PLANT-DERIVED COMPOUNDS: ACUTE TOXICITY, SYNERGISM, AND EFFECTS ON INSECT ENZYME ACTIVITY AND FLIGHT MOTOR RESPONSES

by

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Abstract

Botanical extracts may contain compounds that have insecticidal properties that may be developed as inexpensive insecticides. In this thesis, I used a series of techniques to identify the acute toxicities and modes of action of plant-derived compounds against the Yellow Fever mosquito *Aedes aegypti* and the blowfly *Phaenicia sericata*.

Initially I evaluated the acute toxicity of 16 phytochemicals on aquatic and terrestrial insects alone or with the synergist piperonyl butoxide (PBO) to quantify their lethal and sublethal effects. From this study 5 compounds, thymol, eugenol, pulegone, *α*-terpineol and citronellal, were selected for further study. I then evaluated the biochemical mechanisms underlying the activity of these phytochemicals and the basis of their increased toxicity in the presence of PBO. These phytochemicals affected the biotransformational capacity of these insects to detoxify the compounds, and their effects were enhanced by PBO. I then developed an electrophysiological system to evaluate the physiological effects of the plant-derived compounds and several commercially available insecticides on flight muscle impulses and wing beat signals of the blowfly, *P. sericata*. These compounds readily penetrate the insect cuticle and interfere with flight muscle and/or central nervous system function. All 5 compounds depressed flight-associated responses, and acted similarly to compounds that block sodium channels and facilitate Ɣ-amino butyric acid (GABA) action. I compared these responses to those induced by several synthetic insecticides whose mode of action is well known to allow us to make a more precise prediction of how the 5 compounds affect the target insects.

I then evaluated the effect of the 5 phytochemicals, and octopamine on the octopaminergic system of insects by comparing the production of a second messenger molecule, cAMP, after treatment. Some monoterpenoids interfere with the octopaminergic system by targeting the octopamine receptors. The acute toxicity observed in *Ae. aegypti* and *P. sericata* may be the collective result of these compounds on complex biological systems in the insect and may depend on their structure, concentration, or exposure time. The overall results indicate that plant-derived compounds directly and indirectly affect aspects of insect physiology and could possibly be developed as new insecticides.

Keywords: Monoterpenoids; acute toxicity; oviposition; modes of action; electrophysiology; biotransformation; synergism; neurotoxicity

Dedication

In memory of my mom and thanks to my father for bringing me up with love and support.

Thanks to my family,

Nilakshi, Thumri, Thunuvi and Padma

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Glossary

Agonist	An agonist is a chemical that binds to a receptor of a cell and triggers a response by that cell. Agonists often mimic the action of a naturally occurring substance.
Antagonist	A receptor antagonist is a type of receptor ligand or drug that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses.
Amplitude	Maximum height of an electrical impulse or a sound wave from its base.
Ataxia	Incoordination and unsteadiness due to the brain's failure to regulate the body's posture and regulate the strength and direction of limb movements.
Bidirectional	Impulses witch move in both direction from its base.
Biotransformation	Biotransformation is the process whereby a substance is changed from one chemical to another (transformed) by a chemical reaction within the body. Metabolism or metabolic transformations are terms frequently used for the biotransformation process.
Electrophysiology	Electrophysiology is the study of the electrical properties of biological cells and tissues. It involves measurements of voltage change or electric current on a wide variety of scales from single ion channel proteins to whole organs.
Flight muscle impulses (FMI)	Electric signals generated due to flight muscle activity.
GABA	Gama amino butyric acid. Main inhibitory neurotransmitter in mammals and in invertebrates.
Grooming	A regular activity used by animals to clean themselves to keep their fur, feathers, scales, or other skin coverings in good condition.

Hyperactive	Hyperactivity can be described as a physical state in which an insect is abnormally and easily excitable or exuberant.
Insecticide	Chemical substance that is used to kill insects.
Isoprene units	Isoprene, or 2-methyl-1,3-butadiene, is a common organic compound with the formula CH2=C(CH3)CH=CH2. Isoprene (C5H8) is the monomer of natural rubber and also a common structure motif to an immense variety of other naturally occurring compounds, collectively termed the isoprenoids. Molecular formula of isoprenoids are multiples of isoprene in the form of (C5H8)n, and this is termed the isoprene rule. The functional isoprene units in biological systems are dimethylallyl diphosphate (DMADP) and its isomer isopentenyl diphosphate (IDP).
Knockdown	Is a quick action which brings the insects to paralysis state.
LC_{50}	Lethal concentration that kills 50% of a given population.
LD_{50}	Lethal dose that kills 50% of a given population.
Microsomal	Small particles in the cytoplasm of a cell, typically consisting of fragmented endoplasmic reticulum to which ribosomes are attached.
Monoterpenes	Monoterpenes are a class of terpenes that consist of two isoprene units and have the molecular formula C10H16. Monoterpenes may be linear (acyclic) or contain rings. Biochemical modifications such as oxidation or rearrangement produce the related monoterpenoids.
Moribund	Near state of death with very less movements of appendages.
Neurohormone	A hormone that is produced and released by neurones.
Neuromodulator	Substance other than a neurotransmitter released by neuron and transmitting information to other neurons.

Octopamine	Biogenic monoamine structurally related to noradrenalin, acts as a nurohormone, neuromodulator and neurotransmitter in invertebrates.
Oviposition	The process of laying eggs by oviparous animals.
Paralysis	Loss of muscle function.
Plant-derived compounds	Compounds of plant origin.
Plant essential oils	Steam distillable fraction of plant tissues.
Tremor	A tremor is an involuntary, somewhat rhythmic, muscle contraction and relaxation involving to-and-fro movements (oscillations or twitching) of one or more body parts.
Unidirectional	Impulses which move to one direction. These impulses have either their positive or negative portion from its base.
Wing beat signals (WBS)	Acoustic signals generated due to wing movements during the flight.
Writhing	Twisting action used by an insect using its fore or hind legs.
Xenobiotic	Any foreign substance taken in by the organism. It may be harmful or beneficial to the organism.

Chapter 1: Introduction

Many insect and mite species cause significant economic damage as pests of agriculture, horticulture, stored products, and forestry. Many insects also serve as vectors of serious diseases such as malaria. There are about 70,000 pest species that contribute to the destruction of agricultural crops and livestock health (Pimentel, 2007). Of this number, 10,000 are insects and mites. 50,000 are plant pathogens and 10,000 are weed species (Pimentel, 2007). To combat insect pests, we apply more than 3000 million Kg of insecticides (Pimentel, 2007). The cost of applying chemical insecticides, biological controls, and other non-chemical controls worldwide costs 35,000 million dollars (Pimentel, 2007). Even with these measures, more than 40% of world crop production, valued at 750 000 million USD, is destroyed by pests (Oerke et al., 1994). Without some control measures, the losses of crops due to pests would be even more severe either in the field (Oerke et al., 1994) or in post harvest storage where an additional 25% of harvested crops are lost to insects. The current estimates are that pests cause a 52% loss of all crops, and food potential, despite all the pest control technologies used (Pimentel, 2007).

Insects will continue to challenge and compete with mankind for these resources, forcing us to review the way we have dealt with insect pests in the past and stimulating the development of new innovative control tactics (Klassen, 2005). The development of very effective and persistent synthetic organic insecticides after the Second World War marked the onset of chemical-based approaches against insect pests. The contribution of these synthetic insecticides towards the green revolution of the 1960s cannot be questioned (Oerke et al., 1995). Chemical control provided an effective and economical means to deal with a multitude of arthropod problems; to quell outbreaks, to reduce

populations of vectors that transmit parasites and pathogens to humans and livestock, to suppress mites in honeybee hives, and to control numerous household pests in urban ecosystems (Pimentel, 2007). Without chemical pest control, global agricultural productivity would have been significantly less, food prices much higher and the available food of lower quality (Pimentel, 2007).

Drawbacks to the widespread use of these broad-spectrum insecticides were recognized soon after their introduction (Carson, 1962). They may contaminate soils and water, may affect many non-target and beneficial insects, leading to outbreaks of secondary pests, may sicken the farmers who apply them, and may bioaccumulate in the food chain (Repetto and Baliga, 1996). Governments, and the general public, want a constant, predictable, and low priced food supply. However, the public also recognizes the problems associated with insecticide resistance and environmental contamination. These concerns have promoted the search for more environment-friendly pest control options. The growing demand for "natural" products has intensified in the past 20 years; biologically active and rapidly degrading compounds are being sought for use in alternative and sustainable integrated pest management programs (Rattan, 2010). This includes the use of naturally occurring plant-based compounds.

1.1 Plant-derived insecticidal compounds

The use of natural, plant-derived compounds to control insect pests is gaining greater attention by general public and the scientific community. These compounds have evolved in plants for, among other reasons, defense against phytophagous insects. Their insecticidal, fungicidal, bactericidal, antiviral, antifeedant and insect growth retardant properties (Benner, 1993; Sing et al., 1989; Wilson et al., 1997) are often the result of

synergistic interactions among different biologically active components such as terpenoids, alkaloids and phenolics (Singh et al., 1989). At present, there are four major commercially available plant-based compounds (pyrethrum, rotenone, neem and essential oils) and three limited use compounds (ryania, nicotine and sabadilla) for insect control (Isman, 2006). Essential oils, the natural plant products that give rise to the characteristic plant flavors and fragrances, are the steam distillable fractions of plant tissues and are grouped as monoterpenes, sesquiterpenes, and aliphatic compounds (alkanes, alkenes, ketones, aldehydes, acids and alcohols) (Kordali et al., 2007; Isman, 1999).

1.2 Biosynthesis of plant-derived compounds

The biosynthesis of plant-derived compounds is tissue specific and is developmentally regulated in all higher plants. The pathways and the genes that produce these compounds are tightly regulated and are closely associated with environmental, seasonal or external triggers. The principal constituents of plant-derived compounds are derived mainly from fatty acid, phenylpropanoid, or isoprenoid pathways (Daviet and Schalk, 2010). Subsequent modifications to the basic terpene backbone have produced the thousands of terpenoid compounds found in nature (Acamovic and Brooker, 2005). The isoprenoid biosynthetic pathway (Figure 1-1) leads to the synthesis of plant-derived compounds such as borneol, camphor, citronellal, cineol, geraneol, limonene, linalool, myrcene, pulegone, α-terpineol, and thymol. To date, over 23,000 naturally occurring isoprenoids have been identified, and new compounds continue to be discovered (Sacchettini and Poulter, 1997). The production of these compounds is not new; hapanoids, a form of isoprenoids, have been discovered in sediments dating back 2.5 billion years (Summons et al., 1999).

Isoprenoids and their derivatives play many key roles in plants as constituents of membranes, vitamins, antimicrobial agents, mating pheromones, reproductive hormones, components of signal transduction pathways, and constituents of electron transport and photosynthetic systems (Humphrey and Beale, 2006). Although the final chemical structures of the terpenes are as diverse as their functions, all terpenes are derived from a sequential assembly of molecular building blocks known as isoprene units that consist of a branched chain of five carbon atoms (Dewick, 2001) (Figure 1-1). Initially it was thought that the terpenes were assembled from isoprene (Ruzicka, 1953), hence their alternative name of isoprenoids (Humphrey and Beale, 2006). It is now known that the actual five-carbon building blocks (isoprene units) are the interconvertible isomers of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Humphrey and Beale, 2006). Condensation of these two building blocks in a sequential fashion by the action of prenyltransferases (Humphrey and Beale, 2006) produces geranyl, farnesyl and geranylgeranyl pyrophosphates, squalene and phytoene, which are the direct precursors of the major families of terpenes (Humphrey and Beale, 2006). Subsequent modifications to the carbon backbone by enzyme-catalysed cyclization, oxidation and skeletal rearrangement steps give rise to the multitude of iseprenoid structures illustrated in Figure 1-2.

In higher plants, two separate enzymatic systems are responsible for terpene biosynthesis. One system, in the cytosol, generates most of the sesquiterpenes, the triterpines and sterols; the other, in the plastids, generates the essential oil monoterpenes, the diterpenes and carotenoids (Figure 1-2). Understanding this segregation, and the cross-talk between plastidic and cytosolic biosynthetic machinery, is a developing field

(Humphrey and Beale, 2006; Litchtenthaler, 1999; Eisenreich et al., 2004). The Following section describes the isoprenoid pathway in detail.

1.2.1 Isoprenoid biosynthetic pathway: production of monoterpenoids

The isoprenoid pathway and its products are shown in Figures 1-1 and 1-2. β-Hydroxymethylglutaryl coenzyme A (HMG-CoA) is converted to mevalonate (Ferguson et al., 1959) via the NADPH-requiring enzyme HMG-CoA reductase (HMGR). Phosphorylating mevalonate by mevalonate kinase (MK) yields 5-phosphomevalonate (Tchen, 1958). Further phosphorylation and decarboxylation of 5-phosphomevalonate forms isopentenyl pyrophosphate (IPP) (Tada and Lynen, 1961). Isopentenyl pyrophosphate isomerase isomerizes IPP to dimethylallyl pyrophosphate (DMAPP) (Agranoff et al., 1950). DMAPP serves as the isoprene donor in the production of isopentenyl adenine (Milstone et al., 1978) and in the synthesis of cytokinins in plants (Taya et al., 1978). IPP and DMAPP are condensed to form the 10-carbon geranyl pyrophosphate (GPP) which serves as the precursor for the synthesis of all monoterpenes. The addition of another IPP unit to GPP yields the 15-carbon (sesquiterpene) farnesyl pyrophosphate (FPP). The enzyme FPP synthase catalyzes the synthesis of both GPP and FPP in mammals (Poulter and Rilling, 1978), whereas in plants a separate GPP synthase has been identified (Croteau and Purkett, 1989). FPP sits at the branch-point between sterol and longer-chain nonsterol synthesis. The enzyme squalene synthase catalyzes the head-to-head condensation of two FPP molecules to form the sterol precursor squalene (Humphrey and Beale, 2006; Beytia et al., 1973). Subsequent cyclization steps lead to sterol synthesis. Plants also use FPP as a substrate for sesquiterpene synthesis whereas in insects, FPP is the precursor for juvenile hormone (JH) production (Koyama et al., 1985).

Geranylgeranyl pyrophosphate (GGPP) synthase catalyzes the addition of IPP to FPP to form the 20-carbon product GGPP (Kandutsch et al., 1964). In plants, GGPP serves as the precursor for carotenoids, diterpenes, and chlorophylls, and in some instances, is used to make longer-chain products. FPP and GGPP also serve as isoprene donors in the isoprenylation of proteins catalyzed by the enzymes farnesyl protein transferase (FPTase) and geranylgeranyl protein transferase (GGPTase) I and II (Reiss et al., 1990; Moomaw and Casey, 1992; Yokoyama and Gelb, 1993; Armstrong et al., 1993). Long isoprenyl diphosphate synthase (IDS) produces the side chains of ubiquinone and produces chicle and gutta-percha, comprising approximately 100 and 700 isoprene units respectively (Wang and Ohnuma, 2000). Plants have additional Z-IDS, which can catalyze the production of very long isoprene species, including natural rubber, composed of over 1000 isoprene units (Ibata et al., 1983).

Plants also have a mevalonate-independent deoxy-D-xylulose 5-phosphate (DOXP) pathway (Humphrey and Beale, 2006) that condenses pyruvate and D-glyceraldehyde-3-phosphate to form 1-deoxy-D-xylulose 5-phosphate (DXPS) (Sprenger et al., 1997; Lange et al., 1998; Eisenreich et al., 1998) and the subsequent reactions to produce IPP (Flesch and Rohmer, 1988; Lange and Croteau, 1999).

The compartmentalization of isoprenoid biosynthesis in higher plants is such that sterols, sesquiterpenes, triterpenes, and polyterpenes are synthesized in the cytosol through the mevalonate pathway, whereas monoterpenes, diterpenes, carotenoids, plastoquinones, and the prenyl side chain of chlorophyll are synthesized in the plastid through the DOXP pathway (Lichtenthaler et al., 1997).

The ten-carbon isoprenoids tend to be colorless, volatile oils with highly distinctive aromas and flavors (Sangwan et al., 2001; Mahmoud and Croteau, 2002), and are best known as components of the essential oils of flowers and herbs. The biosynthesis and diversity of the monoterpenoids used in these studies is shown in Figures 1-3 to 1-5. These monoterpenes are synthesized and stored in specialized structures such as resin ducts, specific cells within leaf blades or in glandular trichomes (Sangwan et al., 2001; Humphrey and Beale, 2006; Turner et al., 1999).

1.3 Exploitation of plant-derived compounds

Plants produce a wide range of compounds that have insecticidal, fungicidal, bactericidal, antiviral, antifeedant, or insect growth retardant properties (Singh et al., 1989; Benner, 1993; Wilson et al., 1997). Essential oils of *Thymus serpyllum* and *Oreganum majorama* are toxic as fumigants against the bean weevil (*Acanthoscelides obtectus*) (Regnault et al., 1993). Clove extracts (*Syzygium aromaticum*), and Star anise (*Illicium verum*) are lethal or have antifeedent effects on the red flour beetle (*Tribolium castaneum*) and the maize weevil (*Sitophilus zeamais*) (Huang et al., 1998). Essential oils of cumin (*Cuminum cyminum*), anise (*Pimpinella ansium*), oregano (*Origanum syriacum* var. bevanii) and eucalyptus (*Eucalyptus camaldulensis*) are used as fumigants in greenhouses against the cotton aphid (*Aphis gossypii*) and the carmine spider mite (*Tetranychus cinnabarinus*) (Tuni and Sahinkaya, 1998). Insecticidal activity of monoterpenoids against the western corn rootworm (*Diabrotica virgifera*), the two-spotted spider mite (*Tetranychus urticae*) and the housefly (*Musca domestica*), (Lee et al., 1997) and antifeeding effects of monoterpenoids against the European corn borer (*Ostrinia nubilalis*) have been documented (Lee et al., 1999). Hough-Goldstein (1990),

reported the antifeedent effects of sesquiterpenes isolated from the family Asteraceae against the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), whereas Sharma and Saxena (1974), showed their effectiveness as growth inhibitors on house flies, *Musca domestica* L. Thymol shows both repellent and toxic effects against the twospotted spider mite, *Tetranychus urticae* Koch (Gengaihi et al., 1996), and thymol and citronellic acid are toxic to the common housefly; western corn rootworm, *Diabrotica vergifera vergifera* LeConte; and the twospotted spider mite (Lee et al., 1997). Thyme oil is toxic to the tobacco cutworm *Spodoptera litura* F. (Isman et al., 2001), and rosemary oil shows repellent properties against the onion aphid, *Neotoxoptera formosana* (Takahashi) (Masatoshi and Hiroaki, 1997) and the green peach aphid, *Myzus persicae* (Sulzer) (Masatoshi, 1998). Contact and fumigant toxicities of eugenol and methyl eugenol have been demonstrated on the American cockroach, *Periplaneta americana* (L.) (Ngoh et al., 1998). Citronellal, an acyclic monoterpene has become a popular natural alternative to N,N-ethyl-m-tolumide (DEET) for personal protection against mosquitoes and biting flies (Karr and Coats, 1988; Isman, 1999). A summary of some of these compounds, their insect targets, the mode of application and their effects on the insects is presented in Table 1-1.

There is conflicting data in the literature on whether specific essential oils are repellent, antifeedent, or toxic to a particular species. These effects may vary depending on the concentration of specific oils used. Many essential oils, however, contain multiple constituents, and the individual compounds responsible for toxicity have not been purified, making it difficult to estimate a concentration of the lethal component found in each essential oil.

1.4 Toxicity of plant derived-compounds

Plants produce thousands of chemicals that are not necessarily involved in their primary metabolism, but are likely to be involved in plant defence, communication and competition (Isman, 1999). These chemicals may be stored within plant tissues, e.g. to act as a defence against herbivorous predators, or may be actively released into the surrounding environment (Isman and Aktar, 2007). Natural chemicals can enter the environment through volatilization, exudation from roots, leaching from plant material and decomposition of plant residues, and also through direct transfer via root and shoot grafts, mycorrhizal fungi or haustorial connections of parasitic vascular plants (Rice, 1984). Once released into the soil, these chemicals have the potential to positively or negatively affect the environment (soil structure, nutrient availability) and the resident organisms in an exposed area.

Essential oils and their constituents tend to have minimal mammalian toxicity. Some pure essential oil components are slightly toxic, with rat acute oral LD_{50} values of 2-3 g/Kg (carvacrol, pulegone), but many of the essential oils and their constituents are commonly used as culinary herbs and spices. Pesticide products containing essential oils are exempt from toxicity data required by the US EPA. Static water toxicity tests using juvenile rainbow trout (*Oncorhynchus mykiss*) indicated that, based on 96 h-LC_{50} values, eugenol is approximately 1500 times less toxic than the botanical insecticide pyrethrum, and 15,000 times less toxic than the organophosphate insecticide azinphosmethyl (Stroh et al., 1998; Table 1-2).

Many of these compounds do not persist in fresh water or in soil: the half-life for α-terpineol ranges from 30-40 h at 23^0C, with complete degradation by 50 h (Misra and

Pavlostathis, 1997). Eugenol is completely broken down to common organic acids by soil-borne bacteria (Rabenhorst, 1996). Concerns for residues of essential oil pesticides on food crops should be mitigated by the growing body of evidence that many essential oil constituents acquired through the diet are actually beneficial to human health (Huang et al., 1994). Low mammalian toxicities and rapid breakdown have spurred their development as a new realm of insecticides.

1.5 Mode of action of plant-derived compounds

Although plant-derived compounds have been demonstrated to have antifeedent, growth retardant, repellent and acute toxicity effects, their modes of action are not well characterized (Enan, 2001; Kostyukovsky et al., 2002). It has been suggested that the lipophilic fraction of volatile oils bind to the lipid components of cell membranes, and modify the activity of the calcium ion channels (Svoboda and Hampson, 1999). At higher doses volatile oils saturate the membranes and show effects similar to those of local anesthetics. They can interact with the cell membranes by means of their physiochemical properties and molecular shapes, and can influence enzymes, ion channels and receptors (Svoboda and Hampson, 1999). Some of the physiological effects of essential oils on humans include brain stimulation, anxiety-relieving, sedation, and antidepressant activities, as well as increasing the cerebral blood flow. Plant-derived compounds also may have effects on cognition, memory, and mood. The inhaled fragrant constituents of essential oils are able to cross the blood-brain barrier and interact with receptors in the central nervous system (Svoboda and Hampson, 1999). Bioassays used to describe and explain the action of volatile oils are usually carried out on mice, rats and toads e.g. the influence of peppermint oil on intestinal transport (Beesley et al., 1996); the ability of

volatile oils to penetrate skin (Abdullah et al., 1996); the effect of essential oils on skeletal muscle fibres (Fogaca et al., 1997); screening essential oils for analgesic properties (Aydin et al., 1996).

Although many essential oil compounds have been shown to be toxic or repellent to a range of insects, structure-activity studies have provided few, if any, insights into the mode of action of these compounds (Kostyukovsky et al., 2002). Treatment with certain essential oils or their purified constituents results in visible symptoms that suggest a neurotoxic effect (Enan, 2001). These symptoms include hyperactivity, convulsions and tremors followed by paralysis and are similar to those produced by organophosphate and carbamate insecticides (Kostyukovsky et al., 2002). Some plant-derived compounds, mainly monoterpenes, are competitive inhibitors of acetylcholinesterase (Kostyukovsky et al., 2002).

1.5.1 Effects on second messengers (cAMP)

Various target sites have been proposed for the activity of phytochemicals, and one such target site of essential oils is the insect octopaminergic system. Octopamine (OA) is a multi-functional, naturally occurring biogenic amine that plays a key role as a neurotransmitter, neurohormone and neuromodulator in invertebrate systems, with a physiological role analogous to that of norepinephrine in vertebrates (Enan, 2001). Octopamine modulates physiological functions such as the proboscis extension response (Cnaani et al., 2003), sting response (Schulz and Robinson, 2001), juvenile hormone release from the corpora allata (Rachinsky, 1994; Lorenz and Gade, 2009), and the discrimination of nestmates from unrelated bees (Robinson et al., 1999; Ismail et al., 2008).

The physiological function of OA is mediated by the different subclasses of OA receptors found in different tissues and species (Enan, 2001; Blenau and Baumann, 2001; Kostyukovsky, 2002), which are coupled to different second messenger systems (Blenau and Baumann, 2001). Four different classes of octopamine receptors (OCT-1, -2A, -2B and -3) have been identified (Blenau and Baumann, 2001).

OCT-1 receptors modulate the myogenic rhythm of contraction in the locust extensor-tibiae via changes in intracellular calcium concentrations. OCT-2A and OCT-2B receptors are mediated via the activation of adenylate cyclase activity. More recently, octopamine receptors that mediate changes in cyclic AMP (cAMP) levels in the locust central nervous system have been distinguished pharmacologically as OCT-3 receptors. Because the OCT-2 receptor subtype does not exist in vertebrates, these receptors represent potential targets for the development of insecticides with low/no vertebrate toxicity (Enan, 2001).

OA binds to specific membrane protein receptors that belong to a superfamily of G-protein-coupled receptors (GPCRs) (Roeder and Nathanson, 1993). Activated GPCRs transmit signals to intracellular trimeric GTP-binding (G) proteins. Activated G proteins either stimulate or inhibit specific target proteins, which causes changes in the concentration of intracellular second messengers such as cAMP. Activated second messenger-dependent enzymes modify the functional properties of various cytosolic, membrane-bound, or nuclear proteins. G protein subunits may also regulate ion channel activity directly (Blenau and Baumann, 2001). Depending on the type of GPCR that is activated, an increase or decrease in the intracellular concentration of cAMP and/or Ca2+ takes place. If the receptor binds to a Gs-type (=stimulatory) receptor, the activated Gαs

subunit will interact with adenylase cyclase in the plasma membrane leading to an increase in cyclase activity and production of cAMP. Increased cAMP activates cAMP-dependent protein kinase (PKA) whose phosphorylation of serine and/or threonine residues may modify substrate molecules including cytosolic proteins, ligand-gated and voltage dependent ion channels, and transcription factors (Blenau and Baumann, 2001). Several biogenic amine receptors inhibit adenyl cyclase activity. This effect is mediated by interaction of the receptor with inhibitory G protein (Gi). Interaction of adenylyl cyclase with activated Gαi subunits most likely competes with binding of activated Gαs subunits and thereby interferes with cyclase activation (Blenau and Baumann, 2001). cAMP levels affect cardiovascular (Frank and Kranias, 2000), nervous system (Tasken and Aandahl, 2004), immune response (Latour and Veillette, 2001), and cell growth and differentiation functions, (Tasken and Aandahl, 2004). Any interruption in cAMP production may interfere with general insect homeostasis and may altering the pathogenicity and virulence of disease organisms or toxins.

1.5.2 Effects on host biotransformation enzyme systems

All xenobiotics are dealt with by the insect's detoxification response. The type of change that occurs depends on the species, chemical structure of the compound, and site of entry. Some very polar or insoluble compounds may be excreted unchanged. Four major types of chemical changes can occur: oxidation, reduction, hydrolysis, and synthesis of new compounds (Williams, 1959). The two major steps of detoxification of xenobiotics are a primary step involving oxidative, hydrolytic, and other enzymatic processes to produce polar end-products (nonsynthetic process), and a secondary phase producing water-soluble conjugates (synthetic process). A variety of oxidases, reductases,

esterases, epoxide hydrolases, and group transferases are used by insects to detoxify and eliminate toxic phytochemicals (Skrinjaric-Spoljar et al., 1971). Three major enzyme systems are used by plant feeding insects to deal with potentially lethal compounds.

1.5.2.1 Mixed function oxidases (MFOs).

The NADPH-requiring general oxidation system is commonly referred to as the mixed-function oxidase system. MFOs are located in the microsomal portions of various tissues and require NADPH as a cofactor (Feyeresen, 1999). MFOs oxidize many different substrates (i.e. substrate nonspecificity) (Feyeresen, 1999). A key component of this broad-spectrum oxidation system is a family of hemoproteins called cytochrome P450 that binds with oxygen and the substrates. A component of the oxidase system is NADPH-cytochrome-c-reductase, a flavoprotein that mediates the transport of electrons from NADPH, oxidizing cytochrome P450, which then binds to substrates. The cytochrome P450 complex comprises many hemoproteins, each of which possesses a different substrate preference, allowing the system as a whole to handle many types of xenobiotics. P450s also play a significant role in the biosynthesis of biosignal molecules and secondary metabolites, the adaptive assimilation of unusual carbon sources (Yoshida and Aoyama, 1994) and mediate many oxidative reactions involved in the biosynthesis of secondary metabolites such as flavonoids, terpenoids, alkaloids and antibiotics (Boyer et al., 2006). P450s also are highly involved in the development of resistance to insecticides (Enayati et al., 2005).

1.5.2.2 Glutathione transferase

Glutathione S-transferases (GSTs) belong to a superfamily of enzymes found in arthropods as well as mammals (Enayati et al., 2005). The primary function of GSTs is to

detoxify endogenous and xenobiotic compounds either directly or via secondary metabolism of compounds oxidized by the P450s (Zhu et al., 2007). GSTs catalyze the conjugation of glutathione to hydrophobic substrates such as herbicides and insecticides to facilitate their removal (Yu, 1996; Yu, 1993; Wadleigh, 1988; Hodnick et al., 1996; Zaman et al., 1994). In these reactions one molecule of reduced GST binds to a substrate to form a thioester (Enayati et al., 2005). The activation of the thiol group of GST initiates subsequent nucleophilic attack by the anionic glutathione on the bound hydrophobic compound (Enayati et al., 2005; Atkins et al., 1993). The overall reaction protects cellular components, and renders the products more water soluble and therefore more readily excreted (Enayati et al., 2005).

Insect GSTs induce the expression of other detoxifying enzymes, which enhances the overall defense response, which may contribute to the development of insecticide resistance (Carlini et al., 1995; Hinkle et al., 1995). Much research on insect GSTs has focused on their role in initiating or maintaining insecticide resistance (Boyer et al., 2006); high levels of GST activity and GST dependent metabolism in *Musca domestica* and *Lygus lineolaris* is directly correlated with insecticide resistance (Zhu et al., 2007).

1.5.2.3 Esterases

Esterases are enzymes that catalyze the hydrolysis of carboxylic, thio-, phospho-, and other ester substrates to their component alcohols or acids via the addition of water. In insects, esterases function in proteolysis, nervous system function, hormone metabolism, and xenobiotic metabolism (Flores et al., 2005). Insect esterases are found in both soluble and membrane-bound forms (Maa and Liao, 2000). The carboxyl esterases of several insect species have been studied extensively because of their involvement in

the resistance to carbamte and organophosphate insecticides (Flores et al., 2005; Elizabeth et al., 2004).

1.6 Electrophysiological techniques to detect mode of action of insecticides

Classical electrophysiology techniques involve placing electrodes into various preparations of biological tissue and recording electrical activity. The principal types of electrodes are:

1) simple solid conductors, such as discs and needles (singles or arrays, often insulated except for the tip)

2) tracings on printed circuit boards, also insulated except for the tip

3) hollow tubes filled with an electrolyte, such as glass pipettes filled with potassium chloride solution or another electrolyte solution.

Small electrodes may be inserted into a single cell, allowing for direct observation and recording of the intracellular electrical activity. Such invasive procedures reduce the life of the cell and cause a leak of substances across the cell membrane. Intracellular activity also may be observed using a specially formed (hollow) glass pipette containing an electrolyte. In this technique, the microscopic pipette tip is pressed against the cell membrane, to which it tightly adheres by an interaction between glass and lipids of the cell membrane (Miller, 1979). The electrolyte within the pipette may be brought into fluid continuity with the cytoplasm by delivering a pulse of pressure to the electrolyte in order to rupture the small patch of membrane encircled by the pipette rim (whole-cell recording).

Rather than inserting the electrode into a cell, the tip may remain in the extracellular space. If the tip is small enough, such a configuration may allow indirect observation and recording of action potentials from a single cell, and is termed single-unit recording. Depending on the preparation and precise placement, an extracellular configuration may pick up the activity of several nearby cells simultaneously, termed multi-unit recording. As electrode size increases, the resolving power decreases. Larger electrodes are sensitive only to the net activity of many cells, termed local field potentials. Still larger electrodes, such as uninsulated needles and surface electrodes used by clinical and surgical neurophysiologists, are sensitive only to certain types of synchronous activity within populations of cells numbering in the millions. Other classical electrophysiological techniques include single channel recording and amperometry.

1.6.1 Electrophysiological recordings from moving insects

I used this technique to record the electophysiological responses of blowflies treated with plant derived compounds. In this method electrical activity of nerves or muscles were recorded by inserting electrodes into specific muscles or tissues. Initially we measured the neuromuscular activity of control blowflies to establish baseline neurophysiological parameters. The easiest and most accessible neuromuscular system to measure is that of flight (Miller, 1979). Adult insects can be held and flown in static air, and flight behavior can be analyzed. Similar studies have used the locust (Camhi, 1970; Wilson, 1968), moths (Kammer, 1971; Obara, 1975), flies (Nachtigall and Wilson, 1968; Mulloney, 1970; Heide, 1975) and bees (Bastian, 1972). Some motor output activity in insects is strongly influenced by ascending sensory information and other motor activity

is slightly affected or not affected all by sensory information (Delcomyn, 1973). Therefore the ability to record sophisticated nervous activity from insects, which are intact and performing near normal functions, is important (Miller, 1979). For this reason, we conditioned all the blowflies before they were used in the bioassay.

The indirect dorsoventral flight muscles of the blowfly *Phaenicea sericata* are arranged vertically and the dorsolongitudinal flight muscles are arranged horizontally. Both types of indirect flight muscle are of the fibrillar type and consist with large multinucleate muscle cells (Miller, 1979). Each of the left and right dorsolongitudinal muscles comprises six fibrillar muscle fibers (Miller, 1979). The position of the dorsolongitudinal muscles can be determined easily by the position and pattern of bristles on the dorsum of the thorax, which allow us to locate the recording muscles (dorsolongitudinal muscle 4 and 5) and insert the electrodes directly into the correct location (Fig. 1-6).

Copper recording electrodes, 50 μm diameter, were inserted into the left and right dorsolongitudinal muscles (DLM5) from the anterior of the pterothorax. A common reference ground electrode was inserted between the left and right dorsolongitudinal muscles 4. Muscle insertions were located by referencing their locations to prominent dorsal setae (Adams and Miller, 1980). Once positioned correctly, the electrodes did not hamper the insects from resting on the substrate or flying freely. Electrical impulses from flight muscles (FMI) were fed into a differential preamplifier with an input impedance of 100MΩ and a gain of 100 and frequency response of 100 KHz (Fig. 1-7). Output from the preamplifier was recorded on a NI USB-6008 data logger (National Instruments, Vaudreuil-Dorion, Quebec, Canada) (indicated as DAQ in Fig. 1-7). Simultaneously,

acoustic signals from wing beats of the blowfly (WBS) were recorded with a miniature microphone (Realistic 33-1052) connected to an amplifier speaker (Archer Model 277-1008 B, Taiwan) and the signal was recorded by the same data logger (Fig. 1-7).

1.7 Study compounds

Initially we selected 16 different plant-derived compounds to assess their toxicity to larvae and adults of *Aedes aegypti* (see Chapter 2). We used these data to select a subset of 5 compounds (Fig 1-8) that showed significant effects on the insects for further evaluation using the electrophysiological apparatus.

1.8 Thesis goals and approaches

The objective of my doctoral studies is to study the activity and identify the mode of action of plant-derived compounds. Although data on the acute toxicity and sublethal effects of phytochemicals have been available for many years, few studies have addressed their modes of action. If these compounds are to be developed as novel insecticides it is paramount that we understand their mode of action and effect on non-target organisms. We used *Aedes aegypti* as the model organism in acute toxicity studies. This mosquito species is the main vector of dengue viruses, which cause significant human mortality and morbidity; over 100 million new cases of dengue occur worldwide each year (Gubler and Clark, 1995). We also used the blowfly (*Phaenicia sericata*) as a more robust insect into which we could place electrodes and measure, with our electrophysiologcal apparatus, the effects of the various compounds on electrical impulses, wingbeat frequencies, and other parameters in these insects in response to the

topical application of our phytochemicals and available insecticides for which the mode of action is well known.

In Chapter 2 we describe acute toxicity studies on *Aedes aegypti* and sublethal effects on adult behaviour and oviposition after exposure to 16 plant based compounds. Based on these data we selected 5 compounds for further evaluation.

In Chapter 3 we measured the effect of these phytochemicals and the enhanced toxicity of the compounds in the presence of the synergist piperonyl butoxide, on the production and activity of biotransformational enzymes that should eliminate or detoxify lethal compounds.

In Chapter 4 we measured the effects of 5 compounds selected in Chapter 2 on flight motor activity and wingbeat frequency using the blowfly, *Phaenicia sericata*, as our model target.

In Chapter 5, we characterized the electrophysiological responses of eugenol, pulegone, α-terpineol and citronellal and compared these with the sodium channel blocker RH3421.

In Chapter 6 we evaluated the effects of our 5 selected phytochemicals on the octopamingenic system as determined by their effects on the production of secondary messenger signals (cAMP) that normally would break down the phytochemicals.

Chapter 7 summarizes the major findings of the thesis and the avenues that this research has opened for discussion and further research.

1.9 Figures

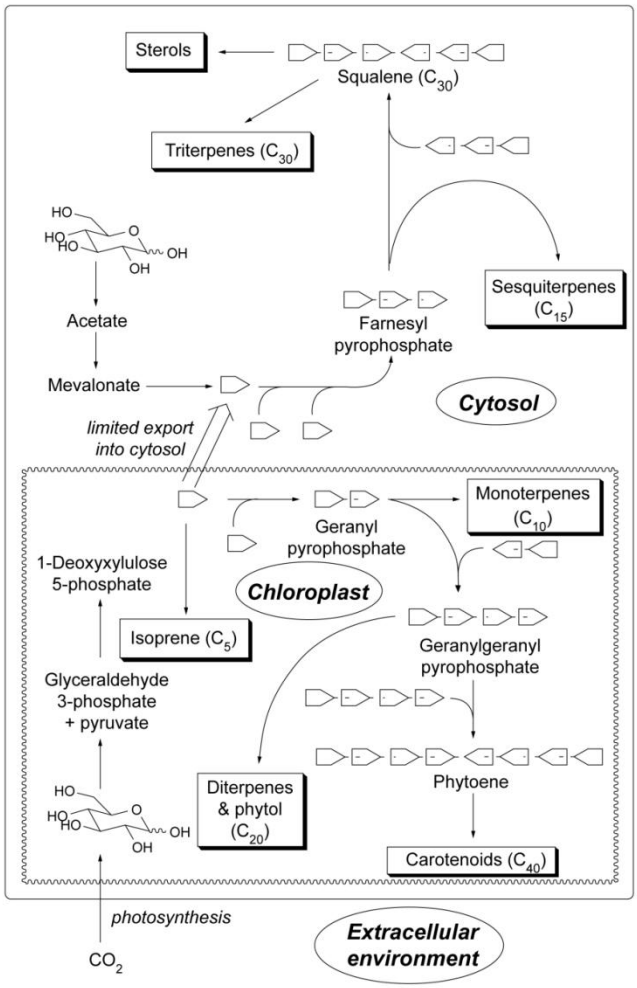
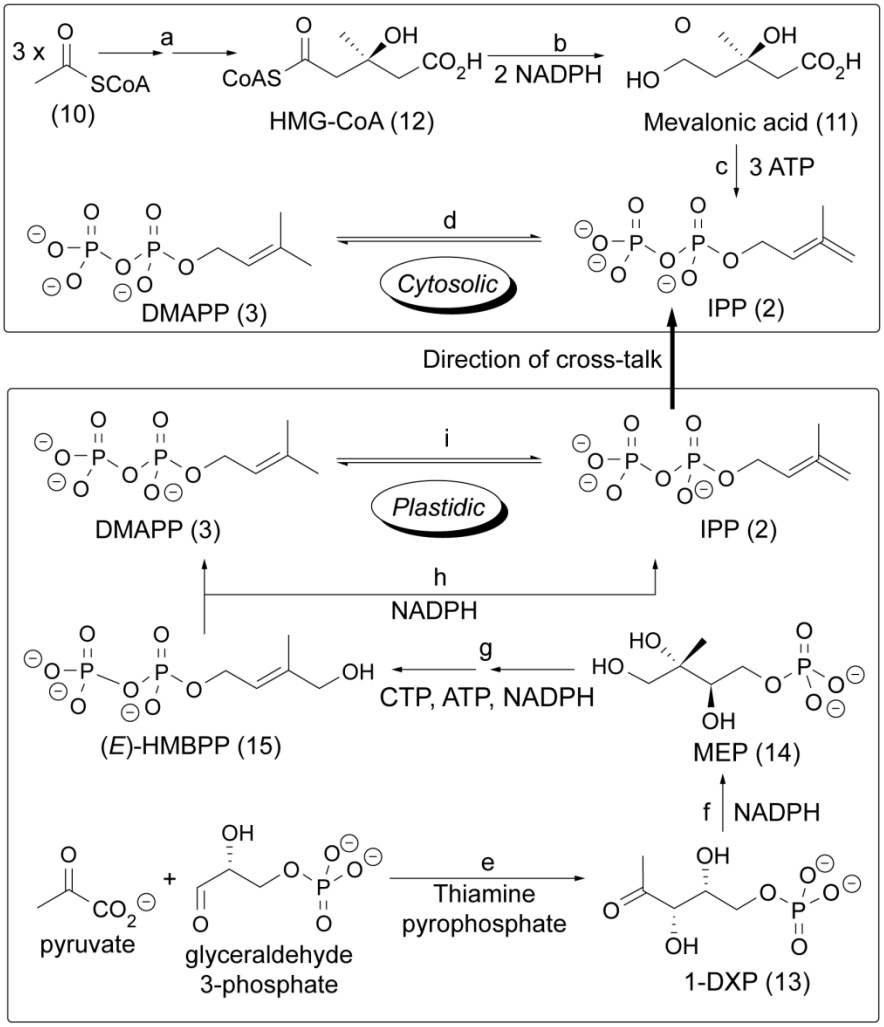


Figure 1-1 Schematic representation of terpene biosynthesis in higher plants.
Blocks (⬠) represent 5-carbon isoprenyl units (IPP or DMAPP) (Reprinted from Plant Secondary Metabolites (2006), A. Crozier, M.N. Clifford and H. Ashihara (Editors) with the permission of Wiley-Blackwell Publishers, UK).



