Neurophysical effects of essential oil constituents in insects and molluscs: Do they target octopamine receptors?

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Neurophysiological effects of essential oil constituents in insects and molluscs: Do they target octopamine receptors?

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Abstract

Essential oils and their constituents are potential pesticides whose mechanism of action is unknown. Some, such as eugenol, are claimed to work at micromolar doses in insects by activation of neuronal octopamine receptors, though there is no physiological evidence. The actions of eugenol, citral and geraniol on the neurophysiology of cockroaches and snails were examined, focusing on comparisons with octopamine.

Cockroaches were killed by topical application or intra-abdominal injection of the oils. Minimum lethal injection doses indicated haemolymph concentrations exceeding 5mM. Eugenol depressed impulse activity recorded extracellularly in the abdominal nerve cord whereas citral and geraniol produced biphasic effects (low-dose excitation). Similar effects on spiking occurred in dorsal unpaired median (DUM) neurons, recorded intracellularly in the isolated terminal abdominal ganglion. The oils reduced spike undershoot and decreased excitability of depolarized DUM neurons, and eugenol induced plateau potentials. Octopamine did not reduce spike undershoot or produce plateau potentials; it had opposing effects to eugenol on DUM neurons and foregut activity, and its effects on DUM neurons were not blocked by eugenol. Thus eugenol did not activate or block octopamine receptors.

Snails were killed by the oils dissolved in the aquarium water (ca. 5×10^{-4}M). Citral and geraniol mimicked octopamine in activating rhythmic movements of the buccal mass and initiating burst activity in intracellularly recorded neurons in isolated buccal ganglia (fictive feeding). Eugenol generally reduced spike activity, acting like a local anaesthetic. Octopamine, applied by perfusion or iontophoresis, hyperpolarized an identified giant neuron, whereas the oils excited it. Metoclopramide blocked excitatory responses to octopamine but not to the oils. Thus the oils did not appear to activate either depolarizing or hyperpolarizing octopamine receptors. Below 10^{-3}M the oils did not block octopamine (or dopamine) receptors, or affect electrotonic coupling, though acetylcholine receptors were blocked at 5×10^{-4}M.

The results indicate no specific targeting by essential oils of octopamine receptors in insects or snails, and the high doses required to produce effects suggest that a range of non-specific factors could contribute to their pesticidal actions.

Declaration and Statements

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed.... .....(candidate)

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Contents

Figures

Tables

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Publications

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Chapter 1

General Introduction

1.10 Essential oils: secondary metabolites and chemical defence

Many organisms produce compounds that often have no apparent function as an energy source or specific storage product, and are classified as 'secondary metabolites' (Luckner, 1984; Mann, 1992). Initially, they were considered to be waste products (Coats et al, 1991; Rice and Coats, 1994; Regnault-Roger, 1997), but it is now known that many of them have important ecological functions. Some act as attractants to aid plant pollination or to attract mates (Mann, 1992) whereas others provide a mode of defence against natural enemies (Rhoades, 1979; Vickery and Vickery, 1981; Pawlik, 1983).

Plants have evolved a wide range of strategies to help defend themselves against herbivores (Arimura et al, 2005) and secondary metabolites have a major role in defending many species, often influencing herbivore feeding behaviour by possessing repellent and toxic properties (Luckner, 1984; Isman, 2000). Some of the main compounds that have been investigated for these properties are essential oils and their major constituents (Ngoh et al, 1997), many of which have insecticidal (Pavela, 2005), molluscidal (Singh et al, 1997), nematocidal (Vasudevan et al, 1997) and anti-microbial (Amin et al, 2005) activity.

1.11 Essential oils and their constituents

Essential oils are volatile plant compounds (Huang et al, 2002; Cheng et al, 2003) that are found in families such as Lauraceae, Rutaceae, Myrtaceae, Lamiaceae, Asteraceae, Apiaceae, Cupressaceae, Poaceae, Zingiberaceae and Piperaceae (Enan, 2001; Bhat et al, 2005). The volatility of these oils enables them to be extracted relatively easily *via* water vapour (Regnault-Roger, 1997). Crude extracts of the oils consist of many different compounds that include terpenes, hydrocarbons, benzene

derivatives and miscellaneous substances (Huang et al, 2002). Many essential oils are used commercially as pharmaceuticals, flavourings and perfumes (Cheng et al, 2003).

1.12 Activity against insects

Essential oils have traditionally been used to protect stored grain and to repel flying insects; however, it is only relatively recently that controlled, scientific studies into their activities have been made (Isman, 2000). The insecticidal effects of these compounds cover a broad range of taxa and include species from many insect orders, including Anoplura (Yang et al, 2004), Blattodea (Ngoh et al, 1998), Coleoptera (Lee et al, 2003), Diptera (Prajapati et al, 2005), Hemiptera (Sampson et al, 2005), Hymenoptera (Enan, 2001), Isoptera (Park and Shin, 2005) and Lepidoptera (Hummelbrunner and Isman, 2001). In addition to the toxic effects, repellent properties have also been shown against many insect species; for example, spathulenol, intermedeol and callicarpenal (extracted from Beautyberry *Callicarpa americana* and *Callicarpa japonica*) have repellent activity against the mosquitoes *Aedes aegypti* and *Anopheles stephensi* (Cantrell et al, 2005). Antifeedant effects have also been reported; for example thymol (from garden thyme *Thymus vulgaris*) deterred feeding in the Tobacco cutworm *Spodoptera litura* (Hummelbrunner and Isman, 2001).

1.13 Activity against molluscs

Molluscicidal effects have been studied less than insecticides, but certain essential oil constituents are toxic to aquatic snails such as *Bulinus truncatus* (Lahlou and Berrada, 2001), *Biomphalaria glabrata* (De Souza et al, 1991), *Lymnaea acuminata* and *Indoplanorbis exustus* (Singh et al, 1997). Many of these species act as vectors for disease-causing parasites in vertebrates; for example, *L. acuminata* is the intermediate host of *Fasciola hepatica* and *F. gigantica*, the causative agents of fascioliasis (Rao and Singh, 2001) and *B. glabrata* is the major intermediate host of *Schistosoma mansoni* (Barbosa et al, 2005). Effects on terrestrial molluscs do not appear to have been examined.

