velocity data for subsequent dose-response and reversibility studies were collected from the third sector.

![Graph showing conduction velocity comparison](image)

** indicates significant differences at P = 0.01 level

Figure 8. LGF conduction velocity in four body regions. Each sector represents one-fourth (ca. 25 segments) of the length of the worm. Sector 1 is the most anterior. For sector 1 n=7, for sector 2 and 3 n=8, and for sector 4 n=7
The effect of acute concentrations on conduction velocity for four different exposure durations are shown in Figures 9 and 10. Paired t tests indicated that after only 0.25 hr exposure, MGF and LGF velocities decreased significantly \((P < 0.01)\) from their pretreatment values in both the 10 ppm and 42.1 ppm treatments. The magnitude of the decreases were directly related to exposure duration. After 0.5 and 1.0 hr of exposure, conduction velocities decreased significantly \((P < 0.01)\) from their pretreatment values in all three concentrations. The relationships between concentration and relative conduction velocity for all three exposure durations were best described by logarithmic models and were moderately to highly dependent, with \(r^2\) values of 0.98, 0.90, and 0.73 for 0.25 hr, 0.5 hr, and 1.0 hr exposures, respectively, in the MGF. In the LGF, \(r^2\) values for 0.25 hr, 0.5 hr, and 1.0 hr exposures were 0.59, 0.86, and 0.97, respectively.

Besides the significant slowing of conduction velocities, other neurotoxic effects were commonly observed in animals exposed to 42.1 ppm for 0.5 or 1.0 hr. The amplitude of giant motor neuron spikes and muscle potentials, which normally accompany MGF spikes, were diminished (Figure 11A). Additionally, propagation of LGF spikes was often completely blocked (Figure 11B) at posterior recording sites. This blocking was generally accompanied by a reduction in LGF responsiveness to tactile stimulation in the posterior half of the worm. Animals exhibiting impulse conduction blocking in the LGF often autotomized in the regions of the blockage within 24 hr following exposure.
Figure 9. Effects of d-limonene concentration and exposure duration on relative conduction velocity in the MGF.

Figure 10. Effects of d-limonene concentration and exposure duration on relative conduction velocity in the LGF.
Figure 11. Effects of acute d-limonene exposure (42.1 ppm for 30 min) on MGF and LGF activity. In A four MGF spikes (open circles) were initiated by anterior touch. Note that giant motor neuron spike potentials and muscle potentials were reduced or absent. Recording sites 2 and 3 were the same as in Figure 6B and 6C. In B two LGF spikes were evoked by touch near the anterior limit of the LGF sensory field. These spikes failed to conduct to the posterior recording site.
After removal from the treatment vials and placement in clean, untreated vials, worms exposed to all d-limonene concentrations and exposure durations were electrophysiologically retested to assess speed and extent of recovery from neurotoxic effects. Except for six of ten animals at 42.1 ppm (1 hr exposure), one of ten animals at 42.1 ppm (0.5 hr exposure), and one of ten animals at 10 ppm (1 hr exposure), all animals survived the acute exposures for the 48 hr interval following the cessation of d-limonene exposure. During the recovery period many of the animals that had been exposed to 42.1 ppm and 10 ppm for 1 hr and 42.1 ppm for 0.5 hr exhibited autotomy of the posterior 1 to 1.5 cm of their bodies. Behavioral and morphological symptoms subsided in most animals by 3 hr and in all animals by 12 hr in the recovery vials.

As expected, recovery from conduction velocity effects was most rapid in animals exposed to the 2.6 ppm concentration. In both the MGF (Figure 12) and LGF (Figure 13) pathways, t tests indicated that no differences ($P < 0.05$) between pre- and post-treatment conduction velocities existed by 3-6 hr in animals in both the 0.5 hr and 1 hr exposure duration groups. Velocities in worms exposed to 10 ppm (Figures 14 and 15) for 0.25 hr were not different from the pretreatment values by 3 hr, whereas those exposed for 0.5 hr recovered by 6 hrs, and those exposed for 1 hr recovered by 12 hr. As mentioned earlier, many worms that were exposed to the 42.1 ppm d-limonene concentration died during the recovery period and the survivors of the lower concentrations required a considerable recovery period before their conduction velocities returned to
Figure 12. Time course of recovery of MGF conduction velocity following exposure to 2.6 ppm \textit{d}-limonene

Figure 13. Time course of recovery of LGF conduction velocity following exposure to 2.6 ppm \textit{d}-limonene
Figure 14. Time course of recovery of MGF conduction velocity following exposure to 10.0 ppm d-limonene

