AN ABSTRACT OF THE THESIS OF

Steven H. Harwood for the degree of Master of Science in Entomology presented on December 14, 1987.

Title: Toxicity of Peppermint Monoterpenes to the Variegated Cutworm, *Peridroma saucia* Hübner

Redacted for Privacy

Abstract approved: _____

The toxicity of menthol, menthone, pulegone, limonene, and α-pinene, five monoterpenes found in peppermint (*Mentha piperita* L.), to the variegated cutworm (*Peridroma saucia* Hübner) was characterized by *in vivo* and *in vitro* methods.

Pulegone and menthone caused slower growth over six days when incorporated into an artificial diet and fed to fifth stadium larvae. Reduced growth was likely the result of the significant antifeedant properties observed for these compounds.

Menthol, the dominant monoterpene found in peppermint leaves caused molting and pupation inhibition when incorporated in the diet over the dosage range comparable to that found *in planta* (0.05% to 0.2%).

Limonene at a dose of 0.2% caused significant inhibition of pupation, but otherwise had little effect on growth or survival. α-Pinene had no effect on pupation, growth or survival over a dosage range of 0.05% to 0.2%.

All five monoterpenes were metabolized *in vitro* by the cytochrome P-450 dependent polysubstrate oxidase system, however piperonyl butoxide did not synergize their toxicity *in vivo*.

Neonate larvae were more sensitive to oral administration of these monoterpenes than were fifth stadium larvae.

These experiments suggest that monoterpenes in peppermint leaves could cause mortality to neonate variegated cutworm larvae.

Toxicity of Peppermint Monoterpenes to the Variegated Cutworm, *Peridroma saucia* Hübner

by

Steven Hunter Harwood

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed December 14, 1987

Commencement June 1988

APPROVED:

Redacted for Privacy

Professor of Entomology in charge of major

Redacted for Privacy

Head of Department of Entomology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented: December 14, 1987

Word-processed by the author

ACKNOWLEDGEMENTS

I wish to thank my major professor, Dr. Ralph Berry, for his continuous support and encouragement. My heartfelt appreciation also goes to Dr. Alison Moldenke for her assistance with the PSMO work, thorough editing and lively conversation. Dr. Rene Feyereisen provided his laboratory and support throughout. Assistance and friendship was also extended by Dr. Len Coop and Ken West, their contributions are greatly appreciated.

Finally, my wife Andrea provided love, support, patience, and understanding.

Table of Contents

List of Figures

List of Tables

TOXICITY OF PEPPERMINT MONOTERPENES TO THE VARIEGATED CUTWORM *Peridroma saucia* HüBNER

RATIONALE AND SIGNIFICANCE

The coevolution of plants and insects has been envisioned as an ever-escalating arms race (Feeny 1976). Herbivorous insects exert pressure on plants to develop defensive mechanisms against herbivory; plant defenses in turn exert pressure on insects to develop means to circumvent them. Recently, attention has been given to the effects plant secondary compounds may have on the next trophic level - parasites, parasitoids, and carnivorous insects. These interactions are complex, and have practical and theoretical implications, e.g. how plant secondary compounds affect herbivore fitness, parasitism rates, detoxification systems in both herbivore and parasitoid, etc.

Understanding the chemical ecology of agroecosystems is essential to the implementation of integrated pest management. The tools of integrated pest suppression, such as natural enemies, interplanting, judicious use of chemicals, are all influenced by the interaction between plant chemistry and subsequent trophic levels. The refined manipulation of plant chemistry using a molecular genetic approach is becoming a reality. It is now possible to insert a gene for the production of a given allelochemical or insecticidal compound into a crop plant and have the gene expressed. Without the knowledge of how plant chemistry interacts with agricultural pests and their natural enemies, it will not be possible to predict what the consequences of such manipulation may be.

Peppermint, *Mentha piperita* (L.), is an important crop in the Pacific Northwest. The leaves of *M. piperita* have oil glands containing over 30 monoterpenes, many of

Peridroma saucia Hübner, feeds on mint and is the major pest in commercial peppermint of Western Oregon (Berry and Shields 1980).

Parasitism of *P. saucia* by the braconid wasp, *Meteorus communis* Cresson drastically lowers leaf consumption and survival of *P. saucia* (Coop 1987, Coop and Berry 1987). However, the effects of mint monoterpenes on *P. saucia* or *M. communis* are largely unknown. Previous work has demonstrated induction of detoxication mechanisms in *P. saucia* by peppermint monoterpenes (Yu *et al.* 1979, Berry *et al.* 1980, Moldenke *et al.* 1983). Nothing is known about how host plant and parasitoid interact, or what interactions may occur between plant nutritional quality and toxicity of allelochemics in herbivore or parasitoid. This is therefore an ideal system for exploring multitrophic interactions between plant, herbivore, and parasitoid.

This thesis project focused on characterizing the toxicity of menthol, menthone, pulegone, limonene, and -pinene to the variegated cutworm and has attempted to document metabolism of these monoterpenes with the cytochrome P-450-dependent polysubstrate monooxygenase enzyme system (referred to hereafter as PSMO). This information is critical before the more complex interactions of nutritional and multitrophic factors can be considered.

General Plant-Herbivore Theory

Plants often possess a complicated array of chemicals, representing a significant investment of energy, that have no known direct role in metabolism or reproduction. This has led to the suggestion that such compounds exist as defenses against herbivory (Feeny 1976).

The guiding paradigm in recent years for conceptualizing plant-insect interaction was formulated independently in the mid-1970's by Paul Feeny (Feeny 1976) and David Rhoades and Rex Cates (Rhoades and Cates 1976). Although the two models used slightly different assumptions, they generated similar predictions (Fox 1981).

Feeny (1976) categorized plants as belonging to two distinct groups: apparent and nonapparent. Apparent plants are large, occur in relatively homogeneous communities and have persistent life histories. Compared to nonapparent plants, apparent plants expend a relatively small amount of their energy budget on reproduction. Being "apparent" in space and time, they are easily found by both specialist and generalist herbivores. Chemical defenses of apparent plants tend to be of low toxicity but occur in high concentrations, causing slow growth and feeding rates. Feeny referred to this type of chemical defense as quantitative.

Nonapparent plants, in contrast, are small, occur in relatively heterogeneous communities, and tend to have annual life histories. They grow rapidly and invest a large proportion of their energy budget for reproduction. Theoretically, because of their critical energy budget, such plants would be defended by small amounts of toxic compounds that involve little metabolic cost. Toxic compounds should exert a high selective pressure on herbivores. Thus, it would be expected that a few eventually would evolve means to circumvent the specific toxins. Compound causing toxic reactions in herbivores are termed qualitative.

The model of Rhoades and Cates (1976) is similar except that the basic unit of apparency is at the tissue level instead of the whole plant. Ephemeral tissues, on either apparent or nonapparent plants are defended by small amounts of toxins of diverse chemistry. Predictable tissues on either apparent or nonapparent plants are protected by large amounts of digestibility reducing compounds of convergent chemistry.

The plant apparency model has had great appeal and its predictions are consistent with current ecological theory, experimental data, and observations of natural phenomena. It works particularly well for comparisons between plants reflecting extremes between apparent and nonapparent. But as with most dichotomous models, apparency and non-apparency is an oversimplification. Plants exist in a continuum of community complexity, size, lifespan, energy budgeting, and defense mechanisms.

Defense Mechanisms of Plants and Insects

Although proof that secondary plant compounds exist for the sole purpose of plant defense is often lacking, many have biological activity towards herbivores. I will discuss two examples of plant defense, one typical of nonapparent plants and one typical of apparent plants.

A good example of qualitative plant defensive compounds are those which bind to DNA, such as the furocoumarins. Furocoumarins are commonly found in the Umbelliferae and Rutaceae. Berenbaum (1981) has shown a relationship between insect community structure and the presence or absence of furocoumarins. She found that species composition on umbellifers was more homogeneous than on plants lacking furocoumarins. When activated by light in the near ultra-violet range, these compounds alkylate and intercalate macromolecules such as DNA and proteins. The linear conformation is normally more phototoxic than the angular conformation, because linear geometry allows for more complete alkylation of DNA.

Comparison of the susceptibility of the semi-specialist *Papilio polyxenes* Fabr. and the generalist *Spodoptera frugiperda* J.E. Smith to xanthotoxin led Ivie *et al.* (1983) and Bull *et al.* (1984, 1986) to conclude that increased oxidative cleavage of the furan ring is responsible for the greater tolerance of the former species towards furocoumarins. *Popillio polyxenes* metabolized xanthotoxin at a much higher rate, with 50% of an administered dose of radiolabeled compound appearing in the excreta within 24 hours, compared to 1% in the psoralen sensitive *S. frugiperda.* Analysis of metabolites indicated that the rate of oxidation was responsible for this difference. Further studies (Bull *et al.* 1986) suggested that the PSMO system was responsible for the oxidation. Ivie *et al.* (1986) compared toxicity of linear and angular furocoumarins to *P. polyxenes.* The differential toxicity of angular furocoumarins to this species is due at least in part to decreased oxidative metabolism compared to that found with the linear series.

The angular conformation appears to be a more recent evolutionary development, and Berenbaum (1982) and Berenbaum and Feeny (1981) argue that the development of angular furocoumarins allowed species of the Umbelliferae to expand their range, since the angular conformation has greater toxicity towards *P. polyxenes*, constituting observable coevolution.

The classic example of quantitative plant defensive compounds are tannins and phenolics. Tannins, and phenolics in general, have received much attention as digestibility reducers, because of their ability to complex with dietary proteins. Phenolics reduce growth rates and increase mortality in some insects (e.g. *Heliothis zea* Boddie: Elliger *et al.* 1981, Isman and Duffey 1982, Reese *et al.* 1982, and *P. polyxenes*: Berenbaum 1983). However, other investigators have questioned this mode of action. Bernays (1978) found that the toxic effects of hydrolysable tannin were not the result of reduced digestion in *Locusta migratoria* (L.). Hydrolysable tannin caused perforation of the gut wall and peritrophic membrane, resulting in damage to the midgut epithelia and gastric caeca. Berenbaum (1983), found that tannins did not affect digestion, growth rate, or nitrogen utilization in either *P. polyxenes*, a specialist that normally does not encounter tannins, or *P. glaucus* (L.), a generalist that does. This suggests that caution needs to be exercised when generalizing about insect/plant interactions mediated by tannins.

Other examples of plant defenses and insect counter-defenses can be found in a recent review by Brattsten (1986).

Plant Monoterpenes

Monoterpenes are widespread in the plant kingdom. Many have antifeedant or insecticidal properties, leading to speculation about a defensive role for these compounds (Brattsten 1983).

Commercial peppermint, *Mentha piperita*, contains over 30 monoterpenes. Menthol and menthone dominate the monoterpene profile. Pulegone, -pinene, and limonene are also present (DeAngelis *et al.* 1983, Formacek and Kubeczka 1982, Embong *et al.* 1985). Raffa *et al.* (1985) found that α-pinene and limonene vapors were the most toxic terpenes in the phloem of grand fir to *Scolytus ventralis* LeConte and they concluded these monoterpenes were significant in the resistance of this tree to bark beetles. Limonene is toxic to the cat flea, *Ctenocephalides felis* Bouche', and is registered with the Environmental Protection Agency for control of this species on dogs and cats (Hink and Fee 1986, Collart and Hink 1986). Limonene also was reported to have insecticidal properties against the bean weevil, *Callosobruchus phasecoli* Gyll. (Taylor and Vickery 1974). Oil of pennyroyal, *Mentha pulegium* L., inhibited feeding of *S. frugiperda* when included with an otherwise palatable food source (Zalkow *et al.* 1979). Gunderson *et al.* (1986) also reported that pulegone was an antifeedant for the fall armyworm, and they found that pulegone was four times more acutely toxic to the southern armyworm (*Spodoptera eridania* Cramer) than to the fall armyworm.

Specific experiments characterizing the toxicity of monoterpenes to the variegated cutworm are discussed in a later chapter.

Insect Detoxication Mechanisms

Insects avoid potential toxicity of diverse plant chemicals by a variety of mechanisms. These include metabolic transformation (Ahmad *et al.* 1986), sequestration (Duffey 1980), excretion, insensitive target site (Berenbaum 1986), morphology (Mullin 1986), and behavior (Tallamy 1986). Of these, only metabolic transformation will be discussed.

Metabolic detoxication

One of the primary mechanisms of insects for circumventing plant chemical defenses is metabolic transformation and ultimate excretion, traditionally visualized as

occurring in two stages. Phase 1 reactions involve the insertion of oxygen and subsequent reduction to make the molecule more hydrophilic and readily excretable. Phase 2 reactions conjugate the toxicant, usually to glutathione or a sugar, again making the complex more excretable. A complete review of metabolic enzymes and metabolic pathways used in biotransformation of xenobiotic compounds can be found in Ahmad *et al.* (1986) and Brattsten (1986). Cytochrome P-450-dependent polysubstrate monooxygenases (PSMO), are discussed in detail below.