(**a**) acetoacetyl-CoA thiolase, HMG-CoA synthase; (**b**) HMG-CoA reductase; (**c**) mevalonate kinase, phosphomevalonate kinase, mevalonate 5-phosphate decarboxylase; (**d**) IPP isomerase; (**e**) 1-deoxyxylulose 5-phosphate synthase; (**f**) 1-deoxyxylulose 5-phosphate reductoisomerase;(**g**) CDP-methylerythritol synthase, CDP-methylerythritol kinase, methylerythritol cyclodiphosphate synthase, (*E*)-4-hydrosy-3-methyl-2-butenyl diphosphate synthase; (**h**) IPP/DMAPP synthase; (**i**) IPP isomerase (plastidic)

Figure 1-2 Biosynthesis of IPP and DMAPP in higher plant cells**.**
(Reprinted from Plant Secondary Metabolites (2006), A. Crozier, M.N. Clifford and H. Ashihara (Editors) with the permission of Wiley-Blackwell Publishers, UK).

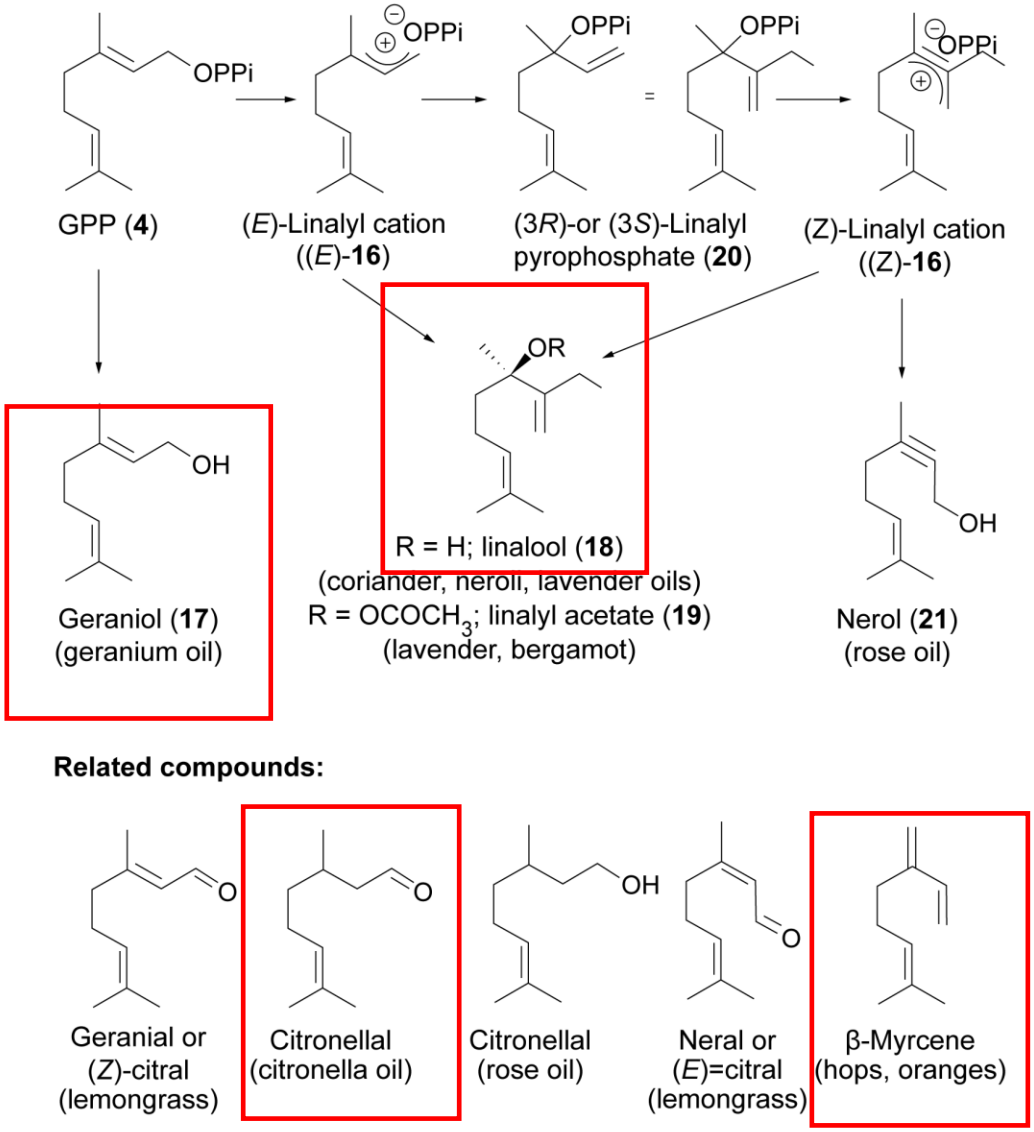


Figure 1-3 Monoterpenes: the geraniol/linalool family.
(Reprinted from Plant Secondary Metabolites (2006), A. Crozier, M.N. Clifford and H. Ashihara (Editors) with the permission of Wiley-Blackwell Publishers, UK). Compounds utilized in this thesis are boxed in red.

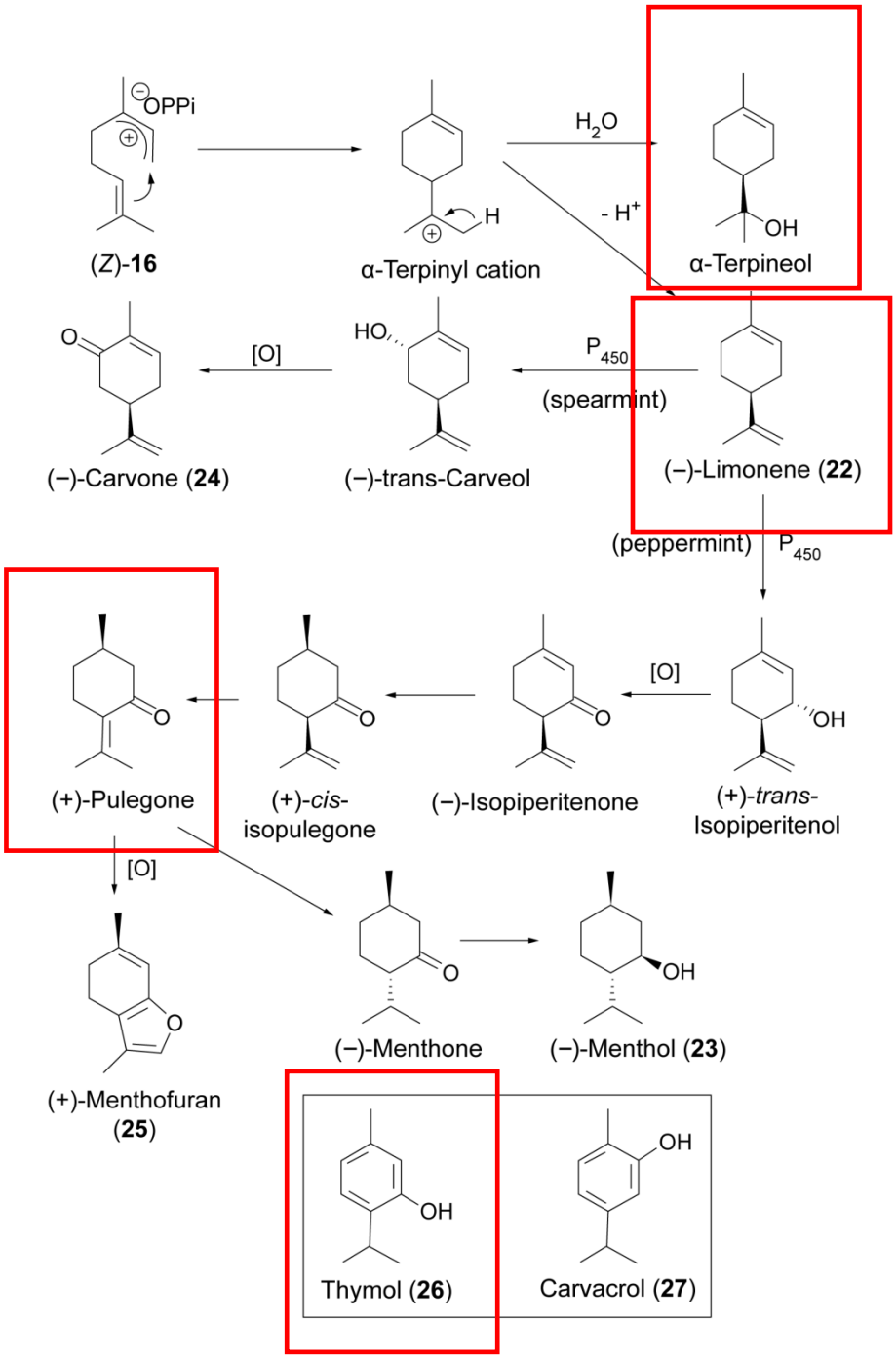


Figure 1-4 Monoterpenes: the menthane family.
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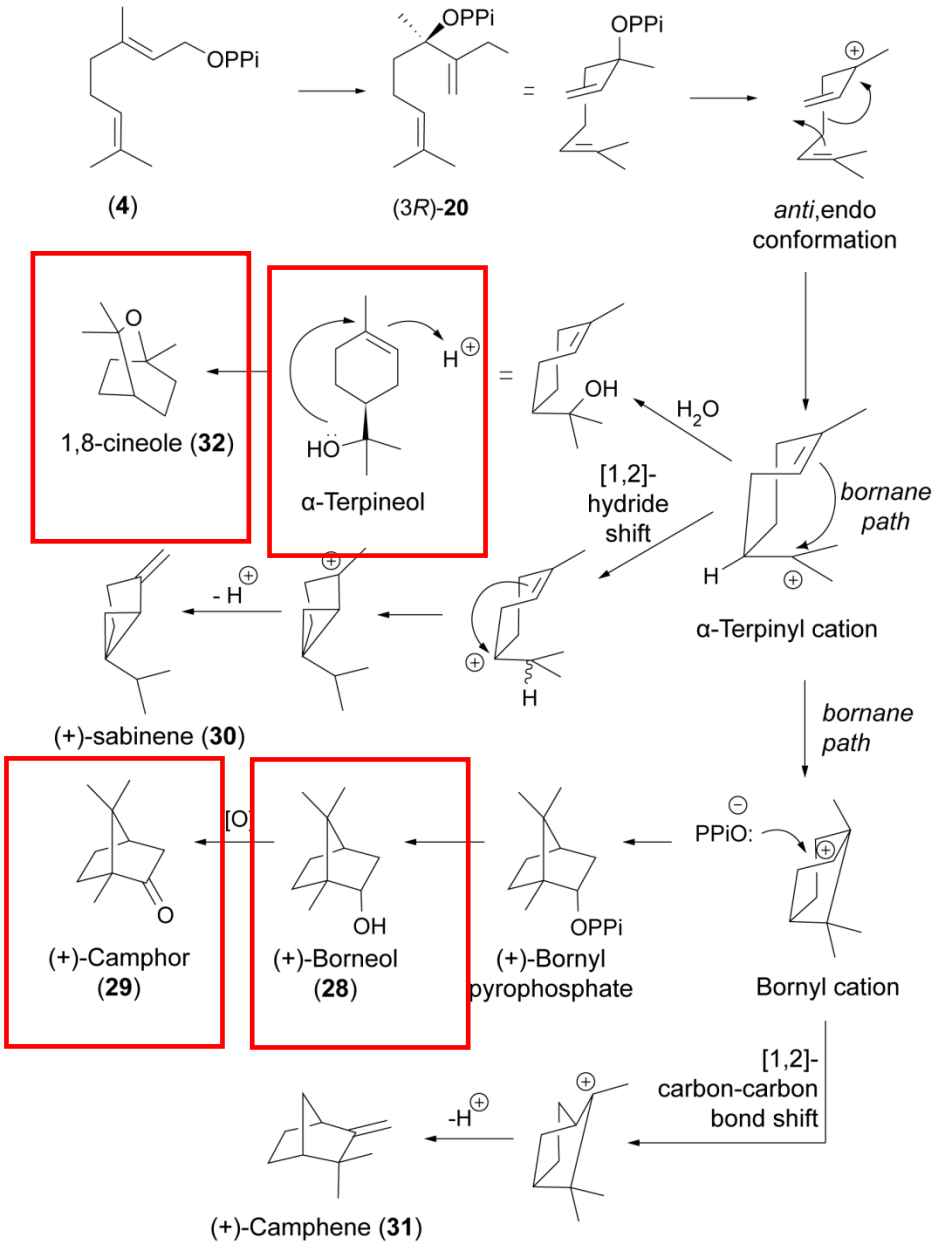

Figure 1-5 Monotepenes: the (+)-bornane family.
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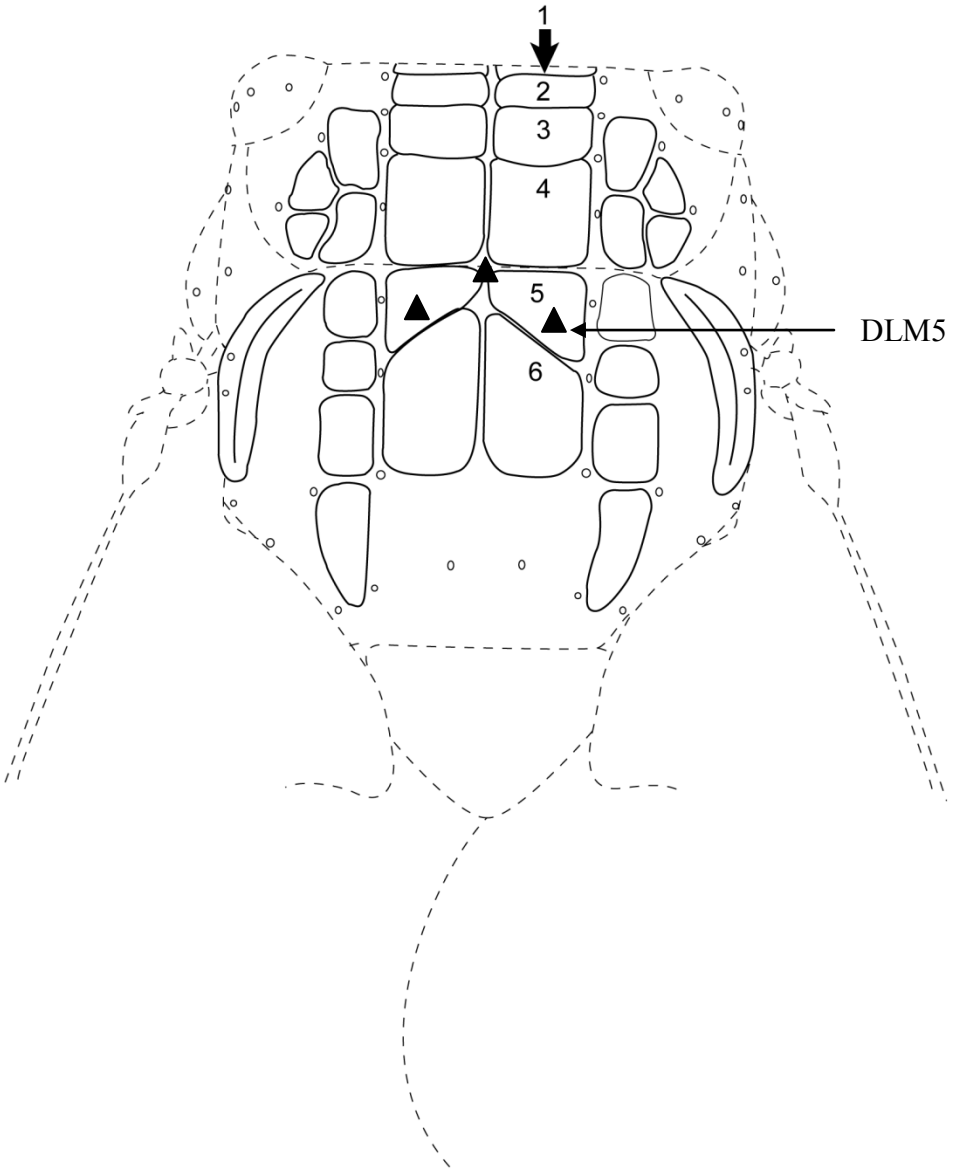


Figure 1-6 Representation of the dorsologitudinal flight muscles (DLM 1-6) in the thorax of *Phaenicia sericata*.
The ▲ indicates the position of the electrodes. Electrodes were inserted into the left and right DLM5 to measure changes in muscle impulses after the application of phytochemicals.

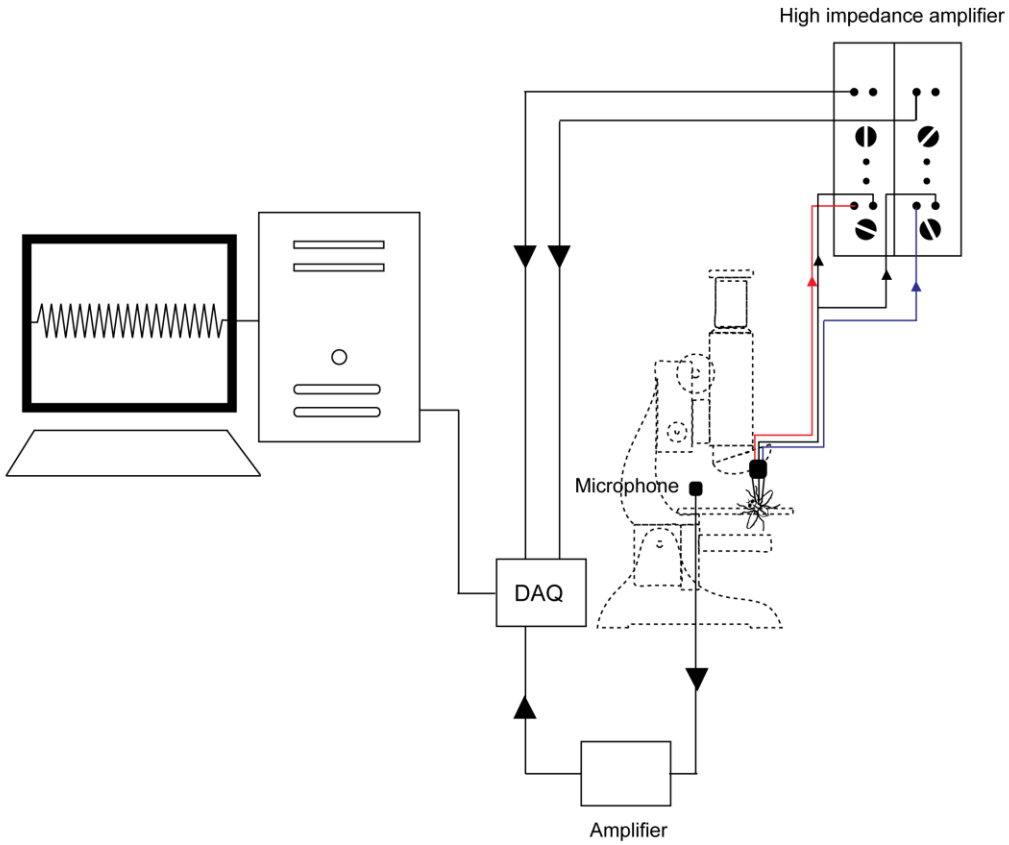
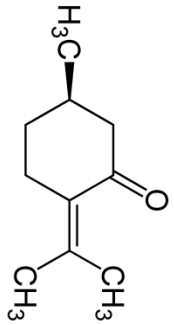
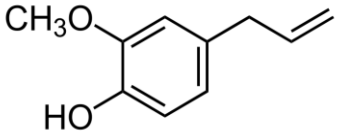


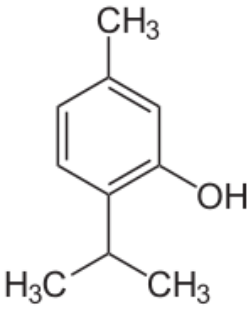
Figure 1-7 Electrophysiological apparatus used to measure flight muscle impulses (FMI) and wingbeat sounds (WBS) in blowflies (*Phaenicia sericata)* treated with test compounds.
FMI from the left and right flight muscles are captured by a high impedance amplifier. The analog data from the amplifier are directed to the Data Acquisition Unit (DAQ) where they are converted to digital data. These data are captured by the computer for analysis with LABVIEW 8.5 software. Simultaneously, sounds (WBS) are recorded by the microphone, transferred to an amplifier, and then to the DAQ and computer for analysis with LABVIEW 7.0 software.



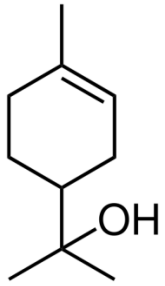
Thymol



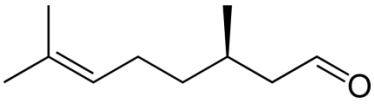
Eugenol



Pulegone



α-terpineol



Citronellal

Figure 1-8 Chemical structures of 5 selected compounds for evaluation of toxicity, and for studying their topical application on the electrophysiological responses of insects after topical application.

1.10 Tables

Table 1-1 Comparison of some plant based chemicals in terms of target, effect, and delivery system reported in the literature.

Compound	Target species	Effect	Delivery	Reference	Compound
α-Pinene	*Culex pipiens* *Botrytis cinerea* *Tribolium castaneum,* *Sitophilus zeamais*	Larvicidal Antifungal Antifeedent, Growth inhib	Solution Topical, Ingestion	Traboulsi et al., 2002. Wilson et al., 1997. Huang et al., 1998. Lancelle et al., 2009.	Pure (1S+1R) Mixture Mixture Mixture
β-Pinene	*Botrytis cinerea*	Antifungal	Topical	Wilson et al., 1997.	Mixture
Borneol acetate	*Aedes aegypti*	Larvicidal	Contact	Waliwitiya et al., 2009.	Pure
Borneol	*Aedes aegypti*	Larvicidal	Contact	Waliwitiya et al., 2009.	Pure
Camphor	*Botrytis cinerea*	Antifungal	Topical	Wilson et al., 1997.	Mixture
Cineol	*Culex pipiens* *Botrytis cinerea*	Repellent Antifungal	Topical Topical	Traboulsi et al., 2005. Wilson et al., 1997.	Pure (1S+1R) Mixture
Citronellal	*Musca domestica* *Diabrotica virgifera*	Insecticidal Insecticidal	Topical Topical	Lee et al., 1997. Lee et al., 1997.	Pure Pure
Eugenol	*Periplanata americana*	Insecticidal	Topical, Fumigant	Ngoh et al., 1998.	Pure
Linalool	*Bactcera dorsalis, B. cucurbitae*	Insecticidal	Topical	Chang et al., 2009.	Pure
Myrcene	*Botrytis cinerea*	Antifungal	Topical	Wilson et al., 1997.	Mixture
p-Cymene	*Culex pipiens*	Repellent	Contact	Park et al., 2005.	Pure
Pulegone	*Musca domestica*	Insecticidal	Topical	Palacios et al., 2009.	Mixture
Rosemary oil	*Neotoxopteraf ormosana* *Myzus persicae*	Repellent Repellent	Topical Topical	Masatoshi and Hiroaki, 1997. Masatoshi 1998.	Mixture Mixture
trans-Anethole	*Bactocera dorsalis, B. cucurbitae*	Insecticidal	Topical	Chang et al., 2009.	Pure
Terpineol	*Culex pipiens* *Acanthoscelides obtectus* *Musca domestica* *Ostrinia nubilalis*	Repellent Insecticidal Insecticidal Growth inhib Antifeedent	Topical, Fumigant Ingestion Ingestion	Traboulsi et al., 2005. Regnault et al., 1993. Lee et al., 1997. Sharma&Saxena, 1974. Lee et al., 1999.	Pure Mixture Mixture Pure Pure
Thymol	*Culex pipiens* *Musca domestica* *Tetranychus urticae* *Spodoptera liteura*	Larvicidal Insecticidal Insecticidal Repellent	Solution Topical Topical Topical	Traboulsi et al., 2002. Lee et al., 1997. Gengaihi et al., 1996. Isman et al., 2001.	Mixture Mixture Mixture Pure
Azadirachtin / Neem	*Anthonomus grandis grandis* *Melanotus communis* *Nasonovia ribis-nigri, Myzus persicae*	Feeding, Oviposition deterrent, Repellent Growth inhibition Insecticidal	Ingestion, Contact Ingestion	Showler et al., 2004. Cherry and Nuessly, 2010. Lowery and Isman, 1994.	Mixture Mixture Mixture
Nicotene	*Helicoverpa annulipes* *Cotesia congregata*	Growth inhib Larval eclosion	Ingestion Ingestion	Barbosa et al., 1985. Barbosa et al., 1985.	Mixture Mixture
Pyrethrum	*Musca domestica*	Insecticidal	Topical	Sawicki 1962.	Mixture
Rotenone	*Ae. aegypti*	Larvicidal	Contact	Yenesew et al., 2003.	Mixture

Table 1-2 Toxicity of selected compounds against juvenile rainbow trout in static water tests (modified from Stroh et al., 1998).

Compound/Product (% active ingredient)	**96h LC_{50} (ppm)**
Eugenol (90%)	60.8
Thyme oil (90%)	16.1
alpha-terpineol (90%)	6.6
Emulsifier	18.2
Neem (3% azadirachtin)	4
Pyrethrum (20% pyrethrin)	0.04
Rotenone (44%)	0.03
Azinphosmethyl (94%)	0.004
Endosulfan (96%)	0.001

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Connecting statement 1

In the introductory chapter, I presented the background and rationale for my thesis and described the use of plant-derived compounds in insect pest control and the current knowledge on the modes of action of plant-derived compounds. In the next chapter I describe the acute toxicities and oviposition-altering activity of 16 plant-derived compounds on *Aedes aegypti*.

Chapter 2: Larvicidal and Oviposition-Altering Activity of Monoterpenoids, *trans*-Anithole and Rosemary Oil to the Yellow Fever Mosquito *Aedes aegypti* (Diptera: Culicidae)

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2.1 Abstract

Aedes aegypti L. is the major vector of dengue fever and dengue hemorrhagic fever. In an effort to find effective tools for control programs to reduce mosquito populations, the authors assessed the acute toxicities of 14 monoterpenoids, *trans*-anethole and the essential oil of rosemary against different larval stages of *Ae. aegypti*. The potential for piperonyl butoxide (PBO) to act as a synergist for these compounds to increase larvicidal activity was also examined, and the oviposition response of gravid *Ae. aegypti* females to substrates containing these compounds was evaluated in behavioral bioassays.

Pulegone, thymol, eugenol, *trans*-anethole, rosemary oil and citronellal showed high larvicidal activity against all stages of *Ae. aegypti* (LC_{50} values 10.3–40.8 mg L^{-1}). The addition of PBO significantly increased the larvicidal activity of all test compounds (3–250-fold). Eugenol, citronellal, thymol, pulegone, rosemary oil and cymene acted as oviposition deterrents and/or repellents whereas borneol, camphor and *β*-pinene increased the number of eggs laid in test containers.

This study quantified the lethal and sublethal effects of several phytochemicals against all larval stages of *Ae. aegypti*, providing information that ultimately may have potential in mosquito control programs through acute toxicity and/or the ability to alter reproductive behaviors.

2.2 Introduction

Mosquitoes are the vectors of important human pathogens, including those responsible for causing malaria, dengue, filariasis and yellow fever (Paul et al., 2006). Malaria, with 300–500 million new cases annually and approximately 2.5 million annual deaths (Lowenberger et al., 1999) is one of the most devastating diseases affecting humans. *Aedes aegypti* (L.) is the main vector of dengue viruses, which cause more human mortality and morbidity than any other arthropod-transmitted viral disease, and rank second only to malaria among mosquito-transmitted infections (Paul et al., 2006). An estimated 2.5 billion people are at risk, and over 100 million new cases of dengue occur worldwide each year (Gubler, 2003).

Vector control using insecticides has been the primary means of reducing dengue virus transmission (Rodriguez et al., 2001), but wide-scale applications of synthetic pesticides can lead to environmental contamination and adverse effects on non-target species, including humans (Chaton et al., 2002; Schulze et al., 2001). In addition, *Ae. aegypti* has developed resistance to organochlorine, organophosphate, carbamate and pyrethroid insecticides in many regions of the world (WHO, 1986; Rawlins and Wan 1995; Georghiou et al., 1997; Rawlins, 1998; Rawlins and Ragoonansingh, 1999), which has hindered control efforts. Plant-based chemicals, or phytochemicals, have been used for many years to control insect pests on agricultural crops (Lee et al., 1997). Their insecticidal, fungicidal, bactericidal, antiviral, antifeedant or insect growth retardant properties (Benner, 1993; Singh et al., 1989; Wilson et al., 1997) often are the result of synergistic interactions among different biologically active constituents such as terpenoids, alkaloids and phenolics (Panella et al., 2005). Phytochemicals degrade

rapidly, are unlikely to persist in soil and leach into groundwater (Isman, 1999), often have a reduced impact on non-target populations and are important components of integrated pest management systems used by organic farmers (Isman, 1999). Many of the active components of phytochemicals are termed essential oils, which refers to the steam-distillable fraction of plant tissues that often are responsible for their characteristic scent or odour (Isman, 1999). Essential oils such as citronella have become popular alternative compounds to N,N-diethyl-m-toluamide (DEET) for personal protection against mosquitoes and other biting flies (Isman, 1999; Karr and Coats, 1988). While essential oils have been demonstrated to have antifeedant (Hough-Goldstein, 1990), growth inhibitor (Sharma and Saxena, 1974) or repellent and toxic effects against various insects (Gengaihi et al., 1996; Isman et al., 2001; Masatoshi and Hiroaki, 1997; Masatoshi, 1998), their modes of action are not well characterized (Enan, 2001). Most insects treated with essential oils display characteristic neurotoxic symptoms including agitation, hyperactivity, paralysis and quick knockdown (Coats et al., 1981; Brattsten, 1983; Grundy and Still, 1985; Isman, 1999). Neem-based products have been used to suppress blood feeding, reduce oviposition and inhibit larval growth in *Culex tarsalis* Coquilles, *Culex quinquefasciatus* Say (Su and Mulla, 1998) and *Ae. aegypti* (Boschitz and Grunewald, 1994). Marigold extract (an essential oil) is lethal to larvae and adults of *Ae. aegypti* and *Anopheles stephensi* Liston (Perich et al., 1994), while some plant essential oils demonstrate oviposition deterrent activities in mosquitoes (Tawatsin et al., 2006). In order to enhance the toxicity of specific compounds to target organisms, some commercial insecticides contain synergists. Piperonyl butoxide (PBO) has been used widely as a synergist to enhance the efficacy of natural pyrethrins and synthetic

pyrethroids (Casida, 1970; Jao and Casida, 1974). PBO is a well-known inhibitor of microsomal monooxygenases which are involved in the metabolism and detoxification of many insecticides (Feyereisen, 1999; Hemingway and Ranson, 2000; Kumar et al., 2002) and thus its use limits the ability of insects to biotransform and detoxify these compounds. In this study, the authors investigated the toxicity of 14 structurally different monoterpenoids, *trans*-anethole and one complex essential oil (rosemary oil from *Rosemarinus officinalis* L., family: Lamiaceae) on first through fourth larval instars of *Ae. aegypti* with the following specific objectives: (1) to quantify the acute toxicities of these compounds to *Ae. aegypti* larvae; (2) to evaluate the possible synergistic effects of PBO on the larvicidal activity of selected compounds; (3) to evaluate selected compounds for their ability to modify the ovipositional activity of *Ae. aegypti*.

2.3 Materials and methods

2.3.1 Insects

Aedes aegypti larvae and adults were raised and maintained as described previously (Lowenberger et al., 1999) at 27^0C and 80–85% relative humidity under a 14 : 10 h light : dark cycle. Adults were provided with a 10% sucrose solution adlibitum. Larvae were raised at densities of 100 larvae L^{-1} distilled water and fed with ground Nutrafin Basix fish food (Rolf C Hagen Inc., Montreal, QC). All bioassays were conducted in a walk-in environmental chamber with these environmental conditions.

2.3.2 Chemicals

1,8-Cineole (95% purity, source *Eucalyptus globules* Labill.), linalool, pulegone and *trans*-anethole (>95% purity) were obtained from Ecosafe Natural Products

(Victoria, BC). Eugenol, p-cymene, bornyl acetate, camphor (>98% purity), α-pinene, β-pinene, α-terpineol, citronellal, thymol, camphene, rosemary oil (>95% purity) and PBO (90% purity) were purchased from Sigma Aldrich (St Louis, MO). Stock solutions of the chemicals were made using aqueous acetone (10% v : v) as the solvent and stored in dark bottles. From the stock solutions, 5-, 10-, 20-, 50-, 100-, 200- and 500- mg L^{-1} treatment solutions were prepared using distilled water. In order to increase the solubility of *trans*-anethole, rosemary oil and the monoterpenoids, and to keep them from binding to test containers, 5 µL of Tween 20 (Uniqema, New Castle, DE) were added to 100 mL of each treatment solution as a solubilizing agent. Control treatments contained distilled water, acetone and Tween 20 in the same concentrations as the test solutions. In the synergist studies, 10 mg L^{-1} of piperonyl butoxide (PBO) was added to treatment solutions after preliminary experiments had indicated that this was the minimum sublethal concentration of PBO that could be used with Tween 20. Control treatments for PBO plus phytochemical solution contained distilled water, acetone and PBO in the same concentrations as the test solutions.

2.3.3 Larval bioassays

All bioassays were carried out in the environmental chamber in which the mosquitoes were raised. In each bioassay, 10 *Ae. aegypti* larvae from each larval instar stage were placed in 140 mL plastic cups (Sunfresh Ltd, ON) containing 50 mL of prepared treatment solution (as described in Section 2.2) and 0.1 g of ground Nutrafin Basix fish food. Cups were covered with mosquito mesh, and larvae were monitored for mortality at 24, 48, 72 and 96 h. Larvae were considered dead if they were immobile and unresponsive to touch with a probe, and dead larvae were removed. In experiments with

PBO, mortality was determined for up to 48 h of exposure because, in the previous experiment, all mortality occurred at time points <48 h. All bioassays were replicated 3 times.

2.3.4 Ovipositional activity bioassay

All oviposition assays were carried out in the environmental chamber under standard rearing conditions. The ovipositional response of adult mosquitoes to essential oils was examined using a binary choice design described previously (Lowenberger and Rau, 1994). Based on preliminary studies, a 20 mg L^{-1} concentration of each test chemical was used in all ovipositional assays. Solutions were prepared as described for acute toxicity bioassays. Two 6 cm diameter pyrex petri dishes containing either 30 mL of treatment solution or 30 mL of control solution (distilled water with acetone and Tween 20) were placed in a cage (45×23×15 cm) containing ten 3– to 4- day-old female and five male *Ae. aegypti*. Mosquitoes were blood fed by keeping the primary author's right hand on the mesh of the rearing cup for 5 min prior to release into the cage. Subsequently, they were blood fed by keeping the primary author's right hand inside the cage for 5 min every 2 days. Cumulative egg numbers were recorded in treatment and control dishes on days 3 and 5. Differential oviposition was measured in duplicate cages for each treatment solution, and each experiment was replicated a minimum of 3 times.

2.3.5 Analysis

Larvicidal bioassay data from three experiments were pooled and analyzed by standard probit analysis (Finney, 1964). LC_{50} values (concentrations that caused mortality in 50% of a sample population) were determined at 48 h because no further mortality

occurred after this time. LC_{50} values were considered to be significantly different ($P \leq 0.05$) from each other if the confidence intervals did not overlap. Abbott's formula (Abbott, 1925) was used to correct the mortality values. The synergistic ratio was calculated as (LC_{50} without PBO)/(LC_{50} with PBO). In oviposition assays, the numbers of eggs laid were counted on days 3 and 5. For each day, and for each cage, the total numbers were converted to proportions of eggs laid $cage^{-1}$ day^{-1}, transformed by arcsine square root and compared using a paired t-test as described previously (Lowenberger and Rau, 1994). Differences in oviposition were considered significant at $P \leq 0.05$. This analysis has been used previously to determine if an individual solution repels/deters or attracts oviposition by gravid females as compared with a control solution. The oviposition activity index (OAI) for each solution was also calculated to generate a global comparison among the test solutions, as described previously (Kramer and Mulla, 1979):

$$OAI = [(T - C)/T + C]$$

where T is the number of eggs collected from the treated dish and C is the number of mosquito eggs collected from the control dish.

2.4 Results

2.4.1 Larval bioassays

The susceptibilities of *Ae. aegypti* larvae to 14 different monoterpenoids, *trans*-anethole and one complex essential oil (rosemary oil) were examined. The range of concentrations used in these bioassays was sufficient to calculate LC_{50} values and their 95% confidence intervals (CIs) for 13 out of 16 (13/16), 5/16, 5/16 and 5/16 of the compounds for larval instars 1 to 4, respectively. For those compounds where LC_{50}

values could be calculated, all test compounds showed increasing mortality with increasing concentration against all four larval instar stages. The acute toxicities of selected compounds with and without PBO to different larval instars of *Ae. aegypti* are presented in Tables 2-1 and 2-2. The least toxic compounds, resulting in no mortality even at the highest concentration, were borneol acetate, camphor, cineol, linalool and myrcene. Rosemary oil (R. oil) was highly toxic to the first-instar larval stage (L1), but was not toxic at the highest concentration tested for L2–L4 stages. Pulegone, *trans*-anethole, thymol, eugenol and citronellal were consistently the most toxic compounds to all larval instars; however, these compounds possessed different levels of toxicity for different stages. The LC_{50} values of these five compounds against first- to fourth-instar larvae of *Ae. aegypti* increased with larval instars, as demonstrated in Tables 2-1 and 2-2. Linear regression analysis of LC_{50} values of these five compounds shows 3.5-, 4.8-, 5.4-, 6.4- and 13.4-fold reductions in toxicity against L4 as compared with L1 instar larvae for thymol, *trans*-anethole, pulegone, citronellal and eugenol, respectively. Exposure to PBO alone did not result in mortality of any larval stage of *Ae. aegypti*, but the addition of PBO to the test solutions significantly increased the larvicidal activity of all chemicals against *Ae. aegypti* larvae (Tables 2-1 and 2-2). The synergistic ratios varied from 3 to 250 for all the test chemicals. Interestingly, the range of LC_{50} values for all 14 different monoterpenoids, *trans*-anethole and rosemary oil against all instar stages was very narrow compared with the very wide range of LC_{50} values in the absence of PBO. For example, the range of values for non-PBO-treated first instars was from 10.3 to >500 mg L^{-1} and from 2 to 5.2 mg L^{-1} for first instars with the addition of PBO. LC_{50} values for second-, third- and fourth-instar larvae ranged from 19.6 to >500, from 39.6 to >500 and

from 48.7 to >500 mg L^{-1}, respectively, for non-PBO-treated larvae, and from 2 to 10.7, from 3.9 to 18.5 and from 8.6 to 99.5 mg L^{-1}, respectively, for PBO-treated larvae.