1.20 Roles in pest control programmes

Concerns over increasing costs, human and environmental safety, and resistance to currently used synthetic pesticides have increased interest in the use of plant-derived compounds to control pests (Clark and Appleton, 1997; Ngoh et al, 1998; Lahlou and Berrada, 2001). The repellent and toxic effects of essential oils and their constituents suggest that they have the potential to be used as effective natural insecticides (Isman, 2000) or molluscicides (Singh et al, 1997) in pest control programmes. Many of the oils are considered to have low mammalian toxicity (Lee et al, 2001; Ketoh et al, 2005), and are relatively cheap and readily obtainable (Isman, 2000). They also have the advantage of being safe for the environment as they biodegrade to non-toxic compounds (Kim et al, 2003; Sampson et al, 2005). Many essential oils are registered as 'Generally Recognised as Safe' (GRAS) by the U.S. Food and Drug Administration (Serrano et al, 2005).

Some essential oil components are already sold as commercial pest control products; for example citronellal (from lemon oil) is the active ingredient in many insect repellent candles (Rice and Coats, 1994) and limonene (also from many citrus oils) is a major constituent of flea shampoos (Karr et al, 1990).

1.30 Mode of toxic action

Few studies on essential oils mention the mechanisms of their toxic action. Most essential oils and their constituents are lipophilic, which is one of the factors responsible for their broad spectrum of biological activities (Lahlou, 2004). They are capable of interfering with many aspects of the biochemistry and physiology of insects (Rice and Coats, 1994).

Treatments with various essential oil constituents often cause symptoms in insects that suggest a neurotoxic mode of action (Coats et al, 1991; Kostyukovsky et al, 2002), with the acute symptoms typified by tremors and hyperactivity, rapid knockdown and eventual death (Coats et al, 1991; Enan, 2001). Scharf (2003) suggested that such symptoms are characteristic of neuroexcitation (in contrast to neuroinhibition, which results in immobility and flaccid paralysis). The affinity of these compounds towards lipid-rich tissues, such as those of the central nervous system, facilitates their passage from the blood (Buchbauer, 1993; Lahlou, 2004).

Karr et al (1990) and Coats et al (1991), using non-invasive recordings, found that limonene, pulegone (from peppermint *Mentha piperita*) myrcene (from bay leaves), α-terpineol (from cinnamon *Cinnamomum verum*) and linalool (from lavender) had neurotoxic effects in the earthworm *Eisenia fetida*; however these studies did not reveal the site or mechanism of action. Neurophysiological effects

have also been studied in the snail *Helix pomatia* (Szabadics and Erdélyi, 2000) (see Chapter 3). Effects on the neurophysiology of insects have not been examined.

Neurotoxic effects in vertebrates have been reported; for example, neuronal excitability was decreased by eugenol (Brodin and Røed, 1984), and linalool and linalyl acetate have local anaesthetic activity in rat phrenic nerve preparations (Ghelardini et al, 1999). The scope of this thesis, however, is restricted to effects on invertebrate neurophysiology.

1.31 Effects of essential oils on octopamine receptors

Some essential oil constituents have a specific agonistic (mimetic) effect on octopamine receptors, and have been claimed to exert their insecticidal properties *via* this action (Enan 2001, 2005b; Kostyukovsky et al, 2002).

Octopamine (*p*-hydroxyphenylethanolamine) is a biogenic monoamine that acts as a neurohormone, neuromodulator and neurotransmitter in invertebrates (Hoyle and Barker, 1975; Evans and O'Shea, 1977; Evans and O'Shea, 1978; Orchard, 1982). It is structurally similar to noradrenaline (see Figure 1, Page 7) and it has been suggested that the octopaminergic systems of invertebrates and noradrenergic systems of vertebrates are homologous (Roeder, 1999). Octopamine has important regulatory roles in many aspects of invertebrate physiology (Achenbach et al, 1997; Bischof and Enan, 2004) including control of endocrine gland activity, mobilization of carbohydrates and lipids, and a peripheral action on muscles to influence motor pattern activity (Stevenson and Spörhase-Eichmann, 1995).

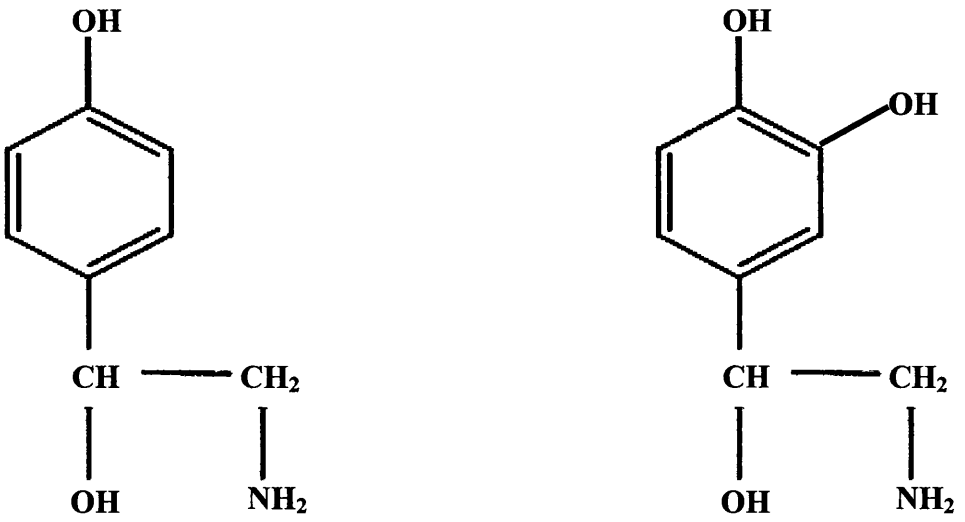


Figure 1. Structural similarities between octopamine and noradrenaline.

Some authors argue that because octopaminergic neurotransmission is mostly restricted to invertebrates, compounds that specifically act on this system should not produce any serious effects in vertebrates because they are largely devoid of the corresponding receptors (Roeder, 1999; Enan, 2001; 2005b). This has been the major 'selling point' of essential oil constituents that are marketed as insecticides. For example, the American company 'EcoSMART Technologies' sells essential oil-based compounds as homeowner insecticides and justifies their safety by the fact that they target a site that "doesn't exist in mammals". Octopamine, however, has been found in the peripheral tissues and brain of rats and other mammals, including humans, but tissue levels are much higher in invertebrates (Axelrod and Saavedra, 1977). There are several receptor subtypes for octopamine in invertebrates (Evans, 1981) and the particular receptor types apparently targeted by essential oils might not occur in

vertebrates. For example, the pharmacological data on the octopamine$_2$ receptor subtype (which mediates many of the physiological functions of octopamine in insects) indicates that it does not conform to the receptor categories found in vertebrates (Nathanson, 1985; Kostyukovsky et al, 2002). Therefore, selective octopamine$_2$ receptor agonists or antagonists could be useful as insecticides with low vertebrate toxicity (provided, of course, that they have no non-specific toxic actions).