Cytochrome P-450-dependent polysubstrate monooxygenases

General Description

The cytochrome P-450-dependent monooxygenases are enzymes instrumental in metabolizing a wide range of endogenous and exogenous substrates in mammals, insects and most other organisms (see reviews by Hodgeson 1985, Agosin 1985, Kulkarni and Hodgeson 1980). They are widely reputed to detoxify plant secondary substances and certain insecticides, and an abundant body of literature speculates on their role in plant-insect coevolution (Krieger 1971, Brattsten 1977, 1979; Gould 1984, Rose 1985).

Cytochrome P-450-dependent monooxygenases are a family of monophyletic isozymes with broadly overlapping substrate specificities (Hodgeson 1980). A wide variety of compounds are substrates for the PSMO system, including insecticides (reviewed by Kulkarni and Hodgeson 1980, Agosin 1985), plant compounds (reviewed by Brattsten 1979, 1983), and endogenous substrates such as methyl farnesoate (Feyereisen *et al.* 1981, Feyereisen 1985).

All cytochrome P-450-dependent monooxygenase reactions can be considered hydroxylations, classified as belonging to one of three categories (Nakatsugawa and Morelli 1976): π-bond oxygenation, aliphatic hydroxylation, and oxygenation at an unshared electron pair. π-bond oxygenations include epoxidation, aromatic hydroxylation, and phosphoester oxidation. Aliphatic hydroxylation involves the

insertion of a hydroxyl group into a saturated C-C bond, which for example, is an initial step in the metabolism of nicotine to cotinine. Sulfoxidation, N-, O-, and S-dealkylations involve oxygenation at an unshared electron pair, and are responsible for a wide range of plant compound and pesticide transformations (Hodgson 1985, Agosin 1985, Kulkarni and Hodgson 1980).

Microsomal oxidations all involve splitting of an oxygen molecule. One atom is added to the substrate, and the other reduced to yield a molecule of water (Fig. I-1).

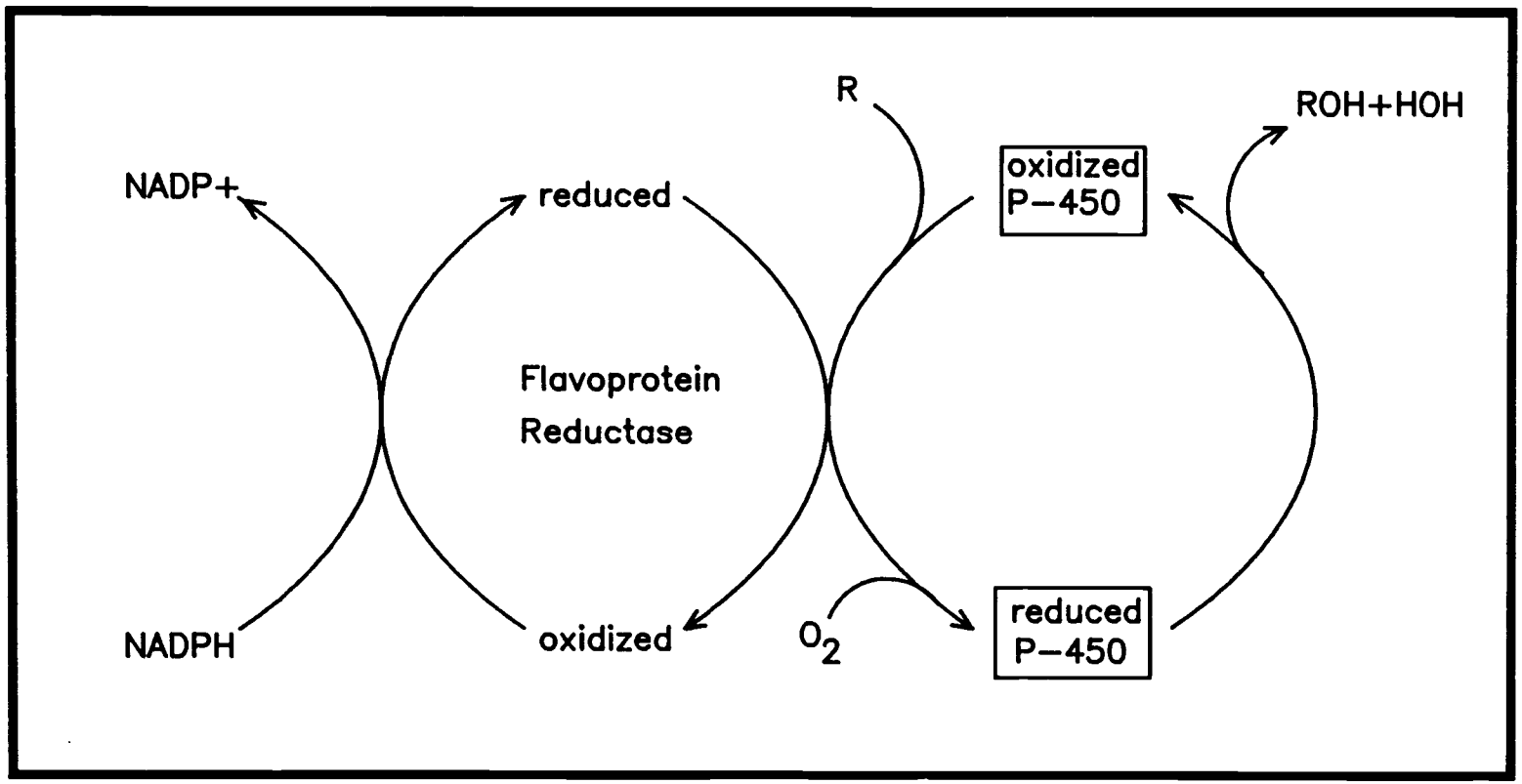

Fig. I-1. Catalytic cycle of microsomal cytochrome P-450

First, the substrate binds to the oxidized ferrous form of the heme. One electron is passed from NADPH through NADPH dependent cytochrome C reductase (flavoprotein reductase), to reduce the heme. By a mechanism not understood, a molecule of molecular oxygen binds to the reduced cytochrome P-450-substrate complex. Another electron from the NADPH-dependent cytochrome P-450 reductase activates the bound oxygen molecule, ultimately causing one atom of oxygen to be inserted into the substrate, and the other is reduced by the two reducing equivilants to form a molecule of water. Cytochrome P-450 is then again in an oxidized form and can bind with another molecule of substrate. The reduced cytochrome P-450-substrate complex can also bind CO: when this occurs, the conformation changes, allowing for detection of the characteristic optical difference spectrum peak at 450 nm.

Phenomenon of Induction

Induction is the sudden increase in enzyme levels and activity as a result of exposure to a xenobiotic compound. It appears to be a relatively non-specific process, in that a wide variety of compounds can cause induction, the only requirement being that they be lipophilic. It now appears that many metabolic enzymes are inducible, including esterases, epoxide hydrolases, GSH-transferases, as well as the PSMO's (see reviews by Terriere 1984 and Yu 1986). A given compound can induce specific isozymes of cytochrome P-450. For example, Collins (1985) working with *Heliothis punctiger* Wallengren, found that α-pinene and tobacco induced forms of cytochrome P-450 which had a low catalytic capacity for aldrin and a high proportion of high-spin P-450, whereas phenobarbitol and lucerne induced forms which had a high catalytic capacity for aldrin and a majority of the cytochrome in the low-spin state.

The fact that plant secondary compounds induce higher levels and activity of PSMO's has generated a tremendous amount of interest and research into the phenomenon of induction, its mechanism, and ecological implications. The notion that an organism could avoid expensive synthesis of a multimeric protein when it is not

needed is appealing. However, the direct proof of an evolutionary role for the PSMO system is difficult to obtain.

Induction occurs when an exogenous inducer enters the cell and binds to a receptor. In mice, the genetic locus of this receptor has been localized and has been designated as Ah (Nebert *et al.* 1984). The Ah receptor binds to the xenobiotic and translocates it to the nucleus. The Ah/xenobiotic complex presumably binds to chromatin components as yet unidentified. This leads to activation and transcription of specific genes which produce high molecular weight precursor nuclear RNA. Within several hours, elevated amounts of cytoplasmic RNA are found, and this RNA is translated on membrane-bound polysomes to produce membrane-bound cytochrome P-450 (Nebert *et al.* 1984).

Induction of PSMO's by dietary compounds has been documented in a variety of insects (Table 1).

Table 1. Plants and plant compounds that induce insect PSMO's.

Insect Species	Host Plant/ Allelochemical	Reference
Popilla japonica	sassafras,	Ahmad 1980
	broccoli,	
	phlox	
Spodoptera eridania	corn,	Yu 1982
	cotton,	
	cowpea,	
	others	
S. frugiperda	terpenoids,	Brattsten
	steroids	*et al.* 1977
	others	
Autographa californica	alfalfa,	Farnesworth
Trichoplusia ni	peppermint,	*et al.* 1981
	broccoli	
Peridroma saucia	peppermint,	Yu *et al.* 1979
	snap-bean,	Berry *et al.* 1980
	terpenes	Moldenke *et al.* 1983
Heliothis punctiger	α-pinene,	Collins
	lucerne,	1985
	tobacco	
H. virescens	wild tomato	Riskallah
		et al. 1986
Anticarsia gemmatalis	monoterpenes,	Christian
	cowpeas,	and Yu 1986
	others	

PSMO involvement in resistance to pesticides

PSMO's have been implicated in the resistance of insects to pesticides. Scott and Georghiou (1986) reported that PSMO mediated detoxication was the major resistance mechanism in the resistance of Learn-PyR houseflies to permethrin. However, DeVries and Georghiou (1981) found that in another permethrin selected strain of housefly (147-R), target site insensitivity was the major mechanism of resistance. McCord and Yu (1987) reported that fall armyworm (*S. frugiperda*) resistance to carbaryl was mediated by PSMO activity. Induction of cytochrome P-450 has been linked to tolerance to diazinon (Riskallah *et al.* 1986), methomyl (Berry *et al.* 1980, Yu 1979), carbaryl (McCord and Yu 1987), and many others. The molecular basis for PSMO-related resistance to pesticides is unknown. Mouches *et al.* (1986) has demonstrated that resistance of *Culex quinquefasciatus* Say to organophosphorous insecticides is due to amplification of an esterase gene. At least 250 times more copies of this gene were present in the resistant strain than in the susceptible strain . With the explosion in new techniques for genetic manipulation, the molecular biology of PSMO induction and PSMO-mediated resistance should be better understood in the near future.

Methods for Study

Cytochrome P-450-dependent monooxygenases have been studied with a variety of *in vivo* and *in vitro* methods.

In vivo methodology

In vivo methods are limited by the inability to monitor cytochrome P-450 activities independently of other cellular and bodily processes. *In vivo* assay is thus primarily limited to use of specific inhibitors of cytochrome P-450.

The discovery that methylenedioxyphenyl (MDP) compounds synergized the toxicity of carbaryl (reviewed by Casida 1970) opened the door for literally hundreds of papers dealing with inhibition of microsomal cytochrome P-450 monooxygenases by various compounds. Synergists have been used to survey PSMO activity in many insects

(Brattsten and Metcalf (1970, 1973). This is accomplished by estimating the median lethal dose (LD_{50}) with and without the application of synergist and expressing the relationship as the ratio of LD_{50}unsynergized to LD_{50}synergized. Numerous investigators have used inhibition of PSMO activity by the MDP compound piperonyl butoxide as an indication of PSMO related activity (Yu *et al.* 1979, 1987, Collart and Hink 1986, DeVries and Georghiou 1981). However, several caveats must be mentioned in relation to uncritical use of synergists as an *in vivo* tool.

First and foremost concerns the multiplicity of cytochrome P-450's. Many investigators have shown that MDP compounds interact with specific isozymes of cytochrome P-450 (Wilkinson *et al.* 1982, Chang *et al.* 1981, Marcus *et al.* 1986, 1987). Complicating this situation is the ability of MDP compounds to induce the PSMO system, and induce different populations of cytochrome P-450 (Yu and Terriere 1974, Thongsinthusak and Krieger 1974, Marcus *et al.* 1986, Cook and Hodgson 1985). Using the synergist ratio as a measure of *in vivo* PSMO activity assumes, *a priori*, that the synergist inhibits the same isozymes that interact with the toxicant.