Figure 15. Time course of recovery of LGF conduction velocity following exposure to 10.0 ppm d-limonene
pretreatment values. Animals receiving 0.25 hr exposures recovered by 24 hr but those receiving 0.5 hr exposures were not recovered until 48 hr. For all concentrations and exposure durations, recovery of LGF velocity tended to be more rapid than MGF velocity.
DISCUSSION

Results obtained in LC_{50} and LT_{50} trials indicated that in Eisenia fetida, d-limonene was a fast-acting toxicant with a very narrow mortality range. The compound appears more toxic to worms than to insects, since the LC_{50} value for vapor exposure in insects was ca. 20 ppm (Karr and Coats 1988) compared to the 6.0 ppm calculated for worms. It is possible that these differences in toxicity were due to dissimilarities in d-limonene metabolism, as indicated by the contrasting effects of synergists in the two groups. The compound was not synergized significantly by either sesame oil or PBO in the earthworm. However, PBO had a significant synergistic effect in both the house fly and the German cockroach (Karr and Coats 1988). It is generally accepted that the MFO system is present in earthworms (Nakatsugawa and Nelson 1972, Nelson et al. 1976) and, as in vertebrates and other invertebrates, functions by oxidizing xenobiotics to more soluble, and thus more excretable forms. That application of the enzyme system inhibitors did not significantly alter the toxic effects of d-limonene in earthworms suggests that either d-limonene is not metabolized by the earthworm MFO system, or the dermally-applied inhibitor or the d-limonene did not reach the MFO system. The latter seems more plausible in view of the results obtained by Nelson et al. (1976), who examined xenobiotic metabolism in earthworms and found that the highest oxidative activity was associated with the gut wall. Thus, it could be argued that the MFO system represents a primary defense against orally ingested toxins but a relatively inaccessible system for interactions with cutaneously administered xenobiotics or inhibitors of
the MFO system.

Rapid onset of a stereotyped sequence of morphological, behavioral, and neurotoxicity effects was observed in *Eisenia fetida* following *d*-limonene exposure beginning at 0.25 hr and continuing through 48 hr. This compares well with observation that, in insects, the compound exhibited rapid knockdown followed by convulsions and subsequent paralysis (Hink and Fee 1986). The swift action suggests that, as a vapor, *d*-limonene quickly penetrates the moist cuticle and epidermis of the earthworm to reach some yet unknown site(s) of action. Since different time courses were evident for each of the various toxicity effects, the results underscore the importance of selecting appropriate end-points and employing a flexible testing protocol in assessing the effects of this compound. For example, an arbitrary selection of a single exposure duration may cause underestimation of toxic effects or may fail to detect the toxic impact on the nervous system given the fast-acting and transient effects of *d*-limonene.

Some types of neurobiological aberrations observed in *Eisenia fetida* following *d*-limonene exposure have been previously observed in earthworms. For instance, rebound spiking, similar to that in Figure 6, was reported following exposure to fluorene (Drewes et al. 1984). Fluorene also induced reductions in MGF and LGF conduction velocity and decreases in MGF-mediated muscle potentials, but it did not induce spontaneous LGF spiking or blocking of LGF spikes as did *d*-limonene. Spontaneous spiking was observed in earthworms exposed to the chlorinated hydrocarbon insecticide dieldrin (Drewes and Vining 1984); however, this was observed in
the MGF rather than in the LGF pathway. Like d-limonene, dieldrin induced decreases in conduction velocity, but these changes required exposure durations of at least 90 minutes. Furthermore, dieldrin intoxication induced hypersensitivity in giant fiber responses to tactile stimulation, which was not observed following exposure to d-limonene. Previously, no single toxicant has been reported to exert the specific combination of neurotoxic effects observed following d-limonene treatment.

Both acute and chronic sublethal d-limonene exposures induced a series of behavioral, morphological, and neurological effects that appeared to be totally reversible given adequate recovery time. During chronic exposures, the recovery occurred in spite of maintained exposure. Several factors could account for this observation. First, the worms may have gradually depleted their immediate environment (the closed vials) of d-limonene through normal metabolic processes. Second, through prolonged, sublethal exposure worms may have built up a tolerance to the compound, perhaps via enzyme induction and subsequent enhancement of metabolic capacity. Finally, it is possible that the opening of the vial at 24 hr and removal of the worm for weighing and electrophysiological monitoring released sufficient vapors that the effective concentration of d-limonene was reduced.