1.32 Effects of essential oils on acetylcholinesterase

Some essential oils and their constituents reduce the activity of acetylcholinesterase (AChE) (an enzyme that cleaves the neurotransmitter acetylcholine, ACh), and its insecticidal activity has been attributed partly to this action (Ryan and Byrne, 1988; Mills et al, 2004). Kostyukovsky et al (2002) however, found that inhibition of AChE by essential oil components only occurred at doses that were much higher than those that caused mortality, so the killing effect could not be attributed to this. Furthermore, Lee et al (2001) found that the insecticidal activity of essential oil terpenoids (such as menthone and menthol from *Mentha arvensis* L *var piperascens*) was not correlated with inhibition of AChE activity; the most toxic compound was a weak inhibitor of AChE, whereas the least toxic compound was a strong inhibitor. Grundy and Still (1985) also showed that inhibition of AChE did not correlate with symptoms in the intact animal.

1.40 Experimental oils

Two terpenoids, geraniol [3,7-dimethyl-2,6-octadien-1-ol] and citral [3,7-dimethyl-2,6-octadienal] and one phenylpropanoid, eugenol [2-methoxy-4-(2-propenyl)phenol] were selected for this study.

1.41 Eugenol

The phenylpropenes are a group of small phenolic molecules, derived from shikimic acid (formed from glucose in plants) *via* the phenylpropanoid pathway (Gang et al, 2002). These compounds generally have a 3-carbon chain attached to a benzene ring and are formed from *trans* or (E)-cinnamic acid *via* the elimination of ammonia from L-phenylalanine.

Eugenol (Figure 2A, Page 11) is a yellow oily liquid and is a major component of clove oil, e.g. from *Eugenia caryophyllata* (Yoo et al, 2005). In addition to its use in perfumes and flavourings, eugenol has medicinal applications such as in clinical dentistry (Markowitz et al, 1992) where it is often combined with zinc oxide to form oral cement (Fujisawa et al, 1999). It has antiseptic and local anaesthetic properties (Brodin and Røed, 1984). Eugenol was the main compound used in Enan's (2001; 2005b) octopamine studies.

1.42 Geraniol and citral

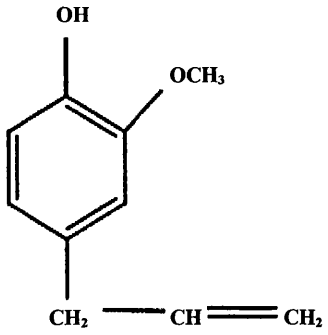
Terpenoids are a structurally diverse group of compounds, representing one of the largest classes of natural products. They are derived from C_5 isopentenoid units (also called isoprene units) and are classified on the number that they contain (Vickery and Vickery, 1981; Bhat et al, 2005). The polymerization of isopentenoid pyrophosphate (3-methylbutenyl pyrophosphate) units gives rise to the different classes: mono- (C_{10}),

sesqui- (C_{15}), di- (C_{20}), sester- (C_{25}), tri- (C_{30}) and tetra- (C_{40}). The polymers of C_5 units undergo many cyclizations, rearrangements and oxidations that yield thousands of structurally unique terpenoids. The biogenetic pathway that gives rise to terpenoids (the mevalonic acid pathway) generally follows the order: acetate → mevalonate → isopentenyl pyrophosphates → geranyl pyrophosphate → farnesyl pyrophosphate → $(C_5)_n$ compounds (terpenoids) (Mabry and Gill, 1979).

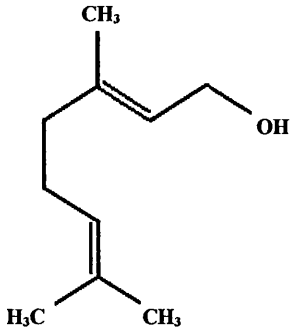
Geraniol is a monoterpenoid alcohol (Figure 2B, Page 11) used in flavourings and perfumes. It is a clear to pale yellow oil and is a constituent of palmarosa (from *Cymbopogon martini*) and geranium oils such as that of the rose-scented geranium (*Pelargonium* species) (Rajeswara Rao et al, 2002; 2005). Citral is monoterpenoid aldehyde (Figure 2C,D, Page 11) commonly used as a flavouring agent and also in the synthesis of vitamin A (Bhat et al, 2005). It is a mixture of the stereoisomers geranial and neral (Nakamura et al, 2003) and is structurally similar to geraniol. Citral is a colourless oil with a strong lemon odour, and is a major constituent of oils from plants in the *Cymbopogon* genus such as lemongrass (*Cymbopogon citrates*) (Wilson et al, 2002).

1.43 Occurrence of essential oils in animals

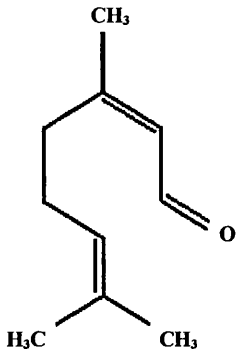
Interestingly, some of these oils are also found in animals; for example, citral and geraniol occur in some sessile marine invertebrates such as the cheilostomate bryozoan *Flustra foliacea* (citral gives it the odour of lemons), where they are thought to have a defensive role (Dyrynda, 1986). Some insects also produce these compounds as a defence mechanism; for example the ant *Acanthomyops claviger* secretes citral from its mandibular glands (Chadha et al, 1962).



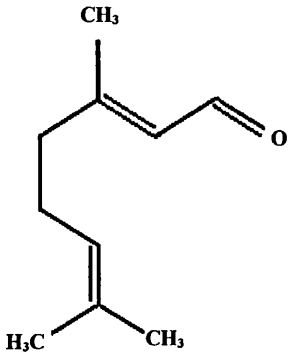
A. Eugenol



B. Geraniol



C. Cis-citral (Neral)



D. Trans-citral (Geranial)

Figure 2. Chemical structures of the essential oil constituents eugenol (a phenylpropanoid), geraniol, and the stereoisomers of citral (monoterpenoids).