Other problems with the use of synergists as *in vivo* tools concern the general relative toxicodynamics between synergist and substrate. Brattsten and Metcalf (1970, 1973) applied carbaryl and PBO in a 1:5 ratio to 74 species of insects from 40 families and 8 orders. The PBO was applied at the same time as the carbaryl. Synergist ratios were calculated and interpreted as "...a measure of the innate detoxifying capacity of the various species." No account was made for differential penetration and transport of carbaryl or PBO. Sun and Johnson (1972) found tremendous differences in synergist ratio depending on the formulation of the mixture of carbaryl and PBO. Particularly for more slowly penetrating insecticides, PBO may in fact alter the permeability of the cuticle and cause a lowering of the LD_{50} not dependent on actual inhibition of cytochrome P-450. Sun and Johnson referred to this problem as quasi-synergism and concluded that the toxicodynamics of topical application could not be ignored when used

in studies of synergism, toxicity, or resistance. In addition, there are no rational criteria for how large the synergist ratio should be before the lower synergized LD_{50} can be concluded to be due to inhibition of the PSMO system. In fact, there has been some argument whether the ratio is the best way to express the degree of inhibition. The difference between synergized and unsynergized LD_{50}'s has been suggested as a better index for bioassay data (Brindley 1977). He based this conclusion on the linear relationship between synergist differences and unsynergized carbaryl LD_{50} for the data in Brattsten and Metcalf (1973). There was no such relationship when synergist ratio was plotted against unsynergized carbaryl LD_{50}. In a later article, Brindley and Selim (1984) suggested other transformations of bioassay data that they claim are better expressions of such data. This debate is best summarized by the following statement contained in Brindley and Selim (1984):

> "Synergism transformations, whether ratios or differences, use estimates of lethality that have their own associated statistical errors in determination. Manipulation of LD_{50} or LC_{50} values may compound those error terms. Furthermore, synergism calculations are not the data; They are heuristic devices for uncovering hypotheses..."

In conclusion, *in vivo* methods should be used in conjunction with *in vitro* methods, and even then there is no assurance that the PSMO system is functioning in a predictable fashion.

In Vitro Methodology

The general protocol used in our laboratory (Moldenke *et al.* 1983) for *in vitro* experiments with the PSMO system is as follows. First, the tissue is homogenized in a suitable buffered medium. Usually this is a phosphate buffer in the pH range of 7.2-7.5 (we use pH=7.5), containing 10% glycerol, EDTA as a metal chelator, dithiothreitol to conserve sulfhydryl groups, and phenymethylsulfonylfluoride added as a proteinase inhibitor. Following motorized homogenization, the tissue is centrifuged at low speed to sediment cell fragments, nuclei, and mitochondria. Depending on the tissue, this is

accomplished by either one spin at 10,000 g for 15 minutes or, if the tissue is particularly coarse, this spin can be preceded by an initial spin at 1000 g for one or two minutes. The pellet is discarded and the supernatant is then centrifuged 100,000-105,000 g for 60-80 minutes to sediment the endoplasmic reticulum containing the cytochrome P-450. The supernatant is discarded and the sedimented ER is resuspended in the above buffer containing 20% glycerol.

A method to determine cytochrome P-450 content was developed by Omura and Sato (1964). Cytochrome P-450 got its name from the characteristic absorbance maximum of the optical difference spectra from the reduced cytochrome-carbon monoxide complex at 450 nm. The difference in absorbance at 450 nm is compared to that at 490 nm. A molar extinction coefficient of 91 $mM^{-1}cm^{-1}$ is then used to estimate the concentration of cytochrome P-450. Because of the presence of cytochrome b_5 and light scattering caused by the microsomal suspension, spectral observations of cytochrome P-450 must be performed by difference spectroscopy. Optical difference spectra are obtained in a dual beam spectrophotometer in which the difference in absorbance between two cuvettes is recorded instead of the absolute absorbance (Fig I-2). This cancels the effects of unwanted endogenous chromophores or hopelessly sloping baselines caused by light scattering

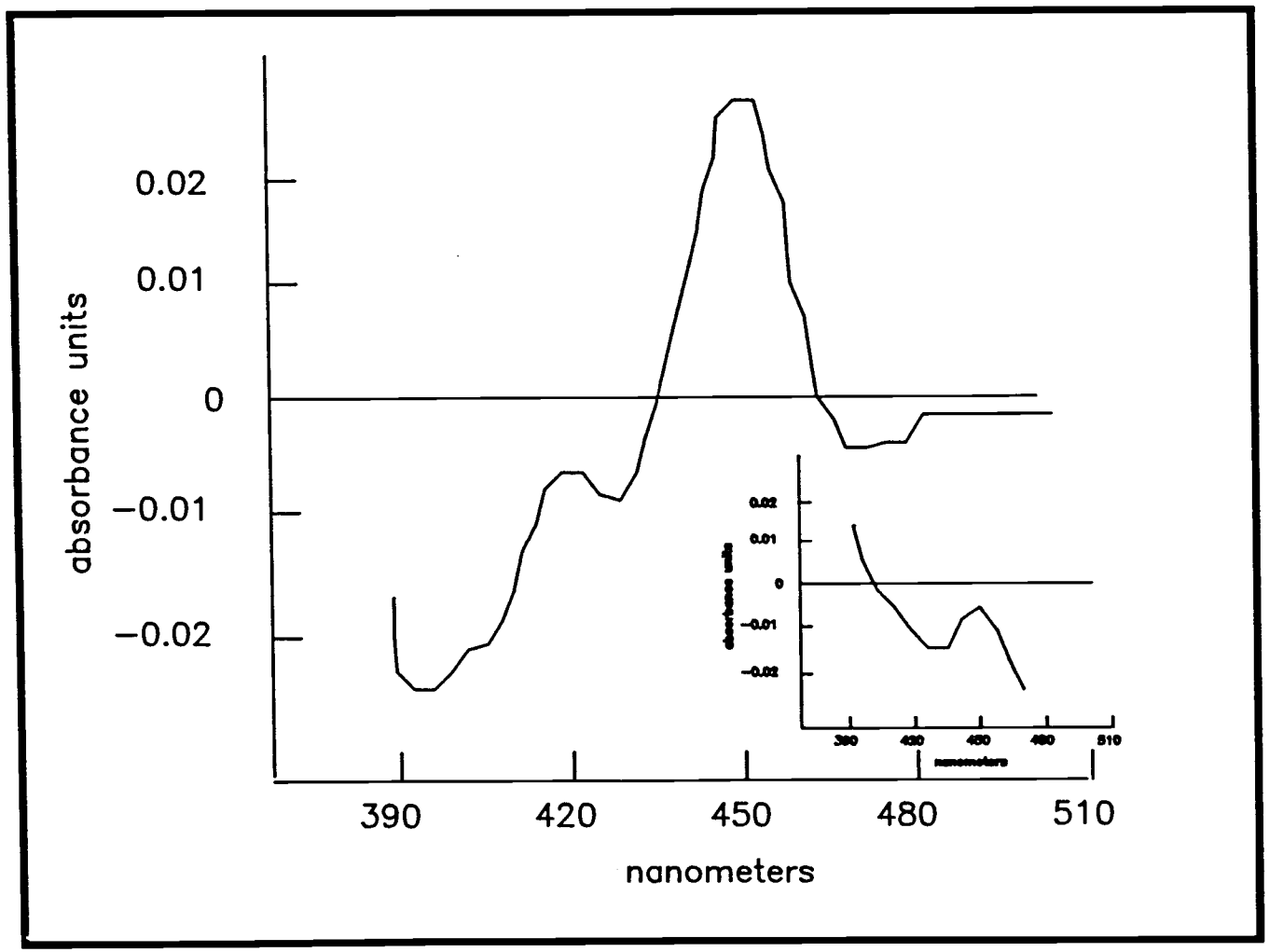

Fig. I-2. Sample P-450 action spectra by difference spectroscopy. Inset shows spectra obtained without using difference spectroscopy

Incubations of microsomal suspensions with substrates are performed with a regenerating system consisting of NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase. This provides a continuous supply of reducing equivalents to the cytochrome. The metabolites are then isolated by appropriate extraction procedures, usually involving a liquid phase extraction with an organic solvent such as hexane. Since any lipophilic molecule will exist in a partition between the lipids in the microsomes and the organic layer, extraction efficiencies should be, but rarely are reported for removal of a given substrate and metabolites.

Another method for following the *in vitro* oxidative metabolism of a putative substrate is the NADPH disappearance method (Yu 1987). NADPH and microsomes are incubated together, and aliquots placed into each of two cuvettes. A baseline is established on a split-beam spectrophotometer, and then substrate is added to the sample cuvette. Decrease in NADPH is monitored by the decline in absorbance at 340 nm. With appropriate controls, such as using boiled microsomes, CO-treated microsomes, or solvent with no substrate, microsomal activity can be assumed with relative certainty.

Evolutionary significance of cytochrome P-450

There has been much debate concerning the ecological significance of the PSMO system since Krieger *et al.* (1971) found that host range was correlated with PSMO activity. Since then, PSMO levels have been surveyed across species, host range, and developmental stage. Brattsten (1983) stated "...it would be charitable to say that the cytochrome P-450 system in insect herbivores does not entirely dictate their interactions with their host plants". Among the evidence suggesting an evolutionary role for the PSMO system are a correlation between host feeding range and PSMO levels, correlation between feeding activity and PSMO levels, and induction by exposure to dietary allelochemicals.

Although these facts suggest an evolutionary role for the PSMO system, the evidence is still largely circumstantial (Gould 1984). For example, while many

substances have been identified as inducers, most have not been shown to be metabolized by microsomal oxidase system. As mentioned before, induction is more correlated with lipophilic character than potential toxicity. Many compounds are in fact activated by the PSMO system (e.g. parathion to paraoxon), a fact which must be kept in mind when talking about detoxication mechanisms. Another weak link in the evidence is the common use of an indicator reaction, such as aldrin epoxidation, to assay for PSMO activity . However, because of the multiplicity of cytochrome P-450's, indicator reactions cannot necessarily be generalized to PSMO activity for a given substrate. Assays ideally should be conducted with the isolated plant compound or host plant extract.

In addition, the evidence that cytochrome P-450 activities are higher in actively feeding insects has been questioned. Gould and Hodgson (1980) reported higher microsomal oxidase activities in sixth stadium *H. virescens* larvae that had ceased to feed, in contrast to the results reported by Krieger *et al.* (1971). Rose (1985) has questioned whether cytochrome P-450 monooxygenase activity is indeed correlated to host feeding range. No correlation could be found between host feeding range and PSMO levels in 62 species of insects collected around Sydney, Australia. He also found no major differences in enzyme activity levels within instars, except a trend towards lower levels in larval lepidoptera preparing to pupate.

Induction, as mentioned earlier, suggests that an organism could avoid the synthesis of microsomal oxidases until they were needed. According to coevolutionary theory, an increase in general fitness should accrue to those organisms able to avoid energy expenditures associated with detoxication. Scriber (1981) concluded, from a study of food utilization parameters, that reduced growth of *S. eridania* feeding on cabbage, as compared to dill or beans, apparently resulted from reduced efficiency of biomass conversion. Scriber considered this effect to result, at least in part, from induction of the PSMO system; however, he did not measure cytochrome P-450 levels or

activities. Neal (1987) recently studied the ergonomic consequences of induction. Using indole-3-carbinol to induce the PSMO system of *H. zea*, he measured food utilization parameters with and without induction. He was unable to demonstrate the expected negative correlation between induction of cytochrome P-450 levels and food utilization parameters.

In conclusion, proof for an evolutionary role for PSMO's is still elusive, in spite of a large body of literature. Although it seems obvious that the PSMO system is involved in the metabolism of a broad spectrum of plant secondary compounds and xenobiotics, more research is needed. Studies comparing the detoxication abilities between closely related species differing in their respective host ranges and feeding behavior, such as those of Bull *et al.* (1984, 1986) are a promising avenue, as are the use of fitness parameters (but correlated with enzyme levels) suggested by Scriber (1981).

TOXICITY OF PEPPERMINT MONOTERPENES TO THE VARIEGATED CUTWORM

Abstract

The toxicity of five peppermint (*Mentha piperita* L.) monoterpenes (menthol, menthone, pulegone, limonene, and α-pinene) to the variegated cutworm (*Peridroma saucia* Hübner) was evaluated. Larvae feeding for six days on a semidefined artificial died (AD) fortified with pulegone (0.05%, 0.1%), menthol (0.1%, 0.2%), and menthone (0.2%) at the onset of the fifth stadium, weighed less than larvae receiving AD without monoterpene. Pupation was inhibited by menthol (0.05%, 0.1%, 0.2%), limonene (0.2%), menthone (0.1%, 0.2%), and pulegone (0.1%). Reduced growth of larvae fed AD containing menthone and pulegone was attributed to feeding inhibition, whereas molting abnormalities caused growth reduction in larvae fed AD containing menthol. Menthol, at a concentration approximately twice that found in peppermint leaves (0.2% wet weight), caused retention of the 5th-instar head capsule during ecdysis to the sixth instar and prevented larvae from feeding. Menthol also had profound effects on pupation; complete inhibition occurred at dosages approximating those found *in planta* (0.05%-0.2% wet weight). Pupal weight was not significantly affected by any of the treatments. Growth, feeding, or pupation were not affected in larvae fed AD containing 0.05% or 0.1% limonene. These parameters were also unaffected by 0.05%, 0.1%, or 0.2% α-pinene.