The recovery following acute exposures clearly indicated that d-limonene's effects on the nervous system were reversible. Significant decreases in conduction velocities and other neurotoxic effects were observed in animals at all three d-limonene concentrations after as
little as 0.25 hr exposure. Without removal from the treatment vials after the maximum 1 hr exposure duration, all animals exposed to 10 ppm or more would have died. However, when exposure ceased before the mortality threshold was crossed, the worms exhibited a steady, progressive recovery from all behavioral, morphological, and neurobiological symptoms, including restoration of conduction velocity values. The reasons behind the recovery are unknown but could indicate that \( d \)-limonene induces either minor, and rapidly reversible lesions, or that it interacts with or binds only temporarily to sites of action before being released or metabolized. The transient, reversible nature of \( d \)-limonene toxicity in the earthworm further demonstrates the importance of understanding the time course of a toxicant's action before designing a protocol for assessing its effects on physiological parameters such as nervous system function.

That the effects of \( d \)-limonene were more pronounced in the third body sector is an important finding from both toxicological and physiological standpoints. It has often been observed that many axial physiological gradients exist in earthworms. For example, Watanabe (1927) described U-shaped gradients for carbon dioxide production, oxidizable substances, electrical potentials, and total solid content along the longitudinal axis of the bodies of \textit{Pheretima hilgendorfi} and \textit{Eisenia fetida}. Axial gradients have also been observed for respiratory rates, muscle glycogen, visceral glycogen, and lipid concentration in \textit{Eisenia fetida} (O'Brien 1957). Additionally, one early toxicological study (Hyman 1916) in aquatic oligochaetes demonstrated that the susceptibility
of tissues to the effects of cyanide was not constant along the length of the body but was most pronounced in the anterior and extreme posterior ends, where the most active metabolic tissues were located. Also, gradients have been shown to exist in the organization and functioning of the earthworm central nervous system, and encompass parameters such as the strength of tactile sensory inputs into giant fibers (Günther 1973, Smith and Mittenthal 1980) and conduction velocity of giant fibers (O'Gara et al. 1982). The experiments in this study were designed to test and, indeed demonstrated, that the impact of d-limonene exposure on conduction velocity and other neurobiological parameters (i.e., rebounding, spontaneous spiking, blocking of conduction) varied between different body regions and was most severe in one specific region along the length of the body, the third sector (ca. segments 50-75).

The predisposition toward neurotoxic perturbations in the third sector may be related to the previously mentioned physiological gradients. For example, it may be possible that penetration through the cuticle is greater in that area or perhaps metabolic or excretory systems responsible for detoxification and removal of d-limonene are less potent in the third sector. Whether d-limonene then acts directly or indirectly on the nervous system is not clear. In the case of possible indirect action, one hypothesis is that region-specific neurophysiological effects result from the previously mentioned axial gradients in metabolic activity. If, for example, respiratory processes cannot keep up with metabolic demands and stress caused by exposure, then carbon dioxide levels in these tissues may rise, leading to acidification (Juel 1980). A decrease
in pH could, in turn, directly and reversibly affect neuronal function by increasing gap junctional resistance and uncoupling electrical synapses (Giaume et al. 1980, Verselis and Brink 1984). Since gap junctions are present at septate partitions in each segment of the Eisenia giant fiber system (Hama 1987), increases in gap junction resistance would translate directly into slower conduction or conduction block in giant fibers (Brink and Barr 1977). Assuming that electrical synapses are also present at the MGF-to-giant motor neuron junction (Günther 1972), then the observed uncoupling between MGF spikes and muscle responses (Figure 11) would also be predicted. It is not evident, however, how effects on electrical synapses could account for other neurobiological symptoms such as induction of rebound spikes after delays of 10-20 ms (Figure 6). Presumably, these effects could involve toxin actions on other, as yet unidentified, circuits in the worm's ventral nerve cord.

In conclusion, this study clearly confirms that non-target, non-arthropod species, such as Eisenia fetida, may serve as valuable models for detecting and assessing in vivo neurotoxicity of candidate insecticides and for elucidating neurotoxic effects difficult or impossible to monitor within a similar context in insect models. The examination of d-limonene in this model system also illustrates that reliable employment of the system depends on a flexible experimental protocol that can easily accommodate unique properties of the chemical and its toxic actions.
REFERENCES CITED


CONCLUSIONS

d-Limonene was found to have a wide range of subtle effects on insects. Whereas it exhibited no residual or oral toxicity, it did show low topical toxicity, moderate fumigant and ovicidal activity, and significant repellency and larvicidal activity. Earthworms, Eisenia fetida, appeared to be about three times more susceptible to topical/vapor exposures than insects. In insects, topical toxicity was significantly synergized with piperonyl butoxide, indicating cytochrome P-450 mixed function oxidase participation in the metabolism of the compound. Significant synergism with piperonyl butoxide was not observed in the earthworm. This implies that earthworms may rely on a different enzyme system for d-limonene metabolism or that the metabolic inhibitors or the d-limonene are not able to reach the MFO system, located principally in the gut wall, when administered cutaneously.