2.4.2 Oviposition assays

Differential oviposition was measured among the various treatment solutions and their controls (Table 2-3). This bioassay does not make it possible to distinguish between a repellent or deterrent activity, so these responses were combined under the term 'deterrent'. There were no significant differences in the numbers of eggs laid on distilled water or on distilled water containing Tween 20 and acetone for day 3 (P = 0.6412) or day 5 (P = 0.1344). Solutions containing *α*-terpineol or *α*-pinene did not receive significantly different numbers of eggs to the control solution, suggesting that they are neither deterrent nor attractive to gravid females. Solutions containing *β*-pinene, borneol acetate, borneol or camphor received more eggs than the controls, while those containing cineol, citronellal, eugenol, linalool, *p*-cymene, pulegone, rosemary oil, *trans*-anethole or thymol received significantly fewer eggs than did their control solutions. The oviposition activity index allows for a global comparison of the attractant or repellent nature of different solutions (Fig. 2-2). The pattern of OAI is maintained for individual solutions on days 3 and 5, except for *α*-terpineol, which had a negative OAI on day 3 but a positive OAI on day 5 (Fig. 2-2). However, there was no significant difference in the numbers of eggs laid on α-terpineol or its control on day 3 (P = 0.939) and day 5 (P = 0.857). In order of strength of activity, as measured by OAI, eugenol, citronellal, thymol, cineol, pulegone, rosemary oil, *p*–cymene, linalool and *trans*–anethole deterred oviposition, whereas *β*-pinene, borneol, camphor and borneol acetate acted as oviposition attractants.

2.5 Discussion

2.5.1 Larval bioassays

Mosquito larvae are attractive targets for pesticide management programs because they are limited to discrete aquatic habitats. In this study, the authors evaluated the toxicities of phytochemicals and an essential oil (rosemary oil) that contains some of the individual compounds tested (α-pinene, 1,8-cineole, camphor, β-pinene and borneol) to compare the relative toxicities of phytochemicals against mosquito larvae. All of the chemicals tested in this study have some larvicidal activity at the tested concentrations against first-instar larvae. Certain compounds, including camphor, linalool and bornyl acetate, had LC_{50} values >500 mg L^{-1} and therefore are less likely to be useful in mosquito control programs. The present results confirm the reports of others that the monoterpenoids thymol and 1,8-cineol are acutely toxic to fourth-instar larvae of *Ae. aegypti* (Silva et al., 2008). The present LC_{50} estimations differ slightly from other reports which probably reflects differences in methodologies and analyses (Kramer and Mulla, 1979; Chantraine et al., 1998; Cheng et al., 2004; Morais et al., 2006; Silva et al., 2008). It is difficult to compare directly the effects of compounds used in different studies because the relative composition of major components in different mixtures may affect the toxicity of the mixture (Kramer and Mulla, 1979; Chantraine et al., 1998; Clements, 1999; Sosan et al., 2001; Cavalcanti et al., 2004; Cheng et al., 2004; Amer and Melhorn, 2006; Morais et al., 2006; Silva et al., 2008).

As such, although two different oils of Brazilian crotons containing α-pinene and β-pinene as major constituents had LC_{50} values of 102 mg L^{-1} and 104 mg L^{-1}, respectively, against third-instar larvae of *Ae. aegypti* (Morais et al., 2006), it is difficult

to compare these data with the present studies using pure α-pinene or β-pinene, or rosemary oil which contains α-pinene or β-pinene along with other compounds. The present data agree with other studies that have evaluated different plant essential oils for toxicity to *Ae. aegypti* larvae (Amer and Mehlhorn, 2006), although there are some differences in absolute values. The reported LC_{50} values of essential oils can vary greatly, depending on their chemical composition, which depends upon the plant species, the plant part extracted, maturity and the extraction method. Essential oils contain many different compounds, which may interact additively, synergistically and even antagonistically, increasing, decreasing or resulting in no change in the larvicidal activity of test oils compared with the purified major active ingredient. Studies on the modes of action or the synergistic interactions of the major constituents of essential oils might help to explain why these combinations are more toxic to larvae. Several phytochemicals have been evaluated against larvae and adults of different mosquito species that occupy very different ecological niches (Kramer and Mulla, 1979; Chantraine et al., 1998; Clements, 1999; Sosan et al., 2001; Cavalcanti et al., 2004; Cheng et al., 2004; Amer and Melhorn, 2006; Morais et al., 2006; Silva et al., 2008). Different species appear to be more susceptible or tolerant to specific compounds. A comparison of the limited available data suggests that *Ae. aegypti* may be more tolerant to the toxic effects of natural and synthetic pesticides than other mosquito species, although more research into this question needs to be done to show this conclusively. The toxicity of most compounds tested varied with the larval instar (Table 2-1). In the cases where toxicity values could be determined, the toxicity of chemicals decreased significantly with increasing larval stage, a trend that has been observed previously (Silva et al., 2008; Cheng et al., 2004; Kramer and Mulla,

1979; Chantraine et al., 1998; Morais et al., 2006; Sosan et al., 2001; Cavalcanti et al., 2004; Amer and Melhorn, 2006; Clements, 1999). A number of factors could attribute to this, including the following:

1. Larger instars present a smaller surface area to volume ratio, and at the same water concentration would absorb less chemical than smaller instars.

2. Alterations in cuticle thickness and composition with increasing size may reduce the permeability of chemicals in larger instars.

3. Detoxification potential is higher in more developed insect larvae, possibly resulting in increased biotransformation of absorbed chemicals.

4. It is possible that, in conjunction with enhanced detoxification ability, increased elimination potential [through various mechanisms such as higher basal expression of xenobiotic efflux pumps (e.g. p-glycoprotein)] may be higher in larger instars.

PBO is a well-known inhibitor of microsomal monooxygenases (cytochrome P450 inhibitor), which are involved in the metabolism and detoxification of a very large number of insecticides (Kumar et al., 2002). Several studies have demonstrated the synergistic effects of PBO with many synthetic insecticides against *Ae. aegypti* larvae and adults (Kumar et al., 2002). The present study, however, is the first to report its synergistic effects with natural phytochemicals. The increases in acute toxicity of individual phytochemicals by the addition of PBO to the test solutions were not directly proportional to the non-PBO-treated values. The toxicity values of all compounds with PBO against first-instar larvae were in the same range (<10 mg L^{-1}), in spite of non-PBO values ranging from 10 to 500 mg L^{-1}. The toxicity of α-pinene to first- and fourth-instar

larvae was increased at least 24-fold (the LC_{50} without PBO could only be estimated as >500 mg L^{-1}), and the toxicity of linalool to first-instar larvae of *Ae. aegypti* was increased 250-fold by the addition of PBO (Tables 2-1 and 2-2). Similarly, the addition of PBO to the test solution containing borneol acetate, which was non-toxic to first-instar larvae without PBO, resulted in a synergistic ratio of 167, and rendered borneol lethal at a low concentration. The synergistic ratios of all compounds are presented in Tables 2-1 and 2-2. The present experiments demonstrate that high mosquito larvae mortality levels can be achieved with relatively low concentrations of some natural plant compounds, and that this lethality can be enhanced further by formulations including PBO. Traditionally, the use of plant-based products has required higher volumes and concentrations of compounds than conventional products. The present data suggest that these volumes can be reduced significantly by using synergists such as PBO. Equally important is that some of the more 'non-toxic' compounds become lethal when combined with PBO, therefore increasing the list of potential natural pesticides. Alternative synergists could be evaluated to determine which ones combine optimally with individual compounds to enhance toxicity, and to reduce the quantity of chemical used, particularly if such compounds are to be used effectively in pest and disease control programs. Compounds used commonly in mosquito control programs include synthetic pesticides, insect growth regulators and chitin inhibitors. Data on the interactions of synergists such as PBO with methoprene (juvenile hormone homolog), dimilin (a chitin inhibitor) and conventional insecticides might produce combinations that reduce the volumes required to achieve control. Whereas many mosquito species have developed resistance to conventional insecticides, combinations of insecticides, phytochemicals, growth inhibitors or juvenile

hormone inhibitors with synergists might provide better control with lower doses and lower costs. Such approaches might render phytochemicals more efficient in practical applications.

2.5.2 Oviposition assay

The choice of an oviposition site by gravid mosquito females is a principal factor that determines species proliferation, population densities and dispersion in different geographical areas (Tawatsin et al., 2006). *Ae. aegypti* breeds in domestic and peridomestic water containers, follows visual and olfactory cues to find appropriate oviposition sites and then uses both physical and chemical factors of the waters to discriminate between suitable sites (Clements, 1999). Oviposition repellents cause mosquitoes to move away from the source (Xue et al., 2003; Bentley and Day, 1989; Clements, 1999), whereas, in the presence of oviposition deterrents, females move towards and land upon a site, assess site quality, but lay few or no eggs before flying away (Xue et al., 2003; Bentley and Day, 1989; Clements, 1999). While attractance could be demonstrated, the present experimental design did not allow for discrimination between oviposition repellence and deterrence of the test compounds. It was demonstrated that gravid females laid significantly more eggs on waters that contained *β*-pinene, borneol acetate, borneol or camphor than their controls and significantly fewer eggs on waters that contained cineol, citronellal, eugenol, linalool, p-cymene, pulegone, rosemary oil, *trans*-anethole or thymol compared with their controls (Table 2-3). Only *α*-terpineol and *α*-pinene did not induce a statistically significant differential oviposition. The oviposition activity index (OAI) represents a global view of the relative preference of a substrate by gravid females. The OAI can be overly influenced by single events in

which there is significantly more oviposition in one cage, as it calculates a value based on total egg counts. Compounds with negative OAI values act as repellents/deterrents, while positive values act as attractants (Fig. 2-1). Thymol, pulegone, citronellal and eugenol showed strong repellent/deterrent activity, whereas *β*-pinene, borneol acetate, borneol and camphor acted as strong oviposition attractants (Fig. 2-1). Rosemary oil, the only essential oil evaluated in this experiment, had a strongly negative OAI. It is important to note that a single concentration was tested, and it is possible that different concentrations may alter the OAI. The specific activity of compounds also may change with time. The repellent/deterrent activity of pulegone, one of the most lethal compounds tested, decreased from day 3 to day 5, whereas the oviposition attractant activity of camphor increased from day 3 to day 5. The other compounds showed consistent oviposition modifying activity over the 5 day period. Repellents/deterrents and attractants could be utilized in mosquito control programs by manipulating the attractiveness of existing oviposition sites. Ideally, attractant compounds could be provided in oviposition traps for use in monitoring programs, while repellent/deterrent compounds could be used to reduce oviposition in specific habitats. Because *Ae. aegypti* predictably lays eggs in peridomestic dark containers, it may be possible to develop ovitraps containing compounds that attract gravid females but kill emerging larvae, and whose effectiveness might be enhanced by the addition of a synergist such as PBO. The present study demonstrates the potential for using natural phytochemicals as larvicides and for altering reproductive behaviors in *Ae. aegypti*. The synergistic effects of PBO with these compounds was also demonstrated, a fact which could be exploited in developing more effective strategies to prevent and control mosquitoes. Furthermore, most of the compounds tested, particularly those that

showed high larval toxicity and oviposition repellency (thymol, eugenol, rosemary oil, pulegone) are listed in the US Food and Drug Administration (FDA) 'generally recognized as safe' (GRAS) list (Waliwitiya et al., 2005), indicating their safe use on food products (Isman, 1999). The positive attributes of these compounds, such as relative safety to non-target aquatic invertebrates, long-term oviposition deterrency and larval control, warrant further research into their potential development as compounds for the control of mosquitoes.

2.6 Figures

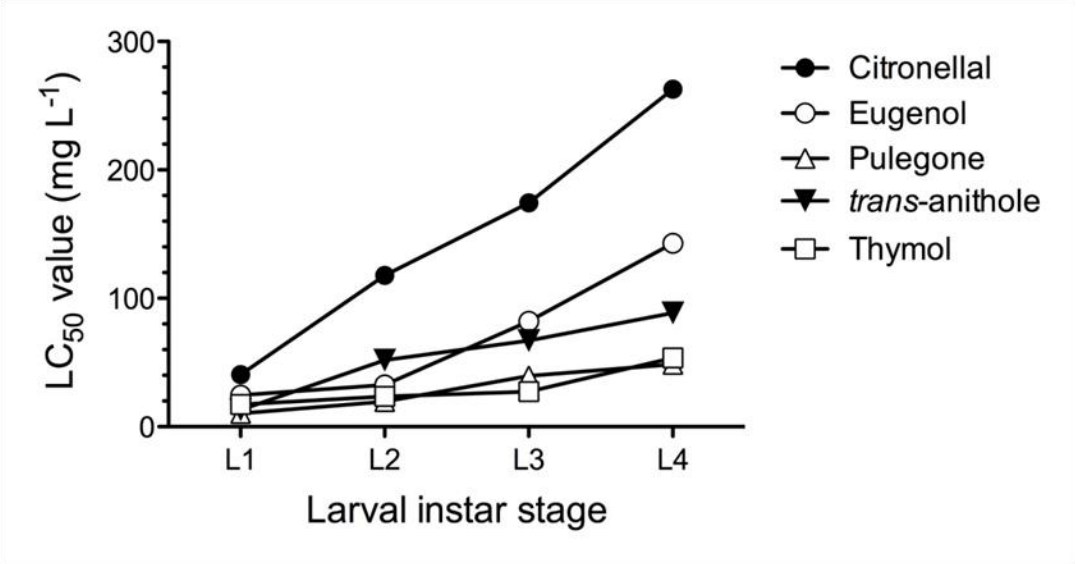

Figure 2-1 Change in acute 24-h LC_{50} values for the five most consistently toxic compounds to first (L1) to fourth (L4) instar larvae of *Ae. aegypti*.

Values are means of n=3 separate bioassays.

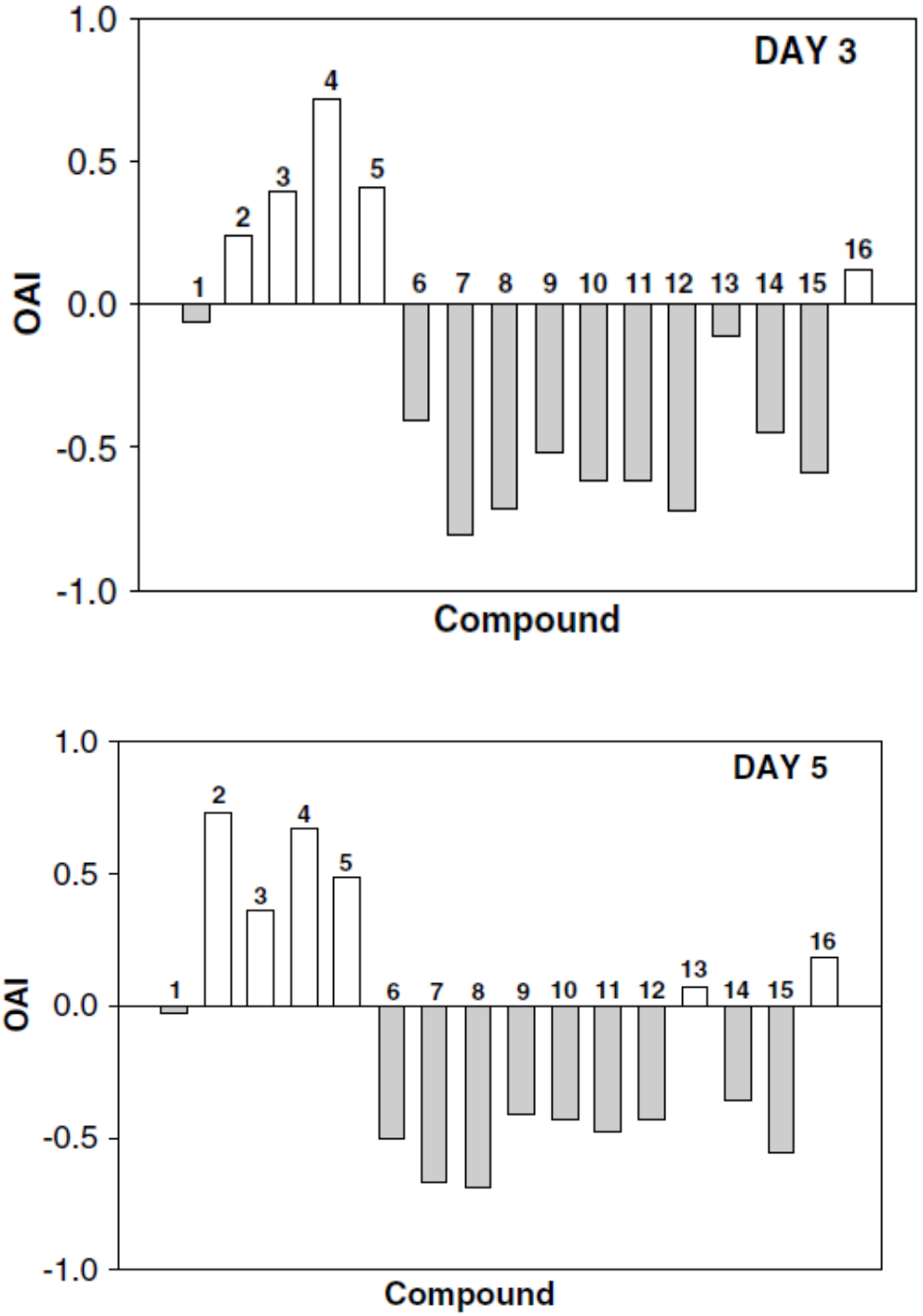

Figure 2-2 Oviposition activity index values for tested compounds on days 3 and 5 (for names of compounds, see table 3).
Compounds 1-16 refer to: α-pinene (1), β-pinene (2), borneol acetate (3), borneol (4), camphor (5), cineol (6), citronellal (7), eugenol (8), linalool (9), p-cymene (10), pulegone (11), Rosemary Oil (12), terpeneol (13), trans-anethole (14), thymol (15), Control (16).

2.7 Tables

Table 2-1 Larvicidal activity of selected monoterpenoids, *trans*-anithole and rosemary oil to first-fourth instar larvae of *Aedes aegypti* exposed for 24 h.

Lethal concentration (LC) values are expressed in mg L^{-1}. All values are means of n=3 experiments. The Synergistic ratio (SR) describes the treatment effect of a compound by itself to treatment effect of compound with PBO. CL=Confidence limit, SR=Synergistic ratio.

Chemical	First Instar Larvae Without PBO				First Instar Larvae With PBO				SR	Second Instar Larvae Without PBO				Second Instar Larvae With PBO				SR
	LC50	95%CL	Slope	X^2 value	LC50	95%CL	Slope	X^2 value		LC50	95%CL	Slope	X^2 value	LC_{50}	95%CL	Slope	X^2 value	
α Pinene	82.3	77-122	1.7	11.07	2.8	2-3	5.91	12.9	29.4	>500	_	-	-	3.3	3-4	2.1	6.1	151
β Pinene	96.2	71-133	1.6	11.04	2.5	2-3	4.9	12.6	38.5	>500	_	-	-	3	1-5	1.2	7.6	167
Borneol Acetate	>500	_	-	-	3	2-4	2.5	1.3	167	>500	_	-	-	6.3	4-9	1.5	3.2	79
Borneol	183.1	113-374	0.96	3.2	2.1	1-4	1.7	2.1	87	>500	_	-	-	2	1-4	0.94	9.4	250
Camphor	>500	341-835	0.83	1.9	2.1	1-3	1.5	3.1	238	>500	_	-	-	3.3	1-6	0.86	2.6	151
Cineol	>500	_	-	-	2.1	1-4	1.3	2.1	238	>500	_	-	-	4.5	2-8	0.96	6.1	111
Citronellal	40.7	30-56	1.5	6.9	2.9	3-4	6.2	12.5	14	117.8	51-188	1.2	14.4	3.2	3-4	5.4	1.6	37
Eugenol	24.5	20-30	2.8	2.9	2.3	4-6	1.4	19.8	11	32.6	24-43	1.7	7.3	4	5-9	1.4	19.7	8
Linalool	>500	_	-	-	2	2-3	3.7	12.6	250	>500	_	-	-	7.2	1-9	1.4	8.5	69
Myrcene	>500	-	-	-	-	-	-	-	-	>500	-	-	-	-	-	-	-	-
p-Cymene	226.2	137-490	0.9	1.8	5.2	4-7	2.2	3.9	44	>500	_	-	-	10.7	8-14	1.5	1.7	47
Pulegone	10.3	9-12	1.1	1.1	3.2	2-4	1.6	2	3	19.6	16-25	2.7	5.4	4.1	3-6	1.7	1.8	4.8
R.Oil	40.8	18-92	2	23.6	2.4	2-3	5.7	12.6	17	>500	_	-	-	7.2	4-12	0.9	1.3	69
trans-anithole	13	9-18	1.7	8.5	2.6	1-4	1.8	2.3	5	51.9	38-72	1.4	10.5	3	1-5	1.3	3.3	17
Terpineol	83.9	63-115	1.6	5.3	3.2	3-4	9.2	12.5	26	>500	_	-	-	4	3-5	6.5	7.7	125
Thymol	17.3	14-22	2.5	3.2	2.7	2-3	7.1	12.9	6	23.7	19-30	3.5	2.4	3.1	1-7	2.1	31	8

Table 2-2 Larvicidal activity of selected monoterpenoids, *trans*-anithole and rosemary oil with 10 mgL^{-1} of PBO to first-fourth instars larvae of *Aedes aegypti* exposed for 48 h.
The LC values are in mgL^{-1}. All values are means of n=3 experiments. Synergistic ratio (SR) describes the treatment effect of a compound by itself to treatment effect of compound with PBO.

Chemical	Third Instar Larvae Without PBO				Third Instar Larvae With PBO				SR	Fourth Instar Larvae Without PBO				Fourth Instar Larvae With PBO				SR
	LC_{50}	95%CL	Slope	χ^2 value	LC_{50}	95%CL	Slope	χ^2 value		LC_{50}	95%CL	Slope	χ^2 value	LC_{50}	95%CL	Slope	χ^2 value	
α Pinene	>500	_	-	-	4.6	3-6	1.8	8.4	109	>500	_	-	-	20.7	15-29	1.4	9.7	24
β Pinene	>500	_	-	-	5.2	3-8	1.3	11.7	96	>500	_	-	-	30.7	22-43	1.4	3.8	16
B. Acetate	>500	_	-	-	12	8-17	1.2	1.4	42	>500	_	-	-	39.3	28-56	1.3	1.6	13
Borneol	>500	_	-	-	7	4-11	0.93	7.9	71	>500	_	-	-	24.6	15-35	1.2	3.2	20
Camphor	>500	_	-	-	6	2-11	0.75	4.5	83	>500	_	-	-	71.8	46-124	0.9	1.9	7
Cineol	>500	_	-	-	11.9	7-18	0.96	4.4	42	>500	_	-	-	96.2	63-162	1	1.8	5
Citronellal	174.3	134-249	4.7	8.2	7.2	6-9	2.6	2.1	24	262.9	232-356	0.5	1.6	15.6	12-20	1.9	9.4	17
Eugenol	82.2	38-223	1.5	16	8.8	7-13	1.6	11.9	9	142.9	101-217	1.4	6.8	52.3	16-36	1.2	5	3
Linalool	>500	_	-	-	18.5	6-12	0.98	7.2	27	>500	_	-	-	99.5	37-77	1.1	2.1	5
Myrcene	>500	-	-	-	-	-	-	-	-	>500	-	-	-	-	-	-	-	-
p-Cymene	>500	_	-	-	9.8	7-13	1.7	2.4	51	>500	_	-	-	23.2	16-33	1.3	5.9	22
Pulegone	39.6	17-86	1.5	14.8	5	4-7	1.9	5	8	48.7	18-79	1.6	13.8	15.1	10-22	1.2	3.7	3
R.Oil	>500	_	-	-	10.7	6-16	0.98	3	47	>500	_	-	-	41.1	29-59	1.2	1.2	12
trans-Anithole	67.1	29-193	1.1	11.7	5.9	2-7	1.1	4.1	11	88.5	75-177	1.2	10.5	25.3	19-34	1.5	8.3	4
Terpineol	>500	_	-	-	3.9	2-7	2.9	54.9	128	>500	_	-	-	8.5	3-18	1.2	16	59
Thymol	27.3	22-34	2.5	3.6	4.2	4-8	1.9	10.8	7	53.5	32-90	2.5	12.6	19.8	11-35	1.5	13.6	3

Table 2-3 Analysis of the proportions of eggs laid in treatment or control waters in binary choice bioassays.

#	Compound	Day 3				Day 5			
		Total # of Eggs	Control%	Treatment%	*P*-Value	Total # of Eggs	Control%	Treatment%	*P*-Value
1	α-Pinene	545	53	47	0.804	1247	54	46	0.29
2	β-Pinene*	550	18	82	0.014	1098	20	80	0.008
3	B.Acetate*	626	31	69	0.045	1114	33	67	0.0001
4	Borneol*	665	15	85	0.017	1338	24	76	0.017
5	Camphor*	508	30	70	0.03	950	31	69	0.034
6	Cineol**	557	70	30	0.033	1058	66	34	0.004
7	Citronellal**	308	88	12	0.035	997	83	17	0.009
8	Eugenol**	410	84	16	0.002	816	83	17	0.001
9	Linalool**	326	75	25	0.004	660	69	31	0.001
10	P-Cymene**	389	73	27	0.003	733	71	29	0.002
11	Pulegone**	449	80	20	0.005	877	76	24	0.0004
12	R.Oil**	872	82	18	0.018	623	74	26	0.0003
13	Terpineol	1391	67	33	0.939	981	48	52	0.857
14	t-Anithole**	438	72	28	0.001	759	71	29	0.001
15	Thymol**	412	79	21	0.004	651	80	20	0.007
16	Control	799	64	36	0.641	757	43	57	0.134

P-value – if < 0.05, egg numbers are significantly difference between the treatment Petri dish and the control Petri dish for the compound.

*Solutions that preferred by gravid females.

**Solutions that repelled gravid females.

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Connecting statement 2

In chapter 2, I evaluated the acute toxicities and sublethal effects of 16 different plant-derived compounds on *Aedes aegypti*. In addition I described the effect of adding PBO to the mix as an efficacy enhancing agent. Based on the acute toxicity data obtained in Chapter 2, I selected 5 of the most toxic compounds for continued evaluation. In the next chapter I examined the effects of these compounds, with and without PBO as a synergist, on the activities of host biotransformational enzyme normally used to eliminate xenobiotic compounds. I used the EROD assay to measure cytochrome P450 activity, *β*-esterase assay to measure esterase activity and glutathione S-transferase to measure the activity of transferases.

Chapter 3: The Synergistic Effects of Insecticidal Phytochemicals and Piperonyl Butoxide on Biotransformational Enzyme Activities in *Aedes aegypti* (Diptera: Culicidae)

A modified version of this chapter has been submitted to Journal of Medical Entomology as:

Waliwitiya, R., R. A. Nicholson, C. J. Kennedy, and C. Lowenberger. The synergistic effects of insecticidal phytochemicals and piperonyl butoxide on biotransformational enzyme activities in *Aedes aegypti* (Diptera: Culicidae).

3.1 Abstract

The biochemical mechanisms underlying the increased toxicity of several plant essential oils (thymol, eugenol, pulegone, *α*-terpineol and citronellal) against 4th instar larvae of *Ae. aegypti* when exposed simultaneously with piperonyl butoxide (PBO) were examined. Whole body biotransformational enzyme activities including cytochrome p450-mediated oxidation (ethoxyresorufin O-dethylase [EROD]), glutathione S-transferase (GST) and *β*-esterase activity were measured in control, essential oil-exposed only (single chemical), and essential oil+PBO (10 mg L^{-1}) exposed larvae. At higher concentrations, thymol, eugenol, pulegone and citronellal alone reduced EROD activity by 5-25% after 16 h post-exposure. *α*-Terpineol at 10 mg L^{-1} increased EROD activity by 5±1.8% over controls. Thymol, eugenol, pulegone, *α*-terpineol and citronellal alone reduced GST activity by 3-20%. PBO exposure alone did not significantly affect the activity of any of the measured enzymes. However, thymol, eugenol, pulegone, citronellal, and *α*-terpineol in combination with PBO reduced EROD activity by 58-76% after 16 h post-exposure and reduced GST activity by 3-85%, respectively. This study demonstrates that essential oils affect the biotransformational capacity of mosquito larvae, and that these effects are enhanced by adding PBO. The data indicate that the toxicity of several of the compounds may be, in part, due to their ability to reduce the activity of biotransformational enzymes.

3.2 Introduction

Phytochemicals such as essential oils have been used for many years as pesticides in agriculture and are important components of integrated pest management systems (Lee et al., 1997; Waliwitiya et al., 2005). Their insecticidal, fungicidal, bactericidal, antiviral, antifeedant and insect growth retardant properties (Benner, 1993; Singh et al., 1989; Wilson et al., 1997) are often the result of synergistic interactions among different biologically active components such as terpenoids, alkaloids and phenolics (Singh et al., 1989). Essential oils, the natural plant products that give rise to the characteristic plant flavors and fragrances, are the steam distillable fractions of plant tissues and are grouped as monoterpenes (hydrocarbons and oxygenated derivatives), sesquiterpenes (hydrocarbons and oxygenated derivatives) and aliphatic compounds (alkanes, alkenes, ketones, aldehydes, acids and alcohols) (Kordali et al., 2007). The biosynthesis of plant-derived compounds is tissue specific and is developmentally regulated in almost all higher plants. In most cases the pathways, and the genes involved in their synthesis, are tightly regulated and may be linked to environmental, seasonal or external triggers. The principal constituents of essential oils are biosynthesized mainly from fatty acids, phenylpropanoids, or isoprenoid pathways (Daviet and Schalk, 2010) and various modifications of the terpene backbone have produced the thousands of terpene compounds found in nature (Acamovic and Brooker, 2005).

The biological activities of plant essential oils and their constituents have been well documented (Isman, 2000). Essential oils of *Thymus serpyllum* and *Oreganum majorama* have been used in fumigants against bean weevil (*Acanthoscelides obtectus*) (Bruchidae) (Regnault-Roger et al., 1993). Extracts of clove (*Syzygium aromaticum*),

and Star anise (*Illicium verum*) have exhibited fumigant properties and antifeedent effects on red flour beetle (*Tribolium castaneum*) and the maize weevil (*Sitophilus zeamais*) (Huang et al., 1998). Essential oils of cumin (*Cuminum cyminum*), anise (*Pimpinella ansium*), oregano (*Origanum syriacum* var. bevanii) and eucalyptus (*Eucalyptus camaldulensis*) have been effective as fumigants against greenhouse pests such as the cotton aphid (*Aphis gossypii*) and the carmine spider mite (*Tetranychus cinnabarinus*) (Tuni and Sahinkaya, 1998). Insecticidal activity of monoterpenoids against western corn rootworm (*Diabrotica virgifera*), the two-spotted spider mite (*Tetranychus urticae*) and the housefly (*Musca domestica*), (Lee et al., 1997) and dietary effects of monoterpenoids against the European corn borer (*Ostrinia nubilalis*) have also been documented (Lee et al., 1999). Because most essential oil solutions contain many constituents, and the individual compounds responsible for toxicity have not been purified, it is difficult to estimate a concentration of individual lethal component found in each essential oil mixture.

The mechanisms underlying the toxic actions of monoterpenoids on insects are not well understood, but the onset of symptoms is usually rapid (Enan, 2001). Hyperactivity and convulsion-like symptoms were seen in wireworms exposed to thymol (Waliwitiya et al., 2005). Cockroaches treated with essential oils also show hyperactivity followed by hyperextension of the legs, as well as fast immobilization and quick knockdown, followed by death (Enan, 2001). In support of a proposed neurotoxic mode of action, several essential oil monoterpenes have been shown to be competitive inhibitors of acetylcholinesterase (AChE) *in vitro* (Miyazawa et al., 1997; Ryan and

Byrne, 1988; Grundy and Still, 1985). In these studies, however, AChE activity *in vivo* was not correlated with AChE activity *in vitro* (Kordali et al., 2007).

Insect resistance to phytochemicals and other toxins is mediated through the enzymatic degradation of the parent compound or its metabolites. A variety of oxidases, reductases, esterases, epoxide hydrolases, and group transferases are used by insects to detoxify and eliminate toxic phytochemicals (Skrinjaric-Spoljar et al., 1971).

The cytochrome P-450-dependent mixed function oxidases (MFOs) play an essential role in this detoxification system (Ahmad, 1986). The P450 superfamily is a large group of hemoprotein monoxygenases participating in the oxidation of various small molecules (Gourley and Kennedy, 2009). P450 demonstrates various substrate specificities and plays a significant role in various metabolic pathways including the biosynthesis of biosignalling substances and secondary metabolites, degradation of toxic xenobiotics, and adaptive assimilation of unusual carbon sources (Yoshida and Aoyama, 1994). P450 mediates many oxidative reactions involved in the biosynthesis of secondary metabolites such as flavonoids, terpenoids, alkaloids and antibiotics (Boyer et al., 2006). Involvement of P450s in insecticide resistance development has been reported in *Ae. aegypti* and *Ae. albopictus* (Enayati et al., 2005) and in the resistance of *An. funestus* to both pyrethroids and carbamates (Casimiro et al., 2006).

Glutathione S-transferases (GSTs) belong to a superfamily of genes recognized in arthropods as well as mammals (Enayati et al., 2005). The primary function of GSTs is generally considered to be the biotransformation of both endogenous and xenobiotic compounds either directly or by catalyzing the secondary metabolism of compounds oxidized by the P450s (Zhu et al., 2007). GSTs catalyze the conjugation of glutathione to

hydrophobic substrates, such as herbicides and insecticides which facilitate the metabolism of target compounds (Yu, 1996; Yu, 1993; Wadleigh, 1988; Hodnick et al., 1996; Zaman et al., 1994). Insect GSTs have been recognized for their role in inducing the expression of other detoxifying enzymes, enhancing the defense machinery, speeding the development of resistance, and causing cross-tolerance to other insecticides (Carlini et al., 1995; Hinkle et al., 1995). Interest in insect GSTs has focused primarily on their role in insecticide resistance (Boyer et al., 2006). High levels of GST activity have been detected in *M. domestica* and *Lygus lineolaris*, and the development of insecticide resistance has been correlated with enhanced GST activity and GST-dependent metabolism (Zhu et al., 2007).

Esterases have diverse functions in insects, including roles in proteolysis, nervous system function, hormone metabolism, and xenobiotic metabolism (Flores et al., 2005). Insect esterases perform both physiological and defensive functions and are found in both soluble and membrane-bound forms (Maa and Liao, 2000). Carboxyl esterases of several pest insects have been studied extensively because of their involvement in insecticide resistance (Devonshire, 1977; Georghiou and Pasteur, 1980), especially in the resistance of mosquitoes to pyrethroids (Ozaki and Kasaai, 1970; Bull and Whiten, 1972; Flores et al., 2005) and red scale resistance to carbamate and organophosphate insecticides (Elizabeth et al., 2004).

In order to enhance the activity of insecticides, and overcome the enzymatic responses of insect described above, synergists are often added to primary insecticidal mixtures. It has been shown that the toxicity of some phytochemicals is enhanced in the presence of piperonyl butoxide (PBO) (Waliwitiya et al., 2009). PBO is a

methylenedioxyphenyl compound that is used routinely as a synergist in insecticide formulations. For example, incorporating PBO with deltamethrin at the ratio of 1:6 significantly increased the larval mortality of *Ae. aegypti*, *Anopheles culicifacies*, *A. stephensi*, *A. vagus* and *Culex quinqufasciatus* (Fakoorziba et al., 2009). Synergysm of PBO with pyrethroids (beta-cyfluthrin, deltamethrin), carbamates (propoxur), organophosphates (chlorpyrifos), phenylpyrazole (fipronil), neonicotinoid (imidacloprid), and oxadiazines (indoxacarb) increased their toxicities against the German cockroach (*Blattella germanica* L.) (Chai and Lee, 2010).

It has been proposed that PBO interferes with the cytochrome P450 and esterase pathways, normally used to detoxify or eliminate toxins, through competitive and noncompetitive inhibition (Brattsten, 1988; Hodgson and Philpot, 1974; Franklin, 1977). This inhibition allows the toxic compounds to act at lower concentrations and may well underlie the synergistic interactions reported in different insects (Hsu et al., 2004; Waliwitiya et al., 2009). In contrast to its effects in insects, PBO has been demonstrated to be an inducer of P450s in fish (Ankley and Collyard, 1995) and in mammals (Erickson et al., 1988) and has been used widely in metabolism studies with mammals, fishes and terrestrial and aquatic invertebrates (Wilkinson et al., 1984).

In vivo toxicity tests using houseflies and cockroaches suggest that PBO functions by inhibiting the oxidative metabolism of insecticides (Sun and Johnson, 1960). PBO and other methylenedioxyphenyl compounds inhibited the microsomal oxidation of many insecticides and other xenobiotics in a number of mammalian and insect species, regardless of whether the PBO was administered prior to, or with the substrate (Casida,

1970), and the inhibition of enzyme by PBO has been shown to be concentration dependent (Gunning et al., 1998).

Most of the terpenoids found in plant essential oils have minimal vertebrate toxicity and have been classified as GRAS (Generally Regarded As Safe) compounds by the US Food and Drug Administration (Kostyukovsky et al., 2002; Waliwitiya et al., 2005). In addition to low non-target toxicity, active components such as the monoterpenes, also are less persistent in soil and water than many synthetic compounds (Isman, 1999). Nonetheless, these compounds do show toxicity to mosquito larvae when applied in an aquatic environment. The aim of this study was to examine the toxicity and biochemical basis for increased acute toxicity of several phytochemicals against 4th instar larvae of *Ae. aegypti* by studying changes in expression of MFOs, GSTs and esterases in the presence and absence of PBO.

3.3 Materials and methods

3.3.1 Insects

Ae. aegypti larvae and adults were raised as described previously (Lowenberger et al., 1999) at 27^0C and 80-85% relative humidity under a 14 h:10 h light:dark cycle. Adults were provided with a 10% sucrose solution ad libitum and larvae were fed with ground Nutrafin Basix fish food (Rolf C. Hagen Inc, Montreal, QC).

3.3.2 Chemicals

Pulegone (>95% purity) was obtained from Ecosafe Natural Products (Victoria, B.C.). Eugenol, α-terpineol, citronellal, and thymol (>95% purity) and PBO (90% purity) were purchased from Sigma Aldrich (St. Louis, MO). Stock solutions of the chemicals

were made as described previously (Waliwitiya et al., 2009). Treatment solutions (nominal 10, 50 and 100 mg L^{-1}) were prepared using distilled water. In order to increase the solubility of the chemicals and to inhibit binding to test containers, 5 µL of Tween 20 (Uniqema, New Castle, DE) were added to 100 mL of each treatment solution as a solubilizing agent. Each treatment solution with PBO contained 10 mg L^{-1}.

3.3.3 Larval exposure

Fourth instar larvae (n=20) were placed in scintillation vials containing 30 mL of prepared treatment solution and provided with 0.1 g of ground Nutrafin Basix fish food. Three replicates were used per treatment (n=60 larvae total). Scintillation vials were covered loosely and larvae remained in the vials for exposure periods of 4, 8 or 16 h. Following exposure, n=20 larvae were removed from the test solutions, placed in 1 mL eppendorf tubes, and stored at -80^{0}C until whole body enzyme analysis was performed. Larvae were exposed to distilled water (controls), PBO only, and to individual monoterpenoids with or without PBO.

3.3.4 Enzyme activity measurements

Whole body cytochrome P450-mediated oxygenase activity was measured using the ethoxyresorufin O-deethylase (EROD) assay as described previously (Fragaso et al., 1998; Gourley and Kennedy, 2009) using resorufin as a standard (Sigma Aldrich, St. Louis, MO). Briefly, 20 larvae were homogenized in 500µl of ice cold phosphate buffer solution ([PBS] 0.05 M, pH 7.2). Homogenates were centrifuged at 9,000 x g for 20 min at 4^{0}C. The supernatant (S9 fraction) was removed and placed in a 500 µl eppendorf tube and stored at -80^{0}C until analysis. A 50µl aliquot of the S9 fraction was added to wells of

a Nunc 96-well tissue culture treated microplate (NUNC, Kamstrupvej, DK). Resorufin standards (0 to 0.5 µg/mL) in DMSO were used to generate a standard curve. Fifty microliters of 7-ethoxyresorufin (22 µM) (Sigma Aldrich, St. Louis, MO) in DMSO diluted 10-fold in HEPES buffer (100 mM, pH 7.8) were added to each well and the plates were incubated in the dark for 10 min. The final concentration of 7-ethoxyresorufin was 2.2 µM in each well. Reactions were started by the addition of NADPH (Sigma Aldrich, St. Louis, MO) in NaH_2PO_4 buffer. The final concentration of NADPH was 24 mM in each well). Fluorescence was measured every min for 15 min, at room temperature (20 ^{0}C) on a Cary Eclipse fluorescence spectrophotometer (Varian Inc, Hansen Way CA) equipped with an excitation filter of 530nm, and an emission filter of 590 nm, each with a 20nm bandwidth. Results were expressed as pmoles of reaction product formed/mg protein/min.