Neonate larvae fed AD fortified with menthol, menthone, limonene, and α-pinene (0.05%), or pulegone (0.025%) did not differ from control larvae feeding on unfortified diet with respect to growth, maximum weight, or pupal weight. However, menthone and pulegone treatments caused significantly higher larval mortality than the controls.

Topical toxicity of menthone, pulegone, limonene, and -pinene was evaluated. The median lethal dosage for pulegone was 1102 μg/g, menthone was 2533 μg/g, and that of α-pinene was 7975 μg/g. Limonene was not sufficiently toxic to generate a probit line. It was not possible to obtain a probit line in the case of menthol, apparently because of limited cuticular penetration. The topical toxicity of menthone and α-pinene to sixth stadium larvae were synergized by prior application of piperonyl butoxide. Synergism of limonene or pulegone was not observed.

All five monoterpenes were metabolized *in vitro* by midgut microsomal suspensions prepared from sixth instar larvae, as monitored by disappearance of NADPH.

Introduction

Monoterpenes are widespread in the plant kingdom. Many have antifeedant or insecticidal properties, leading to speculation about a defensive role for these compounds (Brattsten 1983).

Commercial peppermint, *Mentha piperita* (L.), contains over 30 monoterpenes. Menthol and menthone dominate the monoterpene profile. Pulegone, α-pinene, and limonene also are present (DeAngelis *et al.* 1983, Formacek and Kubeczka 1982). Raffa *et al.* (1985) found that α-pinene and limonene vapors were the most toxic terpenes in the phloem of grand fir to *Scolytus ventralis* LeConte. They concluded that these monoterpenes were significant in the resistance of grand fir to bark beetles. Limonene is toxic to the cat flea, *Ctenocephalides felis* Bouche', and is registered with the Environmental Protection Agency for control of this species on dogs and cats (Hink and Fee 1986, Collart and Hink 1986). Limonene also was reported to have insecticidal properties against the bean weevil, *Callosobruchus phasecoli* Gyll. (Taylor and Vickery 1974). Oil of pennyroyal, *Mentha pulegium* L., inhibited feeding of *Spodoptera frugiperda* J.E. Smith when included with an otherwise palatable food source (Zalkow *et al.* 1979). Gunderson *et al.* (1985) reported that pulegone was four times more acutely toxic to *Spodoptera. eridania* Cramer than to *S. frugiperda.*

The variegated cutworm, *Peridroma saucia* Hübner, is a broadly polyphagous noctuid that is considered one of the most serious pest of commercial peppermint in the Pacific Northwest (Berry and Shields 1980). In addition to peppermint, the variegated cutworm has been reported as a pest on at least 37 other vegetable crops and 85 other host plants (Rings *et al.* 1976). Plant families known for high allelochemical content, such as the Labiatae, Umbelliferae, and Cruciferae, are well represented among the host plants on which this species feeds.

Previous work in this laboratory demonstrated that feeding on peppermint or on isolated monoterpenes in an artificial diet induced the microsomal polysubstrate

cytochrome P-450 dependent monooxygenase system (PSMO) of *P. saucia* (Yu *et al.* 1979, Berry *et al.* 1980, Moldenke *et al.* 1983), and increased tolerance to acephate and methomyl, two insecticides generally metabolized by the PSMO system (Yu *et al.* 1979, Berry *et al.* 1980). Induction of the PSMO system by peppermint and monoterpenes found in peppermint also has been demonstrated with other insects such as *Autographa californica* Speyer and *Trichoplusia ni* Hübner (Farnsworth *et al.* 1981), *S. frugiperda*, and *S. eridania* (Brattsten 1977, 1983), and *Anticarsia gemmatalis* Hübner (Christian and Yu 1986).

While there have been many demonstrations of induction of the PSMO system by plant secondary compounds, (Ahmad 1983, Yu 1982, Yu *et al.* 1979, Christian and Yu 1986, Brattsten *et al.* 1977, Farnsworth *et al.* 1981, Berry *et al.* 1980, Moldenke *et al.* 1983, Collins 1985, Riskallah *et al.* 1986), there have been far fewer demonstrations of actual metabolism by these enzymes (White *et al.* 1979, Trammel 1982, Gunderson *et al.* 1986, Yu 1987). This has prompted some authors to caution against assuming an evolutionary role for PSMO enzymes in herbivore defense against plant secondary compounds (Gould 1984).

(+)-Pulegone has been shown to have a high affinity for acetycholinesterase from a variety of sources. In addition, its 1,2-epoxide was shown to inhibit acetylcholinesterase (AchE) from a variety of sources (Grundy and Still 1985).

The purpose of this study was to characterize the toxicity of specified monoterpenes to the variegated cutworm and to establish whether specified monoterpenes found in peppermint leaves are metabolized by the PSMO system.

Materials and Methods

Insect Culture

Eggs of *P. saucia* were collected each June in a sugar-beet field near Corvallis, Oregon. Eggs were incubated at 24^{o}C in petri plates until hatching. Neonate larvae, unless otherwise indicated, were allowed to feed *ad libitum* on a semi-defined artificial diet (AD) (Bioserv #9000, Frenchtown NJ.) until used in experiments. The diet was prepared as follows. The dry ingredients were added to boiling agar and blended in a waring blender for 5 minutes. The diet was then placed in a 45^{o}C water bath overnight. The following morning, the monoterpenes were blended into 250 g aliquots of diet and 10-12 g of fortified diet were dispensed into each cup.

Chemicals

Monoterpenes used in experiments were at least 98% pure. Pulegone, limonene, menthol, menthone, and piperonyl butoxide (Techn.) were obtained from Fluka. Alpha pinene was obtained from Aldrich. Paraoxon, acetylthiocholine, and dithiobisnitrobenzoate (DTNB) was a gift from H.E. Van De Baan. NADP, NADPH, Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, phenylthiourea, phenylmethylsulfonylfluoride, and dithiothreitol were obtained from Sigma. Carbaryl was a gift from Union Carbide.

Oral toxicity experiments.

Feeding experiment 1. Larvae selected during the last hours of the fourth stadium (diagnosed by slippage of the head capsule) were placed into individual 2.1 ml plastic creamer cups with tight fitting plastic caps without food. The following morning, as fifth-instars, they were placed randomly into cups (20 larvae per treatment), containing 10-12 g of AD fortified with the following concentrations of monoterpene: pulegone: 0.025%, 0.05%, 0.1%; limonene, menthone, menthol, and α-pinene: 0.05%, 0.1%, 0.2%. Caps were opened each day. Larvae were weighed daily beginning four days following the fifth molt until they formed prepupae. Larvae pupated in the cups.

Once prepupae were formed, cups were disturbed as little as possible. The following parameters were measured: weight 6 days post fifth molt, maximum weight attained (larvae were said to have reached maximum weight if they subsequently developed into prepupae, as distinguished from larvae which simply stopped growing; in practice there was little ambiguity), percent mortality (those dying without reaching maximum weight), feeding inhibition (those not feeding during the first 24 hours), percent pupation (no. pupae/no. larvae reaching maximum weight x 100), and pupal weight. Pupae were weighed within 36 hours of pupation. To eliminate effects of variation caused by different sub-populations in the laboratory culture, a randomized block design was used. The experiment was replicated at three different times to sample different subpopulations. Thus, there were 20 larvae per treatment, 16 treatments, and three blocks. A Fisher-protected least significant difference (FPLSD) multiple comparison was used to test differences between terpene treatments and control and within terpene comparisons. Differences were considered significant when $P<0.01\%$.

Feeding experiment 2. To relate these data to the effects of monoterpenes on younger larvae, the experiment was repeated with neonate larvae, (10 per treatment) using the lowest dose tested for each compound listed above. The following parameters were measured: weight of 15 day-old larvae, maximum weight attained (defined as above), % mortality, % pupation, and pupal weight. Cups were opened daily. This experiment also was analyzed as a randomized block design. Each block comprised larvae from a different egg mass, and neonate larvae from a given egg mass were randomly assigned to treatments within blocks. The Fisher protected LSD was used to test differences between control and terpene treatments. The significance level was $P<0.01\%$.

Topical toxicity experiments

Larvae were reared as described above. Newly molted fifth-instars were placed in 2.1 ml creamer cups with perforated plastic lids (five per cup) and provided with six

2-g cubes of unfortified AD, and allowed to feed *ad libitum* until needed. To minimize differences in fat-body content and surface-to-volume ratio between larvae of widely varying size, only larvae weighing between 600 and 800 mg were used. In addition, to assure that no prepupae were used, larvae were used within four days of the sixth molt. Median lethal dosages were calculated on the basis of at least four dosages per regression line and 25 larvae per dose. Each dosage was repeated three times. Synergist ratios were calculated as the ratio of synergized LD_{50} to unsynergized LD_{50}. For the synergized treatment, 2.5 µl of a 12.5% solution of piperonyl butoxide (PB) in acetone, was applied to the thorax two hours before terpene administration. Monoterpenes were applied to the thorax in a volume of approximately 4.5 µl, delivered with a micrometer-driven, 500 ul Hamilton gas-tight syringe. The exact volume delivered was based on the weight of each larva. Each treated larva was put into a 20x150mm test-tube with a wad of absorbent Kim-wipe^{R} in the bottom, and were examined 20-24 hours later. Larvae were considered dead if unable to perform a characteristic curling movement in response to tapping with a pencil eraser (modified from Bloomquist and Miller 1985).

Inhibition of *P. saucia* acetylcholinesterase by pulegone

Acetylcholinesterase activity was measured by a method modified from Ellman (1961). Forty variegated cutworm heads were homogenized in 20 ml of 64 mM potassium phosphate buffer (Van de Baan *et al.* 1985) with five mM phenylthiourea to inhibit melanization (Shapiro *et al.* 1985). The homogenate was centrifuged at 1000g for one minute, then 10,000g for 15 minutes. The pellet was discarded and the supernatant was used for subsequent assays. Protein content was determined by the method of Bradford (1976) using gamma-globulin as standard. Tissue homogenate (1.8 ml, 3.8 mg protein) was preincubated with 200 µl 0.1M pulegone in methylcellosolve, 200 µl 10^{-6}M paraoxon in methylcellosolve, or 200µl methylcellosolve (control) for 20, 40, or 80 minutes. Each incubate of 2ml was performed in triplicate. The incubate (100ul) as added to 850 µl of the homogenization buffer, 25 µl 0.02M dithiobisnitrobenzoate, and 25

l 0.01M acetythiocholine in the sample cuvette of a Aminco Chance DW-2 split beam spectrophotometer. The reference cuvette contained the same amount of acetylthiocholine and DTNB in 950 µl buffer, with no tissue incubate. Increase in absorbance was monitored at 412 nm.

Preparation of microsomes

Larvae selected during the last few hours of the fifth-instar were placed into 2.1 ml. with fresh diet, either unfortified or fortified with 0.1% menthol, and allowed to feed *ad libitum* for 60 hours. Larvae were then washed in water, blotted dry, and their midguts removed. Midguts were separated from the peritrophic membrane and gut contents, washed in 1.15% KCl, and placed in the grinding buffer (of 0.1% potassium phosphate pH 7.5, 10% glycerol, 1mM EDTA, 0.1mM DTT, and 400 µM PMSF). The washed midguts were homogenized in 15 ml of fresh grinding buffer by 12 passes over a motor-driven teflon pestle. The homogenate was strained through cheesecloth into 30 ml Sorval^{R} centrifuge tubes and centrifuged at 10,000g for 15 min to remove nuclei, mitochondria, and cell debris. The supernatant was poured through glass-wool and spun in a Beckman^{R} L8-80 ultracentrifuge at 100,000g for 77 min ($3.56x10^{10}$ w^2t). The supernatant was discarded and pellets were resuspended in resuspension buffer, identical to the grinding buffer except that it contained 20% glycerol. The suspension was dispensed into 1 ml aliquots and stored at -70^{o}C until use.

Protein and Cytochrome P-450 Measurements

The microsomal suspension was thawed and protein was measured by the method of Bradford (1976), using bovine serum albumin as standard. The suspension was diluted to 2 mg protein per ml with incubation buffer (identical to grinding buffer except that it lacked PMSF and had 30% glycerol). Cytochrome P-450 was measured by the method of Omura and Sato (1964). Briefly, 1 ml of microsomal suspension was placed in each of two cuvettes. Absorbance was read on an Aminco-Chance DW-2a dual beam spectrophotometer. Following establishment of a baseline, carbon monoxide

was bubbled through the sample cuvette for 1 min. Sodium dithionite was added to both cuvettes and the absorbance scanned from 375 nm to 500nm. Cytochrome P-450 was estimated on the basis of triplicate scans, using a molar extinction coefficient of 91 mM^{-1} cm^{-1}.