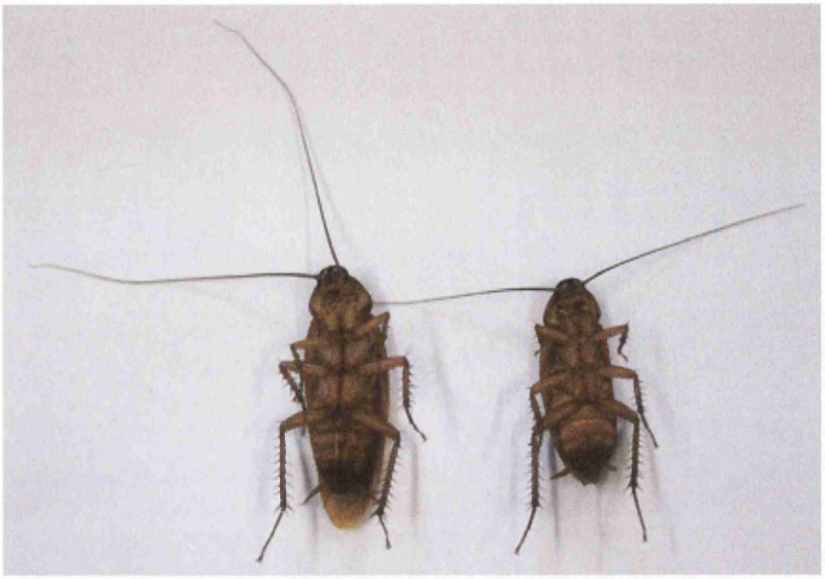
1.50 Experimental animals

1.51 Insects

The American cockroach, *Periplaneta americana* and the West Indian leaf cockroach, *Blaberus discoidalis* were used in the study (Figure 3, Page 13).

P. americana was chosen because its general physiology has been well-documented, and many studies have been carried out on the nervous system (e.g. Kerkut et al, 1968; Crossman et al, 1971; Arikawa et al, 1984; Tanaka and Washio, 1988; and Ritzmann and Pollack, 1997). Rowan and Chambers (1982) reported that neurotoxicity of chemicals could be easily detected and analysed in the isolated ventral nerve cord of *P. americana*. Also, this species was studied by Enan (2001, 2005b) in his experiments on effects of essential oils on octopamine receptors, and this allowed direct comparisons to be made with his results. Cells within the ganglia of cockroaches (e.g. Dorsal Unpaired Median or 'DUM' cells) respond to octopamine (Washio and Tanaka, 1992) and the effects of the oils on these cells were studied. *B. discoidalis* was used as a comparison to *P. americana* to investigate any species differences.

A. Adult *P. americana*



B. Adult *B. discoidalis*

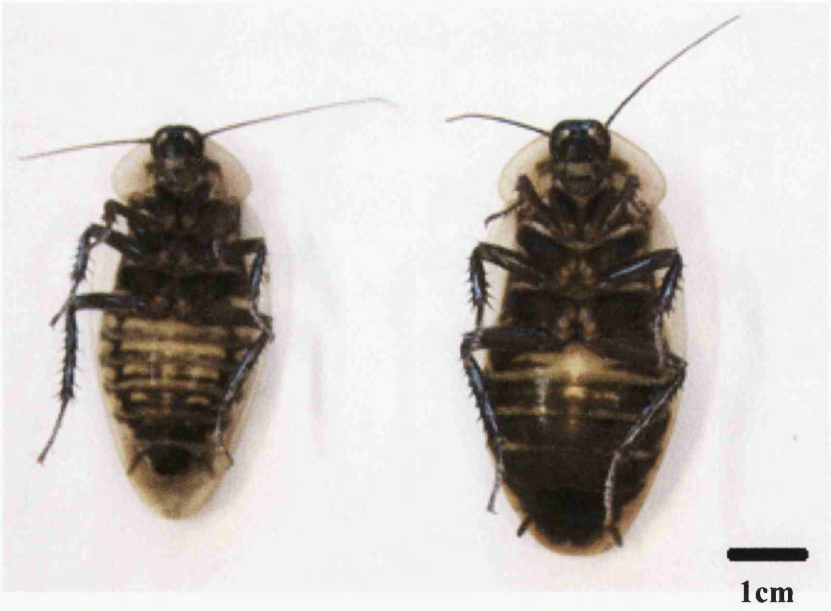


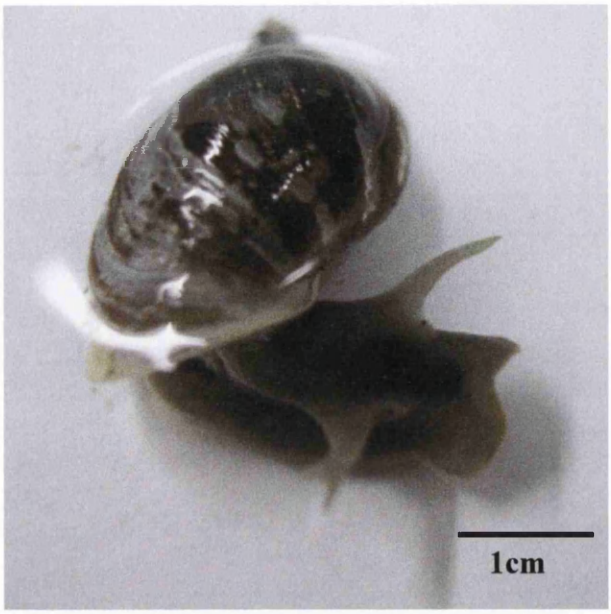
Figure 3. Ventral view of cockroaches used in the study (males on the left-hand side). A) The American cockroach, *P. americana*. B) The West Indian leaf cockroach, *B. discoidalis*. Scale bar is the same for both species.

1.52 Molluscs

Two species of aquatic gastropod were used: the freshwater whelk *Lymnaea stagnalis* and the Ramshorn snail *Planorbis corneus* (Figure 4, Page 15).

Much is known about the general behaviour and neurophysiology of these animals (e.g. Arshavsky et al, 1988a; b; c; Elliott and Susswein, 2002), and how behaviour patterns are generated and controlled by the nervous system (Benjamin and Rose, 1979; Rose and Benjamin 1979, 1981a; b; Benjamin 1983; Elliott and Benjamin, 1985a; b; McCrohan & Kyriakides, 1992). The effects of octopamine in the central nervous system of *L. stagnalis* are well documented (e.g. Vehovszky and Elliott, 2001; Elliott and Vehovszky, 2001; Vehovszky et al, 2004; Vehovszky et al, 2005), providing a good background for the current studies. Again, two species were used, partly to determine any species-specific effects but also because each had its own advantages. For example, *L. stagnalis* was more useful in the toxicological and feeding experiments because control animals were more active and ate more than *P. corneus* (therefore, any effects of the oils were more obvious). In *P. corneus* a specific neuron that responded to octopamine could be readily identified. Where possible, both species were used.