GST and esterase activities were measured using the method described by Boyer et al., (2006). Homogenates were prepared similar to the method described in EROD assay. GST activity was determined as described (Boyer et al., 2006) in individual reaction mixtures containing 8 µl of S9 fraction of larval body extract, 180 µl of 50mM sodium phosphate buffer (pH 7.2), 2 µl of 20 mM reduced glutathione (Sigma Aldrich, St. Louis, MO), and 10 µl of 30 mM 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma Aldrich, St. Louis, MO). The mixtures were incubated at 30^{0}C in a 96 well plate. The change in optical density within the wells was measured at a wavelength of 340 nm at 1 min intervals for 5 min using a scanning spectrophotometer (BioTek Powerwave 340, BioTek Instruments Ltd., Winooski, VT). Results were expressed as pmoles of CDNB conjugated/mg protein/min.

Esterase activities were determined by measuring the production of β-naphthol from β-naphthyl acetate as described (Boyer et al., 2006). Briefly, aliquots (90 µl) of larval homogenates and 90 µL of 50 mM potassium phosphate buffer (pH 6.5) containing 0.1mM β-naphthyl acetate were added to wells of a 96 well plate. Following a 10 min incubation period, 20 µl of 0.02 M o-dianisidine solution was added to each well (200 µl final volume). After 2 min, the change in absorbance was determined at 15 sec intervals for 10 min at a wavelength of 540 nm using a microplate scanning spectrophotometer. Results were expressed as pmoles of β-naphthol produced/mg protein/min. The protein concentrations of all samples were determined using the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

3.3.5 Statistical analysis

EROD, GST and esterase activities were normalized to S9 fraction protein content. Results are expressed as means ± S.E.M. The assumptions of equal variances and normal distributions in residuals were tested and data were log transformed as necessary. Statistical analysis was performed with JMP 7 software (JMP, Version 7. SAS Institute Inc., Cary, NC, 1989-2007) and the log-transformed data were subjected to a general linear model (GLM, repeated measures) procedure to test for significant differences in enzyme activities between individual chemical exposures, the presence and absence of PBO, and time. Least square means were compared using Turkey's HSD test.

The Enzyme Activity Index (EAI) was calculated for each compound as the percent change in enzyme activity calculated as:

$$\frac{\text{(Control Enzyme Activity – Enzyme Activity with Test Chemical present)}}{\text{Control Enzyme Activity}}$$

The EAI compares the enzyme activity of a treatment to the baseline enzyme activity of distilled water controls. Negative values indicate an induction of enzyme activity while a positive value indicates a reduction in enzyme activity. If PBO has synergized the activity of a compound, further reducing enzyme activity, the EAI of the mixture will have a higher positive value than the compound by itself.

3.4 Results

The specific monoterpenoid, the length of exposure, and co-exposure with PBO all had significant effects on the whole body biotransformational enzyme activity of 4th instar larvae. The EROD, GST, and β-esterase activity of larvae exposed to PBO alone, in the absence of monoterpenoids, was not significantly different from the activity levels of these enzymes in control larvae.

3.4.1 EROD activity

Baseline whole-body EROD activities in the control larvae ranged from 3.08-3.18 (±0.1) pmol/min/mg protein over a 16 h period. With all monoterpenoids, there were non-significant trends towards decreased larval EROD activity with time at all concentrations used. There were no significant differences in EROD activity between control and treatment values with eugenol, α-terpineol and citronellal when each time point was compared (e.g. 4 h control v. 4 h treatment, $p>0.05$) (Figs. 3-1B, D, E). When larvae were exposed to thymol alone there was a significant decline (23% reduction) in EROD activity ($p<0.0001$) at the highest concentration used (100 mg L^{-1}) at the 16-h time point. With applications of pulegone, EROD was significantly reduced at a concentration

of 50 mg/L at the 16-h time point, however, significant declines were seen earlier by 8 h at the highest concentration of 100 mg L^{-1} (13.1% decline at 8 h and 13.3% decline by 16 h).

The addition of PBO to the test compounds caused significant reductions in EROD activity compared with the effects of the compounds by themselves. EROD activity in larvae exposed to thymol and PBO was significantly lower than EROD activity of thymol alone at 50 mg L^{-1} (16 h time point) and at 100 mg L^{-1} (8 and 16 h time points, $p<0.0001$, Fig. 3-1A). At the lowest concentration of eugenol (10 mg L^{-1}), the differences in EROD activity between eugenol alone and eugenol plus PBO were significant at the 16 h timepoint ($p=0.0001$). At all other concentrations, and at all timepoints, the EROD activity of eugenol plus PBO was significantly reduced when compared to eugenol exposure alone (Fig. 3-1B). Pulegone plus PBO significantly reduced EROD activity, compared with pulegone alone at 50 mg L^{-1} at the 8 h time point, and this was maintained until 16 h (Fig. 3-1C). EROD activity, in pulegone (100 mg L^{-1}) plus PBO-treated larvae was significantly lower at all time points compared to pulegone alone (Fig. 3-1C). EROD activity of α-terpineol plus PBO was only significantly different from α-terpineol alone at the 8 and 16 h time points at all tested concentrations (Fig. 3-1D). Significant reductions in EROD activity by citronellal plus PBO, compared to citronellal alone required the highest concentrations (50 and 100 mg L^{-1}) and the longest exposure periods (8 and 16 h, Fig. 3-1E).

An enzyme activity index (EAI) was calculated for each phytochemical with respect to its control. Negative values indicate an increase in enzyme activity while positive values indicate inhibition. The EAIs of phytochemicals with or without PBO are

shown in Table 3-1. The EAIs of all the compounds were negative at the 4 h timepoint at 10 mg L^{-1} indicating induction of EROD activity. For all the other concentrations and time points, EAI values were positive indicating reductions in enzyme activity compared with the controls. The addition of PBO caused greater reductions in EROD activity, which was enhanced with increasing concentration and longer exposures for all compounds. The highest EAI (0.77) was observed with eugenol at 100 mg L^{-1} at the 16 h time point indicating the greatest reduction (75%) of EROD activity.

3.4.2 GST activity

Baseline GST activities in the control larvae ranged from 5.9-6.2 (±0.14) pmol/min/mg protein over the 16 h experimental period. Unlike the data presented above for EROD activity, changes in GST activity in larvae exposed to individual monoterpenoids was not consistent with time or concentration. Initially there is a trend of increasing GST activity with thymol, eugenol pulegone and citronellal at 10 mg L^{-1} at the 4 h time point. Although a pattern of slightly reduced GST activity was seen with all compounds as concentrations and exposure time increased, none of the differences from controls was significant.

The addition of PBO to the test compounds caused significant reductions in GST activity compared to the activity in larvae exposed to the compounds alone. The GST activity of thymol plus PBO, at all concentrations and at all time points, was significantly lower than the GST activity of thymol alone ($P<0.0001$). There were no significant differences in the GST activity in larvae treated with the lowest concentration of eugenol (10 mg L^{-1}) plus PBO except at the latest time point. With higher concentrations of 50 and 100 mg L^{-1}, GST activity with PBO was significantly lower than eugenol alone at all

time points. Pulegone plus PBO significantly reduced GST activity compared with pulegone alone at 10 mg L^{-1} at the 8 and 16 h time points and at all time points in the 50 and 100 mg L^{-1} treatment groups. The largest reduction in GST activity occurred with pulegone at the highest concentration at 16 h (43% reduction). The GST activity of α-terpineol plus PBO and pulegone plus PBO followed a similar pattern. At the lowest concentration of α-terpineol and pulegone (10 mg L^{-1}), the differences in EROD activity between monoterpenoids alone were significant at 8 h ($p=0.0001$). At all other concentrations, at all timepoints, the EROD activity of monoterpenoid plus PBO were significantly lower than the compound alone in exposed larvae (Fig. 3-2C, D). The GST activities of citronellal plus PBO compared to citronellal alone were not significant at any concentration or time point.

The EAIs for GST activity are shown in Table 3-1. Thymol, eugenol, pulegone and citronellal (10 mg L^{-1}) exposure resulted in negative EAIs at the 4h time point, indicating an induction of GST activity. In the absence of PBO, there are only minor changes in the EAI values of all compounds. In general, the addition of PBO increased the EAI of all compounds. EAIs increased with concentration and longer exposures, indicating that PBO acts in conjunction with these compounds in reducing GST activity. The highest EAI was found with thymol (100 mg L^{-1}) plus PBO at 16 h (0.85, 84.2% reduction) compared with an EAI of 0.18 in the absence of PBO.

3.4.3 β-esterase activity

Baseline β-esterase activities in the control larvae ranged from 6.03 to 6.21 (±0.34) pmol/min/mg protein over the 16-h treatment period. Initially, a trend in increasing β-esterase activity with thymol, α-terpineol and citronellal at 10 mg L^{-1} at the

4-h time point occurred, but this activity subsequently decreased with time (Fig. 3-3A, D, E). No significant differences in β-esterase activity between control larvae or those treated with the study compound were seen. The addition of PBO to any of the test compounds did not result in any significant changes in β-esterase activity compared to the β-esterase activity of the compounds alone.

3.5 Discussion

In a previous study (Waliwitiya et al., 2009) the acute toxicity of 16 plant-based compounds against 1st-4th instar larvae of *Ae. aegypti* was increased significantly by the addition of piperonyl butoxide (PBO) to the exposure treatments. The mechanisms underlying this enhanced toxicity were unknown, as few studies have evaluated the efficacy-enhancing effects of PBO with plant-derived compounds. Because other studies have indicated that *Ae. aegypti* larvae eliminate xenobiotic compounds with detoxifying or biotransformational enzymes (Boyer et al., 2006), the present study was undertaken to examine changes in the activities of three insect biotransformation enzymes to determine a potential role of enzyme alteration in the enhanced toxicity of these phytochemicals with the addition of PBO to treatment solutions.

In the detoxification of potentially lethal compounds, several enzyme systems have been identified as important for insects in general and mosquito larvae in particular. Cytochrome P450-mediated transformations have been reported to be used by mosquito larvae to detoxify ingested toxins (David et al., 2006) or in the resistance to specific allelochemicals (Brattsten, 1988; Scott et al., 1998). For example, tobacco hornworm larvae fed on tobacco leaves show increased cytochrome P450 activity to purportedly reduce the effects of ingested nicotine (Snyder and Glendinning, 1996).

GSTs are enzymes that provide protection through the catalysis of reactions which conjugate reduced glutathione (a tripeptide) to electrophilic portions of lipophilic compounds (Bernays, 1991), and are activated by the presence of many potentially lethal compounds (Yu, 1996). GSTs have been linked to insecticide resistance (Vontas et al., 2005; Prapanthadara et al., 1993) and in neutralizing damage caused by the production of chemically-generated reactive oxygen species (Sawicki et al., 2003).

The present results suggest that these plant phytochemicals, which are larvicidal to mosquitoes (Waliwitiya et al., 2009, 2010), interact with the mixed function oxidases (EROD) and GST but have no effect on β-esterase activity in mosquito larvae. Initially, many of the compounds tested at low concentrations, and at early time points, increased the activities of the selected enzymes, suggesting that these compounds induce biotransformation capability (Scott, 1999). With higher concentrations, however, or with longer exposure times, overall enzymatic activities were generally reduced. The greatest effects of the phytochemicals were seen on EROD and GST activities with little or no effect on β-esterase activity.

Increasing monoterpenoid concentration, or increasing exposure time, enhanced the reductions in enzymatic activity (EROD and GST). This trend varied by compound, its concentration and exposure time. The lowest EROD and GST activities were generally found at the highest concentrations and longest exposure times. There is little literature available on the affect of allelochemicals or natural plant extracts on biotransformation enzyme activity. In some insects, the consumption of allelochemicals induces P450 activity and reduces the toxicity of natural and synthetic toxins (Zeng et al., 2007), and *in vitro* studies indicate that commercial herbal extracts can reduce activity of human

cytochrome P450 activity *in vitro* (Budzinski et al., 2000; Foster et al., 2003). Human GST activity is inhibited *in vitro* by curcumin and ellagic acid (Hayeshi et al., 2007) and by thonningianin (isolated from *Thonningia sanguinea*, an African medicinal plant) (Gyamfi et al., 2004). Plant phenols effectively inhibit the *in vitro* GST activity of *Trichopulsia ni* and *Papilio polyxenes* (Lee, 1991), and other plant compounds have been shown to inhibit GST activity (Ata et al., 2007).

The present study clearly demonstrates that combining PBO with phytochemicals enhances enzyme modulatory effects in *Ae. aegypti* larvae. The addition of PBO caused significant reductions in EROD and GST activity. These reductions were statistically significant at the lowest concentration (10 mg L^{-1}) by the 8-h exposure period, and became more intense with increasing concentration and exposure time. None of the monoterpenoids alone, or combined with PBO, had significant effects on β-esterase activity. Thymol, α-terpineol and citronellal initially appeared to induce β-esterase activity, which then decreased as concentration and exposure time increased, but these changes were not statistically significant.

While PBO did not alter β-esterase activity, it significantly reduced EROD and GST activities. Few studies exist on the synergistic effects of PBO and plant compounds on biotransformation in insects. Feeding PBO to *Manduca sexta* lowered the cytochrome P450 induction as well as reduced the intake of tobacco leaves and nicotine, a compound that is detoxified by P450 (Snyder and Glendinning, 1996). Pretreatment with PBO has been shown to inhibit P450 activity and cause significant increases in the toxicity of compounds to the parasitic nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis in vitro* (Kotze et al., 2006). Boyer et al. (2006) observed an induction of

cytochrome P450, GST and esterase activities in *Ae. aegypti* larvae exposed to plant leaf litter suggesting that ingested xenobiotics induce the expression of enzymes used to detoxify them. PBO and other compounds such as diethyl malate (DEM) and triphenyl phosphate (TPP) are added to insecticides to enhance their efficacy (Yu, 1993) and affect MFOs, GSTs and non-specific esterases (Tang et al., 2010; Straus and Chambers, 2006).

The results of this study indicate that the addition of PBO to the monoterpenoids significantly enhances reductions in GST and EROD activity, but the molecular mechanism remains unknown. PBO has been examined for its effects on xenobiotic penetration, metabolism and excretion in insects, alone or in conjunction with insecticides (Farnham, 1998). The site of action and the role of PBO in enhancing insecticide toxicity are not well understood (Farnham, 1998). Casida (1970) concluded that methylenedioxyphenyl (MDP) synergists inhibit oxidative metabolism of co-applied toxicants. As well, PBO has been shown to facilitate pyrethroid penetration through the cuticle of resistant *H. armigera* (Gunning et al., 1998). There is no evidence of a monoxygenase-mediated resistance mechanism (Kennaugh et al., 1993; Gunning et al., 1998), although PBO may act as an inhibitor of the pyrethroid resistance-related esterase in *H. armigera*. In the present study, it is unclear if the monoterpenoids and PBO enter the larvae via the alimentary tract or across the cuticle. Until this is clarified, and the targets of the synergist and monoterpenoid are identified, definitive determinations of their modes of action cannot be made.

The EAI refers to the synergistic effects of PBO with a compound with respect to its acute toxicity. The EAI values (Table 3-1) indicate either the inhibition or induction of EROD or GST enzyme activities either by individual compounds or compounds plus

PBO compared to their respective distilled water control. Based on the EAI values, a concentration of 10 mg L^{-1} monoterpenoid stimulated EROD and GST activities at the 4-h time point. For all the tested compounds the EAI increased with time in a concentration dependent manner, indicating reduced enzyme activity. Combining monoterpenoids with PBO caused greater reduction in enzymatic activity at lower monoterpenoid concentrations as indicated by higher EAI values.

The present data suggest that the increase in acute toxicity after exposing mosquito larvae to binary mixtures of plant compounds and PBO (Waliwitiya et al., 2009) may involve synergistic effects on biotransformation enzymes. While most compounds without PBO reduced EROD and GST activities in a concentration and time dependent manner, these differences were not always significantly different, even though some of these compounds were lethal to the larvae (Table 3-1). The effects were far more dramatic with the addition of PBO. This suggests that these monoterpenoids may have several toxic modes of actions that contribute to the acute toxicity to *Ae. aegypti* larvae.

The changes seen in enzyme activities in this study can serve as a starting point for further research into identifying the putative molecular mechanisms behind the acute toxicities of monoterpenoids to insect larvae. It is suspected that these compounds exhibit their toxicities by acting on other biological targets in mosquito larvae as well, including the nervous system. The significant reductions in EROD and GST activities may contribute to increased acute toxicity caused by the binary mixtures containing PBO through reduced detoxification of parent monoterpenoids or potentially toxic metabolites. Further studies are required to investigate the interactions between PBO, monoterpenoids, and the insect detoxifying mechanisms and their role in acute and chronic toxicity.

3.6 Figures

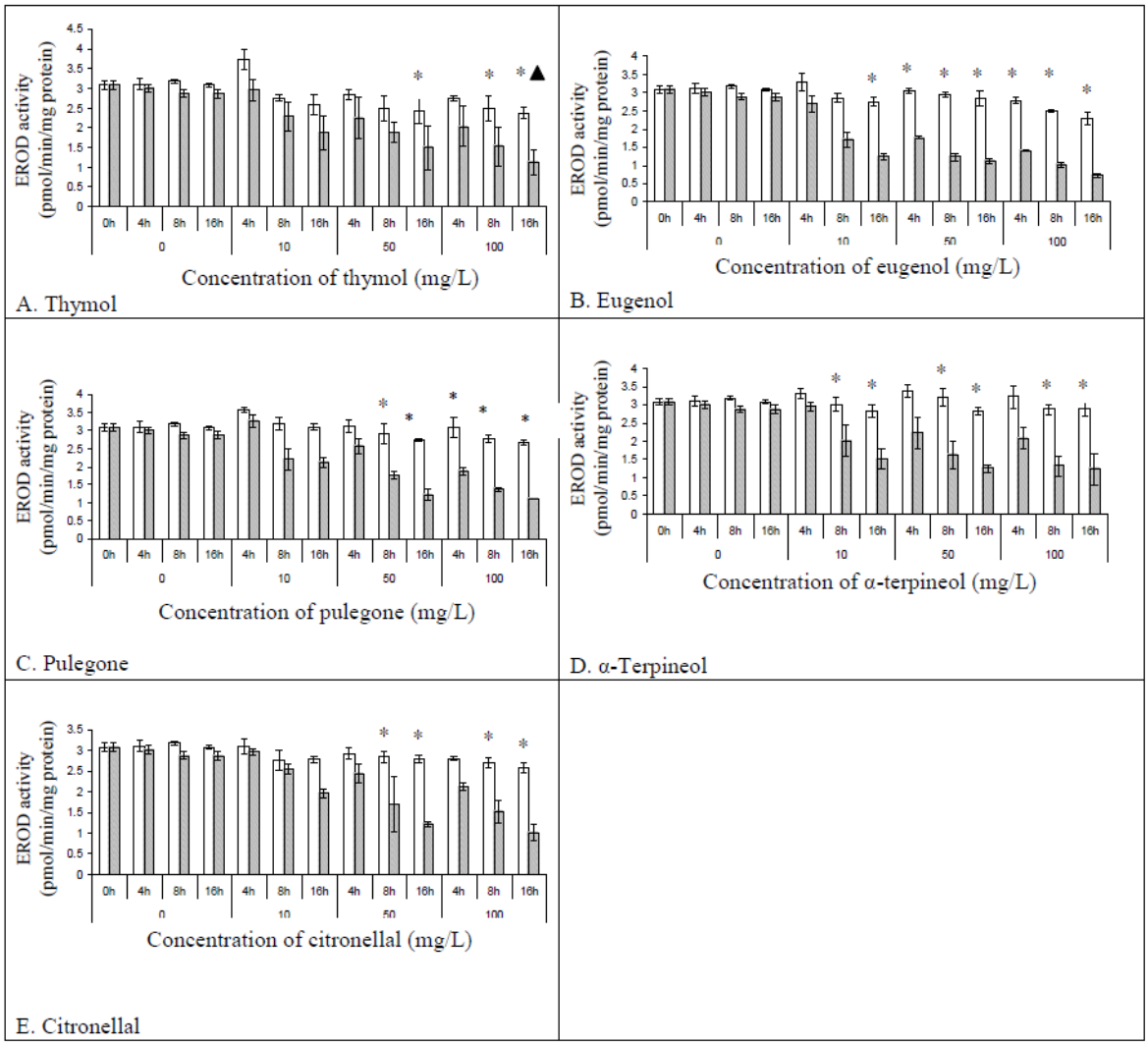

Figure 3-1 EROD activity of thymol, eugenol, pulegone, *α*-terpineol and citronellal in 4th instar larvae of *Aedes aegypti* with and without PBO at 3 different concentrations after 4, 8 and 16 h.

▒ - shows the EROD activity of the compound with PBO and the □ - shows the EROD activity of the compound without PBO. In the control group, the grey bar refers to the EROD activity with PBO itself and the clear bar shows the EROD activity of larvae in distilled water. Charts A-E show the EROD activities of thymol, eugenol, pulegone, *α*-terpineol and citronellal, respectively. The error bars represents the standard errors of the three replicates. An asterisk (*) denotes that EROD activity of the compound is significantly different from EROD activity of the compound+PBO and ▲ denotes EROD activity of a compound is significantly different from its distilled water control at a particular timepoint at a probability level of 0.05.

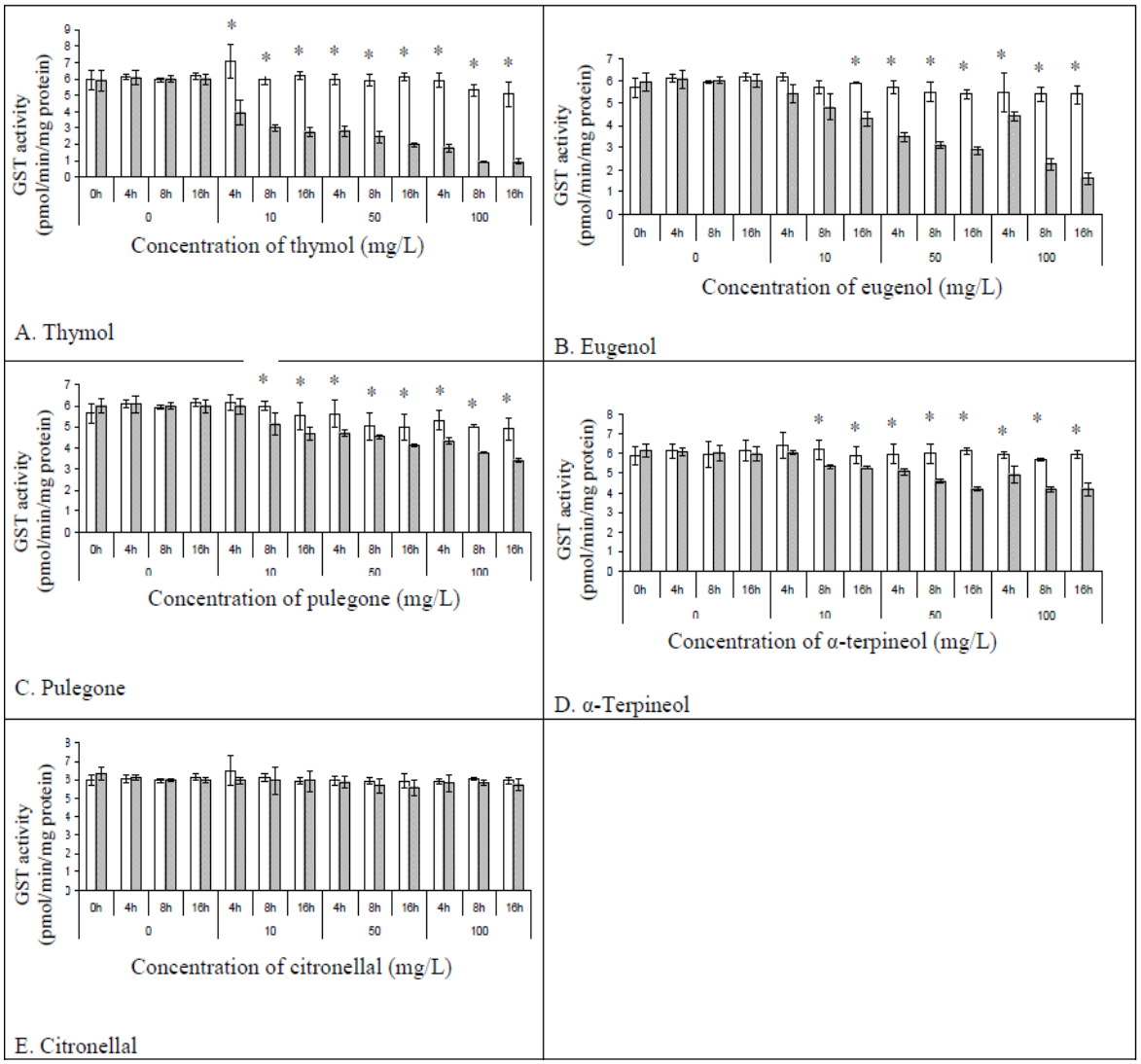

Figure 3-2 GST activity of the 4th instar larvae of *Aedes aegypti* treated with thymol, eugenol, pulegone, *α*-terpineol and citronellal with and without PBO at 3 different time points.

The ▒ - shows the GST activity of the compound with PBO and the □ - shows the GST activity of the compound without PBO. In the control group the grey bar refers to the GST activity with PBO itself and the clear bar shows the GST activity of larvae in distilled water. Charts A-E show the GST activities of thymol, eugenol, pulegone, *α*-terpineol and citronellal, respectively. The error bars represents the standard errors of the three replicates. An asterisk (*) denotes that GST activity of the compound is significantly different from GST activity of the compound+PBO at a particular timepoint at a probability level of 0.05.

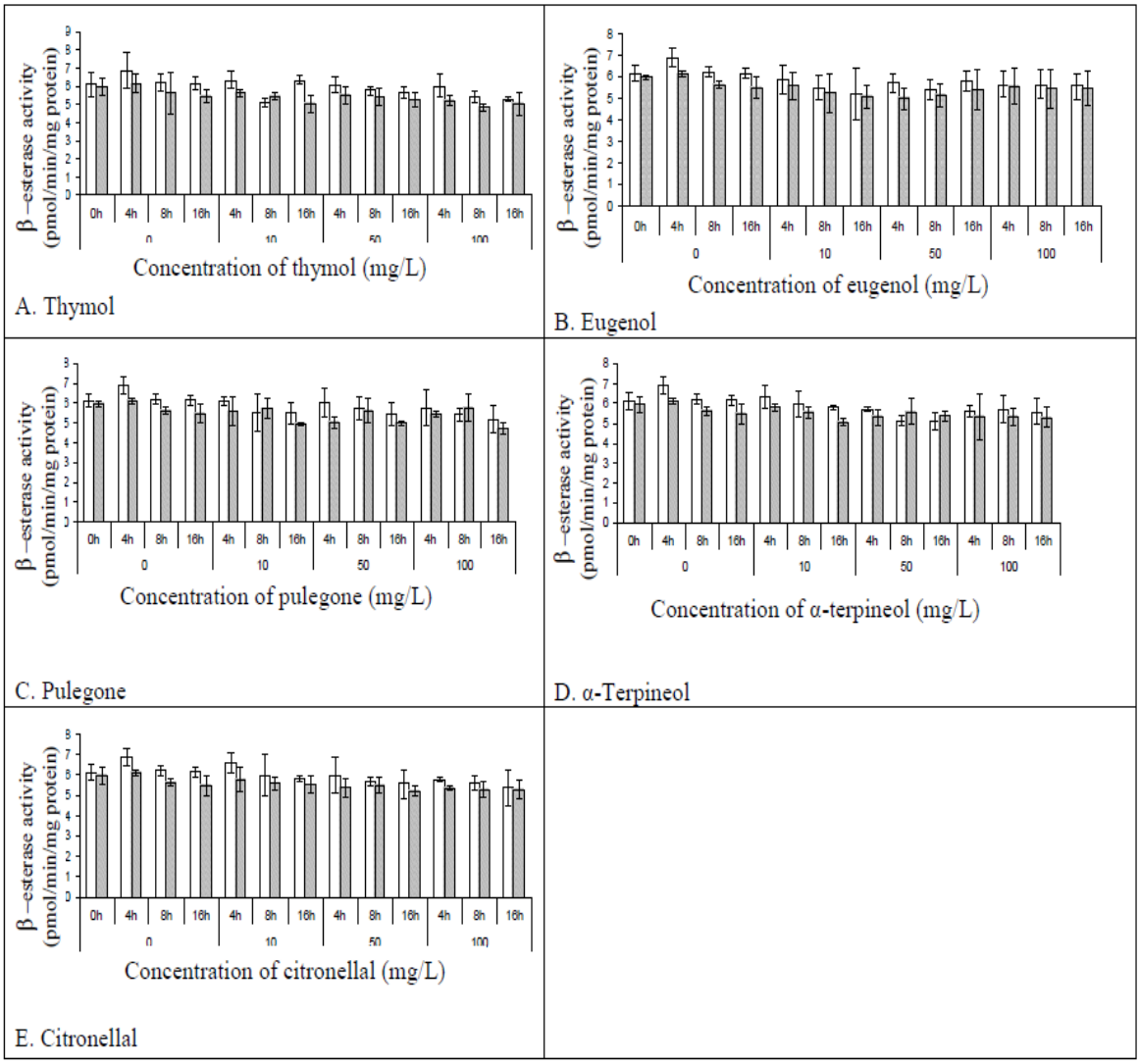

Figure 3-3 *β*-Esterase activity of the 4th instar larvae of *Aedes aegypti* treated with thymol, eugenol, pulegone, *α*-terpineol and citronellal with and without PBO at 3 different time points.

▒ - shows the *β*-esterase activity of the compound with PBO and the □ - shows the *β*-esterase activity of the compound without PBO. In the control group the grey bar refers to the *β*-esterase activity with PBO by itself and the clear bar shows the *β*-esterase activity of larvae in distilled water. Charts A-E show the *β*-esterase activities of thymol, eugenol, pulegone, *α*-terpineol and citronellal, respectively. The error bars represent the standard error of the three replicates.

3.7 Tables

Table 3-1 Inhibition or stimulation of EROD and GST enzyme activities by various phytochemicals alone, or in the presence of PBO compared with respective distilled water control.

Negative values indicate an increase in enzyme activity while positive values indicate inhibition of enzyme activities. Synergistic ratios of thymol, eugenol, pulegone, *α*-terpineol and citronellal with and without PBO in 4th instar larvae of *Aedes aegypti* are shown at the bottom and of the table and they were calculated using LC_{50} values of previously published data (Waliwitiya et al., 2009).

Concentration (mg/L)	Time (mg/L)	Thymol				Eugenol				Pulegone				Terpineol				Citronellal			
		With PBO		No PBO		With PBO		No PBO		With PBO		No PBO		With PBO		No PBO		With PBO		No PBO	
		EROD	GST	EROD	GST	EROD	GST	EROD	GST	EROD	GST	EROD	GST	EROD	GST	EROD	GST	EROD	GST	EROD	GST
10	4	0.05	0.36	-0.2	-0.16	0.14	0.12	-0.06	-0.01	-0.05	0.02	-0.15	-0.01	0.05	0.02	-0.07	0.05	0.04	0.02	-0.002	-0.07
	8	0.28	0.5	0.13	0.00	0.47	0.19	0.11	0.04	0.31	0.13	0.0	-0.01	0.37	0.11	0.05	-0.04	0.2	0.0	0.13	0.03
	16	0.39	0.55	0.16	0.00	0.6	0.31	0.11	0.05	0.32	0.24	-0.01	0.1	0.51	0.15	0.08	0.04	0.36	0.04	0.1	0.04
50	4	0.28	0.55	0.09	0.03	0.44	0.43	0.02	0.07	0.17	0.23	-0.01	0.08	0.28	0.17	-0.09	0.02	0.21	0.1	0.06	0.01
	8	0.41	0.59	0.22	0.01	0.62	0.48	0.08	0.08	0.45	0.24	0.08	0.15	0.49	0.23	-0.01	-0.01	0.47	0.05	0.11	0.0
	16	0.51	0.68	0.21	0.01	0.64	0.53	0.08	0.13	0.61	0.33	0.11	0.19	0.59	0.32	0.08	0.01	0.6	0.09	0.09	0.04
100	4	0.34	0.71	0.11	0.03	0.54	0.28	0.1	0.11	0.4	0.3	0.0	0.13	0.33	0.2	-0.04	0.03	0.32	0.04	0.1	0.02
	8	0.52	0.84	0.22	0.11	0.68	0.62	0.22	0.09	0.56	0.36	0.13	0.16	0.58	0.3	0.1	0.04	0.52	0.02	0.15	0.0
	16	0.63	0.85	0.23	0.18	0.77	0.74	0.26	0.13	0.64	0.45	0.13	0.21	0.6	0.33	0.07	0.04	0.67	0.07	0.16	0.04
Synergistic ratio		2.7				2.73				3.23				58.82				16.85			

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Connecting statement 3

Determining the biochemical activity and host target system of novel compounds based solely on biotransformational enzyme assays that I chose based on predictions of how these molecules might act could be a long and expensive process. In the next chapter I describe a different approach in which I designed and built a system that can measure the electrophysiological responses of target insects to the topical application of the five selected compounds that exhibited acute toxicities in Chapter 2. In this system I monitored the flight muscle impulses (FMI) and wingbeat signals (WBS) of the blowfly *Phaenicia sericata*, after the application of thymol, eugenol, pulegone, α-terpineol and citronellal. These were compared with the responses to several commercial insecticides, whose mode of action has been well characterized, to allow predictions on host targets of these plant based chemicals.

Chapter 4: Effects of the Essential Oil Constituent Thymol and Other Neuroactive Chemicals on Flight-Related Electrophysiology and Wing Beats in Blowflies *Phaenicia sericata*

A modified version of this chapter has been published as:

Waliwitiya, R., C. P. Belton, R. Nicholson, and C. Lowenberger, 2010. Effects of the essential oil constituent thymol and other neuroactive chemicals on flight-related electrophysiology and wing beats in blowflies *Phaenicia sericata*. Pest Management Science, 2010. 66 (3): 277-289.

4.1 Abstract

I evaluated the effects of the plant terpenoid thymol and 8 other neuroactive compounds on flight muscle impulses (FMI) and wing beat signals (WBS) of tethered blowflies (*Phaenicia sericata*).

The electrical activity of the dorsolongitudinal flight muscles was closely linked to the WBS of control insects. Topically-applied thymol inhibited WBS within 15-30 min and reduced FMI frequency. Octopamine and chlordimeform caused a similar, early onset bursting pattern that decreased in amplitude with time. Desmethylchlordimeform blocked WBS within 60 min and generated a profile of continuous but lower frequency FMI. Fipronil suppressed WBS and induced a pattern of continuous, variable frequency spiking which diminished gradually over 6 hrs. Cypermethrin and rotenone-treated flies had initial strong FMI that declined with time. In flies injected with Ɣ-amino butyric acid (GABA), the FMI were generally unidirectional and frequency was reduced as was seen with thymol.

Thymol readily penetrates the cuticle and interferes with flight muscle or central nervous function or both in the blowfly. The similarity of the action of thymol and GABA suggests that this terpenoid acts on GABA receptors of intact blowflies.

4.2 Introduction

In the search for new chemical agents to control pest populations, natural products such as plant-based extracts and essential oils hold substantial promise (Lee et al., 1997; Isman, 1999; Waliwitiya et al., 2009). Thymol (Fig. 4-1) is a monoterpenoid found in *Thymus vulgaris* (Lamiaceae) that exhibits antibacterial (Cosentino et al., 1999; Venturini et al., 2002), antioxidant (Aeschbach et al., 1994), molluscicidal (Singh et al., 1999), antifeedant (Gonzalez-Coloma et al., 2002) and insecticidal activity (Lee at al., 1997; Gonzalez-Coloma et al., 2002; Mansour et al., 2000; Hummelbrunner and Isman, 2001). In wireworms, thymol causes initial hyperactivity followed by abdominal segment hyperextension, extended paralysis and then death (Waliwitiya et al., 2005).

Priestley et al. (2003) compared the GABA-modulating and GABA-mimetic activities of thymol on human $GABA_A$ and fruitfly (*Drosophila melanogaster*) homomeric RDLac GABA receptors expressed in *Xenopus* oocytes. Thymol enhanced the GABA-dependent chloride currents in oocytes expressing various human $GABA_A$ receptor isoforms as well as the insect GABA receptor. Consistent with its action on mammalian $GABA_A$ receptors, thymol also potentiated the binding of [^{3}H] t-butylbicycloorthobenzoate at this complex (Tong and Coats, 2007). Likewise, ivermectin increases chloride ion permeability in invertebrate muscle and nervous tissue through positive modulation of GABA-gated and glutamate-gated chloride channels (Bloomquist, 1996) and paralytic effects have been reported in dipterans (Botham and Nicholson, 1985; Alves et al., 2004). In contrast, fipronil blocks GABA-activated chloride influx (Cole et al., 1993; Le Corronc et al., 2002), and glutamate-activated chloride currents in

insect neurons (Zhao et al., 2004) causing spontaneous electrical activity to increase in the central nervous system (Durham et al., 2001).

Our understanding of the mode of action of terpenoids is incomplete (Enan, 2001), but they may interfere with the octopaminergic system of insects (Enan, 2001; Price and Berry, 2006). In insects, octopamine functions as a neuromodulator, a neurotransmitter and a neurohormone (Nathanson and Greengard, 1973; Orchard, 1982; Roeder, 2004; Farooqui, 2007). Octopamine regulates and desensitizes sensory inputs, exites nerves, and maintains rhythmic and more complex processes such as learning and memory. The octopamine receptor agonist chlordimeform (CDM) was used widely for control of insects and mites (Hollingworth, 1976), but has been withdrawn due to potential carcinogenicity (Costa et al., 1988). In arthropods, CDM causes loss of appetite and cessation of feeding (Hirano et al., 1972; Watanabe et al., 1975; Matsumura and Beeman, 1976; Lund et al., 1979) hyperactivity and incoordination (Doane and Dunbar, 1973), detachment and mortality of parasitic acarines (Stone et al., 1974). When applied topically, CDM and its active metabolite N-desmethylchlordimeform (DMCDM) activate light output from the lantern of the firefly *Photinus pyralis* (Hollingworth and Murdock, 1980), thus mimicking the endogenous agonist octopamine in this insect (Carlson, 1969; Robertson and Carlson, 1976). DMCDM also competes with mianserin (an octopamine antagonist) for binding sites in the cockroach nerve (Kinnamon et al., 1984). In contrast to CDM, rotenone causes paralysis in insects (Fukami, 1985; Hein et al., 2003) and only weakly affects nerve conduction in insects (Yamasaki and Ishii, 1951).

The dipteran flight motor system has provided considerable insight into the physiological actions of pyrethroids such as tetramethrin that elicit repetitive discharge

activity and uncoupling of the flight motor pattern (Hart et al., 1978; Adams and Miller, 1980). Our objective was to characterize the effects of thymol on flight muscle impulse (FMI) responses and wing beat signals (WBS) in the blowfly and, by comparing it with neuroactive substances with known modes of action, gain insight on the mechanisms by which thymol may cause toxicity in insects. In this investigation the effects of thymol were compared with those of octopamine, CDM, DMCDM, rotenone, cypermethrin, fipronil, ivermectin and GABA.

4.3 Materials and methods

4.3.1 Insects

A colony of blowflies (*Phaenicia sericata*) has been maintained at Simon Fraser University for over 20 years at 25^0C, 80% relative humidity, and a 12h:12h light:dark cycle. Pupae were collected and placed in a separate cage and provided with sugar cubes and water ad libitum. Three to five-day old adult female blowflies were selected for electrophysiological studies. The food source was removed from experimental insects 4 h prior to the experiments. Flies had access to sugar cubes for 30 mins prior to experiments to condition the insects for the experiment.