Substrate-Dependent Oxidation of NADPH

Substrate dependent disappearance of NADPH was performed by a method modified from Yu (1987). The incubation mixture contained 0.5 mg protein, 200 µl 1mM NADPH, and incubation buffer in 2.5 ml. One ml aliquots were dispensed into each cuvette and the absorbance at 340 nm was monitored in the absence of substrate for 3 min at 30°C. There was no change in absorbance in the absence of substrate over this period of time. Five 1 of a 50 mM solution of substrate in methyl cellosolve was added to the sample cuvette, and the decrease in absorbance was monitored for six min. The following reaction controls were performed: Five µl methyl cellosolve without monoterpene, incubation with 0.5mg of boiled microsomal protein, microsomal suspension bubbled with carbon monoxide for 1 min, and addition of PB during terpene incubation. The change in absorbance over the two minute period beginning three minutes after addition of substrate was used to determine the amount of NADPH oxidized, assuming a molar extinction coefficient of 6200 M^{-1} cm^{-1} (Dawson *et al.* 1969).

Results

Oral Toxicity

Fortification of the semidefined artificial diet with menthol, menthone, and pulegone significantly altered growth, feeding, and pupation in *P. saucia* larvae.

In feeding experiment 1, in which larvae began feeding on fortified diet at the onset of the fifth stadium, significant growth reduction occurred with menthol (0.1% and 0.2%), menthone (0.2%), and pulegone (0.05% and 0.1%) (Table 2 and Fig. A-2). There

was a significant dosage response over the 0.1% and 0.2% dosages for menthol, the 0.2% dose of menthone, and the 0.05% and 0.1% doses of pulegone. Sixty percent of larvae that fed on menthol fortified diet at 0.2% had difficulty shedding the 5th stadium exuviae during the 5th-6th molt. The 5th stadium head capsule remained attached, and larvae were unable to feed and eventually starved to death. This is reflected in the significantly lower weights of larvae in Table 2, and the resulting heavily skewed distributions shown in Figs. A-2 and A-3 for the 0.1% and 0.2% menthol treatments. Feeding on diet fortified with α-pinene and limonene did not result in reduced growth at any dosage tested (Tables 2 and 3; Figs.A-2 and A-3).

Only pulegone (0.1%), menthone (0.2%), and menthol (0.1%) significantly reduced maximum weight. Because of the molt abnormalities associated with the 0.2% dosage of menthol, this treatment was not included in the analysis.

The 0.2% and 0.1% dosages of menthone and pulegone, respectively, resulted in significant feeding inhibition, and a majority of these larvae subsequently died without feeding (Table 4). Feeding on monoterpene-containing AD did not affect pupal weight for any of the dosages tested (Table 5); however, pupation success was affected. Limonene (0.2%), menthol (0.05%, 0.1%, 0.2%), menthone (0.2%), and pulegone (0.1%) resulted in significantly reduced pupation percentage relative to the controls (Table 6).

Significant differences in mortality were observed when neonate larvae fed on the lowest dosages of monoterpene used in feeding experiment 1 (Table 7). Menthone and pulegone caused 43% and 93% mortality, respectively, compared to 13% observed for the controls. Those larvae which died did not molt to the second stadium, although some remained alive for up to 2 weeks. Larval weights after 15 days did not differ from the controls (Table 8). Similarly, terpene treatments did not differ from controls with regard to maximum weight (Table 9), pupal weight (table 10), or pupation percentage (Table 11).

Table 2. Weight of *P. saucia* larvae after feeding on terpene-fortified artificial diet for six days after 5th molt.

Treatment	N	Mean Weight $(mg)^{1/2}$	Standard Error
Control	60	819	18.9
Limonene 0.05%	59	838[a]	19.1
Limonene 0.1%	59	834[a]	19.1
Limonene 0.2%	59	837[a]	19.1
Menthol 0.05%	59	792[a]	19.1
Menthol 0.1%	60	655[*b]	18.9
Menthol 0.2%	57	326[*c]	19.4
Menthone 0.05%	58	874[a]	19.2
Menthone 0.1%	59	838[a]	19.1
Menthone 0.2%	46	537[*b]	21.6
Pulegone 0.025%	60	841[a]	18.9
Pulegone 0.05%	60	710[*b]	18.9
Pulegone 0.1%	22	316[*c]	31.2
α-Pinene 0.05%	59	943[*a]	19.1
α-Pinene 0.1%	60	886[a]	18.9
α-Pinene 0.2%	58	887[a]	19.2

1. Values followed by asterisk significantly different from control, FPLSD ($P<0.01$)
2. Values followed by same letter not significantly different for within terpene comparison FPLSD ($P<0.01$)

Table 3. Maximum weight attained by *P. saucia* larvae feeding on terpene fortified artificial diet

Treatment	N	Mean Weight (mg.) 1/2	Standard Error
Control	60	1143	22.0
Limonene 0.05%	59	1069[a]	17.2
Limonene 0.1%	59	1124[a]	16.3
Limonene 0.2%	59	1102[a]	18.4
Menthol 0.05%	59	1090[a]	14.4
Menthol 0.1%	60	1013[*a]	31.0
Menthone 0.05%	58	1147[a]	19.5
Menthone 0.1%	59	1144[a]	19.1
Menthone 0.2%	46	974[*b]	27.3
Pulegone 0.025%	60	1162[a]	20.1
Pulegone 0.05%	60	1112[a]	15.6
Pulegone 0.1%	27	1020[*b]	27.2
α-Pinene 0.05%	59	1194[a]	18.9
α-Pinene 0.1%	60	1149[a]	18.5
α-Pinene 0.2%	58	1150[a]	15.5

1. Values followed by asterisk significantly different from control FPLSD (P<0.01)
2. Values followed by same letter not significantly different for within terpene comparison FPLSD (P<0.01)

Table 4. Percent mortality of *P. saucia* larvae feeding on terpene fortified diet since the 5th stadium.

Treatment	N	Larvae	Percent dead w/o feeding 1,2	Percent not feeding in 24 hrs 1,2
Control	3	60	0	0
Limonene 0.05%	3	60	2^{a}	2^{a}
Limonene 0.1%	3	60	0^{a}	0^{a}
Limonene 0.2%	3	59	0^{a}	0^{a}
Menthol 0.05%	3	60	2^{a}	2^{a}
Menthol 0.1%	3	60	0^{a}	0^{a}
Menthol 0.2%	3	60	3^{a}	3^{a}
Menthone 0.05%	3	60	3^{a}	3^{a}
Menthone 0.1%	3	60	3^{a}	3^{a}
Menthone 0.2%	3	60	37^{*b}	43^{*b}
Pulegone 0.025%	3	60	0^{a}	0^{a}
Pulegone 0.05%	3	60	0^{a}	0^{a}
Pulegone 0.1%	3	60	68^{*b}	93^{*b}
α-Pinene 0.05%	3	59	0^{a}	0^{a}
α-Pinene 0.1%	3	60	0^{a}	0^{a}
α-Pinene 0.2%	3	60	0^{a}	0^{a}

1.Values followed by asterisk significantly different from control. FPLSD ($P<0.01$)

2. Values followed by same letter not significantly different for within terpene comparison FPLSD ($P<0.01$)

Table 5. Pupal weights for *P. saucia* larvae feeding on terpene-fortified diet since 5th stadium.

Treatment	N	Mean weight (mg)[1]	Standard error
Control	37	458_a	9.3
Limonene 0.05%	38	461_a	6.8
Limonene 0.1%	27	478_a	10.9
Limonene 0.2%	12	490_a	13.5
Menthol 0.05%	5	518_a	14.8
Menthone 0.05%	31	483_a	9.3
Menthone 0.1%	20	482_a	15.7
Menthone 0.2%	7	435_a	16.1
Pulegone 0.025%	39	483_a	6.9
Pulegone 0.05%	25	455_a	12.6
α-Pinene 0.05%	42	484_a	7.3
α-Pinene 0.1%	31	467_a	7.2
α-Pinene 0.2%	40	484_a	6.3

1.Values followed by same letter not significant from control or within terpene FPLSD ($P<0.01$)

Table 6. Percent pupation of *P. saucia* larvae feeding on terpene-fortified artificial diet

Treatment	N	Larvae	%pupation[1/2]
Control	3	60	62a
Limonene 0.05%	3	59	66[a]
Limonene 0.1%	3	58	47[a]
Limonene 0.2%	3	59	20[*a]
Menthol 0.05%	3	59	8[*a]
Menthol 0.1%	3	60	0[*a]
Menthol 0.2%	3	40	0[*a]
Menthone 0.05%	3	56	55[a]
Menthone 0.1%	3	56	36[*b]
Menthone 0.2%	3	38	18[*b]
Pulegone 0.025%	3	60	65[a]
Pulegone 0.05%	3	58	43[a]
Pulegone 0.1%	3	33	0[*b]
α-Pinene 0.05%	3	59	71[a]
α-Pinene 0.1%	3	60	52[a]
α-Pinene 0.2%	3	58	69[a]

1. Values followed by asterisk significantly different from control FPLSD ($P<0.01$)
2. Values followed by same letter not significantly different for within terpene comparison FPLSD ($P<0.01$)

Table 7. Percent mortality for neonate *P. saucia* larvae feeding on terpene-fortified artificial diet.

Treatment	N	Larvae	Percent Mortality[1]
Control	3	30	13.3[a]
Menthol	3	30	36.7[a]
Menthone	3	30	43.3[b]
Limonene	3	30	10.0[a]
α-Pinene	3	30	33.3[a]
Pulegone	3	30	93.3[b]

1. Values followed by same letter not significant from control or within terpene FPLSD ($P<0.01$)

Table 8. Weights for *P. saucia* larvae feeding on terpene-fortified artificial diet for 15 days following egg hatch.

Treatment	N	Mean weight (mg)[1]	Standard error
Control	26	273_a	16.3
Menthol	19	248_a	22.5
Menthone	17	309_a	26.7
Limonene	27	326_a	24.0
α-Pinene	19	292_a	22.2
Pulegone	2	217_a	51.0

1. Values followed by same letter not significant from control or within terpene Fisher FPLSD ($P<0.01$)

Table 9. Maximum weight attained by *P. saucia* larvae feeding on terpene-fortified artificial diet since egg hatch.

Treatment	N	Mean weight (mg)[1]	Standard error
Control	26	1118_a	22.7
Menthol	19	1086_a	31.8
Menthone	17	1111_a	33.4
Limonene	27	1087_a	22.4
α-Pinene	19	1114_a	27.5
Pulegone	2	941_a	61.4

1. Values followed by same letter not significant from control or within terpene FPLSD ($P<0.01$)

Table 10. Pupal weights of *P. saucia* larvae feeding on terpene-fortified artificial diet artificial diet since egg hatch.

Treatment	N	Mean weight (mg)[1]	Standard error
Control	14	462_a	11.1
Menthol	5	453_a	13.4
Menthone	10	451_a	21.7
Limonene	18	451_a	8.4
α-Pinene	7	435_a	13.1
Pulegone	2	425_a	3.0

1. Values followed by same letter not significant from control or within terpene FPLSD ($P<0.01$)

Table 11. Percent pupation of *P. saucia* larvae feeding on terpene-fortified artificial diet since egg hatch

Treatment	N	Larvae	Percent pupation[1]
Control	3	26	50.0_a
Menthol	3	19	26.3_a
Menthone	3	17	58.8_a
Limonene	3	27	66.7_a
α-Pinene	3	20	35.0_a
Pulegone	3	2	100.0_a

1. Values followed by same letter not significant from control or within terpene FPLSD ($P<0.01$)

Dermal Toxicity

Topical application of menthol, menthone, pulegone, limonene, or α-pinene resulted in the LD_{50} shown in Table 12. Pulegone was the most toxic, followed by menthone, and α-pinene. Limonene was not sufficiently toxic, even at doses which completely saturated the cuticle, to generate a synergist ratio . Menthol apparently did not readily penetrate the cuticle, as large crystals of menthol were visible on the cuticle 24 hours following application.