A. *Lymnaea stagnalis*



B. *Planorbis corneus*

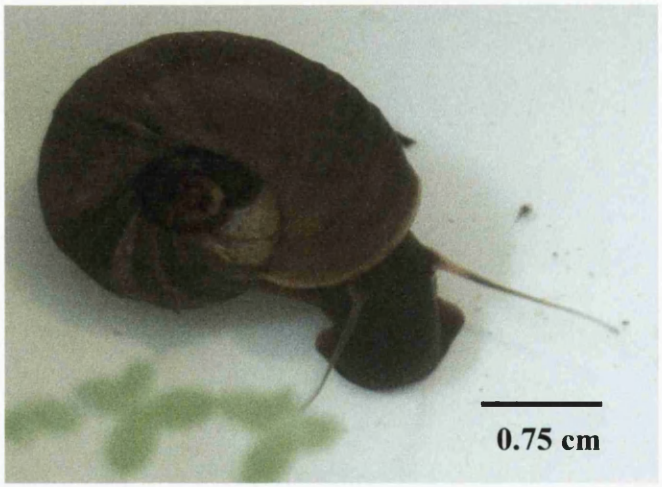


Figure 4. Aquatic gastropod molluscs used in the study. A) The freshwater whelk *L. stagnalis*. B) The ramshorn snail *P. corneus*.

1.60 Aims of the study

The principal aim was to analyse the neurophysiological effects of essential oil constituents in insects and molluscs. Previous studies on insects are all of a biochemical nature (e.g. Enan, 2001), and this is the first time that electrophysiological effects of essential oils have been studied in the insect nervous system. In molluscs, there are a few reports on neurophysiological effects (e.g. Szabadics and Erdélyi, 2000) but these are largely restricted to demonstration of local anaesthetic-like actions of essential oils. A variety of effects were examined in the current study, but as a starting point (and major focus) the effects of the oils were compared to those of octopamine in an attempt to establish whether they target octopamine receptors, as suggested by Enan (2001, 2005b) and Kostyukovsky et al (2002) in insects.

In parallel to the experiments on the nervous system, the chemotactic, antifeedant and lethal effects of the oils were assessed, to confirm their behavioural and toxic effects and possibly provide clues to their mode of action. For example, feeding in *L. stagnalis* was significantly reduced by some of the oils, which directed much of the neurophysiological work towards the feeding network of the buccal ganglia.

The overall aim was to enlarge on the scarce literature on neurophysiological effects of essential oils in invertebrates, and to provide a balanced view on the extent to which these effects contribute to the mechanisms of insecticidal and molluscicidal action.

Chapter 2

High doses of essential oil constituents are needed to kill cockroaches: an insight into the mechanisms of their insecticidal actions?

2.10 Introduction

Many essential oil constituents have contact and fumigant toxicity against insects (Isman, 2000), and this has been shown for the compounds used in this study (eugenol, citral and geraniol). For example, the phenolic compound eugenol is effective against various species of Coleoptera (Huang et al, 2002), Hymenoptera (Enan, 2001), Isoptera (Park and Shin, 2005) and many others. The insecticidal effects of the monoterpenoids citral and geraniol have been less well studied; however, citral is toxic to the Mediterranean fruit fly *Ceratitis capitata* (Salvatore et al, 2004) and the Caribbean fruit fly *Anastrepha suspensa* (Coats et al, 1991), and geraniol to the mosquitoes A*edes aegypti, A. albopictus* and *Anopheles quadrimaculatus* (Xue et al, 2003).

Effects of citral and eugenol on cockroaches have been documented but geraniol does not appear to have been examined. For example, Ngoh et al (1998) found that benzene derivatives such as eugenol were more toxic and repellent to *P. americana* than other terpenes, and that they had strong contact toxicity but low fumigant toxicity. They suggested that eugenol enters the body and exerts its killing effects *via* ingestion or penetration of the integument. Enan (2001) demonstrated that eugenol was toxic to *P. americana* and the German cockroach *Blatella germanica*, with greater potency than two other essential oil constituents (α-terpineol and cinnamic alcohol). Vartak et al (1994) showed that both eugenol and citral had repellent properties against *P. americana.*

2.11 Aims

Before studying the neurophysiological effects of eugenol, citral and geraniol on cockroaches, the toxic effects of these compounds are assessed in this chapter with the aim of providing insights into their mechanisms of action. The major difference between this research and previous studies is that the effects of the oils were assessed using several different routes of application. For example, the internal concentrations that cause death were estimated by injecting the oils directly into the abdomen of the cockroach. It was hoped to distinguish between the possible mechanisms of action responsible for mortality, such as low-dose specific targeting of receptors for neurotransmitters (as suggested by Enan, 2001) or high-dose, non-specific effects on perhaps a variety of targets. The oils were also applied topically to an area of thick (pronotum) or thin (ventral abdomen) integument to assess the effectiveness of these lipophilic compounds at penetrating insect cuticle. The toxicity of the vapour of the oils was also analysed because some of these compounds have fumigant toxicity against insects, particularly stored product pests such as beetles (Regnault-Roger and Hamraoui, 1995; Lee et al, 2003). Food consumption was assessed because some natural plant extracts act as antifeedants against insects; for example some diterpenoids have activity against the Potato beetle *Henosepilachna vigntioctopunctata* (Govindachari et al, 1999). Possible repellent (or attractant) effects were also studied. None of the oils seem to have been previously tested on *B. discoidalis*.

2.20 Materials and Methods

2.21 Experimental animals

P. americana were purchased from Blades Biological Supplies Ltd (UK) and maintained in an insectarium at constant temperature (25°C) and relative humidity (60%) under a 12L:12D photoperiod. They were provided with cat biscuits and water *ad libitum*. *B. discoidalis* were obtained from the departmental breeding colony and maintained under the same conditions. Adult males, weighing 1.0-1.5g (*P. americana*) and 4.0-4.5g (*B. discoidalis*) were used in experiments.

2.22 Toxicity tests

Topical application of oils

Citral (95%), geraniol (98%) and eugenol (99%) were purchased from Sigma-Aldrich (UK). Individual oils were dissolved in methanol (99%; Fisher Scientific, UK) and were deposited onto the cockroaches using a micropipette (Finnpipette digital). The chemicals were placed on to either the dorsal thorax (pronotum) or ventral surface of the abdomen (between the 3rd and 5th sternite) (total volume of 10µl in each case). Both species were treated with the same absolute amounts of 0.5, 1, 2.5, 5 and 10mg (1mg is approximately 1µl) of the compounds. Control animals were treated with methanol (10µl) or olive oil (10µl, presumably inactive), or were untreated. After treatment, each insect was placed for 1 h in a small glass chamber (length 6cm, width 3cm, height 3cm for *B. discoidalis*; 4 x 2 x 3cm for *P. americana*) to limit its movements, thus decreasing the spread of the chemical to other parts of the cuticle and allowing time for the oil to be absorbed. The animal was then placed into a separate glass container (14 x 8 x 7.5cm) containing dried cat biscuits and moist tissue paper.