4.3.2 Chemicals

Thymol, octopamine hydrochloride (OA), GABA, rotenone and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). Technical grade chlordimeform (CDM), desmethylchlordimeform (DMCDM), ivermectin (IVM), cypermethrin and fipronil were from bona fide industrial sources. Cypermethrin, rotenone, ivermectin, CDM, DMCDM and thymol were dissolved and

administered in DMSO. OA and GABA were dissolved and administered in distilled water. Control insects were treated with DMSO or water.

4.3.3 Dosing and data recording

Blowflies were briefly anesthetized using CO_2. Copper electrodes (live and reference electrodes) were inserted into the dorsolongitudinal muscles (DLM5) and prothorax respectively using micromanupulators. Ten min after recovering from CO_2 anesthesia, 50 µg of thymol, CDM, DMCDM, rotenone, cypermethrin, IVM or fipronil were topically applied to the tip of the abdomen or 50 µg of octopamine or GABA were injected into the abdomen using a microsyringe. Control insects were topically treated or injected with 5 µL of DMSO or water. Recordings began immediately. The electrophysiological responses (FMI) were amplified and signals recorded using a NI USB-6008 data logger (National Instruments, Vaudreuil-Dorion, Quebec, Canada). At the same time acoustic signals (WBS) from wing beats of the blowfly were recorded using a miniature microphone (Realistic 33-1052) connected to an amplifier speaker (1.8 V, ACS 340, Altec Lansing, Archer, Taiwan) and the signal was recorded by the same data logger.

Firing rates (impulses/s) were calculated by counting the number of action potentials that occurred after the application of the test compound. The mean responses and their standard errors were determined, and amplitudes were calculated with their standard errors using LABVIEW 8.5 (National Instruments, Quebec, Canada). At least 3 blowflies were tested with each compound.

4.4 Results

4.4.1 Baseline wing beat and dorsolongitudinal muscle electrical activity

The control FMI from dorsolongitudinal muscles were typically bidirectional with asynchronous spikes presenting as positive and negative amplitude deflections (Fig. 4-2). This normal pattern of activity is consistent with other reports (Hart et al., 1978). WBS were always closely coupled to FMI with the ratio of FMI:WBS averaging 1:9±1 during the 4-h recording period. Under our experimental conditions control blowflies (water or DMSO) flew for extended periods, but not continuously.

4.4.2 Effects of thymol

The effects of topically applied thymol (50 μg) on FMI and the resulting WBS are shown in Fig. 4-3. The initial (first 15 min) FMI of dorsolongitudinal muscles were similar to the control signals (Fig. 4-3A), after which a transient train of lower frequency FMI appeared (Fig. 4-3F). By 30 min, wings were motionless but sporadic bursts of FMI with mostly unidirectional spikes were observed over the next 3 hrs (Fig. 4-3B, F). At 4 hrs, FMI recovered to a more normal pattern (Fig. 4-3C, F) but wings remained motionless (Fig. 4-3F). Amplitude changes are shown in Fig. 4-3C. The ratio of FMI:WBS ranged from 1:8±1.5. Treated flies groomed extensively after treatment and showed slight tremors by 20 min. None of the treated flies died within 4 hrs.

4.4.3 Effects of octopamine

The effects of 50 μg octopamine (OA) on FMI and accompanying WBS after injection are shown in Fig. 4-4. An abnormal pattern of activity started approximately 10 min after treatment and bursts of FMIs became shorter and higher in frequency over the

next 3 hrs (Fig. 4-4B, F). WBS remained robustly linked to FMI until 186 min (Fig. 4-4F), when FMI amplitude was reduced dramatically and the WBS stopped after 260 min (Fig. 4-4C, F). The amplitude of the FMI typically increased 4-fold at 10 min of OA application and then declined to almost zero (Fig. 4-4C). Similarly, the mean number of impulses per second increased at 10 min, and this frequency was generally maintained until the end of the experiment (Fig. 4-4F). Following OA application, the ratio of FMI:WBS was 1:7±2. Blowflies treated with OA groomed their abdomen and wings at the beginning of the experiment, but extended their probosces during the later stages. No flies died during the experimental period.

4.4.4 Effects of chlordimeform (CDM)

Normal FMI and WBS were observed immediately after dosing blowflies with 50 μg CDM (Fig. 4-5A). At 10 min the bursts of FMI were shorter in duration and by 30 min WBS ceased (Fig. 4-5F). Between 30 and 90 min a weaker pattern of discontinuous FMI activity occurred. The mean frequency of spikes increased immediately after treatment (Fig. 4-5F). By 150 min after treatment, FMI activity became continuous (Fig. 4-5B) but the amplitude declined gradually to zero by 300 min (Fig. 4-5C). The ratio of FMI:WBS averaged 1:4±1 after CDM treatment. CDM-treated blowflies showed increased grooming, tremors, paralysis and all died.

4.4.5 Effects of desmethylchlordimeform (DMCDM)

The initial discontinuous trains of FMI and WBS after dosing blowflies with 50 μg DMCDM were similar to controls (Fig. 4-6A). At 10 min there were intense continuous FMI of increased amplitude and WBS remained tightly linked to this activity

(Fig. 4-6C, F). Brief reversion to an atypical discontinuous FMI at 60 min preceded a final phase of continuous firing which declined to zero amplitude by 190 min (Fig. 4-6C). No WBS were observed after 60 min (Fig. 4-6F). FMI frequency increased within the first hour and then declined (Fig. 4-6F). DMCDM-treated flies had a ratio of FMI:WBS of 1:5±2. DMCDM symptoms were similar to those of CDM including complete mortality.

4.4.6 Effects of rotenone

The initial discontinuous trains of FMI and WBS immediately after treatment with 50 µg rotenone (Fig. 4-7A) were similar to those of control insects. At 24 min after treatment, sporadic bursts of FMI of increased amplitude occurred and WBS remained tightly linked to this activity (Fig. 4-7C, F). After 24 min, the wings stopped beating (Fig. 4-7F). At this time the amplitude of the FMI dropped below the initial amplitude and continued to drop over the duration of the experiment (Fig. 4-7C). In parallel to amplitude reduction, the absolute frequency of both WBS and FMI dropped (Fig. 4-7F). At 154 min continuous weak FMI appeared and continued for a few minutes (Fig. 4-7C, F). Rotenone gave a ratio of FMI:WBS of 1:7±1. Rotenone-treated blowflies groomed initially then became paralyzed and died.

4.4.7 Effects of cypermethrin

The initial trains of FMI and WBS just after application of 50 µg cypermethrin (Fig. 4-8A) closely paralleled those of controls. At 5 min there were intense multiple FMI of high amplitude and WBS remained tightly linked to this activity (Fig. 4-8C, F). Intense high frequency FMI were still present 44 min after treatment (Fig. 4-8F). However 5 min

after application, the amplitude of the FMI fell below its initial level and did not return to pretreatment levels (Fig. 4-8C). At 82 min, FMI and WBS became uncoupled (Fig. 4-8B, C, F). FMI frequency declined markedly and by 226 min both the frequency and the amplitude of the FMI fell to zero (Fig. 4-8C, F). No WBS were observed after 44 min. Cypermethrin produced a ratio of FMI:WBS of 1:6±1. Cypermethrin caused extensive grooming, tremors, convulsions and at 226 min all flies were dead.

4.4.8 Effects of fipronil

The effects of fipronil are shown in Fig. 4-9. Soon after treatment, WBS became less frequent and after 30 min the insects could not fly (Fig. 4-9B, F). Between 10 min and 2 h after dosing a pattern of initially variable frequency FMI that subsequently became continuous were observed (Fig. 4-9B, F). The amplitude of FMI was maximal at 2 h (Fig. 4-9C), but by 6.5 h electrical activity almost ceased entirely (Fig. 4-9C, F) and all flies died. Fipronil produced a ratio of FMI:WBS of 1:4±0.3. Fipronil-treated flies showed grooming and convulsions before death.

4.4.9 Effects of ivermectin

Fifteen min after application, the amplitude and the frequency of FMI increased slightly and then declined below initial levels (Fig. 4-10C, F). Sporadic trains of FMI appeared after 47 min and became more infrequent at 162 min. The ratio of FMI:WBS was 1:8±1. WBS were closely linked to FMI until about 47 min when WBS ceased (Fig. 4-10F). By 162 min, FMI became almost unidirectional (Fig. 4-10B). This effect became more pronounced as time progressed (Fig. 4-10C). Ivermectin-treated flies showed increased grooming and paralysis but no mortality over the timecourse of the experiment.

4.4.10 Effects of GABA

GABA-treated flies showed patterns of activity most similar to those of thymol-treated flies. Typical effects of GABA on the FMI and resulting WBS are shown in Fig. 4-11. WBS were closely linked to the FMI for about 10 min but then wing movement ceased (Fig. 4-11F). By 47 min, the FMI became unidirectional (exclusively negative deflections) and this pattern continued until 162 min (Fig. 4-11B, C). The frequency of the FMI dropped steadily from 47 min after treatment to the end of the experiment (Fig. 4-11F). Following GABA application, the ratio of FMI:WBS was 1:5±1. GABA treatment triggered grooming activity but no mortality.

4.5 Discussion

I examined the effects of the natural product thymol on flight motor-associated electrical activity and wing beat in live, tethered adult female blowflies. The electrophysiological results indicate effects on the central nervous system, the longitudinal flight muscles and neuromuscular junctions. This approach has proved useful for investigating the *in vivo* effects of insecticides and glutamate analogs in dipterans (Hart et al., 1978; Adams and Miller, 1980). Monitoring WBS affords a convenient way of acquiring data pertinent to flight activity (Moore, 1991). I compared the pattern of disruption observed with thymol to those observed with neuroactive substances with known modes of action to shed more light on how thymol interferes with central and peripheral flight pathways in vivo. These experiments can provide information on the ease with which thymol crosses the insect cuticle and gains access to tissues, identified in *in vitro* studies, to possess sites sensitive to the effects of thymol.

The first effect of thymol was a brief succession of lower frequency spikes in the absence of WBS that reverted to control-like activity. Within 30 min of topical application, WBS were fully suppressed and trains of FMI with mostly unidirectional spikes were evident. These results clearly suggest that thymol efficiently penetrates insect cuticle and accesses excitable tissues. Its speed of action, however, is slower than the other topically applied insecticides; even slower than CDM which relies on bioactivation. Similar types of unidirectional FMI occurred in flies treated with ivermectin. The predominantly unidirectional pattern of spiking observed with thymol is more likely to arise through interference with flight motor control centrally rather than from a "side-selective" neuromuscular block. Of all the neuroactive compounds I examined, GABA showed the closest resemblance to thymol which caused strong inhibition of one component of bidirectional spiking. In insects, GABA is an important inhibitory neurotransmitter that plays a key role in the peripheral and central nervous system. Insect neurons exhibit transient hyperpolarizing responses following application of GABA (Rauh et al., 1990). GABA has been reported to block spontaneous spiking in corn borer ventral nerve cord (Durham et al., 2001). Our data indicate that thymol acts on GABA-sensitive sites in vivo, either by mimicking or facilitating the effects of this inhibitory neurotransmitter. Such an *in vivo* action is consistent with a previous *in vitro* pharmacological study that reported a potentiation of GABA responses at insect GABA receptors by thymol (Waliwitiya et al., 2005). Our data clearly distinguish the effects of thymol from that of the GABA receptor antagonist fipronil where continuous bidirectional spiking occurred. Although treatment with ivermectin also showed some activity similar to GABA, ivermectin acts on receptor-operated chloride channels leading

to long-lasting hyperpolarisation or depolarization of the neuron or muscle cell, blocking function (Wolstenholme and Rogers, 2005). GABA-treated flies developed unidirectional impulses much earlier than did ivermectin-treated flies.

A high FMI:WBS ratio indicates efficient coupling of motor output to the thoracic muscle units involved in flight activity. Thymol, ivermectin and OA produced a FMI:WBS ratio that was similar to their controls. However, the ratio was affected by other treatments. For example, flies treated with fipronil, CDM, DMCDM and GABA exhibited the lowest FMI:WBS ratios, while cypermethrin did not reduce the ratio as much. Based on these ratios, fipronil, CDM, DMCDM, GABA and to a lesser extent cypermethrin, affected the excitability of the indirect flight muscles.

An objective of our research was to investigate whether octopamine-like activity occurred with thymol *in vivo* since some essential oil constituents are known to act at octopaminergic sites (Enan, 2001; Lindahl and Oberg, 1961). Because previous reports have suggested that formamidines are OA agonist (Watanabe et al., 1975; Hollingworth and Lund, 1983; Kinnamon et al., 1984; Whim and Evans, 1988) I included CDM and DMCDM in this investigation. CDM, DMCDM and OA affected FMI in a qualitatively similar manner. Bidirectional impulses occurred as discrete bursts of FMI activity and spiking within bursts was always continuous. However, the time of onset, duration and intensity of effects differed among the compounds. Bursts with OA and CDM occurred earlier and were much more obvious than with DMCDM. Also, intense early and late phase continuous spiking was a consistent feature of DMCDM, whereas only the late phase component was observed with CDM, which may be related to a requirement for bioactivation. Although late phase multiple spiking was observed with OA, the FMI were

much smaller in amplitude. Nonetheless, the FMI patterns elicited by the formamidines are considerably different from those of thymol and I conclude that thymol does not act as an octopamine agonist on flight motor-specific pathways *in vivo*.

Voltage-dependent sodium channels represent major sites of pyrethroid action (Soderlund and Bloomquist, 1987). Pyrethroids delay the closing of sodium channels in neurons. All blowflies treated with cypermethrin showed symptoms of hyperactivity and convulsions as described for other pyrethroids (Adams and Miller, 1980). The activity patterns caused by cypermethrin were different from those produced by GABA, ivermectin or thymol (Adams and Miller, 1980). Intense FMI were seen in blowflies treated with cypermethrin which often lasted for over 30 min. In contrast, rotenone inhibits mitochondrial complex I and the changes in electrical activity produced by rotenone were very different than those produced by GABA, ivermectin or thymol.

In summary, this investigation focused on the temporal progression of interference with FMI and WBS after treatment of blowflies with the plant terpenoid thymol and various neuroactive substances. I have devised an electrophysiological system that can measure and compare the effects of different compounds on insects *in vivo* and in so doing can ascribe a putative mode of action of unknown substances. Our data suggest that thymol may interfere with GABAergic control of the dipteran flight motor system in vivo, most likely through a central action. This work reemphasizes the utility of the flight motor system in helping to understand the *in vivo* actions of plant-derived compounds such as thymol with potential applications in pest management.

4.6 Figures

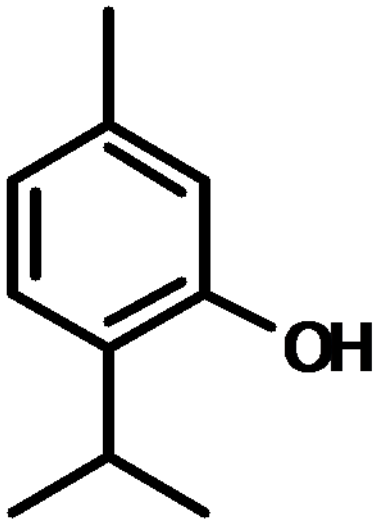

Figure 4-1 The structure of thymol.

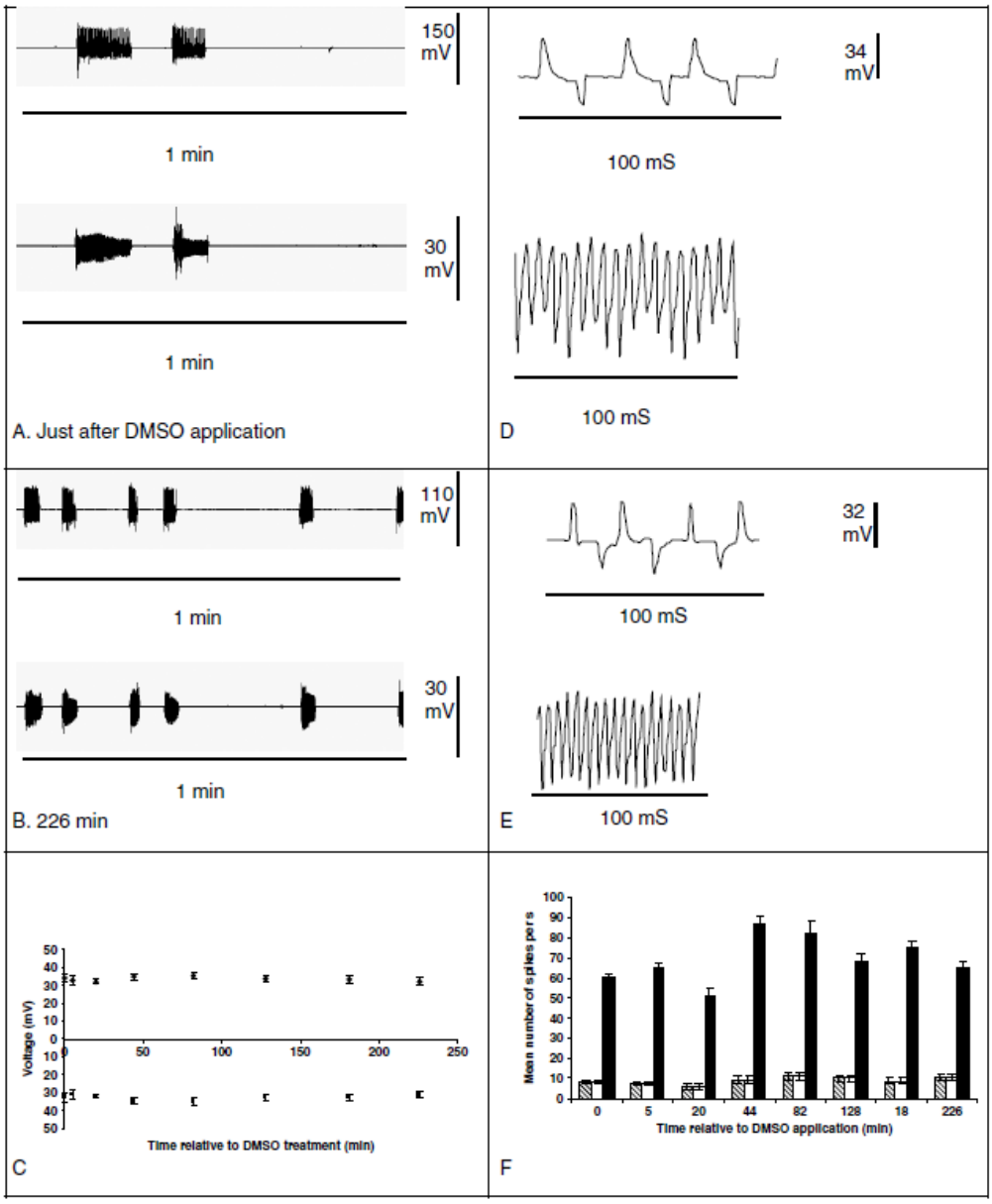

Figure 4-2 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of control (DMSO-treated) blowflies.
A and B: temporal progression of FMI (top panels) and WBS (lower panels) at 1 min and 226 min post application respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D and E: Enlarged sections of FMI (top panels) and WBS (lower panels) from A and B respectively. F: The effect of DMSO on FMI and WBS frequency over the 226 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

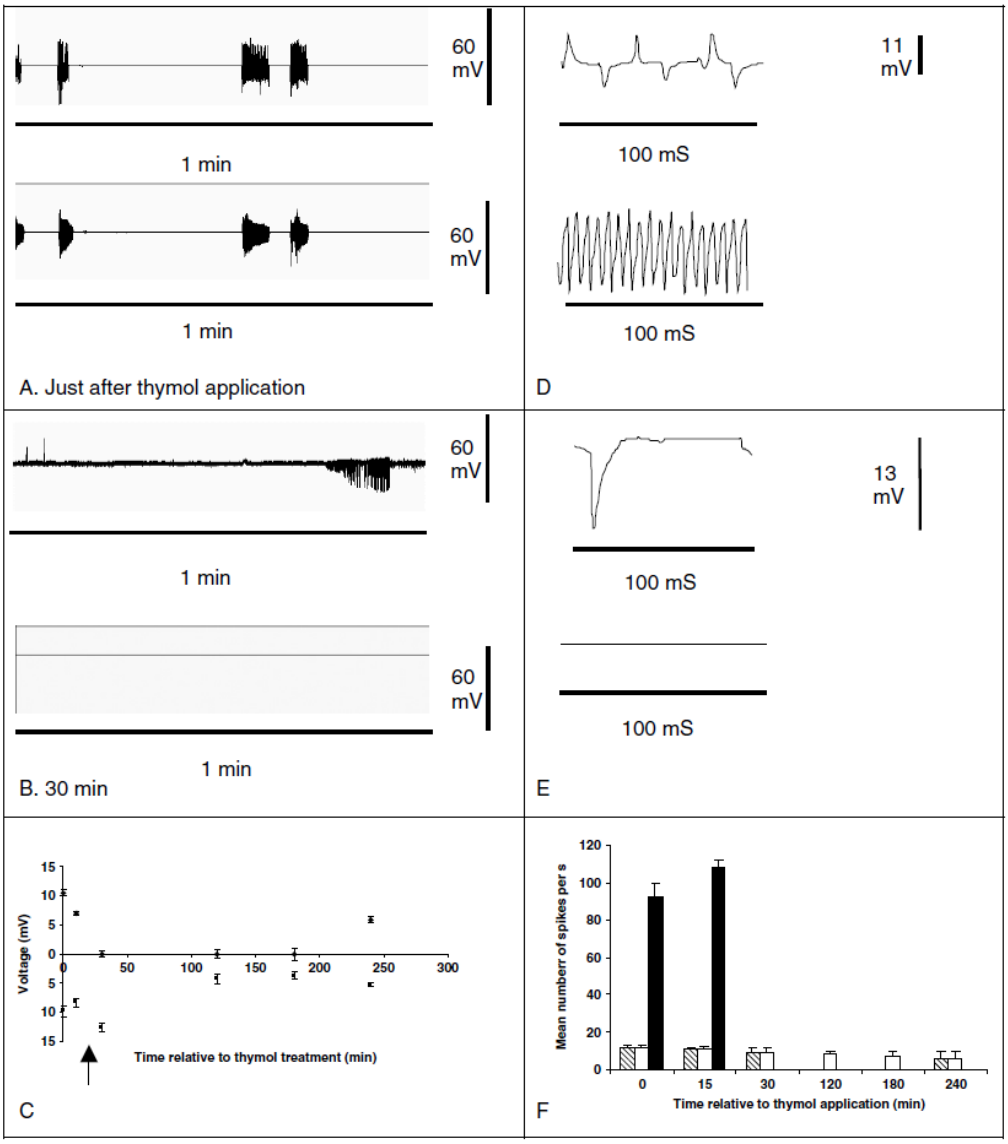

Figure 4-3 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies treated topically with thymol (50 µg).
A and B: temporal progression of FMI (top panels) and WBS (lower panels) at 1 min and 30 min post application respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D and E: Enlarged sections of FMI (top panels) and WBS (lower panels) from A and B respectively. F: The effect of thymol on FMI and WBS frequency over the 240 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

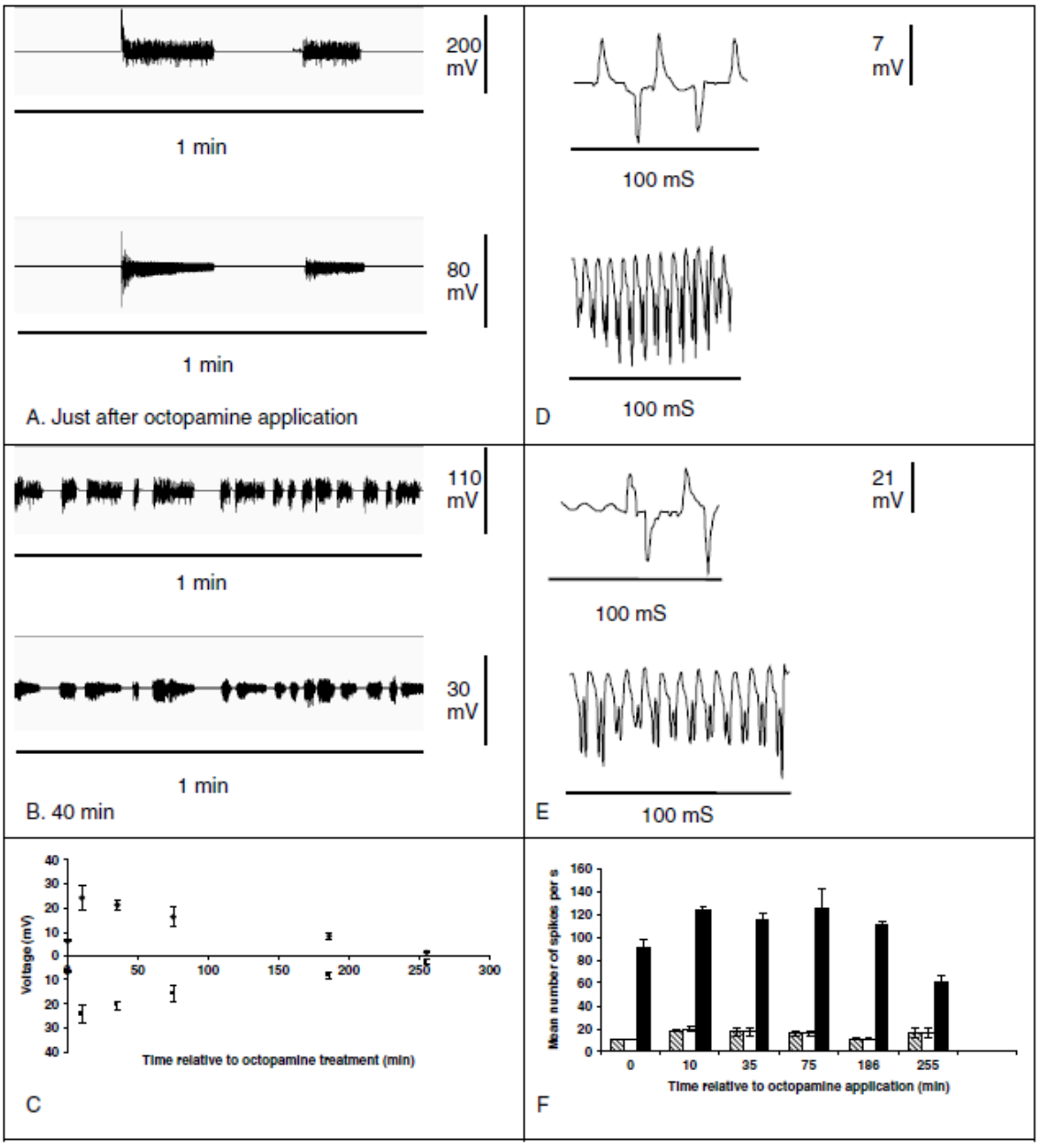

Figure 4-4 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies injected with octopamine (OA; 50 µg).
A and B: temporal progression of FMI (top panels) and WBS (lower panels) at 1 min and 40 min post application respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D and E: Enlarged sections of FMI (top panels) and WBS (lower panels) from A and B respectively. F: The effect of OA on FMI and WBS frequency over the 255 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

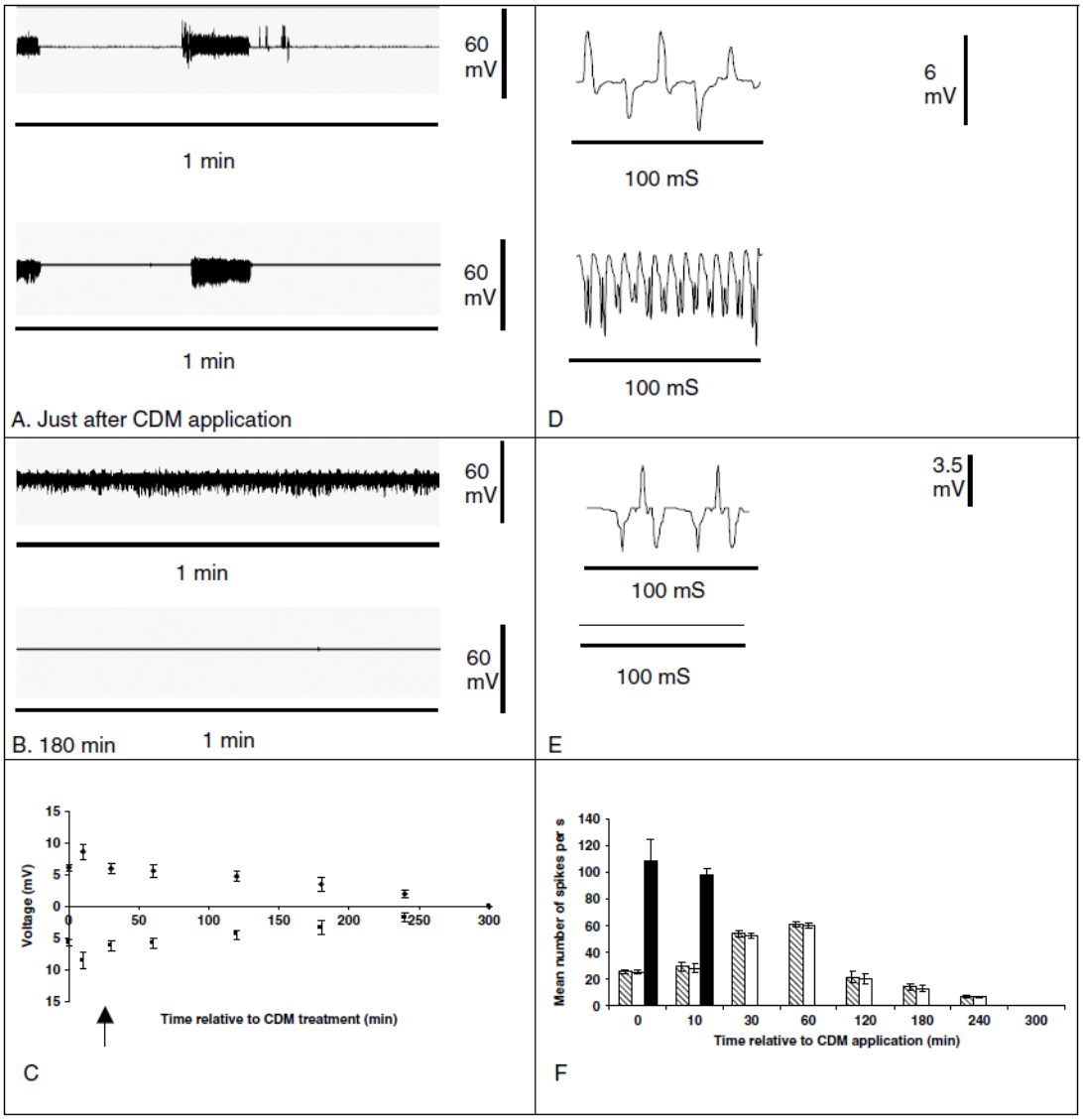

Figure 4-5 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies treated topically with chlordimeform (CDM; 50 µg).
A and B: temporal progression of FMI (top panels) and WBS (lower panels) at 1 min and 180 min post application respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D and E: Enlarged sections of FMI (top panels) and WBS (lower panels) from A and B respectively. F: The effect of CDM on FMI and WBS frequency over the 300 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

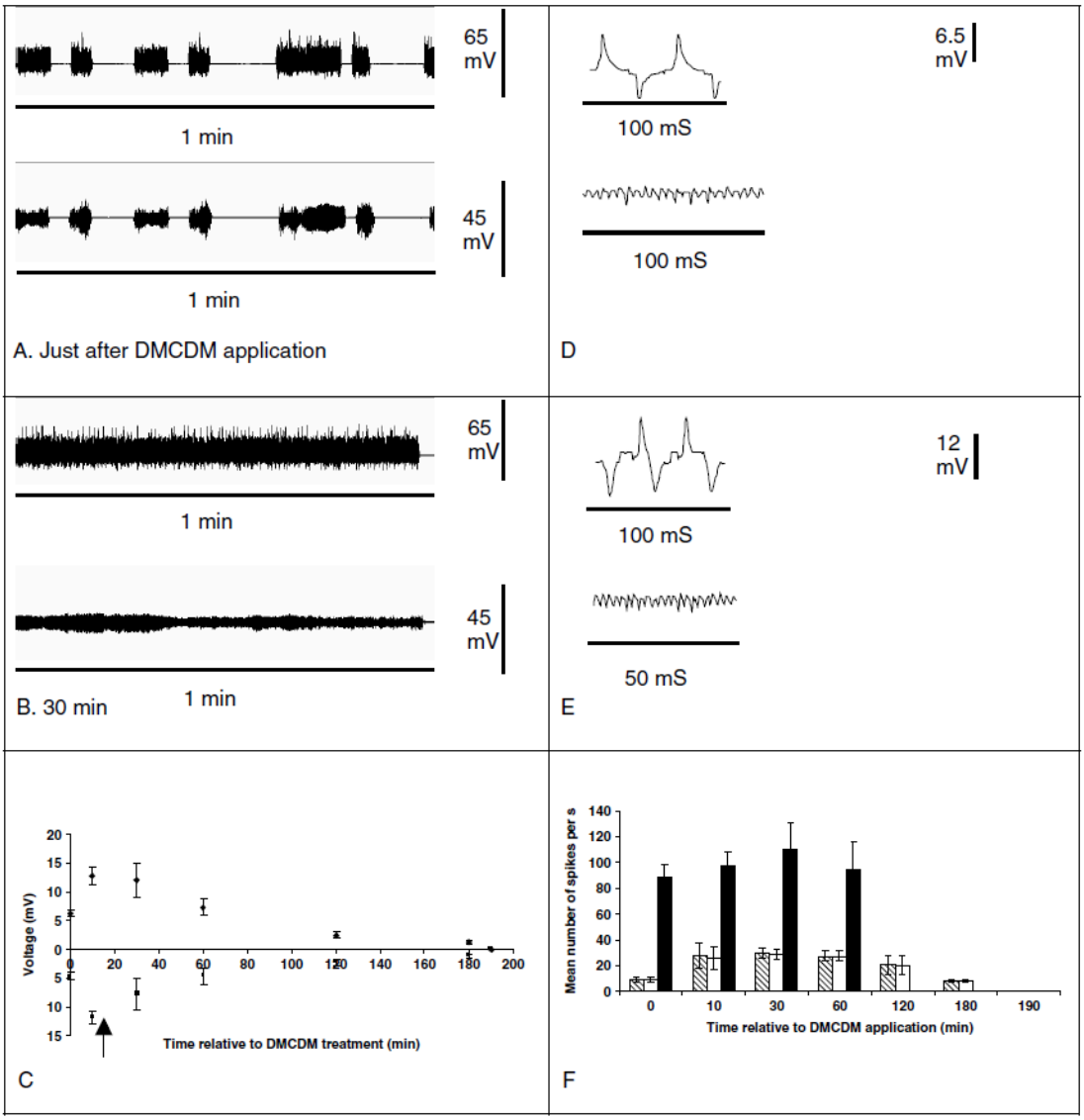

Figure 4-6 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies treated topically with desmethylchlordimeform (DMCDM; 50 µg).
A and B: temporal progression of FMI (top panels) and WBS (lower panels) at 1 min and 30 min post application respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D and E: Enlarged sections of FMI (top panels) and WBS (lower panels) from A and B respectively. F: The effect of DMCDM on FMI and WBS frequency over the 190 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

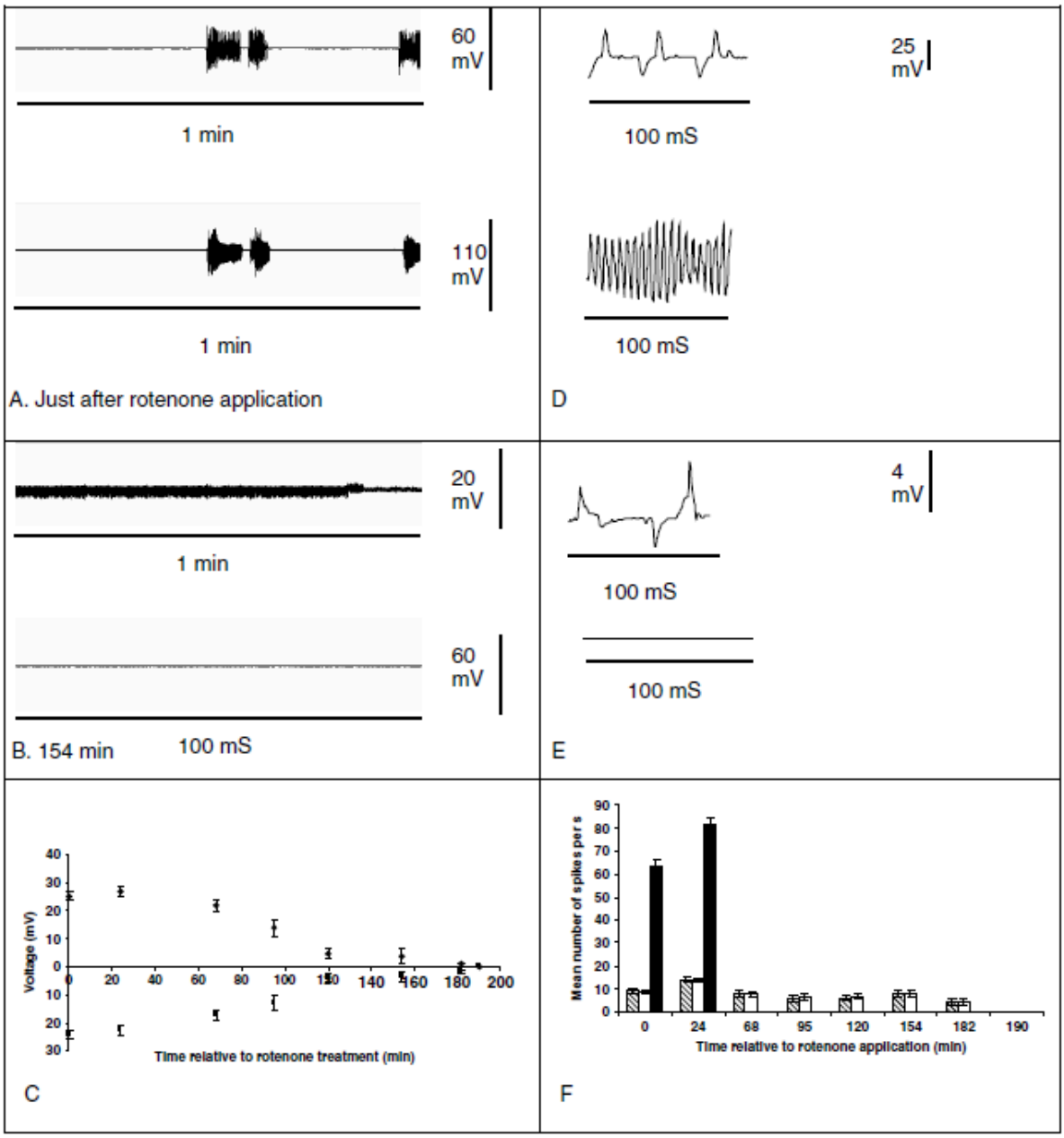

Figure 4-7 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies treated topically with rotenone (50 µg).
A and B: temporal progression of FMI (top panels) and WBS (lower panels) at 1 min and 154 min post application respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D and E: Enlarged sections of FMI (top panels) and WBS (lower panels) from A and B respectively. F: The effect of rotenone on FMI and WBS frequency over the 190 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