Pulegone and menthone exhibited symptoms suggestive of neurotoxicity. Following application, larvae began writhing and spitting within 20 seconds, an activity which continued for approximately one to two minutes. Larvae became quiescent and flaccid. Larvae that ultimately survived became active two to three hours following application, suggesting detoxication. Grundy and Still (1985) reported that the major insecticidal compound isolated from *Lippia steochadifolia* is pulegone-1,2-epoxide, and that this compound inhibits acetylcholinesterase (AchE) from a variety of sources. (+)-Pulegone had the highest affinity for the enzyme of five other structurally related monoterpenes. I therefore tested the inhibitory properties of pulegone towards AchE isolated from *P. saucia.*

Pulegone did not inhibit variegated cutworm AchE (Table 13). As a check treatment, I preincubated the homogenates with paraoxon, which, as expected, did inhibit AchE. Pulegone may be neurotoxic but probably has a different mode of action. Further experiments are needed to determine the mechanism of toxicity for menthone, pulegone and menthol.

Table 12. Toxicity and synergist ratios for monoterpenes applied topically to sixth-instar *P. saucia.*

TREATMENT	R^2	LD_{50} (µg/g)	SYNERGIST RATIO
Carbaryl	.983	238.8	3.7
Carbaryl+PBO	.722	65.0	
Pulegone	.978	1102	1.0
Pulegone+PBO	.999	1117	
Menthone	.982	2533	1.5
Menthone+PBO	.952	1734	
α-Pinene	.913	7975	1.4
α-Pinene+PBO	.961	5868	
Limonene	N/A	>10,000	N/A
Limonene+PBO	N/A	>10,000	
Menthol	N/A	N/A	N/A
Menthol+PBO	N/A	N/A	

Table 13. Inhibition of variegated cutworm acetylcholinesterase (AchE).

Treatment	Time of preincubation	Ache activity (m/min/ug prot. $x10^{-12}$)[1]	%of Control
Control	20	3.7	100
	40	3.5	100
	80	3.9	100
pulegone	20	3.9	110
	40	4.0	110
	80	3.7	95
paraoxon	20	1.0	27
	40	0.7	20
	80	1.0	26

1. Mean of three replications

Interaction of peppermint monoterpenes with the PSMO system of P. saucia.

Prior topical application of PB increased the toxicity of topically applied menthone and α-pinene by factors of 1.5 and 1.4 respectively (Table 12). The toxicity of limonene and pulegone was not synergized. PB synergized the toxicity of carbaryl by a factor of 3.7.

Prior feeding on artificial diet fortified with 0.1% menthol resulted in a two-fold increase in midgut microsomal P-450 from 0.17 nm/mg protein to 0.32 nm/mg protein. This is roughly equivalent to the P-450 content and degree of induction found previously for 0.1% menthol administered to variegated cutworm (Moldenke *et al.* 1983).

All five of the monoterpenes tested caused the oxidative disappearance of NADPH from incubations containing microsomal suspension (Table 14). The reaction was linear for up to 20 minutes. No disappearance of NADPH was detected when incubations were conducted with boiled microsomes or carbon monoxide treated microsomes. No reaction was obtained when the carrier (methyl cellosolve) was added alone. Following incubation of monoterpene, addition of PB caused the reaction rate to decline to zero within 2-3 minutes. From these data it can be assumed that the decline in absorbance at 340 nm was dependent on the presence of monoterpene and active microsomes. The rates obtained are approximately an order of magnitude higher than the rates of epoxidation recorded previously for this species (Yu *et al.* 1979, Berry *et al.* 1980, Moldenke *et al.* 1983).

Table 14. Monoterpene dependent oxidation of NADPH (nm NADPH/mg protein) by sixth instar variegated cutworm midgut microsomal suspensions.

Terpene	Control[1]	Induced[1]	Induction Ratio
Menthol	1.65±0.11	4.35±0.00	2.4
Pulegone	1.69±0.07	3.79±0.16	2.4
Menthone	2.22±0.11	5.08±0.14	2.1
Limonene	1.45±0.18	3.47±0.21	2.9
α-Pinene	1.41±0.18	2.70±0.04	1.9

1. Mean of 3 reps.

Table 15. Relationship of monoterpene concentrations naturally occurring in peppermint plants to those used in feeding experiments.

Terpene	Percentage in		Dosage in diet relative to that found in leaves		
	of oil	of fresh	(low)	(med.)	(high)
Menthol	38.0	0.095	0.5	1.1	2.1
Menthone	17.3	0.043	1.2	2.3	4.6
Pulegone	1.6	0.004	6.1	12.3	24.5
Limonene	2.6	0.007	7.7	15.3	30.7
α-Pinene	1.6	0.004	12.4	24.4	48.8

Discussion

Growth rates were lower when fifth-instar larvae were given AD fortified with menthol, menthone, and pulegone compared to the control larvae. The high dose of menthone and pulegone significantly inhibited feeding. The lower weights after six days on the diet were likely the result of feeding inhibition, since the effects on maximum weight were much less dramatic.

In the case of menthol, lower weights on day 6 probably resulted from molt abnormalities associated with the 0.2% dosage; there were only three larvae that molted abnormally at the 0.1% dosage (notice the three outliers in Fig. A-3). Menthol did not inhibit feeding in these studies. The retention of fifth-instar head capsules observed with the 0.2% dose of menthol is similar to the effects observed when *Bombyx mori* (L.) larvae were fed diet fortified with phytoecdysteroids (Kubo *et al.* 1983).

Menthol had a profound effect on pupation success. Even the low dose of menthol resulted in near total molt failure. The cause of this phenomenon is not readily apparent. Successful pupation of variegated cutworm larvae is apparently not disrupted when they feed on mint leaves in the laboratory (L. Coop, personal communication). Larvae in the field would not be expected to be continually exposed to monoterpenes during prepupal and pupal development as they were in this study which could explain this discrepancy.

Some of the larvae receiving the 0.1% dosage of pulegone died without feeding within the first 24 hours, implying contact or vapor phase toxicity. Newly molted fifth-instars survived for up to 1 week without any food or water (data not shown). However, 0.1% pulegone is a dosage approximately 25 times that found normally in peppermint leaves (Table 15). Pennyroyal, (*M. pulegium*) contains pulegone levels up to 50 times that found in peppermint. Significant mortality occurred when larvae were fed

diet containing 0.1% pennyroyal oil, a concentration which probably approximates that found in the plant (G.C. analysis, data not shown).

In feeding experiment 2, a clear pattern of tolerance or non-tolerance emerged. Neonate larvae were more sensitive to dietary monoterpenes than older larvae. Once larvae fed and molted to the second stadium, they performed roughly as well as the controls. This is demonstrated by the significant differences in mortality between terpene treatments and controls, and lack of significance for the other parameters.

Table 15 relates the dosages used in the feeding experiments to the approximate wet weight concentrations in peppermint leaves (Data compiled from Formacek and Kubeczka 1982, DeAngelis *et al.* 1983, and Coop 1987). As can be seen, the dosage range used for menthol was approximately equivalent to that found in peppermint leaves. However, the monoterpene profile of peppermint leaves varies with the age of the leaf tissue. In young leaves, pulegone, menthone and other keto-isomers predominate. As the leaf matures, more highly reduced monoterpenes such as aldehydes and alcohols appear. In the flowers, menthofuran predominates (Burbott and Loomis 1969). How phenological monoterpene variation affects larval fitness or behavior is unknown.

Pulegone was moderately toxic when applied topically. Limonene, α-pinene, menthone, and pulegone should penetrate the cuticle easily since all four monoterpenes are hydrophobic liquids. Menthol, a crystalline solid at room temperature, probably did not readily penetrate the cuticle. Non-absorbed menthol was visible on the cuticle and bottom of the test-tube 24 hours post-treatment. Very little mortality was observed even with a saturated menthol solution, and a linear dose-response could not be obtained.

The modest synergist ratios obtained for menthone and α-pinene are not clear evidence for *in vivo* microsomal metabolism. The lack of synergism for pulegone is likewise puzzling. The toxicodynamics of xenobiotic compounds can be complex. Differential penetration rates, multiple possible metabolic routes, target site interactions,

etc., make comparisons between synergized and unsynergized treatments difficult to interpret (Brindley and Selim 1984, Sun and Johnson 1972).

The PSMO system of the variegated cutworm is at least capable of metabolizing peppermint monoterpenes. Whether or not the PSMO system is primarily responsible for the metabolism of monoterpenes is unclear. Other enzyme systems could be involved, such as GSH-transferases or epoxide hydrolases.

CONCLUSION

Results from these experiments raise the possibility that monoterpenes contained in peppermint leaves may cause substantial natural mortality among neonate variegated cutworm larvae. As yet, no work has been done on allelochemical mediated survival of the variegated cutworm in peppermint fields, or what effect monoterpenes may have on parasitism rates.

Future work in this laboratory will explore further the metabolic fate of peppermint allelochemicals in the variegated cutworm and extend these experiments to include *Meteorus communis*.

BIBLIOGRAPHY

Agosin M. 1985. Role of microsomal oxidations in insecticide degradation. in "Comprehensive Insect Physiology, Biochemistry, and Pharmacology" (G.A. Kerkut and L.I. Gilbert eds.) 12:647-712.

Ahmad, S. 1983. Mixed function oxidase activity in a generalist herbivore in relation to its biology, food plants, and feeding history. Ecology 64:235-243.

Ahmad, S., L.B. Brattsten, C.A. Mullin and S.J. Yu. 1986. Enzymes involved in the metabolism of plant allelochemicals. in "Molecular Aspects of Insect-Plant Associations (Brattsten and Ahmad eds.). pp. 73-127. Plenum Press, New York, New York.

Berenbaum M. 1981. Patterns of furanocoumarin distribution and insect herbivory in the Umbelliferae: plant chemistry and community structure. Ecology 62:1254-1266.

Berenbaum, M. 1983. Coumarins and caterpillars: a case for coevolution. Evolution 37:163-179.

Berenbaum, M. 1983. Effects of tannins on growth and digestion in two species of papilionids. Ent. Exp. Appl. 34:245-250.

Berenbaum, M. 1986. Target site insensitivity in insect-plant interactions. in "Molecular Aspects of Insect-Plant Associations (Brattsten and Ahmad eds.) Plenum NY, NY. pp. 257-272.

Bernays E.A. 1978. Tannins: an alternative viewpoint. Ent. Exp. Appl. 24:44-53.

Berry R.E. and E.J. Shields. 1980. Variegated cutworm: leaf consumption and economic loss in peppermint. J. Econ. Entomol. 73:607-608.

Berry, R.E., S.J. Yu, and L.C. Terriere. 1980. Influence of host plants on insecticide metabolism and management of variegated cutworm. J. Econ. Entomol. 73:771-774.

Bloomquist, J.R. and T.A. Miller. 1985. A simple bioassay for detecting and characterizing insecticide resistance. Pestic. Sci. 16:611-614.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Bioch. 72:255-260.

Brattsten L.B. 1983. Cytochrome P-450 involvement in the interactions between plant terpenes and insect herbivores. in "Plant Resistance to Insects" (Hedin ed.) pp. 173-195.

Brattsten L.B. 1986. Fate of ingested plant allelochemicals in herbivorous insects. in "Molecular Aspects of Insect-Plant Associations. (Ahmad and Brattsten eds.). Plenum Press. New York, New York. pp. 211-256.

Brattsten L.B. and R.L. Metcalf. 1973. Synergism of carbaryl toxicity in natural insect populations. J. Econ. Entomol. 66:1947-1348.

Brattsten L.B., C.F. Wilkinson, and T. Eisner. 1977. Herbivore-plant interaction: mixed function oxidases and secondary plant substances. Science.196:1349-1352.

Brattsten, L.B. 1979. Ecological significance of mixed function oxidases. Drug Metab. Rev. 10:35-58.

Brattsten, L.B. and R.L. Metcalf. 1970. The synergistic ratio of carbaryl with piperonyl butoxide as an indicator of the distribution of multifunction oxidases in the Insecta. J. Econ. Entomol. 63:101-104.

Brindley W.A. 1977. Synergist differences as an alternate interpretation of carbaryl-piperonyl butoxide toxicity data. Environ. Entomol. 6:885-888.

Brindley W.A. and A.A. Selim. 1984. Synergism and antagonism in the analysis of insecticide resistance. Environ. Entomol. 13:348-353.

Bull D.L., G.W. Ivie, R.C. Beier, and N.W. Pryor. 1986. *In vitro* metabolism of a linear furanocoumarin (8-methoxypsoralen, xanthotoxin) by mixed-function oxidases of larvae of black swallowtail butterfly and fall armyworm. J. Chem. Ecol. 12:885-892.

Bull, D., G.W. Ivie, R.C. Beier, and N.W. Pryor. 1984. Fate of photosythesizing furanocoumarins in tolerant and sensitive insects. J. Chem. Ecol. 10:893-901.

Burbott A.J. and W.D. Loomis. 1969. Evidence for metabolic turnover of monoterpenes in peppermint. Plant Physiol. 44:173-179.

Casida, J.E. 1970. Mixed function oxidase involvement in the biochemistry of insecticide synergists. J. Agr. Food Chem. 18:753-772.