Effect of oil vapour

Oils (10mg, equivalent to maximum dose applied topically) were placed on a $3cm^2$ piece of filter paper and left to dry for 10 min. The filter paper was then placed out of reach from the cockroaches, suspended from the lid of the container, and the animals were assessed for mortality over seven days. The containers were the same as those used previously (14 x 8 x 7.5cm). Holes in the lids of the containers provided ventilation.

Injection of oils

The oils were diluted in physiological saline (160mM NaCl, 3.1mM KCl, 5mM $CaCl_2$, 4mM $MgCl_2.6H_2O$, 5mM HEPES; pH 7.2), stirring vigorously on a magnetic stirrer for 1 h to produce an emulsion which was then used within 1 min before the oil started to separate out. High doses of the compounds were injected to allow for dilution by the body fluids of the insect. Oils were injected laterally through the intersegmental membrane, between the last two abdominal sternites, using a Hamilton syringe with a 21G needle (Figure 5, Page 23). The absolute amounts (0.5, 1, 2.5, 5 and 10mg compound) were the same for both species. Saline was injected as a control, and all injection volumes were 10µL. Olive oil (10µl) was also injected. After injection, the animal was treated in the same way as in the experiments involving topical application.

Estimation of body fluid volume

An estimation of body fluid volume was needed in order to estimate the internal concentrations of the oils required to cause death. A cockroach was killed (using ethyl acetate) and immediately weighed and placed into a drying oven for 24 h at 50°C. The

animal was then weighed again, thus providing a value of the weight of fluid lost through evaporation (dissection revealed that the internal body cavity was devoid of any fluids and, if the animal was left in the oven for a further 12 h, there was no more weight loss). This weight was converted to volume (assuming a specific gravity of 1.0), and the values were similar to those obtained by Smith (1994) using a spectrophotometric technique. The body fluid volume of thirty *P. americana* (0.5-1.5g) was 70-150µl, and for thirty *B. discoidalis* (4-5g) 300-550µl. These values represented total body fluids (intracellular and extracellular) and provided only rough estimates; they were considered to be sufficiently accurate however, in view of the large amounts of injected oils necessary to cause death (any inaccuracies are unlikely to alter the conclusions).

Injection of dye

In separate experiments, Basilen Blue (1mM, in saline) was injected (10µl) to test for possible leakage and to determine the spread of injected compounds within the animal.

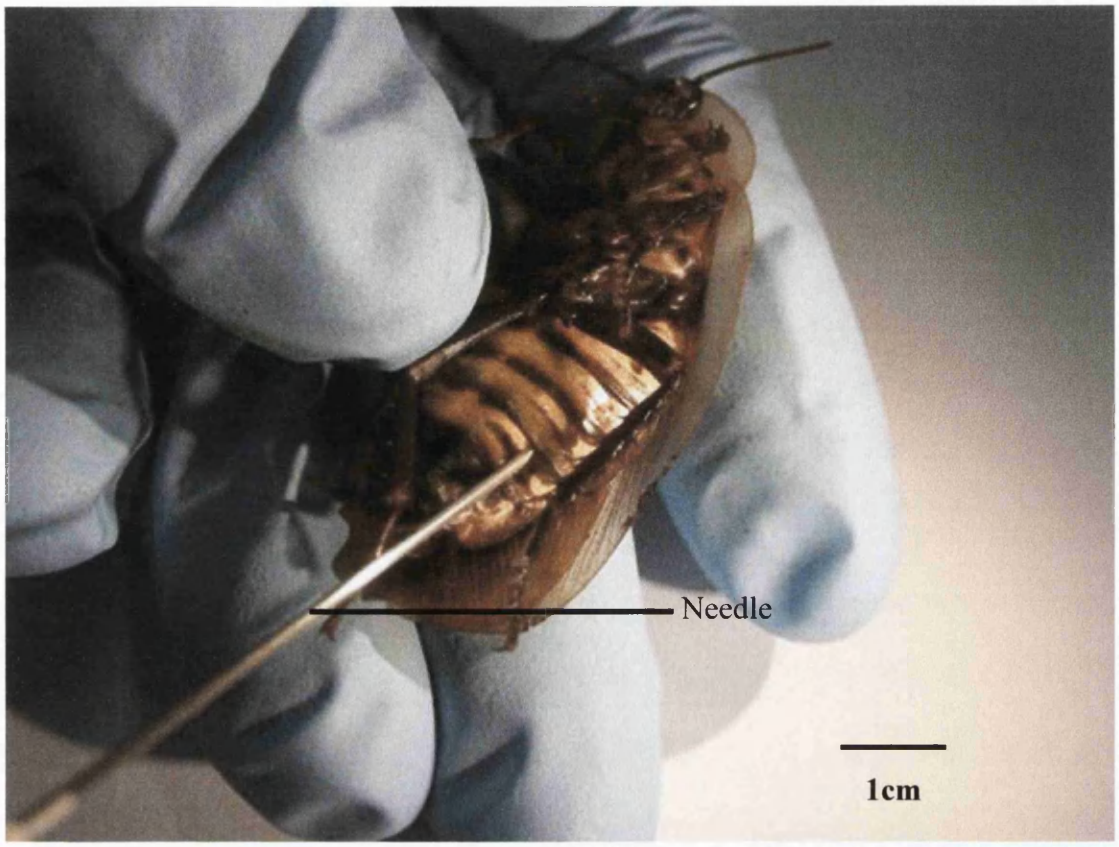


Figure 5. Injection of *B. discoidalis*

Assessment of animals

The condition of the insects was assessed every 15 min for the first 2 h of the experiment to determine the initial effects of the oils. The animals were then assessed every 24 h for 7 days by gently prodding the posterior of each animal with forceps. Healthy animals moved rapidly away. Thirty cockroaches per treatment were used.

2.23 Food consumption

Food consumption was measured only for *B. discoidalis* because untreated specimens of *P. americana* did not consistently consume recordable amounts of food within the experimental period. The cockroaches were starved for 24 h prior to oil application, and then placed in glass boxes containing pre-weighed cat biscuits (approximately 0.25g) that had been oven dried at 50^0C for 1 h. Every 24 h the remaining food was removed from each container, oven dried at 50^0C for 1 h and weighed. Fresh food was added daily. Food consumption was analysed only for individuals that were classified as healthy at the time of recording.