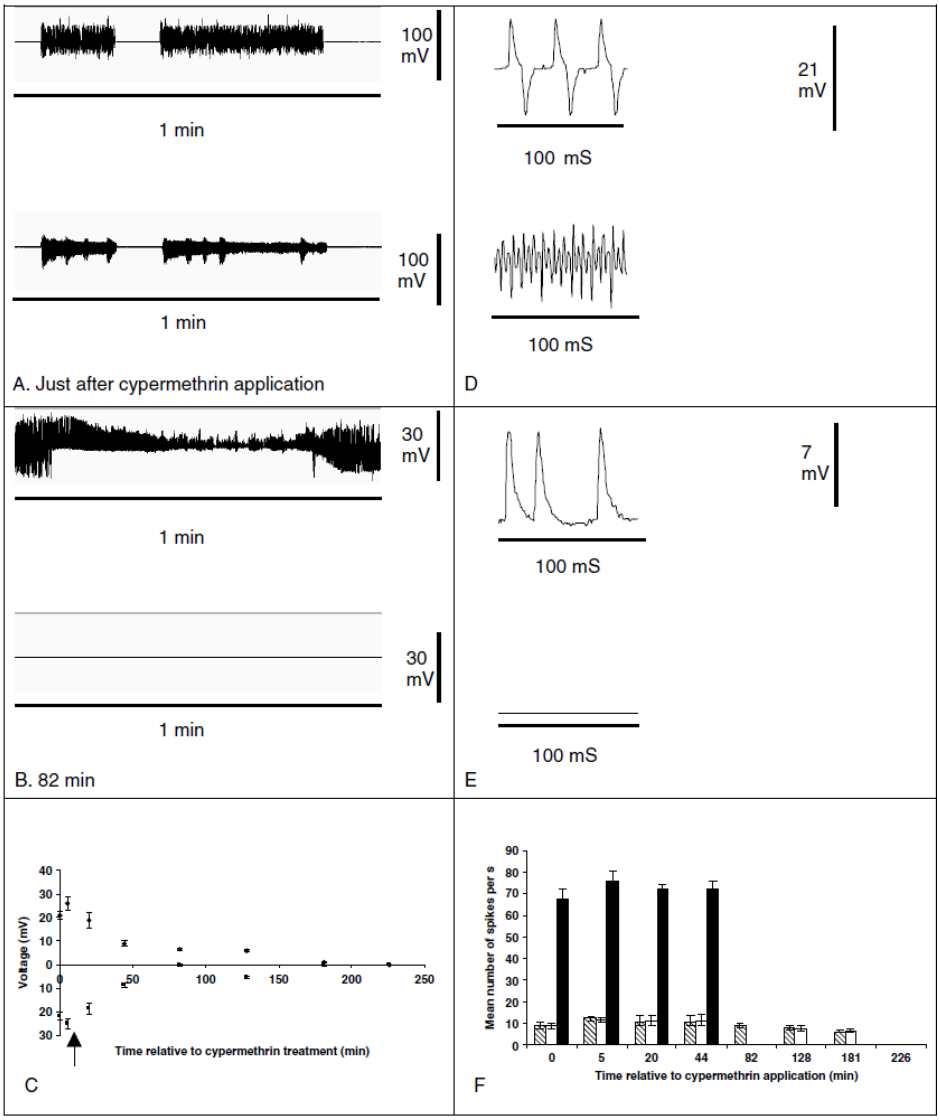

Figure 4-8 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies treated topically with cypermethrin (50 µg).
A and B: temporal progression of FMI (top panels) and WBS (lower panels) at 1 min and 82 min post application respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D and E: Enlarged sections of FMI (top panels) and WBS (lower panels) from A and B respectively. F: The effect of cypermethrin on FMI and WBS frequency over the 226 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

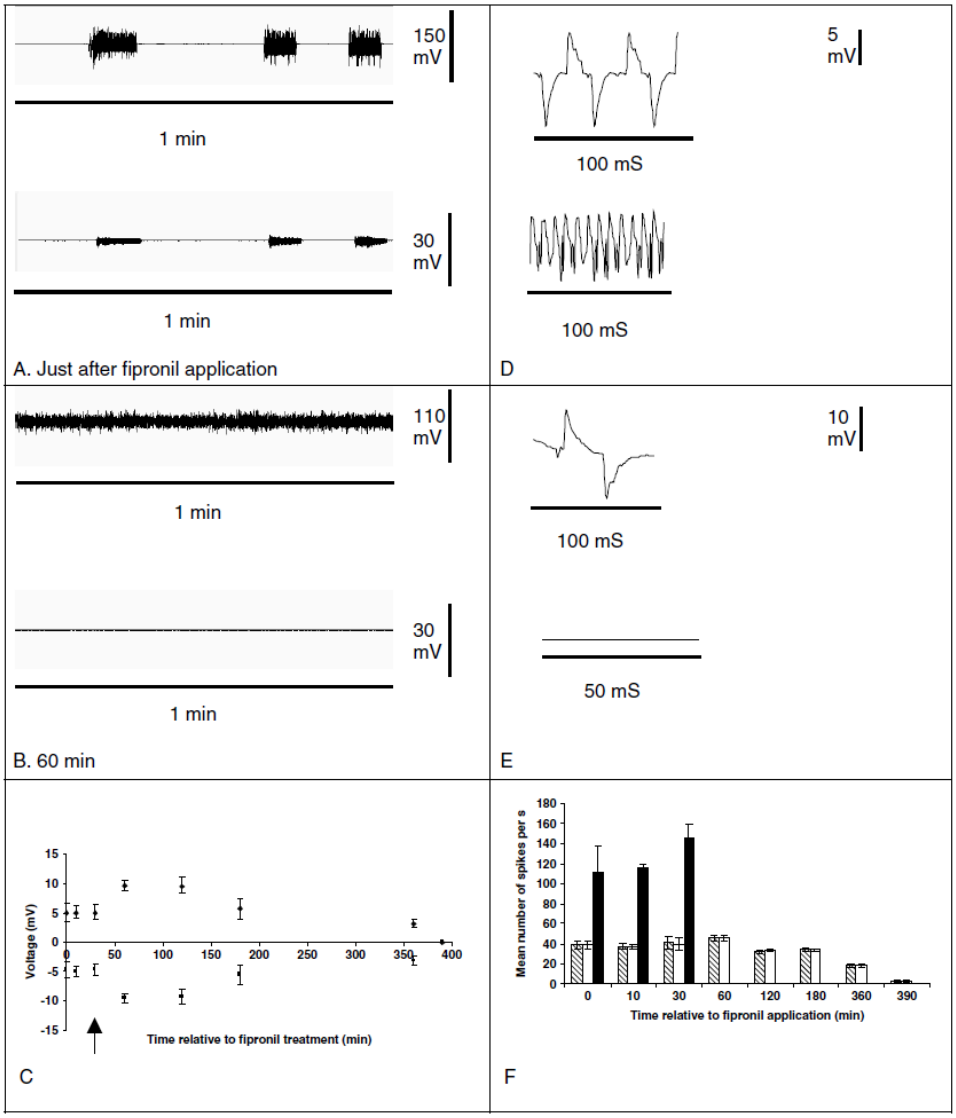

Figure 4-9 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies treated topically with fipronil (50 µg).
A and B: temporal progression of FMI (top panels) and WBS (lower panels) at 1 min and 60 min post application respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D and E: Enlarged sections of FMI (top panels) and WBS (lower panels) from A and B respectively. F: The effect of fipronil on FMI and WBS frequency over the 390 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

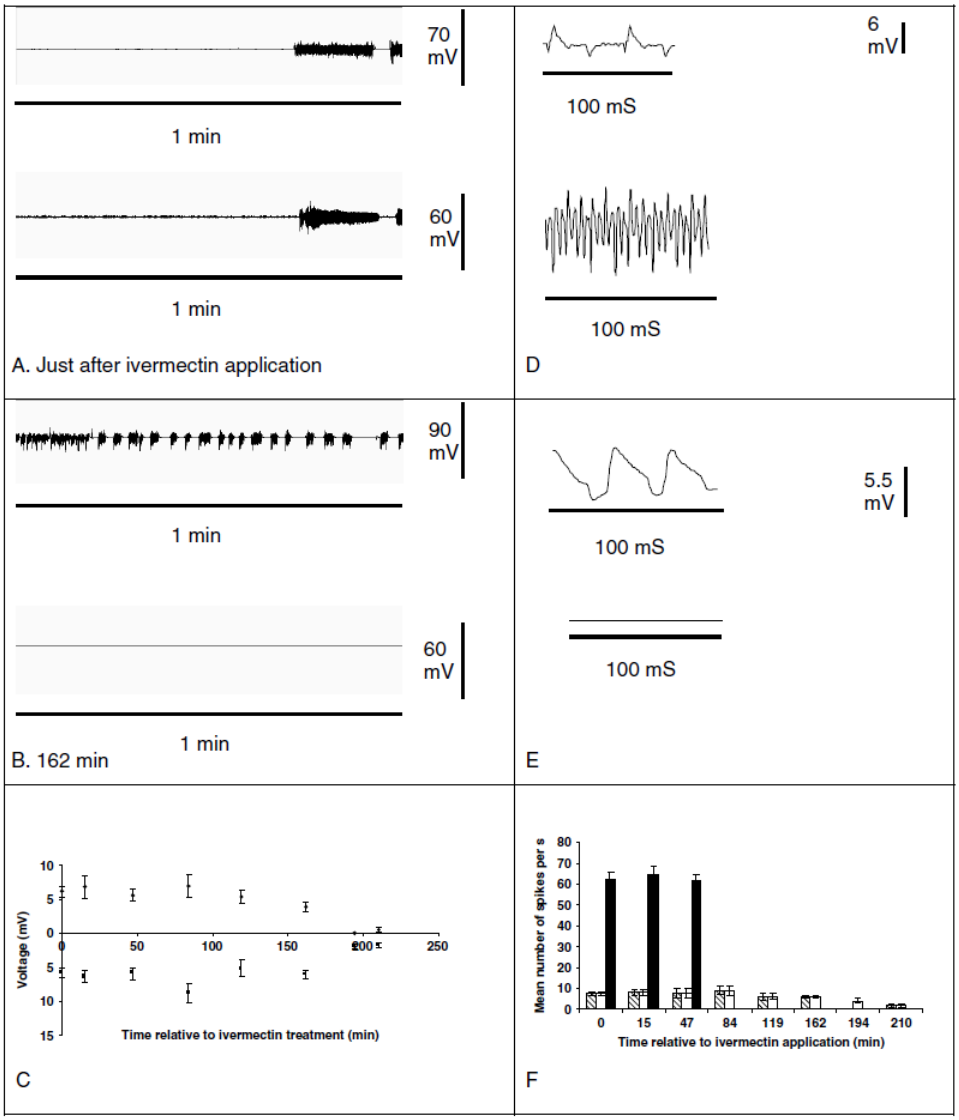

Figure 4-10 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies treated topically with ivermectin (50 µg).
A and B: temporal progression of FMI (top panels) and WBS (lower panels) at 1 min and 162 min post application respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D and E: Enlarged sections of FMI (top panels) and WBS (lower panels) from A and B respectively. F: The effect of ivermectin on FMI and WBS frequency over the 210 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

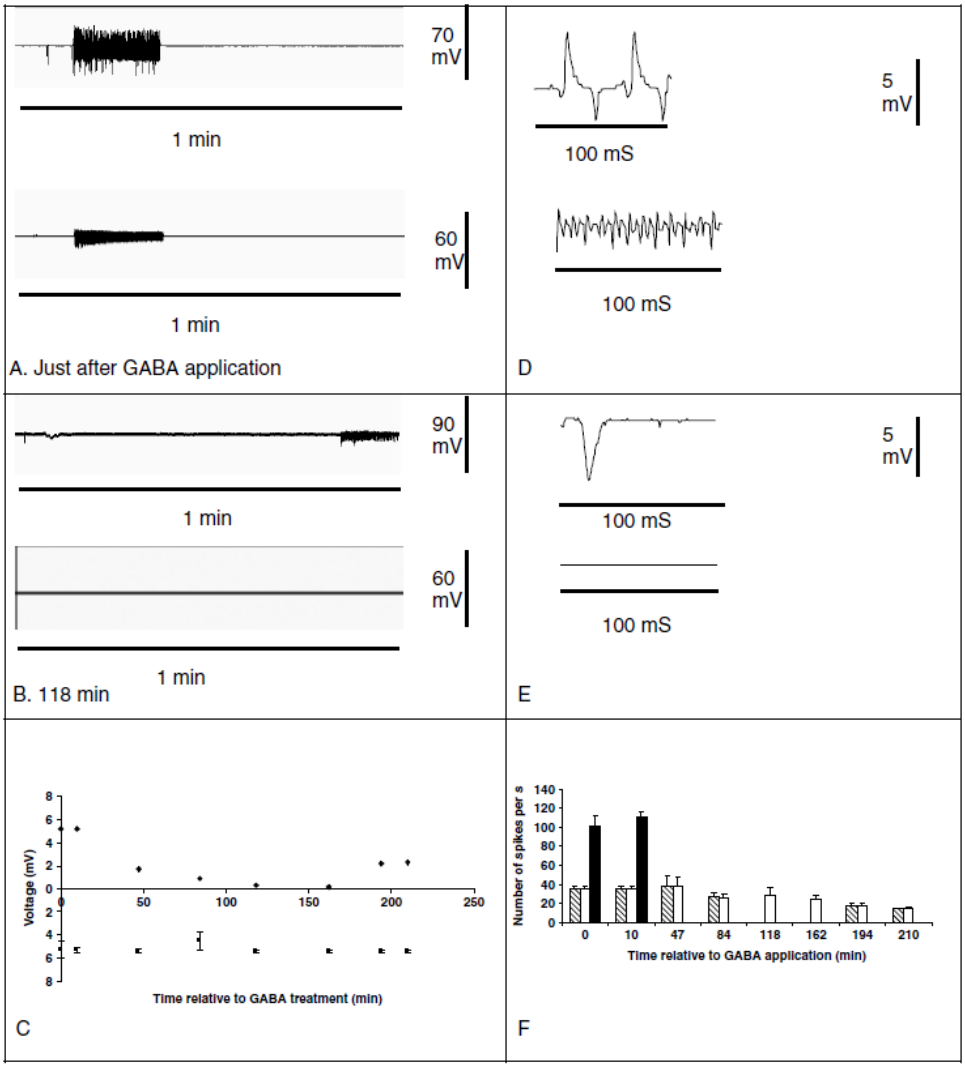

Figure 4-11 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies injected with GABA (50 µg).
A and B: temporal progression of FMI (top panels) and WBS (lower panels) at 1 min and 118 min post application respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D and E: Enlarged sections of FMI (top panels) and WBS (lower panels) from A and B respectively. F: The effect of GABA on FMI and WBS frequency over the 210 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

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Connecting statement 4

In chapter four, I developed an electrophysiological recording system and compared the electrophysiological recordings (FMI and WBS) of plant-derived compound thymol with other known neurotoxic compounds – octopamine, chlordimeform, desmethylchlordimeform, rotenone, cypermethrin, fipronyl, ivermectin and GABA. In the next chapter I expanded these studies to compare the electrophysiological responses of other selected plant-derived compounds (eugenol, pulegone, α-terpineol and citronellal) with dieldrin, malathion and sodium channel blocker RH3421.

Chapter 5: Plant Terpenoids: Acute Toxicities and Effects on Flight Motor Activity and Wing Beat Frequency in the Blowfly *Phaenicia sericata*

A modified version of this chapter has been submitted to the Journal of Economic Entomology as:

Waliwitiya, R., C. P. Belton, R. Nicholson, and C. Lowenberger. Plant terpenoids: Acute toxicities and effects on flight motor activity and wing beat frequency in the blowfly *Phaenicia sericata*.

5.1 Abstract

I evaluated the acute toxicities and the physiological effects of plant monoterpenoids (eugenol, pulegone, citronellal and α-terpineol) and neuroactive insecticides (malathion, dieldrin and RH3421) on flight muscle impulses (FMI) and wing beat signals (WBS) of the blowfly (*Phaenicia sericata*).

Topically-applied eugenol, pulegone, citronellal and α-terpineol produced neurotoxic signs of poisoning, but were less toxic (LD_{50}s: 20.2, 37.5, 18.8 and 83.1 µg/blowfly, respectively) than malathion, dieldrin, or RH3421 (LD_{50}s: 2.3, 1 and 10 µg/blowfly, respectively). After topical application, eugenol, pulegone and citronellal (25 µg/insect) reduced spike amplitude in one of the two banks of blowfly dorsolongitudinal flight muscles within 6-8 min, but with citronellal, the amplitude of FMIs reverted to a normal pattern within one hour. In contrast to pulegone and citronellal, where impulse frequency remained relatively constant, eugenol caused a gradual increase, then a decline in the frequency of spikes in each muscle bank. Electrical activity was completely absent 1-2 hours after dosing with pulegone and eugenol. However, in citronellal-treated blowflies, normal flight muscle spiking was observed at the end of the recording period. Wing beating was blocked permanently within 6-7 min of administering pulegone or citronellal. Eugenol blocked wing beating less rapidly, but wing beat signals failed completely. α-Terpineol-treated blowflies did not beat their wings (between 50-70 min) despite a normal FMI pattern throughout the recording period. The actions of these monoterpenoids on blowfly flight motor patterns are discussed and compared to those of dieldrin, malathion, RH3421 and a variety of other neuroactive substances I have previously investigated in this system.

Eugenol, pulegone and citronellal readily penetrated the blowfly cuticle and interfered with flight muscle central nervous function. Although differences in their actions were apparent, they mainly depressed flight-associated responses, and acted similarly to compounds that block sodium channels and facilitate GABA action. The least toxic monoterpenoid (α-terpineol) showed minimal interference with flight-associated function.

5.2 Introduction

Plants and insects have experienced a long period of reciprocal interactions, mainly as a result of phytophagous insects consuming significant amounts of plant tissues. To repel or incapacitate insects, plants biosynthesize a variety of defense chemicals broadly classified as terpenoids, phenolics and alkaloids (Isman and Akhtar, 2007). Many extracts from higher plants and their constituent compounds have been evaluated against agricultural, garden and domestic pests for possible lethal, repellent and antifeedent properties. However, with notable exceptions (e.g. the pyrethrins, rotenone, nicotine and azadirachtin), few plant-derived substances have been commercialized for arthropod control.

Despite this limitation, there is considerable interest in the potential of developing monoterpenoids such as pulegone, citronellal, α-terpineol and eugenol as new tools to manage arthropod pests. For example, α-terpineol is toxic to the adult human head louse and prevents eggs from hatching (Yang et al., 2009). Citronellal and eugenol are toxic to wireworm larvae (Waliwitiya et al., 2005), and knockdown and repellent effects in mosquitoes have been reported with terpineol and citronellal (Vartak and Sharma, 1993). Eugenol kills or repels various beetle pests of stored grain products (Obeng-Ofori and Reichmuth, 1997), while pulegone is toxic to houseflies and cockroaches as well as stored product coleopteran pests (Lee et al., 2003). These monoterpenes also are common constituents of perfumes, cosmetics, food flavorings and herbal medicines. The compounds themselves, or their parent essential oils, are GRAS- (Generally Recognized As Safe) listed by the US Food and Drug Administration. Monoterpenes tend to

biodegrade rapidly (Misra et al., 1996; Hu and Coats, 2008) but are generally less toxic to the pest than synthetic insecticides (Yang et al., 2009; Lee et al., 2003).

Not a great deal is known about how individual monoterpenoids kill insects but the symptoms suggest considerable impairment to nervous system or neuromuscular function (Coats et al., 1991; Kostyukovsky et al., 2002; Waliwitiya et al., 2010). The neurotoxic action of monoterpenoids is further supported by *in vitro* studies which have identified various neuronal targets sensitive to these compounds. Thymol enhances GABA-activated chloride currents in oocytes expressing fruitfly homomeric GABA receptors (Priestley et al., 2003), and the sensitivity of native GABA receptors of housefly and cockroach CNS to thymol and pulegone has been verified recently using [^{3}H]TBOB binding and ^{36}Cl flux assays (Tong and Coats, 2010). Monoterpene mixtures increase cAMP levels in Lepidoptera, possibly by activating octopamine receptors (Kostyukovsky et al., 2002). In the American cockroach, eugenol and ∞-terpineol increase heart rate in a manner similar to octopamine, produce biphasic changes to brain cAMP levels, and displace the binding of [^{3}H]octopamine to neuronal membranes (Enan, 2001). However, Price and Berry (2006) found that eugenol inhibits spiking of cockroach DUM neurons in marked contrast to octopamine, which increases spike frequency. In contrast to the excitatory action of octopamine, eugenol suppressed cockroach foregut contractions. The effects of other monoterpenes (citral and geraniol) were, however, more octopamine-like in these preparations (Price and Berry, 2006).

Our work has focused on the blowfly flight motor system as an *in vivo* assay of the neuroactive effects of plant monoterpenes on insects (Waliwitiya et al., 2010). This application has provided valuable insights into the actions of both natural products and

synthetic insecticides on the flight motor output (Hart et al., 1978; Adams and Miller, 1980). It can also indicate how rapidly a compound penetrates the host and locates the nervous system after dosing, and how rapidly its effects subside (Hart et al., 1978). Moreover, a comparison of the pattern of flight motor signal disruption induced by plant natural products with those of neuroactive compounds of known modes of action can help show the way plant products act. For example, of the neuroactive compounds I examined, thymol showed the closest resemblance to GABA suggesting a central GABA-like action for thymol (Waliwitiya et al., 2010).

In this study I investigated the acute toxicities and effects of eugenol, pulegone, α-terpineol and citronellal on flight motor impulses and wing beat frequency in the blowfly. Because eugenol has been reported to affect sodium channels and GABA receptors in the rat central nervous system (Aoshima and Hamamoto, 1999; Cho et al., 2008) and monoterpenoids can inhibit acetylcholinesterase (López and Pascual-Villalobos, 2010), I included an acetylcholinesterase inhibitor (malathion), an insect GABA-gated chloride channel blocker (dieldrin) and a blocker of insect sodium channels (RH3421) for comparison.

5.3 Materials and methods

5.3.1 Insects

A colony of blowflies (*Phaenicia sericata*) was maintained as described previously (Waliwitiya et al., 2010) at 25^0C, 80% relative humidity, and on a 12h:12h light:dark cycle. Pupae were collected, placed in cages with access to sugar and water *ad libitum* upon eclosion. Three to five-day old adult females were selected for toxicological and electrophysiological studies. Food was removed 4 h prior to experiments and then

replaced 3.5 hrs later, to encourage insects to feed to repletion just before toxicity and flight motor experiments were carried out.

5.3.2 Chemicals

Eugenol, α-terpineol, citronellal, and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). Pulegone was obtained from Ecosafe Natural Products (Victoria, B.C.). Technical grade malathion, dieldrin and RH3421 were obtained from industrial sources. All the compounds were dissolved and administered topically in DMSO. Control insects were treated with DMSO.

5.3.3 Acute toxicity assay

Acute toxicity assays were conducted to estimate the LD_{50} values of the compounds. Adult female blowflies, selected from the laboratory colony (Waliwitiya et al., 2010), were anesthetized with CO_2. The compounds were applied to the tip of the abdomen using a glass microsyringe. In all cases, 10 blowflies were used per treatment and each treatment was replicated at least 3 times. Blowflies were examined for toxic signs over 24h. At the end of this period, mortality was assessed by gently probing the blowflies with a pair of forceps. Blowflies that showed no movement were considered dead. Blowflies living after 24h were killed with 70% ethanol. Probit analysis was used to calculate LD_{50} values (Finney, 1964).

5.3.4 Recording of blowfly FMI and WBS

The techniques used in his section have been described in more detail (Waliwitiya et al., 2010). Briefly, blowflies were anesthetized mildly using CO_2. Copper recording electrodes 50 μm diameter (fully insulated except for the last 3 mm) were inserted into

the left and right dorsolongitudinal muscles (DLM5) from the anterior of the pterothorax. A common reference ground electrode was inserted between the left and right DLM 4 using micromanupulators. Muscle insertions were located by referencing their locations to prominent dorsal setae (Adams and Miller, 1980). Once positioned correctly, the electrodes themselves supported the blowflies while on the substrate, and allowed them to fly freely. Electrical impulses from flight muscles (FMI) were fed into a differential preamplifier with an input impedance of 100MΩ and a gain of 100 and frequency response level to 100 KHz. Output from the preamplifier was recorded on a NI USB-6008 data logger (National Instruments, Vaudreuil-Dorion, Quebec, Canada). Simultaneously, acoustic signals from wing beats of the blowfly (WBS) were recorded with a miniature microphone (Realistic 33-1052) connected to an amplifier speaker (Archer Model 277-1008 B, Taiwan) and the signal was recorded by the same data logger. Ten minutes after recovery from CO_2 anesthesia, eugenol (25 µg), pulegone (25 µg), citronellal (25 µg) or α-terpineol (75 µg) were applied topically (in DMSO) to the tip of the abdomen. Insecticides (5 µg malathion, dieldrin or RH3421) were administered similarly. Control insects were treated topically with DMSO. Recordings began immediately. Firing rates (impulses/s) were calculated by counting the number of action potentials that occurred after the application of each test compound. The mean responses and their standard errors were determined, and amplitudes ± SEs were calculated using LABVIEW 8.5 (National Instruments, Quebec, Canada). Each compound was tested on a minimum of three blowflies. For any given treatment the results were very similar and representative traces are displayed in the figures.

5.4 Results

5.4.1 Acute toxicity of plant terpenoids and insecticides to blowflies

LD_{50} values of the plant terpenoids and synthetic insecticides are shown in Table 5-1. Plant-derived compounds had larger LD_{50} values than the synthetic insecticides. Citronellal and eugenol were the most acutely toxic monoterpenoids to the blowflies, followed by pulegone and α-terpineol.

Flies treated with eugenol showed difficulty in locomotion and later became uncoordinated. Pulegone-treated flies became hypoactive and after approximately 12 hrs they became uncoordinated and then paralysed. Citronellal-treated flies exhibited ataxia, accompanied by tremors within 30-60 min. Blowflies treated with malathion showed extensive grooming, early onset tremors, and pronounced writhing that continued for nearly 3 h. Blowflies treated with dieldrin initially showed increased grooming, writhing and attempted to fly. Within 30-40 minutes they became hyperactive and later were paralyzed. Blowflies treated with RH3421 showed less vigorous writhing and grooming activity than with malathion or dieldrin. No tremors were observed and within 3-3.5 h flies became paralyzed. Probing of quiescent blowflies treated with RH3421 or pulegone produced temporary excitation, but this response was not observed with eugenol or citronellal. Blowflies treated with α-terpineol showed a low level grooming and writhing behavior followed by paralysis.

5.4.2 Effects on FMI and WBS in control blowflies

Blowflies treated with DMSO had normal muscle activity (Fig 1 A-C) throughout the study period. Very similar control flight motor patterns were observed in blowflies in our previous investigation (Waliwitiya et al., 2010) and other published reports (Hart et

al., 1978; Miller and Kennedy, 1972). In control insects, WBS were regular and always linked closely to FMI (1:7). Under our experimental conditions, control blowflies flew for extended periods, but not continuously.

5.4.3 Effects of eugenol

The effects of eugenol on FMI and WBS are shown in Fig. 5-2. For the first 15 min muscle impulses (Fig. 5-2A) were similar to the controls (Fig. 5-1A) but from 18-49 min these became higher frequency, lower amplitude impulses (Fig.5-2B and C). From 8 min onwards, the amplitude of signals from the left DLM bank (lower deflections) was reduced during periods of electrical activity (see also Fig 5-2D). There were no wingbeats after 8 min of treatment but these returned at 76 min (Fig. 5-2E). No FMI or WBS activity was detected after 117 min (Fig 5-2D and E). The flies remained alive, but inactive, at the end of the 2 h recording period.

5.4.4 Effects of pulegone

Initially, FMI and WBS were similar to controls (Fig. 5-3A). However, by 28 min, the amplitude of impulses from the left bank of muscles had declined to zero, despite maintenance of normal flight motor output from the right DLM bank (upward impulses) until 28 min (Fig 5-3B). At 60 and 75 min after dosing, no FMI were present (Fig. 5-3C and D), but impulses could be elicited by physical probing. Within 6 min of treatment with pulegone there were no wingbeats (Fig. 5-3D), little leg movement, but no mortality occurred during the experimental period.

5.4.5 Effects of *α*-terpineol

Muscle impulses and wingbeats were normal immediately after dosing blowflies with 75 µg of *α*-terpineol (Fig. 5-4A,C, and E). Between 50-70 min WBS ceased, and sporadic FMI occurred (Fig 5-4B). After 70 min FMI and WBS returned to normal patterns for another 25 min. During the experimental period no flies died.

5.4.6 Effects of citronellal

FMI and WBS were similar to controls after dosing blowflies with 25 µg citronellal and WBS remained tightly linked to FMI activity (Fig. 5-5A and D). However there were no wingbeats after 7 min. (Fig 5-5B and D). FMI amplitude declined rapidly in the left bank of muscles but recovered significantly by the end of the experiment (Fig. 5-5B and C). FMI frequency was largely unchanged (Fig 5-5D). No mortality due to citronellal was observed during the experimental period but flies were lethargic.

5.4.7 Effects of malathion

Flight activities were normal immediately after dosing blowflies with 5 µg of malathion (Fig. 5-6A). However WBS ceased by 18 min, and between 18 and 116 min regular bursting of FMI was apparent (Fig. 5-6C) and FMI spike amplitude and frequency were reduced in both muscle banks (Fig. 5-6B-E). No FMI activity was detectable after 140 min (Fig 5-6D) and flies were either dead, or very close to death, by the end of the assessment period.

5.4.8 Effects of dieldrin

Flight activities were normal immediately after dosing blowflies with 5 µg of dieldrin (Fig. 5-3A). At 5 min, the bursts of FMI became more repetitive (Fig. 5-7B).

Wingbeats ceased after 46 min (Fig. 5-7E) at which point continuous FMI activity of higher amplitude also developed (Fig. 5-7C). The amplitude and frequency declined thereafter (Fig. 5-7D and E) in both muscle banks, and by 187 min there was no electrical activity (Fig. 5-7D and E). All flies treated with dieldrin died or were moribund after 3h.

5.4.9 Effects of RH3421

The effects of topically applied RH3421 (25 μg) are shown in Fig. 5-8. Electrical activities of both groups of dorsolongitudinal muscles was similar to the controls for the first 15 mins (Fig. 5-8A) after which trains of unidirectional (upward) impulses became more apparent through 112 min (Figs. 5-8B and C). However their amplitude dropped progressively 25 min after dosing (Fig. 5-8D) and all spiking ceased by 148 min (Fig. 5-8D and E). There were no wingbeats detected after 25 min (Fig. 5-8E). However, probing of blowflies during a quiescent phase induced FMI.

5.5 Discussion

Our results clearly demonstrate that the plant-derived monoterpenoids eugenol, pulegone and citronellal interfere with flight motor-associated electrical activity and wing beat signals of the blowfly in vivo. This supports the idea that, at doses close to LD_{50}, eugenol, pulegone and citronellal penetrate the insect cuticle in a reasonably efficient fashion and gain access to excitable tissues. The response patterns are multifaceted and very much dependent on the monoterpenoid in question. Within ten minutes of topical application, eugenol, pulegone and citronellal produce clear reductions in the amplitude of spikes associated with one bank of dorsolongitudinal flight muscles. With eugenol, this was accompanied by a progressive increase, then a decline in the frequency of spiking in

both muscle banks. Similar transitions have also been observed *in vitro* with eugenol where increased spike activity in depolarized DUM neurons of the cockroach (*P. americana*) and depression of spontaneous and evoked impulses within the nerve cord occur (Price and Berry, 2006).

All flight motor-associated electrical activity was eventually suppressed by eugenol and pulegone. However with citronellal, the amplitude of spikes in the most affected muscle bank recovered and the spike frequency in both muscle banks was relatively constant for the entire experimental period. Citronellal and pulegone quickly stopped wing beats and inhibited flight muscle contraction. Eugenol was less effective in this regard, only inhibiting wing beat signals briefly, although eventually complete loss of WBS occurred. An important *in vitro* effect of eugenol on mammalian neurons is the inhibition of voltage-gated sodium channels (Park et al., 2009), and such an action could help explain the action of this phytochemical in our experiments. Indeed, in the present experiments, eugenol and pulegone produced effects that were more like those of the sodium channel blocker RH3421 than malathion or dieldrin, since with RH3421, attenuation of spike amplitude also was more prominent in one bank of flight muscles before complete inhibition of flight motor-associated electrical responses occurred. Soon after FMIs ceased in flies treated with pulegone, spiking could be reactivated by gently probing the fly. I observed a very similar effect with the sodium channel blocking insecticide RH3421. Such a response is well-established for RH3421 in insects (Salgado, 1990). Also, the characteristic bursting of FMIs observed after dosing blowflies with malathion and dieldrin was much more prominent than with any of the monoterpenoids. These findings tend to argue against inhibitory actions of these natural products on

GABA-gated chloride channels and acetylcholinesterase. Eugenol, pulegone and citronellal also appear to interfere with flight motor responses in a different way to a number of other neuroactive agents (specifically octopamine, chlordimeform, rotenone and the pyrethroid insecticide cypermethrin) characterized in a previous study (Waliwitiya et al., 2010). On the other hand, the early onset reduction in spike amplitude in one fight muscle bank and the cessation of wing beat signals observed here with pulegone and citronellal are quite similar to the pattern of disruption produced by thymol and GABA (Waliwitiya et al., 2010). This suggests that pulegone and citronellal may be capable of positively influencing GABAergic pathways. GABAergic neurons are known to be present in insect central flight motor circuitry (Meldrum-Robertson and Wisniowski, 1988), and it has been reported recently that thymol and pulegone stimulate [^{3}H]TBOB binding and augment GABA-activated ^{36}Cl influx in *in vitro* insect nerve preparations (Tong and Coats, 2010).

The only detectable flight-related response to α-terpineol was a temporary loss of WBS around one hour after dosing. While α-terpeneol was the least acutely toxic of all the monoterpenoids, blowflies exposed topically to this compound displayed obvious signs of neurotoxicity at 24 h. Moreover, α-terpineol produces toxic signs in several species of insect (Vartak and Sharma, 1993; Yang et al., 2009) and blocks the compound action potential of nerves (Moreira et al., 2001). It is possible that even at close to LD_{50} doses, the time course used in our flight motor experiments did not allow α-terpineol to accumulate at sufficient concentrations to adversely affect this system or that a more sensitive alternative target for α-terpineol is present in blowflies.

In summary, this work further highlights the usefulness of the flight motor system in our understanding of the *in vivo* actions of neuroactive substances in insects. Phytochemicals such as eugenol, pulegone and citronellal, interfere with flight-associated responses in the blowfly at close to LD_{50} values, suggesting relevance of this target system in the intoxication process. Muscle bank-selective reductions in spike amplitude and changes in spike frequency may occur as a result of interference with the central flight oscillatory system or its output pathways within the CNS. As well, direct effects of monoterpenoids on peripheral flight motor nerves and flight muscle contractility may be involved. Comparison of the flight motor and wing beat profiles with those of various neuroactive standards suggests that the *in vivo* effects of eugenol, pulegone and citronellal are most similar to GABA-facilitating or sodium channel-blocking depressant drugs.

5.6 Figures

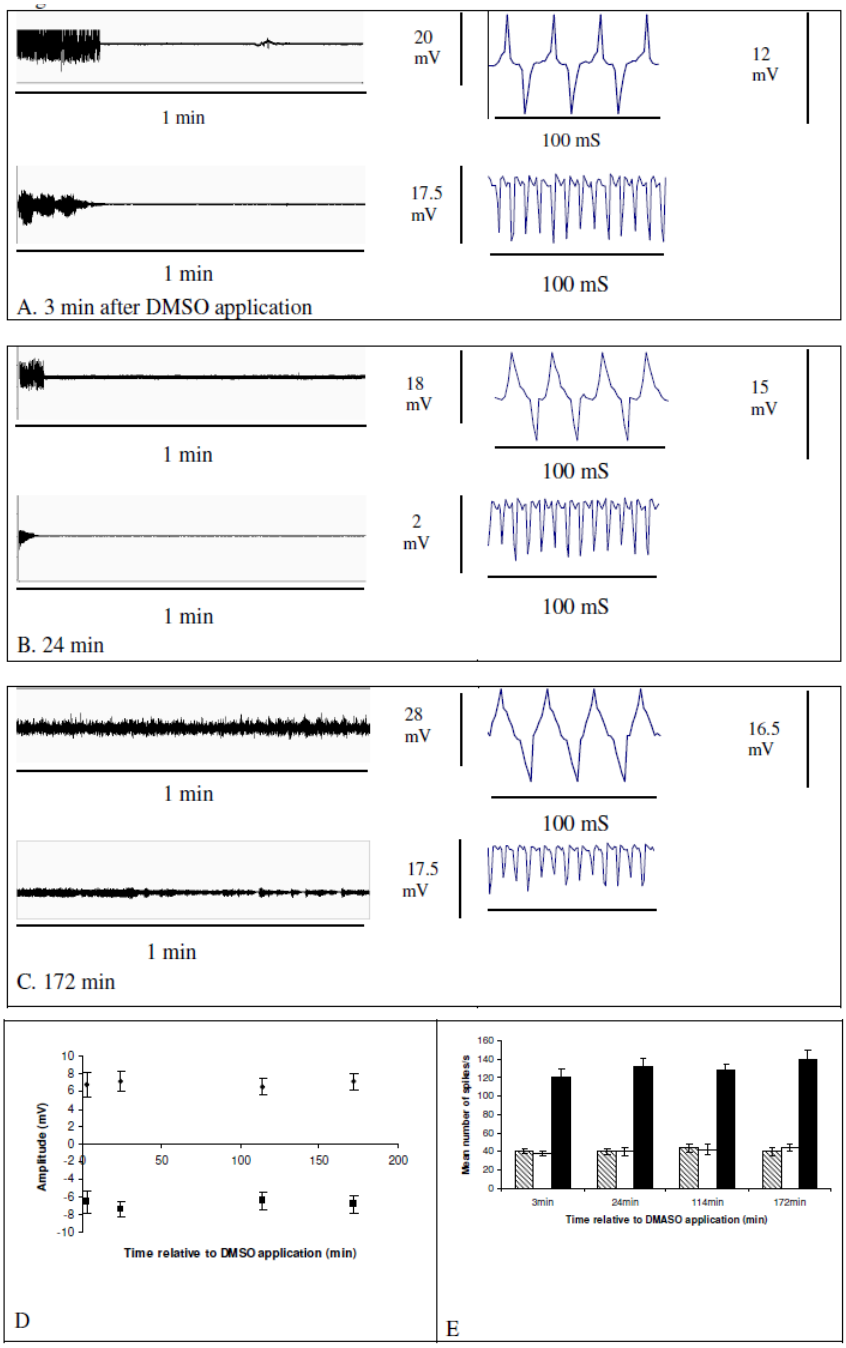

Figure 5-1. Typical control electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies, *Phaenicia sericata*, treated topically with DMSO (5 µg).
A, B and C: temporal progression of FMI (top panels) and WBS (lower panels) at 3, 24 and 172 min post application, respectively, and expanded sections of FMI (top panels) and WBS (lower panels) from these traces. D: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. E: Control FMI and WBS frequency over the 172 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

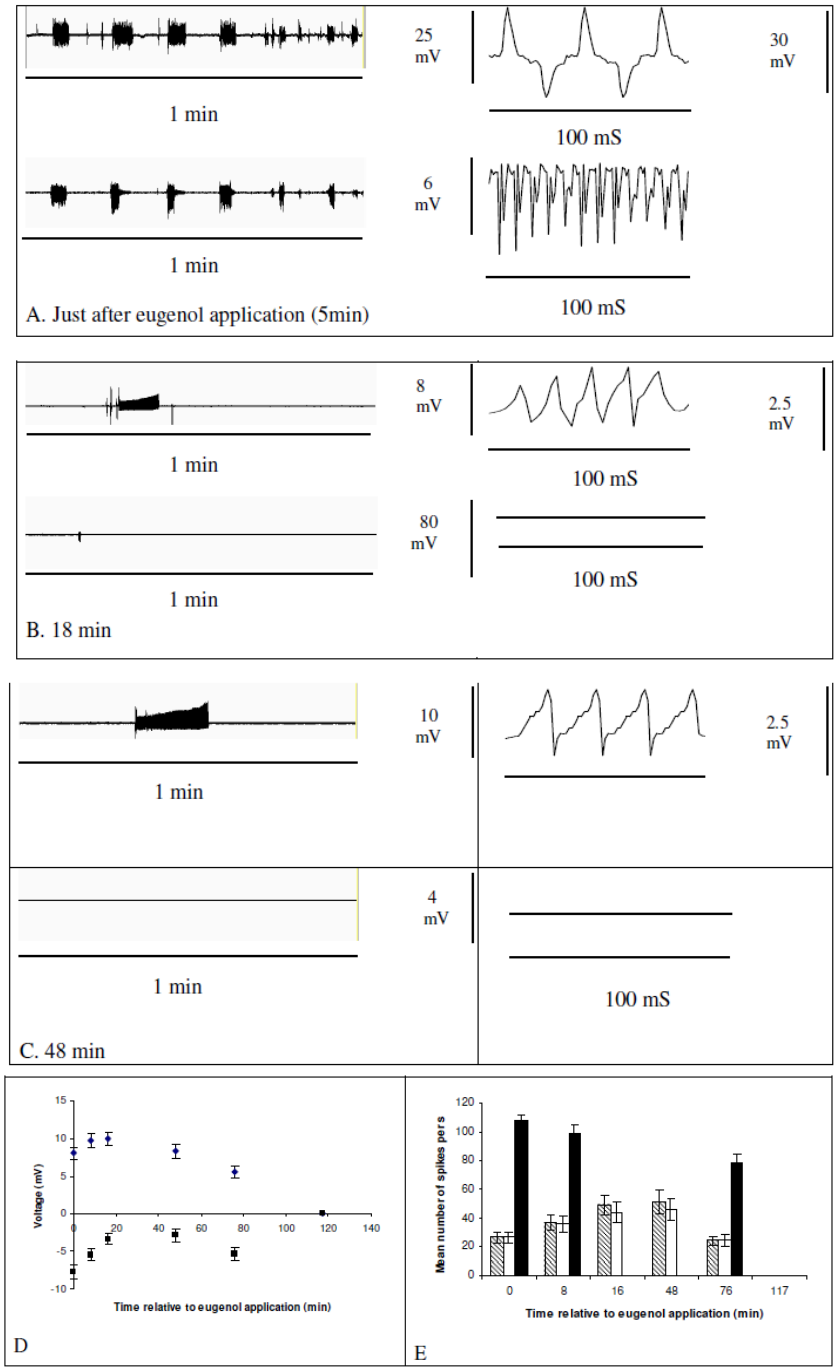

Figure 5-2 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies, *Phaenicia sericata*, treated topically with eugenol (25 µg).
A-C: (left) temporal progression of FMI (top panels) and WBS (lower panels) at 1, 18 and 48 min post application, respectively and (right) expanded sections of FMI (top panels) and WBS (lower panels) from A-C. D: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. E: The effect of eugenol on FMI and WBS frequency over the 117 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