Chang K., C.F.Wilkinson, K. Hetnarski, and M. Murray. 1983. Aryl hydrocarbon hydroxylase in larvae of the southern armyworm (*Spodoptera eridiania*). Insect Biochem. 13:87-94.

Chang, K., C.F. Wilkinson, and K. Hetnarski. 1981. Spectral and inhibitory interactions of methylenedioxyphenyl compounds with southern armyworm (*Spodoptera eridania*) midgut microsomes. Pestic. Biochem. Physiol. 15:32-42.

Christian M.F. and S.J. Yu. 1986. Cytochrome P-450-dependent monooxygenase activity in the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner. Comp. Biochem. Physiol. 83C:23-27.

Collart, M.G. and W.F. Hink. 1986. Sublethal effects of D-limonene on the cat flea, (*Ctenocephalides felis*). Ent. Exp. Appl. 42:225-229.

Collins, P.J. 1985. Induction of the polysubstrate monooxygenase system of the native budworm (*Heliothis punctiger*) Wallengren (Lepidoptera:Noctuidae). Insect Biochem. 15:551-555.

Cook, J.C. and E. Hodgeson. 1985. The induction of cytochrome P-450 by isosafrole and related methylenedioxyphenyl compounds. Chem.-Biol. Interactions 54:299-315.

Coop, L. 1987. Management of Variegated Cutworm in Peppermint. Ph.D. Thesis, Oregon State University.

Coop L.B. and R.E. Berry. 1986. Reduction in variegated cutworm (Lepidoptera:Noctuidae) damage to peppermint by larval parasitoids. J. Econ Entomol. 79:1244-1248.

Dawson R.M.C., D.C. Elliot, W.H. Elliot, and K.M. Jones. 1969. Data for Biochemical Research 2nd ed. Oxford Press, New York.

DeAngelis, J., R.E. Berry, and G.W. Krantz. 1983. Effects of spider mite (Acari:Tetranychidae) injury on essential oil metabolism in peppermint. Environ. Entomol. 12:522-526.

DeVries, D.H. and G.P. Georghiou. 1981. Absence of enhanced detoxication of permethrin in pyrethroid resistant house-flies. Pestic. Biochem. Physiol. 15:242-252.

Duffey, S.S. 1980. Sequestration of plant natural products by insects. Annu. Rev. Entomol. 25:447-477.

Elliger, C.A., Y. Wong, B.G. Chan, and A.C. Waiss Jr. 1981. Growth inhibitors in tomato (Lycopersicon) to tomato fruitworm *Heliothis zea*. J. Chem. Ecol. 7:753-758.

Ellman, G.L., K.D. Courtney, V. Andres Jr., and R.M. Featherstone 1961 A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7:88-95.

Embong M.B., L. Steele, and D. Hadziyev. 1977. Essential oils from herbs and spices grown in Alberta: peppermint oil (*Mentha piperita*) var. Mitcham. J. Inst. Can. Sci. Technol. Aliment. 10:247-255.

Farnsworth, D.E., R.E. Berry, and L.C. Terriere. 1981. Aldrin epoxidase activity and cytochrome P-450 content of microsomes prepared form alfalfa and cabbage looper larvae fed various diets. Pestic. Biochem. Physiol. 15:158-165.

Feeny, P. 1976. Plant apparency and chemical defense. Rec. Adv. Phytochem. 10:1-40.

Feyereisen R. 1985. Regulation of juvenile hormone titer: degradation. in "Comprehensive Insect Physiology, Biochemistry, and Pharmacology" (G.A. Kerkut and L.I. Gilbert eds.). 7:391-429.

Feyereisen, R., G.E. Pratt, and A.F. Hamnett. 1981. Enzymic synthesis of juvenile hormone in locust corpora allata: evidence for a microsomal cytochrome P-450 linked methyl farnesoate epoxidase. Eur. J. Biochem. 118:231-238.

Fox, L.R. 1981. Defense and dynamics in plant herbivore interactions. Amer. Zool. 21:853-864.

Gould, F. 1984. Mixed function oxidases and herbivore polyphagy: the devils advocate position. Ecol. Entomol. 9:29-34.

Grundy, D.L. and C.C. Still. 1985. Isolation and identification of the major insecticidal compound of Poleo (Lippia stoechadifola). Pestic. Biochem. and Physiol. 23:378-382.

Gunderson, C.A., J.H. Samuelian, C.K. Evans, and L.B. Brattsten. 1985. Effects of the mint monoterpene pulegone on Spodoptera eridania (Lepidoptera:Noctuidae). Environ. Entomol. 14:859-863.

Gunderson, C.A., L.B. Brattsten, and T.J. Fleming. 1986. Microsomal oxidase and glutathione transferase as factors influencing the effects of pulegone in southern and fall armyworm larvae. Pestic. Biochem. Physiol. 26:238-249.

Hink, W.F. and B.J. Fee. 1986. Toxicity of D-limonene, the major component of citrus peel oil, to all life stages of the cat flea, *Ctenocephlides felis* (Siphonaptera:Pulicidae). J. Med. Entomol. 23:400-404.

Hodgson, E. 1985. Microsomal mono-oxygenases. in "Comprehensive Insect Physiology, Biochemistry, and Pharmacology" (G.A. Kerkut and L.I. Gilbert eds.). 11:225-322.

Isman, M.B. and S.S. Duffey. 1982. Toxicity of tomato phenolic compounds to the fruitworm, *Heliothis zea* Ent. Exp. & Appl. 31:370-376.

Ivie, G.W., D.J. Bull, R.C. Beier, N.W. Pryor, and E.H. Obertli. 1983. Metabolic detoxification: mechanism of resistance to plant psoralens. Science. 221:374-376.

Ivie, G.W., D.L. Bull, R.C. Beier, and N.W. Pryor. 1986. Comparative metabolism of [^{3}H]psoralen and [^{3}H]isopsoralen by black swallowtail (*Papillio polyxenes* Fabr.) caterpillars. J. Chem. Ecol. 12:871-884.

Kreiger, R.I., P. Feeny, and C.F. Wilkinson. 1971. Detoxification enzymes in the guts of caterpillars: an evolutionary answer to plant defenses? Science. 8:579-580.

Kulkarni, A.P. and E. Hodgeson. 1980. Metabolism of insecticides by mixed function oxidases. Pharmacol. Therap. 8:379-475.

Marcus C.B., M. Murray, K. Hetnarski, and C.F. Wilkinson. 1987. Methylenedioxyphenyl complexes with microsomal cytochrome P-450: (*in vivo* complex formation in rat liver and in midgut tissues of the southern armyworm (*Spodoptera eridania*). Pestic. Biochem. Physiol. 28:140-147.

Marcus, C.B., M. Murray, C. Wang, and C.F. Wilkinson. 1986. Methylenedioxyphenyl compounds as inducers of cytochrome P-450 and monooxygenase activity in the southern armyworm (*Spodoptera eridiana*) and the rat. Pestic. Biochem. Physiol. 26:310-322.

McCord E. and S.J. Yu. 1987. The mechanisms of carbaryl resistance in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). Pestic. Biochem. Physiol. 27:114-122.

Moldenke, A.F., R.E. Berry, and L.C. Terriere. 1983. Cytochrome P-450 in insects V. Monoterpene induction of cytochrome P-450 and associated monooxygenase activities in the larvae of the variegated cutworm -*Peridroma saucia.* Comp. Biochem. Physiol. 74C:365-371.

Mouches, C., N. Pasteur, J.B. Berge', O. Hyrien, M. Raymond, B.R. DeSaint Vincent, M. DeSilvestri, A. 1986. Amplification of an esterase gene is responsible for insecticide resistance in a California (*Culex*) mosquito. Science. 233:778-780.

Mullin C.A. 1986. Adaptive divergence of chewing and sucking arthropods to plant allelochemicals. in "Molecular Aspects of Insect-Plant Associations" (Brattsten and Ahmad eds.) Plenum NY,NY. pp. 175-210.

Nakatsugawa T. and M.A. Morelli. 1976. Microsomal oxidation and insecticide metabolism. in "Insect Biochemistry and Physiology" (C.F. Wilkinson ed.), pp. 61-114.

Neal J.J. 1987. Metabolic costs of mixed-function oxidase induction in *Heliothis zea.* Ent. Exp. Appl. 43:175-179.

Nebert, D.W., H.J. Eisen, and O Hankinson. 1984. The Ah receptor: binding specificity only for foreign chemicals? Biochem. Pharmacol. 33:917-924.

Omura, T. and R. Sato. 1964. The carbon monoxide binding pigment of liver microsomes II. Solubilization, purification, and properties. J. Biol. Chem. 239:2379-2385.

Raffa, K.F., A.A. Berryman, J. Simasko, W. Teal, and B.L. Wong. 1985. Effects of grand fir monoterpenes on the fir engraver, *Scolytus ventralis* (Coleoptera: Scolytidae), and its symbiotic fungus. Environ. Entomol. 14:552-556.

Reese, J.C., B.G. Chan, and A.C. Waiss. 1982. Effects of cotton condensed tannin, maysin (corn), and pinitol (soybeans) on *Heliothis zea* growth and development. J. Chem. Ecol. 8:1429-1436.

Rhoades D.F. and R.G. Cates. 1976. A general theory of plant antiherbivore chemistry. Rec. Adv. Phytochem. 10:168-213.

Rings R.W., B.A. Johnson, and F.J. Arnold. 1976. Host range of the variegated cutworm on vegetables: a bibliography. Bull. Ent. Soc. Amer. 22:409-415.

Riskallah, M.R., W.C. Dauterman, and E. Hodgeson. 1986. Nutritional effects on the induction of cytochrome P-450 and glutathione transferase in larvae of the tobacco budworm, *Heliothis virescens.* Insect Biochem. 16:490-499.

Rose H.A. 1985. The relationship between feeding specialization and host plants to aldrin epoxidase activities of midgut homogenates in larval Lepidoptera. Ecological Entomol. 10:455-467.

Scott J.G. and G.P. Georghiou. 1986. Mechanisms responsible for high levels of permethrin resistance in the house fly. Pestic. Sci. 17:195-206.

Scriber M. 1981. Sequential diets, metabolic costs, and growth of *Spodoptera eridania* (Lepidoptera:Noctuidae) feeding upon dill, lima bean, and cabbage. Oecologica 51:175-180.

Shapiro, J.P., K.S. Kiem, and J.H. Law. 1984. Structural studies on lipophorin, and insect lipoprotein. J. Biol. Chem. 259:3680-3685.

Sun, Y. and E.R. Johnson. 1972. Quasi-synergism and penetration of insecticides. J. Econ. Entomol. 65:349-353.

Tallamy D.W. 1986. Behavioral adaptations in insects to plant allelochemicals. in "Molecular Aspects of Insect-Plant Associations" (Brattsten and Ahmad eds.) Plenum NY,NY. pp. 273-300.

Taylor W.E. and B. Vickery. 1974. Insecticidal properties of limonene, a constituent of citrus oil. Ghana J. Agr. Sci. 7:61-62.

Terriere L.C. 1984. Induction of detoxification enzymes in insects. Annu. Rev. Entomol. 29:71-88.

Thongsinthusak T. and R.I. Kreiger 1974 Inhibitory and inductive effects of piperonyl butoxide on dihydroisodrin hydroxylation (*in vivo*) and (*in vitro*) in black cutworm (*Agrotis ypsilon*) larvae. Life Sci. 14:2131-2141.

Trammell D.J. 1982. *In vitro* metabolism of (+)-pulegone and (-)-carvone by southern armyworm *Spodoptera eridania* microsomes. M.S. thesis Georgia Institute of Technology, Atlanta.

Van De Baan H.E., L.A.M. Kuijpers, W.P.J. Overmeer, and F.J. Oppenoorth. 1984. Organophosphorus and carbamate resistance in the predacious mite *Typhlodromus pyri* due to insensitive acetylcholinesterase. Exp. Appl. Acarol. 1:3-10.

White, R.A., R.T. Franklin, and M. Agosin. 1979. Conversion of -Pinene to -Pinene oxide by rat liver and the bark beetle (*Dendroctonus terebrans*) microsomal fractions. Pestic. Biochem. Physiol. 10:233-242.

Wilkinson, C.F., M. Murray, C. Marcus, and C. Dube'. 1982. Mechanistic studies on the inhibition of cytochrome P-450 mediated mixed function oxidation. in Pesticide Chemistry Academic Press v.3 pp. 463-468.

Yu S.J. 1984. Interactions of allelochemicals with detoxification enzymes of insecticide-susceptible and resistant fall armyworms. Pestic. Biochem. Physiol. 22:60-68.

Yu S.J. 1987. Microsomal oxidation of allelochemicals in generalist (*Spodoptera frugiperda*) and semispecialist (*Anticarsia gemmatalis*) insect. J. Chem. Ecol. 13:423-436.