Symptoms of intoxication following topical or vapor exposure of d-limonene suggest a mode of action linked to nervous system interference in earthworms and insects. However, the growth-stimulating and reproduction-inhibiting qualities observed in German cockroaches intimate an additional mode of action involving hormonal stimulation or interference. Additional research is needed to further elucidate such alternate modes of action.

Toxic effects of d-limonene in the earthworm unequivocally involve neurotoxicity. The compound induced rapid disruptions in the conduction velocities and waveform characteristics in both the medial and lateral giant fiber systems in earthworms. These disruptions, however, were
typically completely and rapidly reversible. Whereas the exact mechanism of d-limonene's disruption of normal nervous system function is not known, the reversibility indicates that the compound probably binds transiently to a site or sites of action in or related to the nervous system, and without inducing permanent lesions. Further research is needed to elucidate the actual sites of d-limonene interference in the earthworm and to determine if the neurotoxicity is a direct or indirect effect of intoxication. Additionally, it would be useful to investigate the physiological parameters that appear to predispose the third body sector of Eisenia fetida to the toxic effects of d-limonene and perhaps other neurotoxins.

Is d-limonene a suitable candidate for widespread employment as a general-use insecticide? Based on the results reported here we must say probably not. With no residual life, low topical toxicity, and no oral effects there is little hope for its successful exploitation as an agrochemical or as a household insecticide. Its use as a mammalian ectoparasite control tool has merit however and will probably continue. Additional uses which further exploit the fumigant activity and possibly the larvicidal properties may also have commercial appeal and should be further investigated. Whereas d-limonene itself is not a particularly potent material, this work has demonstrated that it does interfere with several life processes in insects and earthworms. We can be sure, therefore, that the material does bind at some important sites of action. It may thus be possible to search for related monoterpenoids or synthetic d-limonene analogues that bind at the same sites, but with more per-
manence or tenacity than does d-limonene. Once accomplished, this search may yield a group of new, biorational insecticides unlike any currently in use.

What has this work told us about the biological roles of d-limonene in particular and monoterpenoids in general? This is a question that I ponder often and one to which there is no single or definitive answer. d-Limonene possesses, without a doubt, bioactive properties. But in the plant, are these properties enhanced or even synergized by coexisting allelochems or other plant characteristics that have not yet even been discovered or investigated? This is a question that needs to be kept in mind as we attempt to exploit natural products as insecticides. Out of context, individual secondary plant compounds may have elusive effects or little impact on other organisms. However, the mélange of allelochems that have been carefully and conveniently formulated together in virtually every plant may offer to us the best hints yet for successful development of the ideal biorational insecticide.
ACKNOWLEDGEMENTS

I am grateful to many people for their support, assistance, and
guidance with this research and my graduate career at Iowa State Univer-
sity. I thank my major professor, Dr. Joel Goats, for his support and
for encouraging me to participate in the Toxicology program. I also ack-
nowledge the members of my graduate committee, Drs. Charles Drewes, John
Mutchmor, Kenneth Shaw, and Jon Tollefson for providing valuable input.
Dr. Drewes deserves special mention. His assistance and interest in my
research have been invaluable and I sincerely appreciate all he has done
to enrich me as a researcher and a writer. I also wish to thank Dr.
Larry Pedigo for permitting and encouraging my involvement in the writing
of his textbook and accompanying laboratory manual. It was a valuable
and exciting experience.

Technical assistance in my research was provided by many dedicated
and supportive people including Nancy Weiss, Tracy Hageman, and Dave
Swanlund; I sincerely appreciate their commitment and helpful efforts. I
also wish to acknowledge all my fellow students in the toxicology group,
past and present, for their advice, encouragement, and friendship; my
years here have truly been the "best of times" thanks to all of them. A
special thanks must go to Drs. Wendy Wintersteen and Leon Higley. Their
faithful support and camaraderie have been undying and they are unques-
tionably the best friends I could ask for.

Finally, I thank my husband, James "Giff" Gifford. His friendship,
love, and encouragement were indispensable contributions toward the
completion of my degree.