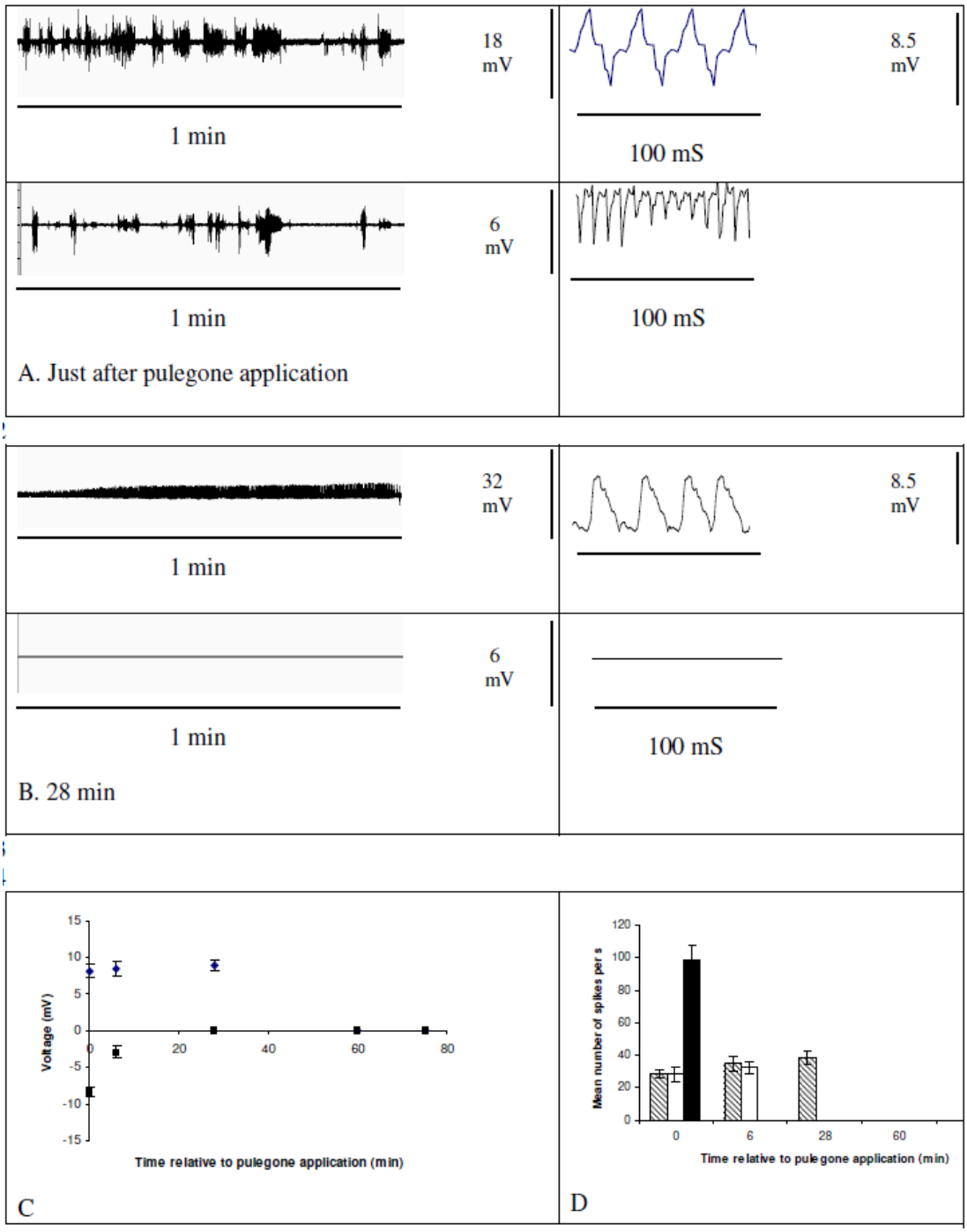

Figure 5-3 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies, *Phaenicia sericata*, treated topically with pulegone (25 µg).
A and B: (left) temporal progression of FMI (top panels) and WBS (lower panels) at 1 and 28 min post application, respectively, with (right) expanded sections of FMI (top panels) and WBS (lower panels) of A and B. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D: The effect of pulegone on FMI and WBS frequency over the 75 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

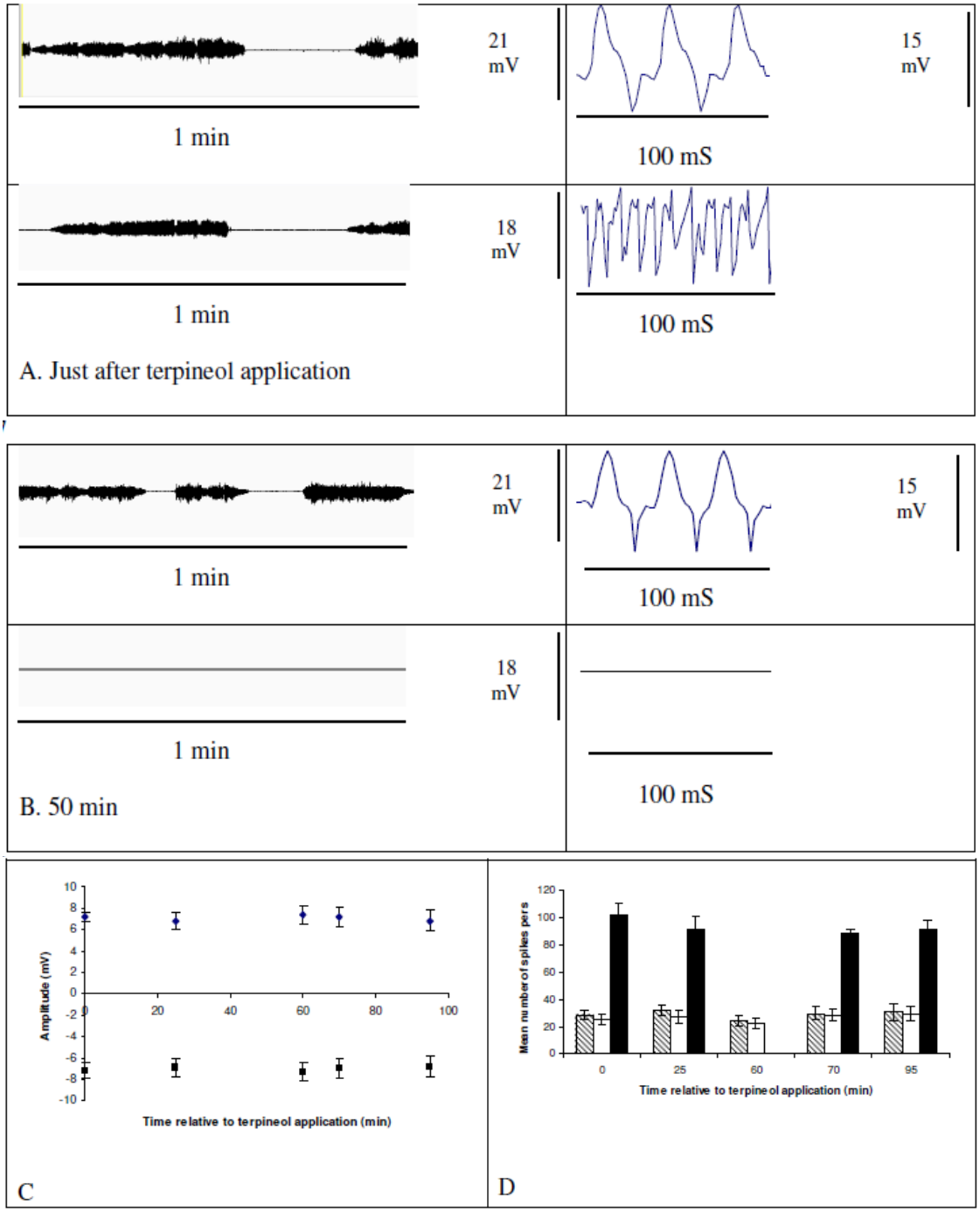

Figure 5-4 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies, *Phaenicia sericata*, treated topically with *α*-terpineol (25 µg).
A and B: (left) temporal progression of FMI (top panels) and WBS (lower panels) at 1 and 60 min post application, respectively, with (right) expanded sections of FMI (top panels) and WBS (lower panels) from A and B, respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D: The effect of *α*-terpineol on FMI and WBS frequency over the 95 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

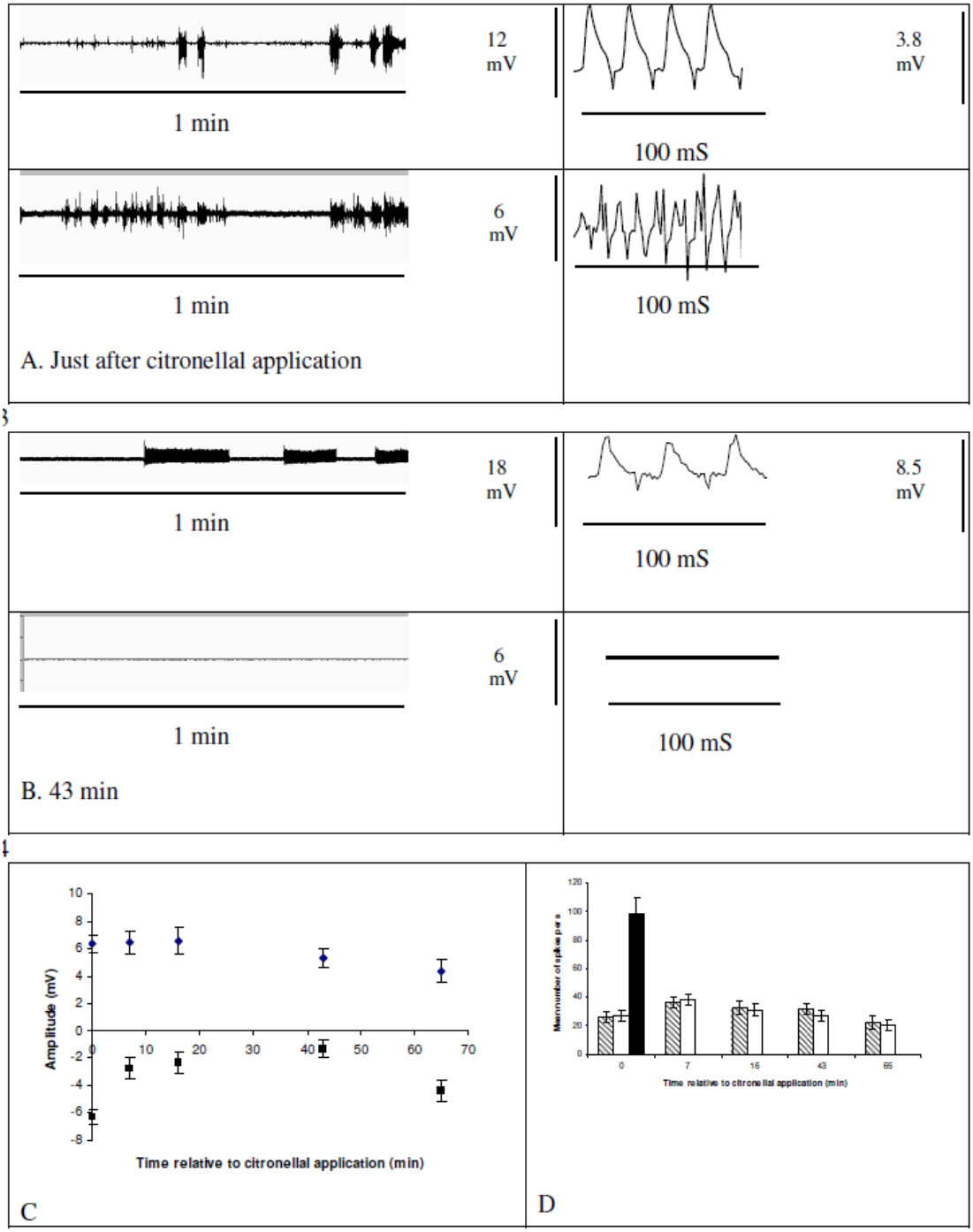

Figure 5-5 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies, *Phaenicia sericata*, treated topically with citronellal (25 µg).
A and B: (left) temporal progression of FMI (top panels) and WBS (lower panels) at 1 and 43 min post application, respectively, with (right) expanded sections of FMI (top panels) and WBS (lower panels) from A and B, respectively. C: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. D: The effect of citronellal on FMI and WBS frequency over the 65 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

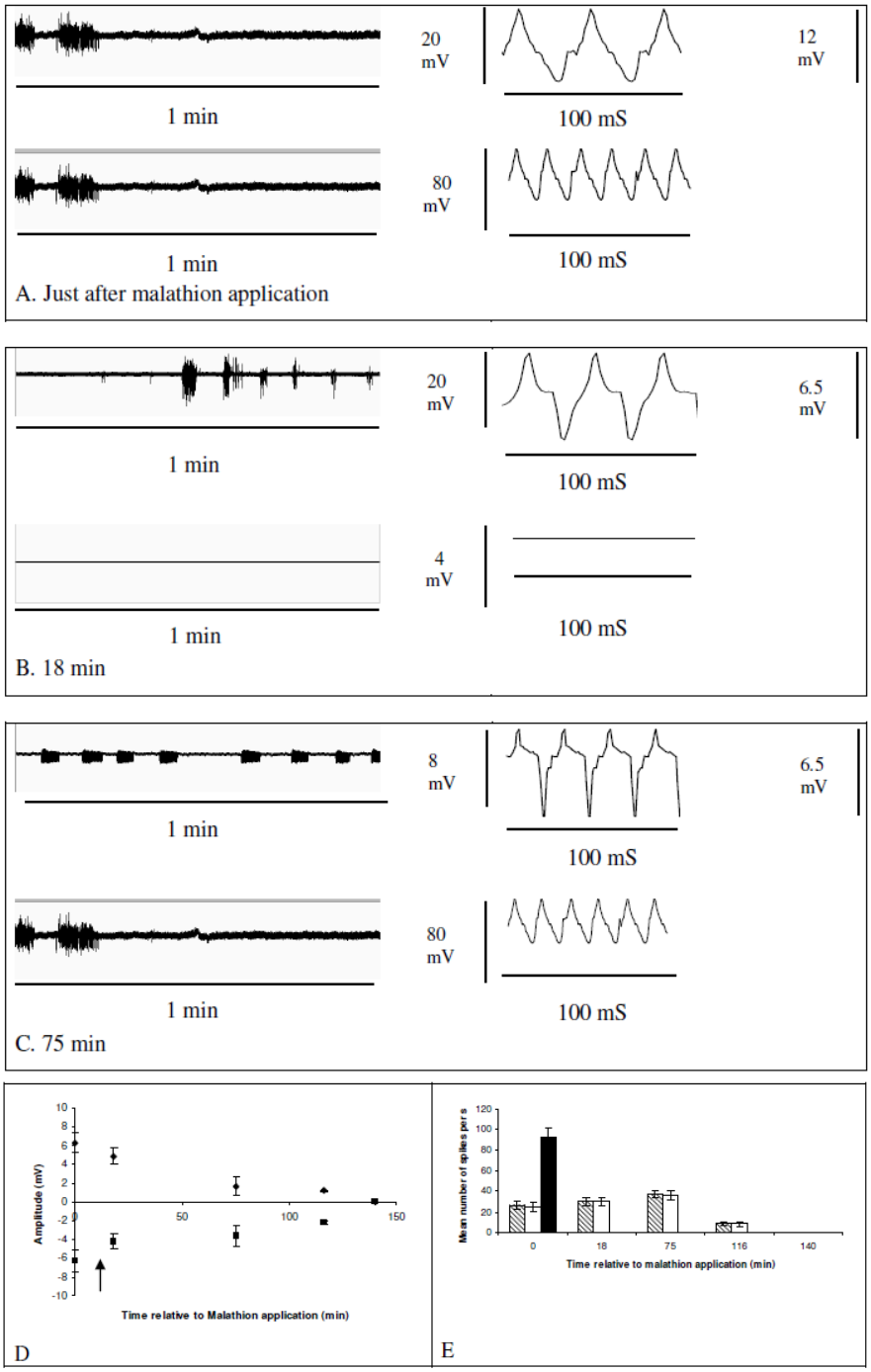

Figure 5-6 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies, *Phaenicia sericata*, treated topically with malathion (5 µg).
A, B and C: (left) temporal progression of FMI (top panels) and WBS (lower panels) at 1, 18 and 75 min post application, respectively, and (right) expanded sections of FMI (top panels) and WBS (lower panels) from A, B and C, respectively. D: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. E: The effect of malathion on FMI and WBS frequency over the 140 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency. In 6D the arrow indicates the timepoint at which tremors first were observed.

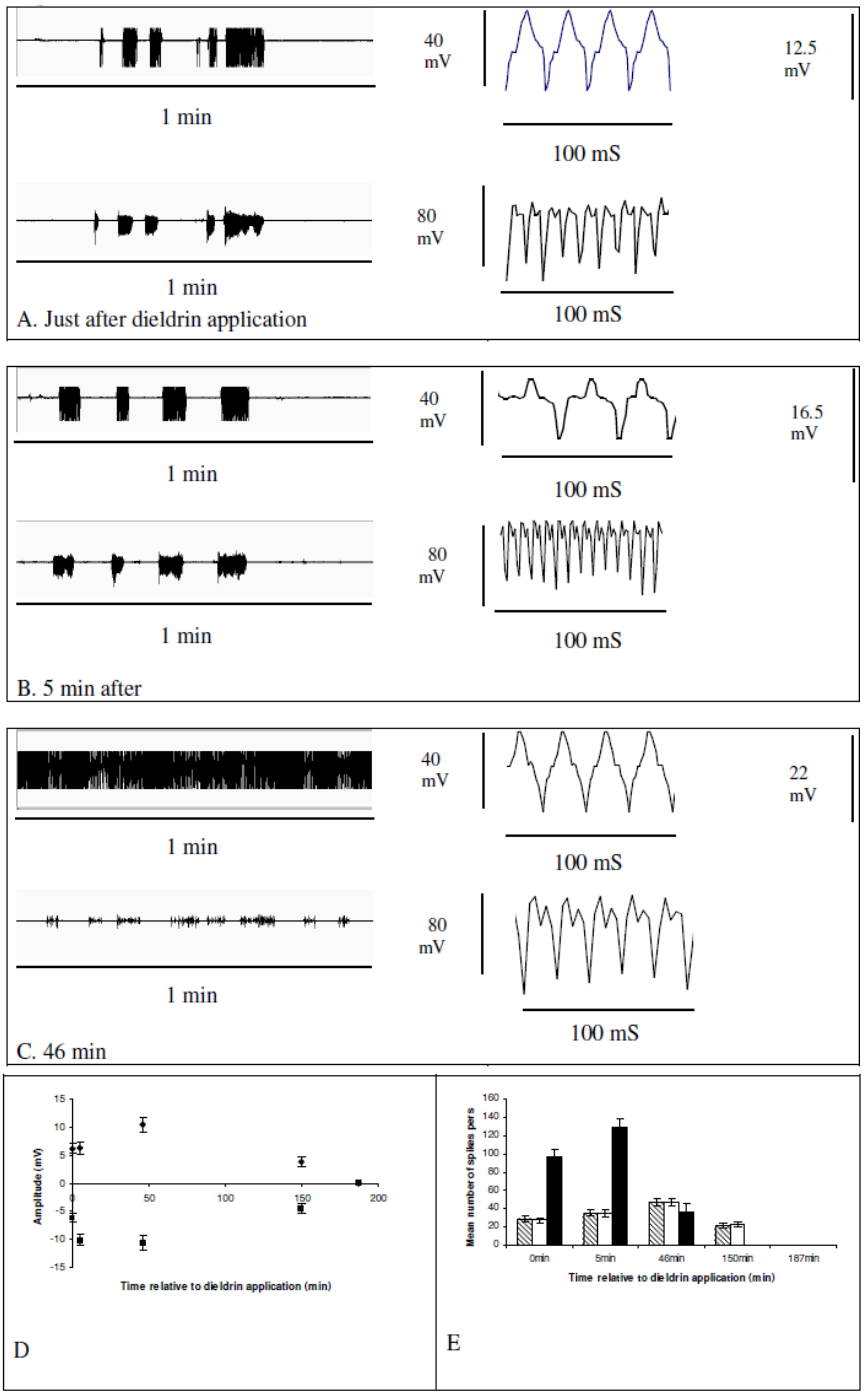

Figure 5-7 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies, *Phaenicia sericata*, treated topically with dieldrin (5 µg).
A, B and C: (left) temporal progression of FMI (top panels) and WBS (lower panels) at 1, 5 and 46 min post application, respectively, with (right) expanded sections of FMI (top panels) and WBS (lower panels) from A, B and C, respectively. D: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. E: The effect of dieldrin on FMI and WBS frequency over the 187 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency. In 7D, the arrow indicates the timepoint at which tremors first were observed.

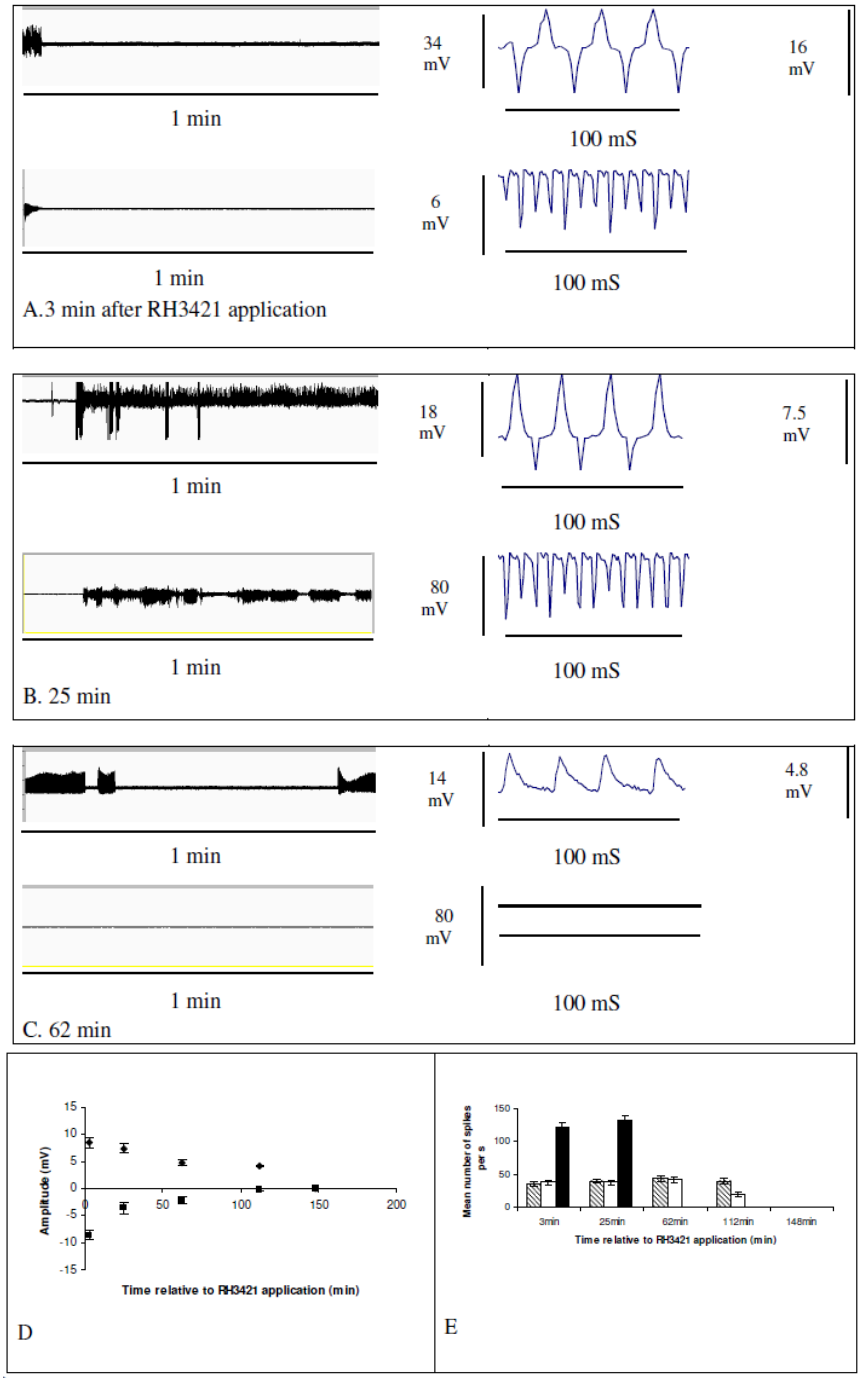

Figure 5-8 Typical electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of blowflies, *Phaenicia sericata*, treated topically with RH3421 (10 µg).
A-E: (left) temporal progression of FMI (top panels) and WBS (lower panels) at 3, 25 and 62 min post application, respectively, with (right) expanded sections of FMI (top panels) and WBS (lower panels) from A–C, respectively. D: Changes in amplitude of the right (♦) and left (■) DLM FMI over time. E: The effect of RH3421 on FMI and WBS frequency over the 148 min recording period. Hatched bar: mean (±SE) number of spikes per second of the right DLM; white bar: mean (±SE) number of spikes per second of the left DLM; black bar represents WBS frequency.

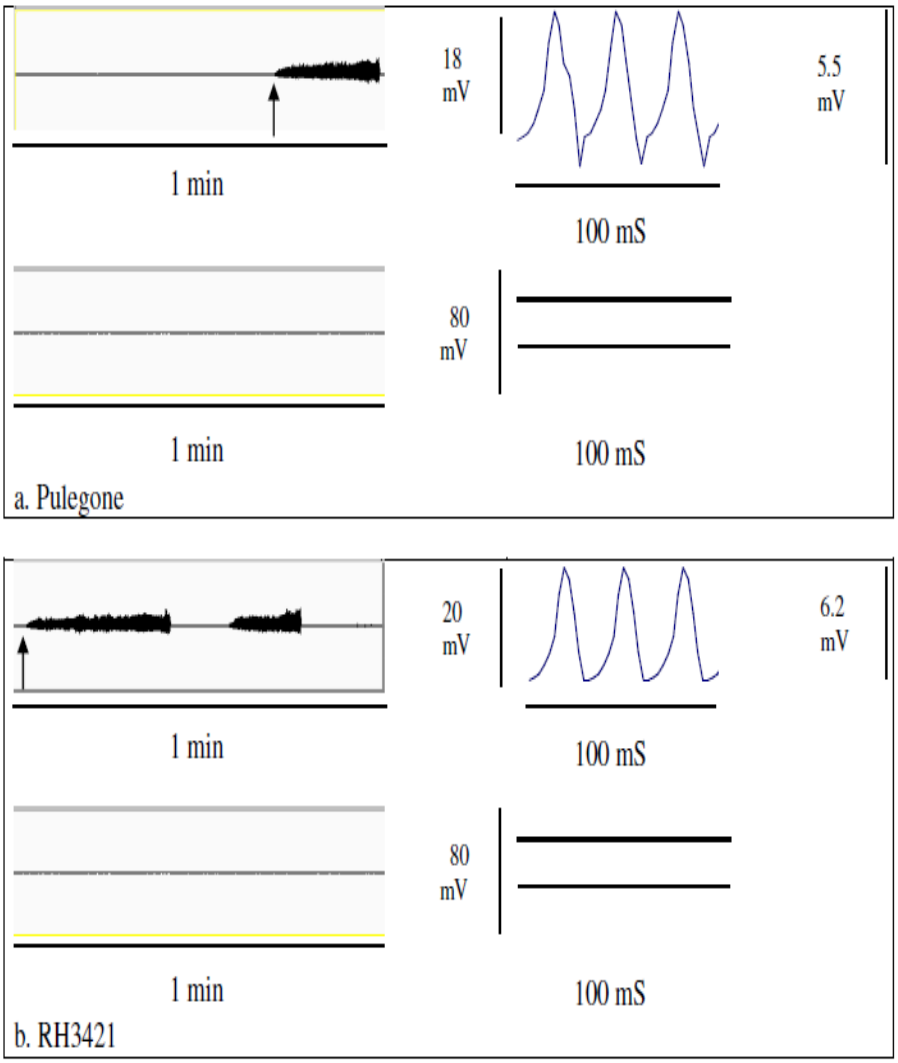

Figure 5-9 The electrophysiological responses recorded from the dorsolongitudinal muscles (DLM) of quiescent blowflies, *Phaenicia sericata*, after probing of bowflies treated with pulegone (Fig. 5-9a) and RH3421 (Fig. 5-9b).
The arrow indicates the timepoint at which physical activation of blowfly.

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Connecting statement 5

In previous chapters I used acute toxicity assays, enzyme assays and monitored electrophysiological responses to observe the effects of plant-derived compounds on different biological systems in *Ae. aegypti* and *P. sericata*. Some data suggested that a potential target for essential oil activity is the octopaminergic system of insects. Octopamine (OA) is a multi-functional naturally occurring biogenic monoamine that plays a key role as a neurotransmitter, neurohormone and neuromodulator in invertebrates similar to norepinephrine in vertebrates. The downstream result of OA binding to its receptors is mediated by specific membrane proteins that belong to a superfamily of G-protein-coupled receptors (GPCRs). The interactions between OA and GPCRs may stimulate or inhibit specific target proteins causing changes in the concentration of intracellular second messengers such as cyclic-adenosine mono-phosphate (cAMP). In the next chapter, I examine the effects of thymol, eugenol, pulegone, α-terpineol and citronellal on cAMP production in 4th instar larvae of *Ae. aegypti* and compared their effects with those of octopamine to determine if similar physiological responses are induced, allowing me to predict a mode of action of these compounds.

Chapter 6: Effects of Octopamine and the Plant Monoterpenoids, Thymol, Eugenol, Pulegone, Citronellal and α-Terpineol, on cAMP Production in 4th Instar Larvae of *Aedes aegypti* (Diptera: Culicidae)

A modified version of this chapter has been submitted to the Journal of Insect Physiology as:

Waliwitiya, R., R. Nicholson, and C. Lowenberger. Effects of Octopamine and plant monoterpenoids to the octopaminergic system of 4th instar larvae of *Aedes aegypti*.

6.1 Abstract

I studied the effects of exposure to essential oil components on the octopaminergic system of insects. Octopamine (OA) is a multi-functional, naturally occurring biogenic monoamine that plays a key role as a neurotransmitter, neurohormone and neuromodulator in invertebrates. I evaluated the interactions between thymol, eugenol, pulegone, α-terpineol and citronellal and OA receptors, in 4th instar larvae of *Aedes aegypti* by comparing the production of a second messenger molecule, cAMP, after treatment. Baseline cAMP levels changed minimally over the time of the experiments. Thymol, eugenol and α-terpineol induced a biphasic response in cAMP production; at the lowest concentration (10 mg L^{-1}) thymol, eugenol and α-terpineol significantly induced cAMP production whereas at higher concentrations (50 and 100 mg L^{-1}), they significantly reduced the cAMP production compared with baseline levels. Pulegone, at 10 mg $L^{-1,}$ did not affect cAMP production while exposure to 50 mg L^{-1} increased the cAMP production and 100 mg L^{-1} significantly reduced cAMP production. Citronellal only altered cAMP levels at the highest concentration (100 mg L^{-1}). OA significantly increased the cAMP production at all tested concentrations. Our results indicate that some monoterpenoids lethal to insects may target the octopaminergic system and that OA receptors may be important targets for these compounds.

6.2 Introduction

Plants have evolved to produce a huge variety of protective compounds against phytophagous insects and many of these compounds have been evaluated for their ability to kill, repel, deter feeding or oviposition, or for their antibacterial and antiviral properties (Benner, 1993; Singh et al., 1989; Wilson et al., 1997; Shaaya et al., 1991; Shaaya, 1998; Wilson and Shaaya, 1999; Wilson and Shaaya, 1998; Regnault-Roger and Hamrouni, 1995; Ngoh et al., 1998; Isman, 1999). Most of these compounds are broadly classified as terpenoids, phenolics and alkaloids (Isman and Akhtar, 2007) and are contained within plant essential oils (Kostyukovsky et al., 2002).

Many monoterpenoids show insecticidal or repellent properties. Alpha-terpineol is toxic to the human head louse (Yang et al., 2009), citronellal and eugenol are toxic to wireworm larvae (Waliwitiya et al., 2005), and terpineol and citronellal can knockdown or repel mosquitoes (Vartak and Sharma, 1993). Eugenol is used to kill or repel various coleopteran pests of stored grain products (Obeng-Ofori and Reichmuth, 1997) and pulegone is toxic to houseflies, cockroaches, and stored product coleopteran pests (Lee et al., 2003). Monoterpenes also are components of perfumes, cosmetics, food flavorings and herbal medicines and have earned the GRAS (Generally Recognized as Safe) designation by the US Food and Drug Administration.

Despite their use in controlling insect pests, the enzymatic modes of action of these compounds are not well understood (Kostyukovsky et al., 2002). Previous studies have indicated that wireworms exposed to monoterpenoids or neurotoxic insecticides exhibit similar symptoms (Waliwitiya et al., 2005) suggesting that these compounds affect the nervous system or neuromuscular function (Kostyukovsky et al., 2002;

Waliwitiya et al., 2010). I have used electrophysiological studies to demonstrate that essential oils can penetrate the insect cuticle and alter central nervous system function in particular the flight motor activity of tethered *Phaenicia sericata* (Waliwitiya et al., 2010). The application of monoterpenoids also affects the production and efficiency of biotransformational enzyme activities in *Aedes aegypti* (Waliwitiya et al., submitted).

Various neuronal targets are sensitive to specific monoterpenoids *in vitro*; thymol enhances GABA-activated chloride currents in oocytes expressing fruitfly homomeric GABA receptors (Priestley et al., 2003) and the sensitivity of native GABA receptors of the housefly and cockroach CNS to thymol and pulegone has been confirmed using [^{3}H]TBOB binding and ^{36}Cl flux assays (Tong and Coats, 2010). Monoterpene mixtures cause increases in cAMP production in Lepidoptera, possibly by activating octopamine receptors (Kostyukovsky et al., 2002), and eugenol and α-terpineol increase the heart rate in cockroaches in a manner similar to the application of octopamine (Enan, 2001). Price and Berry (2006) demonstrated that eugenol inhibits spiking of the cockroach dorsal unpaired median neuron (DUM), which contrasts with the increased spike frequency observed when octopamine is applied to the same tissue.

I demonstrated the effects of several monoterpenoids on the flight motor activity of the blowfly (*P. sericata*) *in vivo* (Waliwitiya et al., 2010). These studies compared the effects of these natural products and synthetic insecticides on the flight motor output signals. I made inferences on the mode of action of individual monoterpenoids based on similarities or differences in the electrophysiological response between monoterpenoids and insecticidal standards whose mode of action is well known. Thymol, for example, affects the insects in a manner most similar to GABA, suggesting a GABA-like mode of

action (Waliwitiya et al., 2010). I also compared the effects of several monoterpenoids on the biotransformational or detoxifying enzymes of *Aedes aegypti* (Walitiwiya et al., unpublished). The activity of these enzymes was reduced with increasing concentration of monoterpenoids, but the precise target and mode of action were not identified (Enan, 2001; Kostyukovsky, 2002).

A potential target for certain essential oil components is the octopaminergic system of insects. Octopamine (OA) is a multi-functional, naturally occurring biogenic monoamine that plays a key role as a neurotransmitter, neurohormone and neuromodulator in invertebrates similar to norepinephrine in vertebrates (Kostyukovsky et al., 2002). In addition to being a putative central transmitter, OA also acts as a peripheral transmitter in higher invertebrates (Woodring et al., 1989; Sporhase-Eichmann et al., 1992). For example, OA-containing axons terminate in the pericardial organs of lobsters where OA is released (Evans et al., 1976), and OA contributes to the heartbeat regulation in many insects (Grega and Sherman, 1975; Florey and Rathmeyer, 1978; Prier et al., 1994, Enan, 2001). OA is known as the insect 'fight or flight' hormone (Orchard, 1982) as OA production correlates with active or stressful behavior and also may be part of a general arousal system to prepare insects for vigorous activity (Evans and Siegler, 1982; Corbet, 1991).

Evans (1981) and Roeder (1992) suggested that the OA exerts its effects through interactions with at least two classes of receptors. OA binds to receptors in muscles, fatbody, oviducts, and other tissues. The downstream result of this binding is mediated by specific membrane proteins that belong to a superfamily of G-protein-coupled receptors (GPCRs) (Roeder and Nathanson, 1992). Activated GPCRs transmit signals to

intracellular trimeric GTP-binding (G) proteins (Blenau and Baumann, 2001) (Fig. 1A). Depending on the specific interactions between receptors and G-proteins, activated G-proteins may either stimulate or inhibit specific target proteins causing changes in the concentration of intracellular second messengers such as cAMP (Gudermann et al., 1996; Gudermann et al., 1997). When the receptor binds to a G_s-type (=stimulatory) receptor protein, the activated $G_{\alpha s}$ subunit engages with adenylase cyclase in the plasma membrane leading to an increase of cyclase activity and production of cAMP. Increases in cAMP then activate cAMP-dependent protein kinase (PKA) which phosphorylates serine and threonine residues of various substrate molecules including cytosolic proteins, ligand-gated and voltage dependent ion channels, and transcription factors (Blenau and Baumann, 2001).

Several biogenic amine receptors are known to inhibit adenyl cyclase activity (Fig. 1B) by preventing the binding of adenylyl cyclase to activated $G_{\alpha i,}$ thereby preventing cyclase activation and stopping the generation of second messengers (Blenau and Baumann, 2001). In mammals numerous hormones, including corticotrophin, luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone, calcitonin, glucagon, vasopressin and parathyroid hormone act through a similar mechanism (Levitzki, 1986; Gilman, 1987). Activation and inhibition of mammalian adenylate cyclase by membrane receptors are mediated by unique GTP-regulatory proteins (G-proteins) (Levitzki, 1986; Gilman, 1987). cAMP has been shown to be involved in the cardiovascular (Movsesian, 1998; Frank and Kranias, 2000) and nervous system (Tasken and Aandahl, 2003), in immune mechanisms (Latour and Veillette, 2001), cell growth and differentiation (Tasken and Aandahl, 2003) and general

metabolism (Lowell, 1996). There remains considerable interest in the measurement of intracellular cAMP in tissues and cell cultures, and this may help to provide an understanding of the physiology and pathology of many disease states.

The present study addresses the possible interaction between thymol, eugenol, pulegone, α-terpineol and citronellal interact with OA receptors in 4th instar larvae of *Aedes aegypti* by comparing cAMP production after treatment with these compounds.

6.3 Materials and methods

6.3.1 Insects

Ae. aegypti larvae and adults were raised as described previously (Lowenberger et al., 1999) at 27^{0}C and 80-85% relative humidity under a 14 h:10 h light:dark cycle. Adults were provided with a 10% sucrose solution *ad libitum* and larvae were fed with ground Nutrafin Basix fish food (Rolf C. Hagen Inc, Montreal, QC).