2.24 Repellent effects

Experiments were performed in a linear olfactometer made of Perspex tubing (length 150cm, diameter 3.5cm). The chemicals (10mg, 1mg or 0.1mg) were dissolved in methanol and the solution (100µl total volume) was spread on to a piece of filter paper (Whatman type 1; $9cm^2$) that was left to air dry (for 1 h) before being placed at one end of the tubing; untreated filter paper was placed at the opposite end. Methanol was tested against an untreated piece of filter paper to assess if it had any repellent (or attractant) properties of its own. The ends of the tube were sealed with gauze that prevented escape of insects but allowed air movement, and the apparatus was left to equilibrate for 1 h before 6 cockroaches were admitted *via* an entrance hole at the centre. The olfactometer was kept in total darkness to eliminate any illumination bias. Each half on either side of the entry portion was considered as a separate section and the number of insects present was counted. The position of the insects was recorded after 15 min, and again after 1, 2, 6, 12 and 24 h. The experiment was repeated five times with different cockroaches (i.e. 30 animals were used for each dose). Animals

found within 15cm of the entry point were not regarded as being clearly in one half, and were omitted from the analysis. A diagram of the apparatus is shown in Figure 6, below.

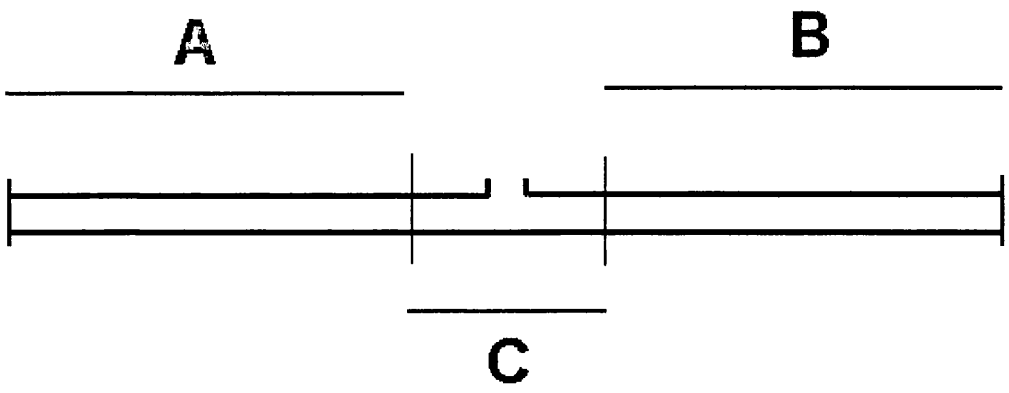


Figure 6. A diagrammatic version of the olfactometer. The tube was split into three sections: Section A contained the oil, section B contained no oil. The number of insects in each section were counted. Section C was labelled as the entry portion and the individuals counted within this section were omitted from the analysis because they were not regarded as being clearly in one half of the tube.

2.25 Statistical analysis

For topical application and injection of oils, values of LC_{50} (the dose required to kill 50% of the animals) and LT_{50} (the time taken for 50% of the animals to die) were calculated using probit analysis (Finney, 1952). Doses are expressed as mg/g body weight, calculated by dividing the absolute application dose by the body weight of each individual used. The mean for each dose was then calculated for the total number of animals used; for example, 10mg oil applied to *B. discoidalis* gave a mean value of 2.43mg (range 2.39-2.47mg/g, N=90).

Mortality was also expressed as mean values (± standard error) because LC_{50} values alone provide little information on any significant differences between variables. Two-tailed Student's *t* tests were used to examine for significant differences between means. Data from the repellence experiments are also expressed as mean values ± standard error, and analysed by two-tailed Student's *t* tests.

2.30 Results

2.31 Mortality

Symptoms of toxicity

All three essential oil constituents had dose-dependent toxic effects on the cockroaches. Initial effects of lethal doses (within 2 h of application) were characterized by hyperactivity compared to control animals; this included excessive cleaning with the limbs, antennae biting (occasionally bitten off), and running around the periphery of the arena. Prior to death, affected individuals were on their backs, had rigid limbs and twitched when prodded (labelled as knockdown). Recovery did not occur, and individuals displaying these symptoms always died by the end-point of the experiment. Since all dead individuals displayed knockdown prior to death, knockdown and mortality data were similar and so only the latter is included to avoid repetition. In contrast to 'knocked-down' insects, dead animals had rigid limbs and did not twitch when prodded.

Analysis of mortality

Tables 1 and 2 (Pages 28 and 29) show (for *B. discoidalis* and *P. americana* respectively) the LC_{50} values after every 24 h; tables 3 and 4 (Pages 37 and 38) show the LT_{50} values for the two highest doses. Figure 7 (Pages 30-36) displays the mean mortality recorded at the end of the experiment (168 h). Only individuals that did not respond to tactile stimuli were included in the analysis. No deaths occurred in untreated insects or in those treated with methanol, olive oil or saline. Exposure to vapour, without direct contact of the oil, also failed to kill any insects.

Table 1. LC_{50} (mg/g body weight) for citral, geraniol and eugenol, applied topically to the dorsal thorax (pronotum) or ventral abdomen, or by intra-abdominal injection in *B. discoidalis*. Measurements of lethality were made every 24 h after a single application.

Chemical and application site	LC_{50} (mg/g) (95% confidence limits) Time after application						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Citral							
Pronotum	4.06[a]	3.23[a]	3.23[a]	3.04[a]	3.04[a]	3.04[a]	3.04[a]
Abdomen	2.22 (1.96-2.60)	2.00 (1.75-2.32)	2.00 (1.75-2.32)	1.90 (1.67-2.19)	1.80 (1.58-2.09)	1.75 (1.54-2.02)	1.75 (1.54-2.02)
Injection	2.18 (1.88-2.64)	1.56 (0.98-3.25)	1.22 (0.76-2.08)	0.86 (0.51-1.58)	0.77 (0.49-1.40)	0.77 (0.49-1.40)	0.77 (0.49-1.40)
Eugenol							
Pronotum	3.04[a]	3.04[a]	3.04[a]	3.04[a]	3.04[a]	3.04[a]	3.04[a]
Abdomen	3.23[a]	2.78[a]	2.31 (2.00-2.80)	1.92 (1.70-2.21)	1.58 (1.13-2.47)	1.58 (1.13-2.47)	1.58 (1.13-2.47)
Injection	2.70 (2.24-3.59)	1.51 (1.03-2.43)	1.23 (0.77-2.19)	0.98[a]	0.85 (0.74-0.98)	0.81 (0.61-1.09)	0.81 (0.61-1.09)
Geraniol							
Pronotum	[b]	3.23[a]	3.23[a]	3.23[a]	3.23[a]	3.23[a]	3.23[a]
Abdomen	2.46[a]	2.37[a]	2.26[a]	2.20[a]	2.09 (1.87-2.30)	2.05 (1.83-2.26)	2.05 (1.83-2.26)
Injection	2.36 (2.10-2.74)	1.37 (1.14-1.67)	1.33 (1.11-1.62)	1.21 (1.01-1.47)	1.21 (1.01-1.47)	1.19 (0.99-1.44)	1.19 (0.99-1.44)

[a]Estimates of 95% confidence limits could not be calculated reliably due to limitations of the dose range.