Yu S.J. and L.C. Terriere. 1974. Bimodal effect of methylenedioxyphenyl compounds on detoxifying enzymes in the housefly. Pestic. Biochem. Physiol. 4:160-169.

Yu, S.J. 1982. Induction of microsomal oxidases by host plants in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). Pestic. Biochem. Physiol. 17:59-67.

Yu, S.J. 1986. Consequences of induction of foreign compound-metabolizing enzymes in insects. in "Molecular Aspects of Insect-Plant Associations" (Brattsten and Ahmad eds.). Plenum Press, New York, New York. pp. 153-174.

Yu, S.J., R.E. Berry, and L.C. Terriere. 1979. Hostplant stimulation of detoxifying enzymes in a phytophagous insect. Pestic. Biochem. and Physiol. 12:280-284.

Zalkow, L.H., M.M. Gordon, and N. Lanir. 1979. Antifeedants from rayless goldenrod and oil of pennyroyal: toxic effects for the fall armyworm. J. Econ. Entomol. 72:812-815.

APPENDIX

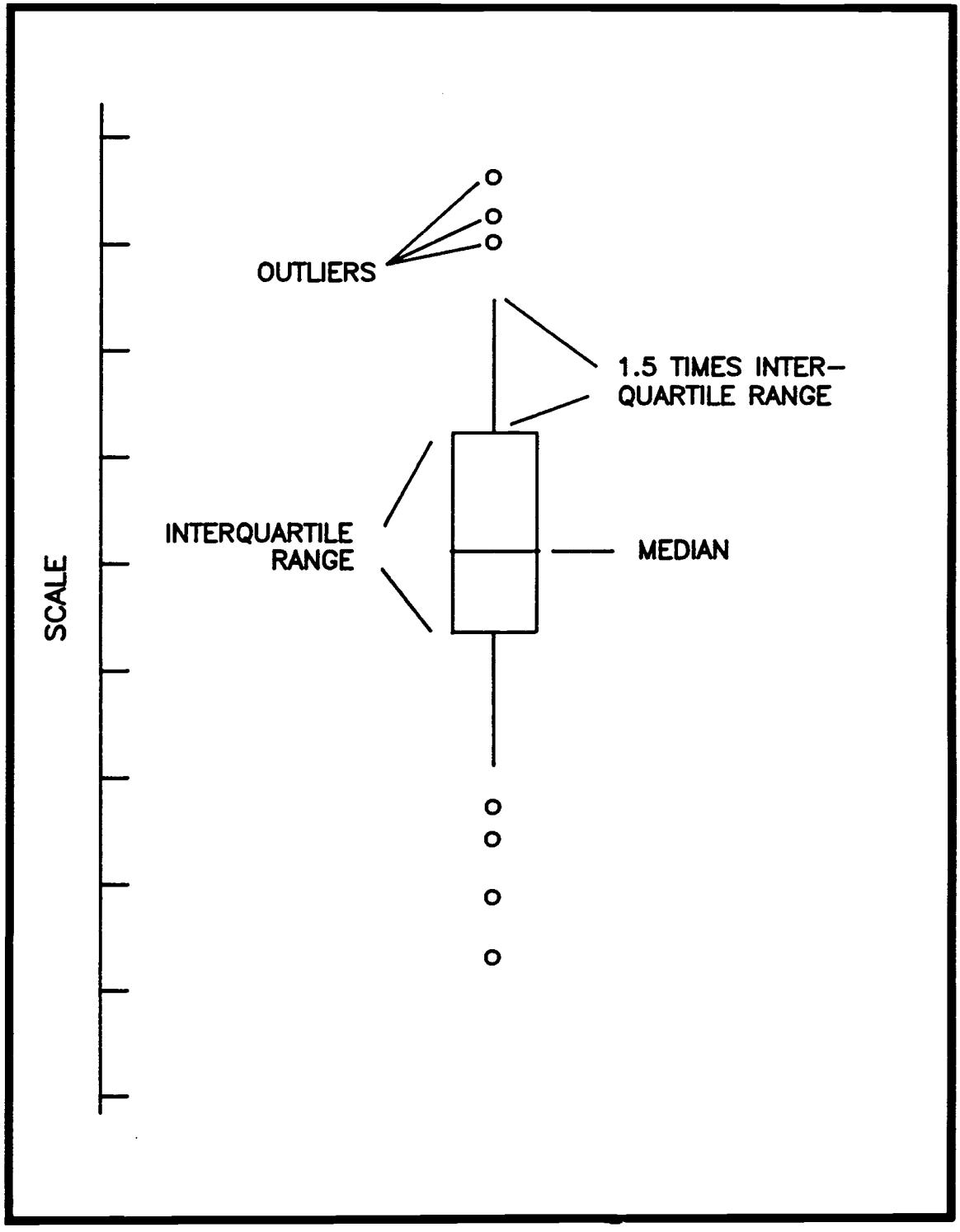

Fig. A-1. Interpretation of Boxplots

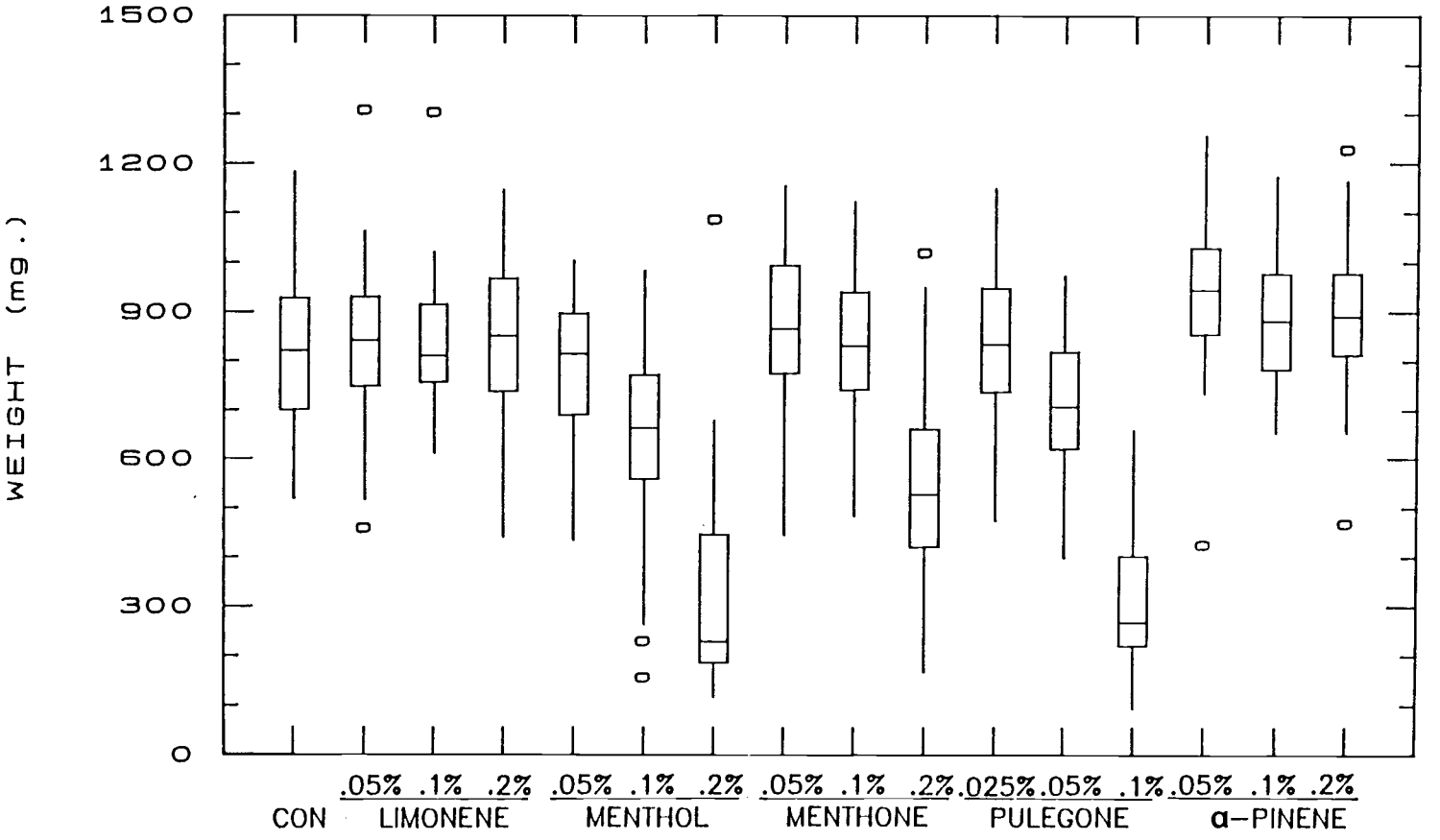

Fig. A-2. Weight of larvae after feeding on terpene-fortified artificial diet for six days post fifth molt.

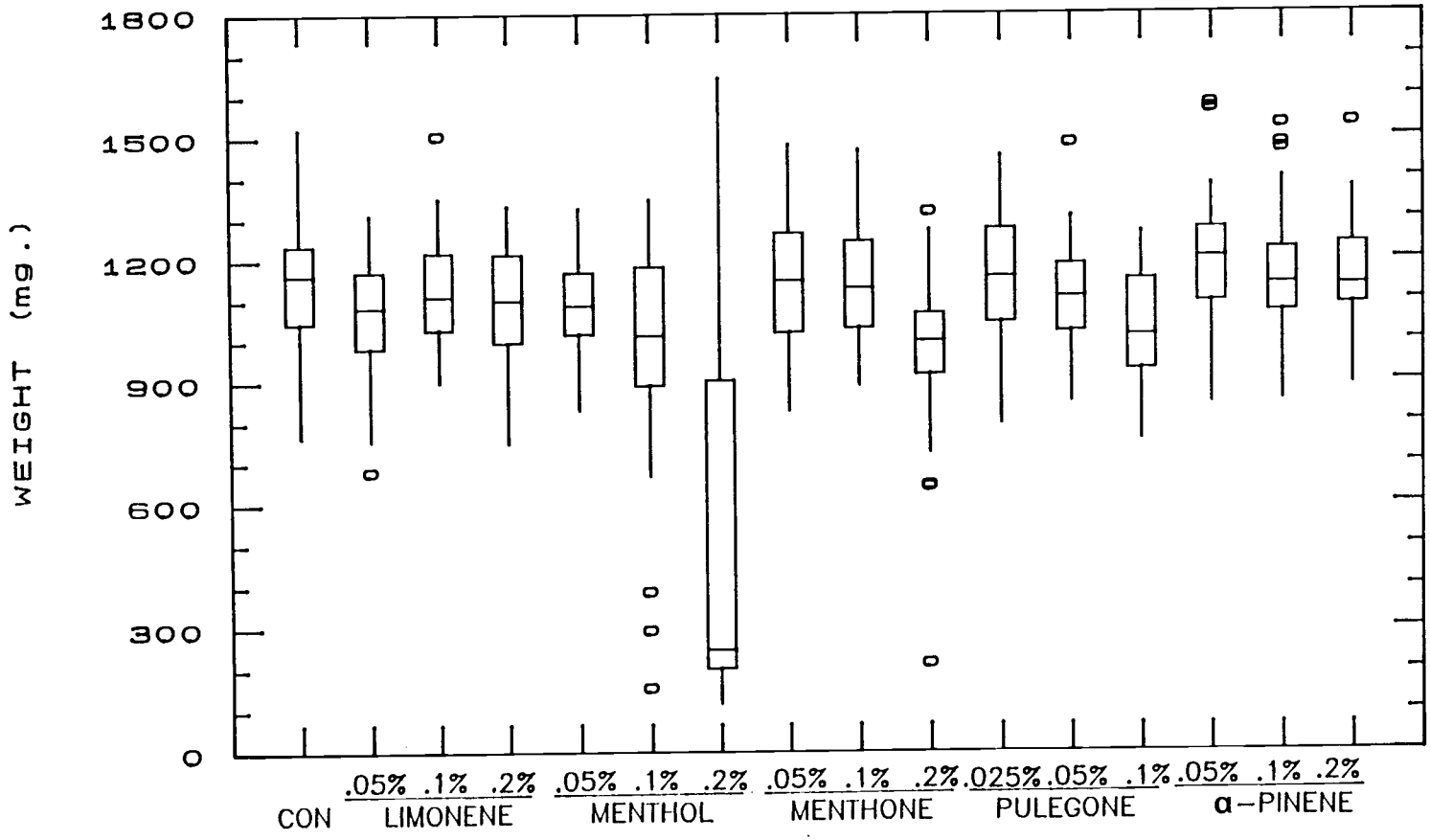

Fig A-3. Maximum weight attained by larvae feeding on terpene fortified artificial diet. Larvae began feeding on fortified diet fifth stadium.