6.3.2 Chemicals

Pulegone (>95% purity) was obtained from Ecosafe Natural Products (Victoria, B.C.). Octopamine hydrochloride (OA), eugenol, *α*-terpineol, citronellal, and thymol (>95% purity) were purchased from Sigma Aldrich (St. Louis, MO). Solutions of 10, 50 and 100 mg L^{-1} were prepared in distilled water.

6.3.3 Exposure of larvae to chemicals

Ten 4th instar larvae were placed in glass scintillation vials containing 10 mL of treatment solution and 0.05 g of ground Nutrafin Basix fish food. Three replicates (30 larvae) were used per treatment. Scintillation vials were covered loosely and larvae kept

for of 2, 4 or 8 h. At each time the larvae were removed from the test solutions, to 1 mL Eppendorf tubes, and stored at -80 ^{0}C.

6.3.4 Assay of cAMP production

cAMP production was measured using the Amersham cyclic AMP (^{3}H) assay kit (GE Health Care, Quebec, Canada) following the manufacturer's instructions. Briefly, 10 larvae were ground in 1 mL of 95% ethanol. The mixture was centrifuged at 9000 g for 10 min and the supernatant was collected. The precipitate was washed with 1 mL ethanol and centrifuged again under the same conditions. The supernatants from each centrifugation were combined and evaporated to dryness under a stream of air. The residue was dissolved in 0.05 M tris: EDTA buffer containing 4 mM EDTA (pH 7.5). cAMP in mosquito extracts was quantified by binding competition between unlabelled cAMP and (^{3}H) labeled cAMP. The amount of labeled protein-cAMP complex formed is inversely related to the amount of cAMP in the sample whose concentration is ultimately extrapolated from a standard curve.

The protein concentrations of all samples were determined using the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

6.3.5 Statistics

cAMP activities of treated larvae were normalized to the S9 fraction protein content. Results are expressed as means ± S.D. The assumptions of equal variances and normal distributions in residuals were tested and data were log transformed as necessary. Statistical analysis was performed with JMP 7 software (JMP, Version 7. SAS Institute Inc., Cary, NC, 1989-2007) and the data were subjected to a general linear model (GLM,

repeated measures) procedure to test for significant differences in cAMP activities between individual chemical exposures and time. Least square means were compared using Tukey's HSD test.

6.4 Results

The three way ANOVA for the experiments (Table 6-1) indicates that the treatment compound, concentration, and length of exposure all contributed to the overall changes in cAMP production in the whole bodies of 4th instar larvae.

6.4.1 Baseline cAMP levels

Baseline cAMP levels of whole body extracts of 4th instar larvae are shown in figure 6-3. During the 8h experiment cAMP levels changed from 92.5-96.1 (±1.9) pmol/mg protein. The changes at different times are not statistically different ($p=0.618$).

6.4.2 Effects of thymol on cAMP production

Figure 6-4 shows the changes in cAMP production caused by different concentrations of thymol. The effect is bi-phasic. At the lowest concentration (10 mg L^{-1}) thymol significantly increased cAMP production while at the highest concentration (100 mg L^{-1}), thymol significantly reduced the cAMP production over the 8h exposure period compared with baseline cAMP level. At all timepoints after exposure to 10 mg L^{-1}, cAMP production significantly increased, up to 151.6% over baseline levels after 8 h exposure. This pattern continued when exposed to 50 mg L^{-1} at 2 and 4 h post exposure. In contrast after exposure to 50 mg L^{-1} for 8 h, cAMP levels were reduced significantly by 58.4% compared with baseline level ($p<0.0001$).

6.4.3 Effects of eugenol on cAMP production

Figure 6-5 demonstrates the changes in cAMP production caused by three concentrations of eugenol. The effects of eugenol exposure were similar to those of thymol. At 10 mg L^{-1} eugenol significantly increased the cAMP production while at the highest concentration (100 mg L^{-1}), it significantly reduced the cAMP production compared with controls at all timepoints. At all timepoints after exposure to 10 mg L^{-1}, and at 2-h post exposure to 50 mg L^{-1} eugenol, cAMP increased significantly, up to 69.7% compared with baseline cAMP level. In contrast, 8 h exposure to 50 mg L^{-1} or 4- to 8- h exposure to 100 mg L^{-1} significantly reduced the cAMP production by as much as 48.1% ($p<0.0001$) compared with the baseline level.

6.4.4 Effects of pulegone on cAMP production

Figure 6-6 shows the changes in cAMP production caused by three concentrations of pulegone. No significant increases in cAMP were measured for 8-h after exposure to 10 mg L^{-1} of pulegone. cAMP production increased significantly 2h after exposure to 50 mg L^{-1} (43.5% increase) but fell significantly by 8h (34.4% reduction). Pulegone at 100 mg L^{-1}, significantly reduced cAMP production at 4 and 8 h (41.4 and 56.4%, respectively) compared with controls ($p<0.0001$).

6.4.5 Effects of *α*-terpineol on cAMP production:

Figure 6-7 shows the changes in cAMP production caused by *α*-terpineol. *α*-Terpineol induced responses similar to those of thymol. At 10 mg L^{-1}, *α*-terpineol significantly induced cAMP production while at 100 mg L^{-1} it significantly reduced it compared with controls at all timepoints. By 8-h after exposure to 10 mg L^{-1} *α*-terpineol, cAMP

production was increased by 112.7% whereas 8h exposure to 100 mg L^{-1} reduced it by 60.2%. At the intermediate concentration, 50 mg L^{-1}, α-terpineol significantly increased cAMP production at 2-H and 4-h (58.4 and 27.5% respectively) while at 8-h it was significantly reduced ($p<0.0001$).

6.4.6 Effects of citronellal on cAMP production:

Figure 6-8 shows the effects of exposure to citronellal. Citronellal did not significantly alter the cAMP production at 10 and 50 mg L^{-1} at any timepoint tested, but reduced it at the 100 mg L^{-1} level at all timepoints (30-38% ±4.3 reduction) compared with the baseline level ($p<0.0001$).

6.4.7 Effects of octopamine (OA) on cAMP production:

Figure 6-9 shows the changes in cAMP production caused by three concentrations of OA. At all three concentrations, and at all times, OA significantly increased the cAMP production compared with baseline level. At 10 mg L^{-1} level, OA increased the cAMP production by 84.2-219.3% and at the 50 mg L^{-1} level cAMP production was increased by 150.3-355.3% from 2-8 h. At the highest concentration of OA (100 mg L^{-1}), cAMP production was increased from 204%-602.4% ($p<0.0001$).

6.5 Discussion

I reported previously the effects of various phytochemicals on the survival, behavior, flight motor activity and wing beat frequency of treated insects (Waliwitiya et al., 2009, 2010). I also measured the effects of several monoterpenoids on the expression of detoxifying enzymes in terrestrial insects (Waliwitiya et al., unpublished). In these papers I discussed problems associated with treating aquatic invertebrates such as

mosquito larvae and delivering a suitable dose to reduce pest populations in aquatic environments, and considered that some of the monoterpenoids exert their activity via induced cAMP pathway. Other studies have shown that octopamine acts as a neurohormone and activates the cAMP pathway (Evans, 1984) and that monoterpenoids also elevate cAMP levels through this pathway (Enan, 2001).

cAMP levels of untreated larvae did not change significantly over the 8-h time period (Fig. 3). Exposure of mosquitoes to all three concentrations of OA increased cAMP production significantly in a concentration- and time-dependent manner. The highest level was 8-h after exposure to 100 mg/L of OA (674.8 pmol/mg protein) and represented a 602.4% increase compared with the baseline level. Similarly, Enan 2001 observed a 255% increase in cAMP production in brain tissues of *Periplaneta americana* treated with 1 nmol/mL OA. Kostyukovsky et al., (2002) observed similar elevations in cAMP production in abdominal tissues of the cotton bollworm *Helicoverpa armigera* treated with OA. Florey and Rathmeyer (1978) observed an increase in amplitude and frequency of the heartbeat of *Astacus leptodactylus* and *Eriphia spinifrons* treated with OA and Prier et al., (1994) reported an increase in cardioacceleratory peptides via cAMP dependent mechanisms. Octopaminergic modulation of cockroach visceral muscles via cAMP was demonstrated by Lange and Orchard in 1986.

In contrast to the OA treatment, our results demonstrate that the monoterpenoids can activate or reduce cAMP production, depending on the concentration or duration of exposure. Thymol and eugenol clearly had biphasic effects on modulating cAMP production. At the lowest concentration (10 mg/L) they significantly increased cAMP production and at the highest concentration (100 mg/L) cAMP production was

significantly reduced. Both the increase and the reduction of cAMP activities are significantly different from the baseline cAMP levels of 4th instar *Ae. aegypti*. Enan (2001) observed a similar biphasic pattern of cAMP production in brain tissues of the American cockroach *Periplanata americana* treated with eugenol. At low concentration (1 nmol/mL), eugenol significantly increased cAMP production (139%) while at 10, 100 and 1000 nmol/mL concentrations it significantly decreased the cAMP production. In the same study α-terpineol at 1-10 nmol/mL significantly increased the cAMP production while 100 and 1000 nmol/mL it significantly reduced cAMP production. In our study α-terpineol at 10 mg/L increased cAMP production throughout the 8-h time period. At 50 mg/L level, cAMP was increased initially and then fell. Similarly, citronellal reduced cAMP production significantly at the 100 mg/L level, whereas Enan (2001) did not observe a significant reduction or induction of cAMP levels at any tested concentrations (1-1000 nmol/mL). Pulegone reduced cAMP production only at 50 and 100 mg/L levels while at the 10 mg/L there was no change. The LC_{50} values of the test compounds are shown in Table 2. Based on the acute toxicity data, the compounds can be ranked from most toxic to least toxic: pulegone>thymol>eugenol>citronellal>α-terpineol. Based on the cAMP data, the compounds can be arranged from greatest to lowest inhibitory effects: α-terpineol>thymol>pulegone>eugenol>citronellal. The toxicity of α-terpineol can therefore not be explained by its low inhibitory action on cAMP production. However, based on these rankings, the toxicities of the other compounds show good agreement with their inhibitory effects on cAMP production.

Monoterpenoids have been studied and used as natural control compounds against many different insect pests (Singh et al., 1989; Shaaya et al., 1991; Benner, 1993;

Regnault-Roger and Hamrouni, 1995; Wilson et al., 1997; Ngoh et al., 1998; Shaaya, 1998; Wilson and Shaaya 1998; Isman, 1999; Wilson and Shaaya, 1999). The data are similar in that all compounds initially increase cAMP production, suggesting that OA receptors may act as important targets of these test compounds. However, OA increased cAMP production to far greater levels than did any of our compounds (602.4% by OA vs 151.6% by thymol). In addition, OA does not, at higher concentrations, reduce cAMP production as was seen with all study compounds except citronellal in *Ae. aegypti* larvae. So, while similarities with OA action exist in terms of activating the cAMP signaling pathway, our data on thymol, eugenol, pulegone and α-terpineol indicate an inherent sensitivity of a component (or components) of this cascade to inhibition as concentrations are raised. Except for eugenol (Enan, 2001), there are few reports in the literature that describe biphasic changes in cAMP levels similar to those I observed after the application of monoterpenoids. Further studies are underway to try to clarify the basis for these positive and negative modulatory effects and to investigate possible interference with other targets in *Ae. aegypti* larvae.

Health, safety and environmental concerns have reduced or restricted the use of a number of synthetic pesticides. Certain of the monoterpenoids discussed here may represent alternatives to synthetic pesticides due to reduced effects on the environment and potential for low non-target toxicity towards mammals since the OA receptors are not found in mammals (Enan, 2001). However these phytochemicals are intrinsically less toxic to pests than many synthetic pesticides and their usefulness ultimately depends on their ability to manage pests effectively under field conditions at environmentally benign application rates. In addition, a more thorough understanding of targets and mechanisms

relevant to the toxic actions of these monoterpenoids is need before their use can be promoted widely as new pest control products.

In summary, our data support the octopaminergic system as a target for several monoterpenoids in *Ae. aegypti* larvae. Whether these or similar phytochemicals can be developed and commercialized as novel mosquito larvicides will require further research on their modes of action in target and non-target organisms and realization of in-field activity at moderate application rates.

6.6 Figures

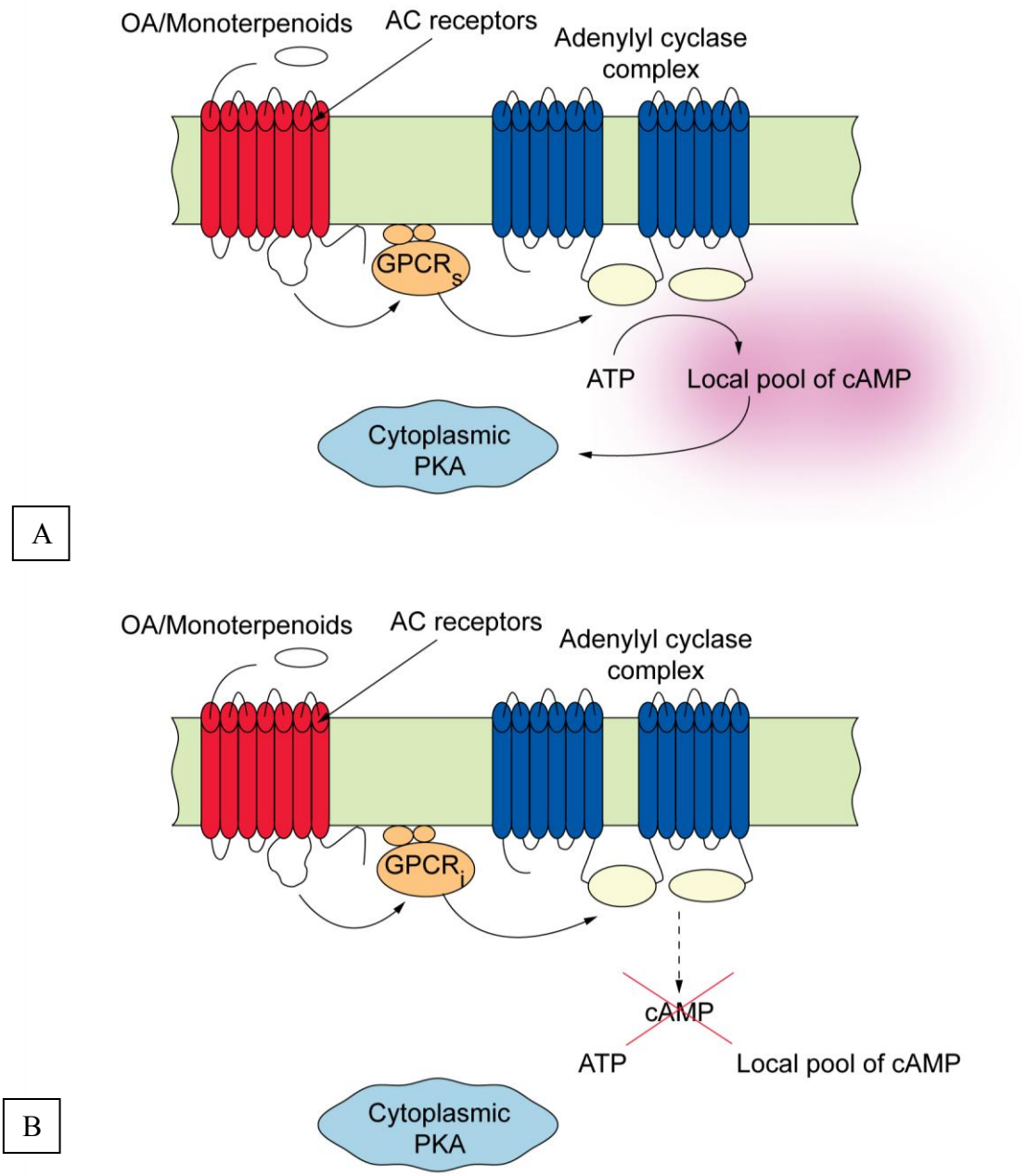
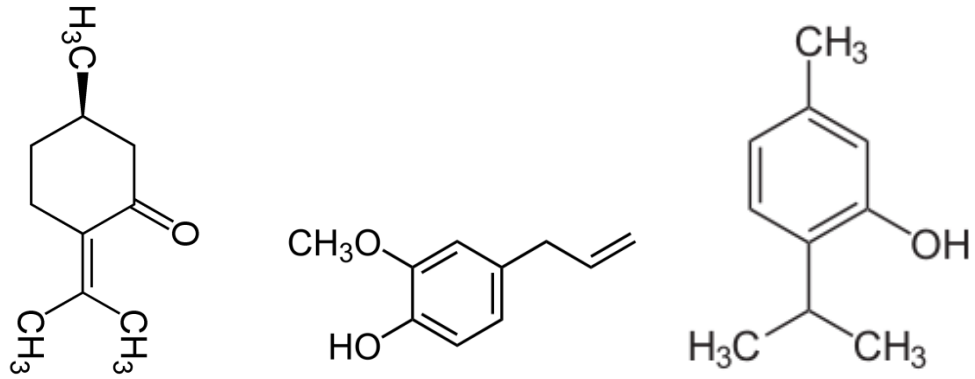
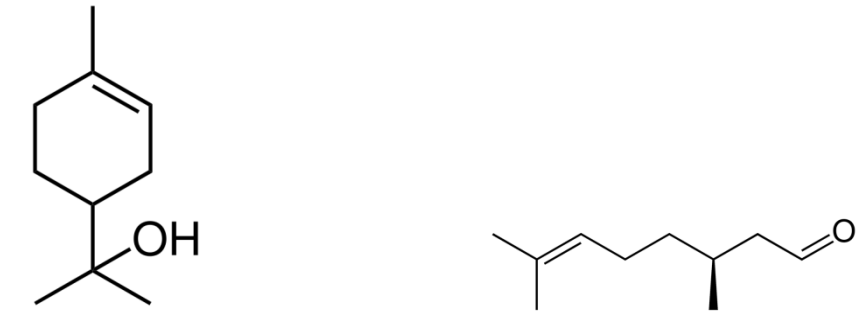


Figure 6-1 Biogenic amine receptors coupled to intracellular cAMP signaling pathways.
A: Biogenic amine receptors are activated by binding of agonists (OA/monoterpenoids). The ligand-bound receptor then activates a stimulatory G protein (Gs), which leads to an increase in the enzymatic activity of adenylyl cyclase (AC). Adenylyl cyclase catalyzes the conversion of ATP to cAMP. As the intracellular concentration of cAMP increases, cAMP-dependent protein kinase (PKA) is activated and phosphorylates different target proteins on serine and threonine residues. B: Several biogenic amine receptors are known to inhibit AC activity via inhibitory G proteins (Gi).



Thymol Eugenol Pulegone



α-terpineol Citronellal

Figure 6-2 Structures of thymol, eugenol, pulegone, α-terpineol and citronellal.

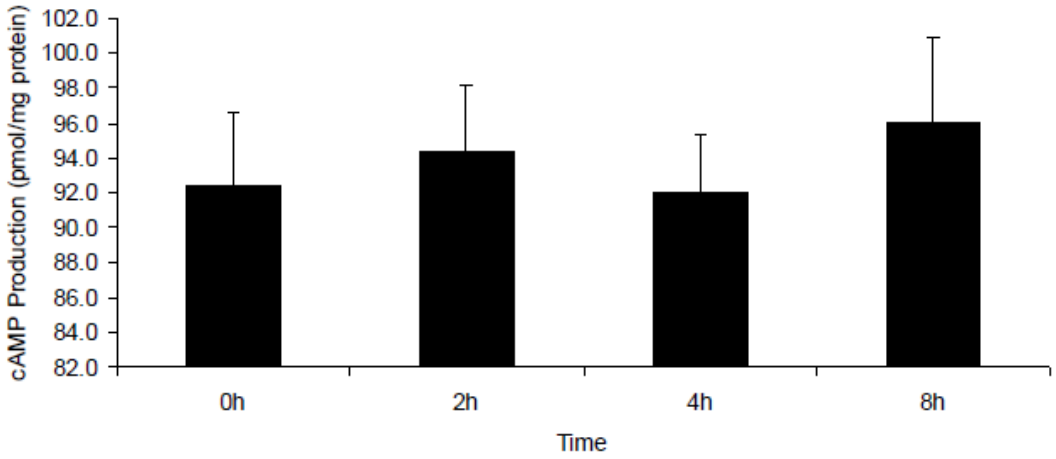


Figure 6-3 Baseline cAMP levels of larvae reared in distilled water. The error bars represents the standard deviation of three independent replicates. No significance differences were observed.

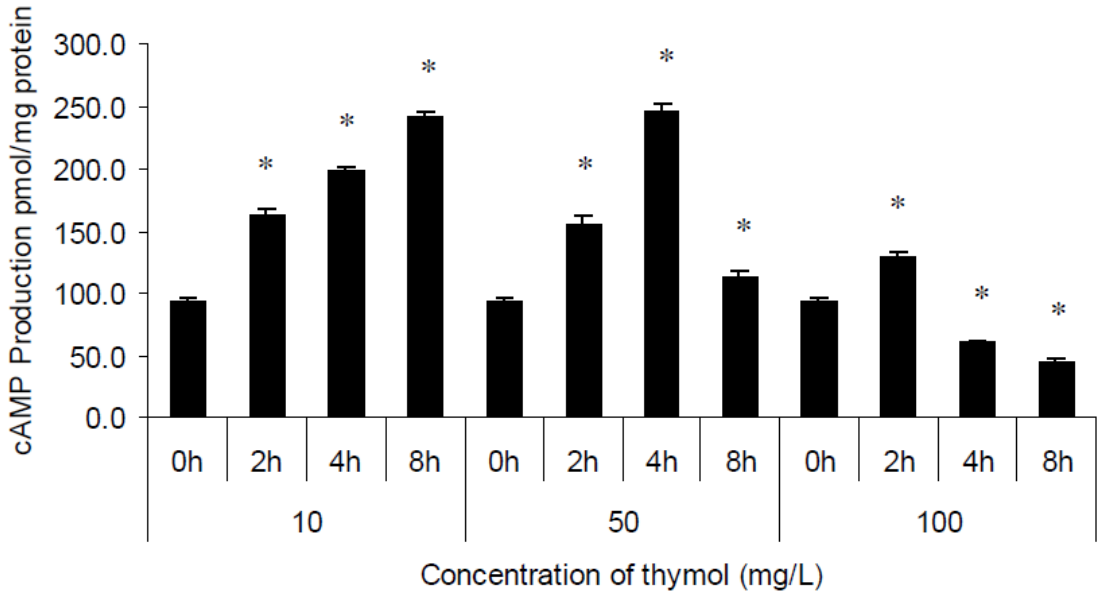


Figure 6-4 cAMP levels of 4th instar larvae of *Aedes aegypti* exposed to 10, 50 and 100 mg L^{-1} thymol for different time periods.
The error bars represents the standard deviation of three independent replicates. An asterisk (*) over a specific timepoint indicates that cAMP levels are significantly different from the control ($p < 0.05$).

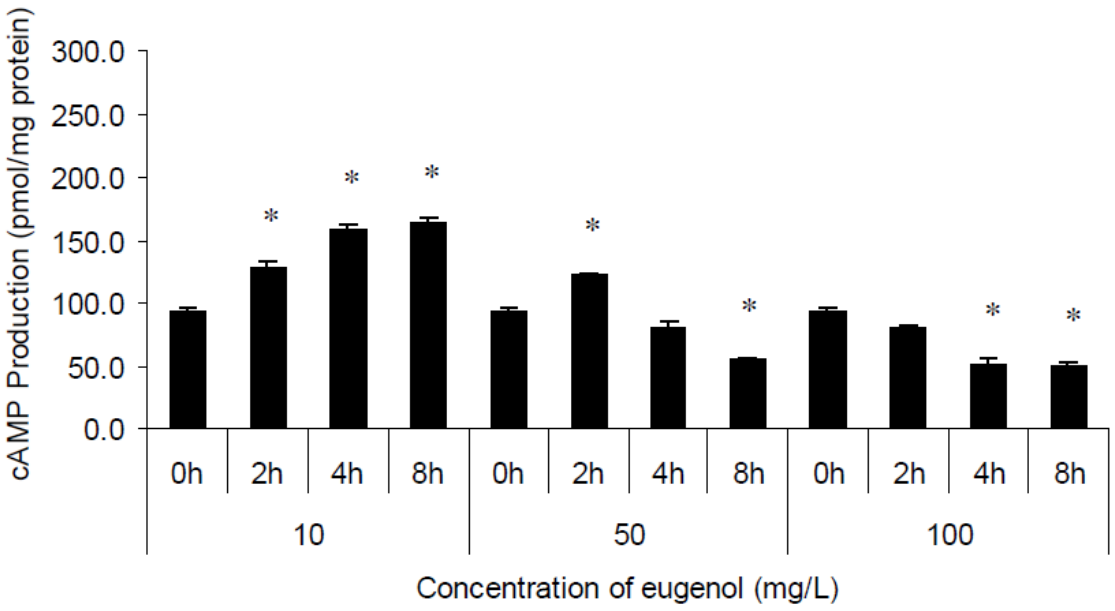


Figure 6-5 cAMP levels of 4th instar larvae of *Aedes aegypti* exposed to 10, 50 and 100 mg L^{-1} eugenol for different time periods.
The error bars represents the standard deviation of three independent replicates. An asterisk (*) over a specific timepoint indicates that cAMP levels are significantly different from the control ($p < 0.05$).

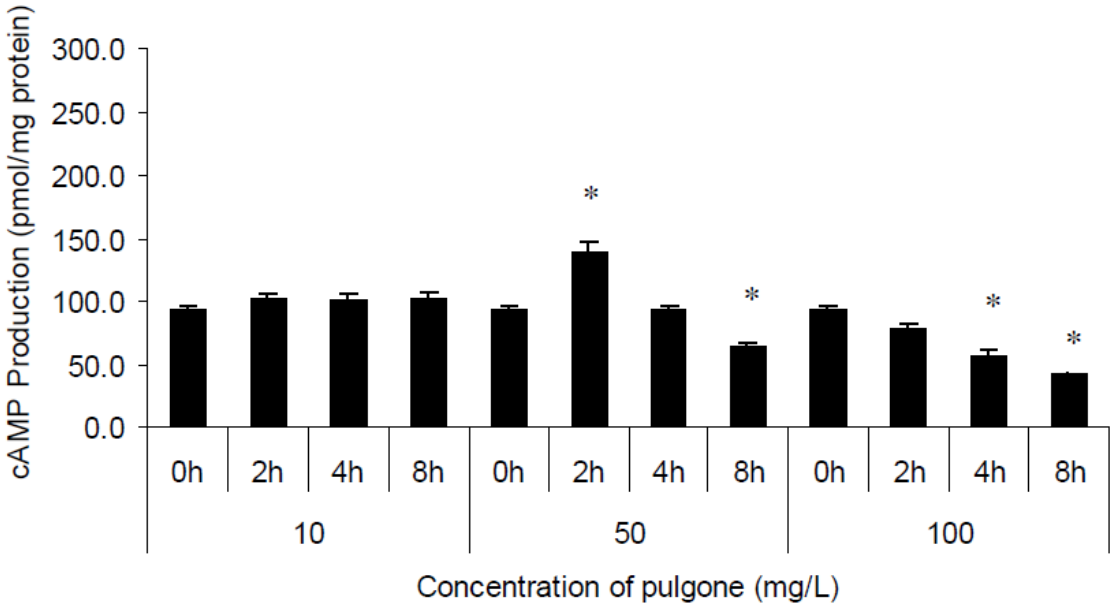


Figure 6-6 cAMP levels of 4th instar larvae of *Aedes aegypti* exposed to 10, 50 and 100 mg L^{-1} pulegone for different time periods.
The error bars represents the standard deviation of three independent replicates. An asterisk (*) over a specific timepoint indicates that cAMP levels are significantly different from the control ($p < 0.05$).

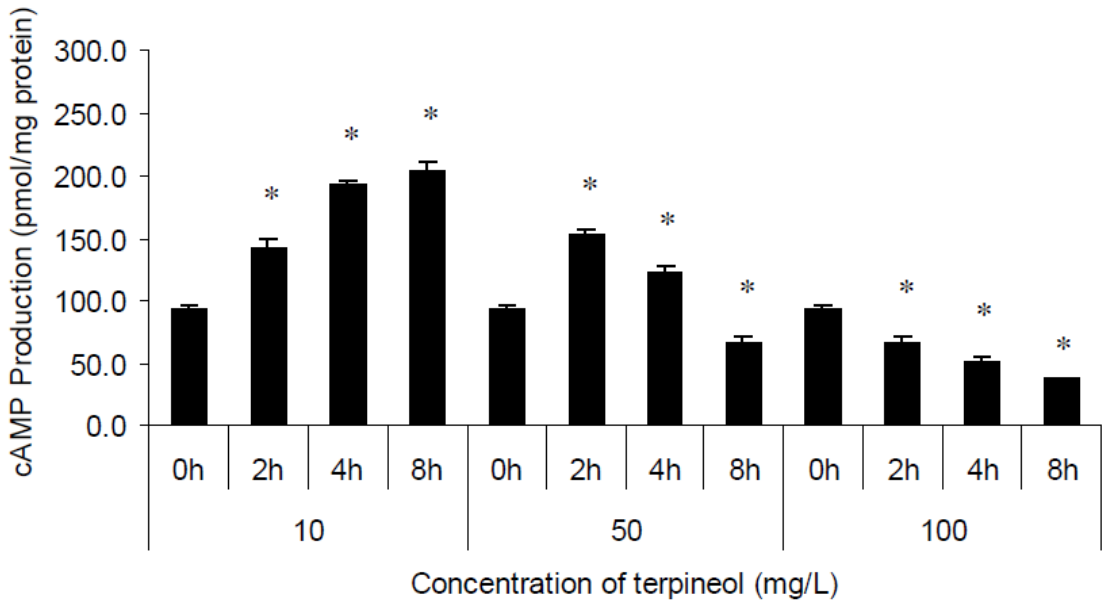


Figure 6-7 cAMP levels of 4th instar larvae of *Aedes aegypti* exposed to 10, 50 and 100 mg L^{-1} α-terpineol for different time periods.
The error bars represents the standard deviation of three independent replicates. An asterisk (*) over a specific timepoint indicates that cAMP levels are significantly different from the control ($p < 0.05$).

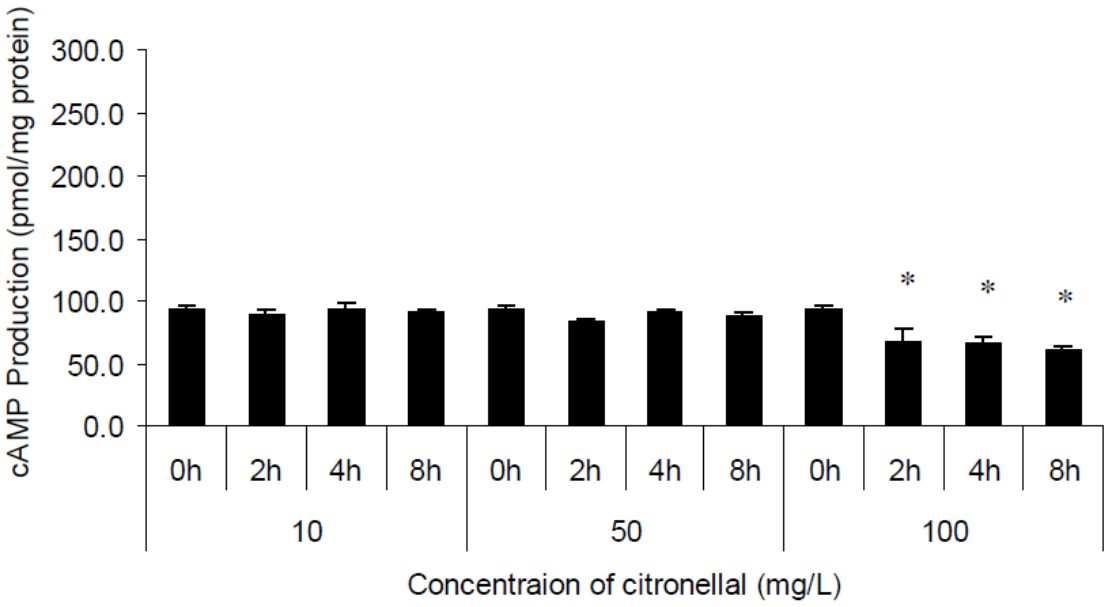


Figure 6-8 cAMP levels of 4th instar larvae of *Aedes aegypti* exposed to 10, 50 and 100 mg L^{-1} citronellal for different time periods.
The error bars represents the standard deviation of three independent replicates. An asterisk (*) over a specific timepoint indicates that cAMP levels are significantly different from the control ($p < 0.05$).

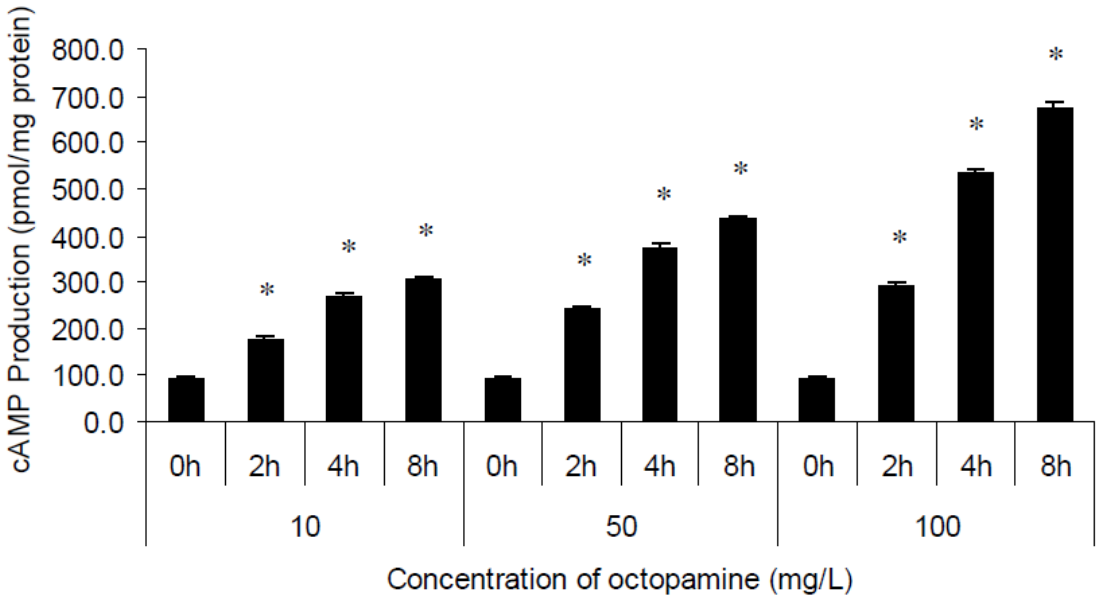


Figure 6-9 cAMP production of 4th instar larvae of *Aedes aegypti* exposed to 10, 50 and 100 mg L^{-1} octopamine for different time periods.
The error bars represents the standard deviation of three independent replicates. An asterisk (*) over a specific timepoint indicates that cAMP levels are significantly different from the control ($p < 0.05$).

6.7 Tables

Table 6-1 The three way Analysis of Variance for the factors.
All three factors have significant effects on cAMP production individually as well as all three factors together.

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Chemical	5	5	911304.26	7569.410	<.0001*
Concentration	3	3	107444.94	1487.419	<.0001*
Time	3	3	117135.96	1621.577	<.0001*
Chemical*Concentration	15	15	665282.85	1841.975	<.0001*
Chemical*Time	15	15	492164.00	1362.659	<.0001*
Concentration*Time	9	9	65754.85	303.4268	<.0001*
Chemical*Concentration*Time	45	45	375866.01	346.8879	<.0001*

Table 6-2 Larvicidal activity of thymol, eugenol, pulegone, α-terpineol and citronellal to fourth-instar larvae of *Aedes aegypti* exposed for 24 h.
Lethal concentration (LC) values are expressed in mg L^{-1}. All values are means of n = 3 experiments (extracted from Waliwitiya et al., 2009).

Compound	LC_{50}
Thymol	53.6
Eugenol	142.9
Pulegone	48.7
Terpineol	>500
Citronellal	262.9

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Chapter 7: Conclusions and future perspectives

Insect pest management is facing economic and ecological challenges worldwide. This is due, in part to an open competition between insects and man to harvest a single resource. To maintain an upper hand we have produced and used synthetic pesticides which have their own inherent environmental hazards (Ratten, 2010). Identifying novel, effective, and inexpensive insecticidal compounds is essential to combat new pests and those that have developed resistance to existing compounds. Botanical extracts containing active insecticidal phytochemicals are promising sources of such molecules to address some of these problems. Their success will require a continuous need to explore new active molecules with different mechanisms of action.

Many plant molecules with insecticidal properties already exist and likely evolved as defenses against phytophagous insects. Plants typically synthesize a wide array of moderately toxic defense compounds or a small number of highly toxic substances (Ratten, 2010). The insect herbivores then feed on a wide range of plant species, potentially encountering toxic substances with relatively non-specific effects on a wide range of molecular targets. These targets range from proteins (enzymes, receptors, ion-channels, structural proteins), nucleic acids, and biomembranes to secondary metabolites with specific or nonspecific interactions (Harborne, 1993). Analogues of secondary metabolites may interfere with vital components of the cellular signaling system, or interfere with the synthesis or function of vital enzymes used in neurotransmission, protein binding, receptor activation and function, or signal transduction among other functions (Wink, 2000).

The knowledge of chemical properties of such novel compounds is necessary to determine the safety and economics of their use. These phytochemical biomolecules, however, may become the basis of future insecticides directly or may form the backbone on which novel compounds might be designed with specific or multiple target sites, while ensuring the economic and ecological sustainability.

The majority of phytochemical insecticide research has studied the lethal, repellent, or deterrent effects of plant extracts and essential oils on insects (Ratten 2010) but fewer studies have studied or elucidated the precise modes of action of these compounds (Lewis et al., 1993; Roeder, 1994; Vanden Broeck et al., 1995; Zafra-Polo et al., 1996; Enan, 1998, 2001; Kostyukovsky et al., 2002; Priestley et al., 2003). In some studies serendipity has been more important than biorational approaches. Determining the target, the biotransformational enzyme response, and the effects of these compounds on non-target organisms is essential if these compounds will ever be used commercially (Casida and Quistad, 1998).

In this thesis I have:

1) evaluated the direct toxicity of 16 phytochemicals on aquatic and terrestrial insects in the absence and presence of the synergist PBO. Five of these compounds, thymol, eugenol, pulegone, α-terpineol and citronellal, were selected for more in depth analysis;

2) developed a system that measures the electrophysiological responses of insects to the topical application of phytochemicals. Changes in flight muscle impulses responses and wing beat signals then were compared with the responses of these insects to common insecticides (octopamine, CDM, DMCDM, rotenone, cypermethrin, fipronil,

ivermectin, dieldrin, malathion, DDT and GABA) whose mode of action is well known. This rapid process allows us to make a prediction on the mode of action of the phytochemicals;

3) evaluated several of the compounds for their ability to interrupt the production and efficiency of host insect biotransformational enzymes to confirm the predictions made from the physiological responses. Different compounds affected the cytochrome p450 enzyme activity and GST activity, and cAMP production, but they did not show effects on β-esterase activity. Enzyme inhibition was greater in the presence of the synergist PBO.

The results indicate that these plant-derived compounds directly and indirectly affect aspects of insect physiology. The acute toxicity may be the collective result of these effects on complex biological systems. The observed effects depend on the type of phytochemical, the concentration, exposure time and the target organism. Their efficacy to control insect pests as well as indirect effects on beneficial organisms and on crop plants, must be evaluated under a broader range of conditions and applications. Whether they can be developed and commercialized as novel insecticides will require further characterization of targets, detailed modes of action, and effects on non-target organisms.

The research described in this thesis has laid the foundation for significant advances in the understanding of the modes of action of plant-based extracts on target insect pests. However there is much more to do. In the short term we must evaluate multiple enzyme systems for targets and modes of action of individual compounds such as the specific receptors using receptor binding assays, the effects of plant-derived

compounds on insect ion channels, and their effects on specific biotransformational enzymes.

Many plant essential oils contain several compounds. Therefore we must move to testing mixtures to evaluate whether our electrophysiological system can be used when evaluating mixtures of compounds that act upon the same target or on different targets within the insect. Will we be able to decipher and interpret our trace data when different effects become superimposed on a single trace? Can we measure the additive or synergistic effects of these compounds on a single target species? Ultimately I would like to establish a database of structure-function-activity relationships for plant derived compounds that can be used to predict modes of action and effects on non-targets. A problem, or consequence, of research is that often more questions are generated than answers, leading to new avenues of research and new questions to address. This truly has been the case for the results of the research carried out and described in this thesis.

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