[b]No mortality.

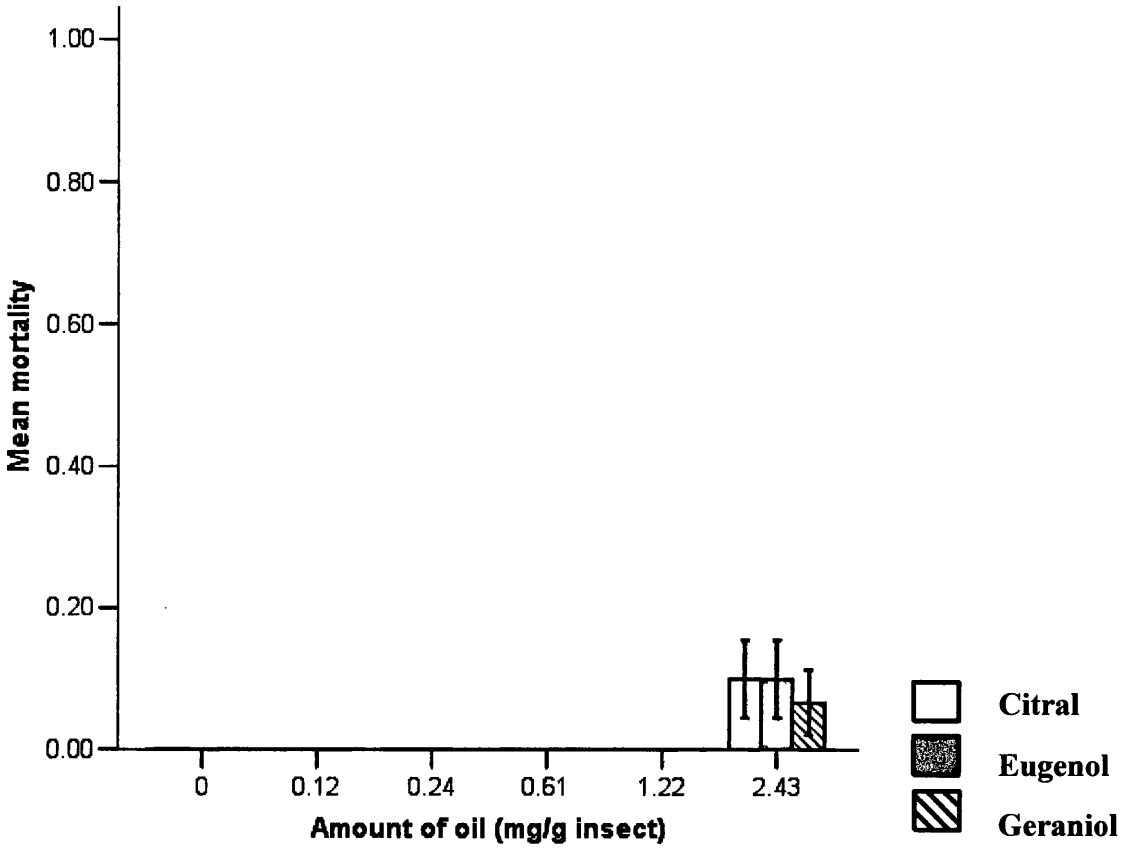
Table 2. LC_{50} (mg/g body weight) for citral, geraniol and eugenol, applied topically to the dorsal thorax (pronotum) or ventral abdomen, or by intra-abdominal injection in *P. americana*. Measurements of lethality were made every 24 h after a single application.

Chemical and application site	LC_{50} (mg/g) (95% confidence limits) Time after application						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Citral							
Pronotum	10.00[a]	9.87[a]	9.63[a]	9.41[a]	9.12 (8.20-10.36)	9.12 (8.20-10.36)	9.12 (8.20-10.36)
Abdomen	10.64[a]	10.44 (8.41-14.52)	7.82 (6.62-9.64)	6.76 (5.73-8.21)	6.55 (5.57-7.90)	6.51 (5.51-7.90)	6.09 (5.18-7.31)
Injection	8.40 (7.45-9.65)	4.20 (3.05-6.18)	3.52 (2.23-6.82)	3.52 (2.23-6.82)	3.45 (1.86-9.37)	3.20 (1.63-8.43)	3.20 (1.63-8.43)
Eugenol							
Pronotum	9.63[a]	8.69 (7.52-10.44)	8.23 (7.18-9.71)	7.62 (6.69-8.82)	6.68 (6.00-8.01)	6.49 (5.68-7.51)	6.49 (5.68-7.51)
Abdomen	6.97 (6.07-8.15)	4.01 (3.39-4.81)	3.34 (2.79-4.08)	3.18 (2.66-3.88)	3.14 (2.62-3.85)	3.14 (2.62-3.85)	3.14 (2.62-3.85)
Injection	7.96 (6.70-9.90)	1.80[a]	1.13[a]	1.01 (0.22-4.53)	0.98[a]	0.98[a]	0.98[a]
Geraniol							
Pronotum	10.14[a]	9.31[a]	9.10[a]	9.00[a]	9.00[a]	9.00[a]	9.00[a]
Abdomen	18.80 (12.29-69.47)	8.21 (5.57-18.43)	6.58 (4.63-11.01)	5.68 (4.05-8.70)	5.11 (3.35-8.69)	5.11 (3.35-8.69)	5.11 (3.35-8.69)
Injection	10.08 (8.69-12.53)	7.63 (5.72-11.73)	6.41 (5.58-7.48)	6.32 (5.45-7.45)	6.32 (5.45-7.45)	6.32 (5.45-7.45)	6.32 (5.45-7.45)

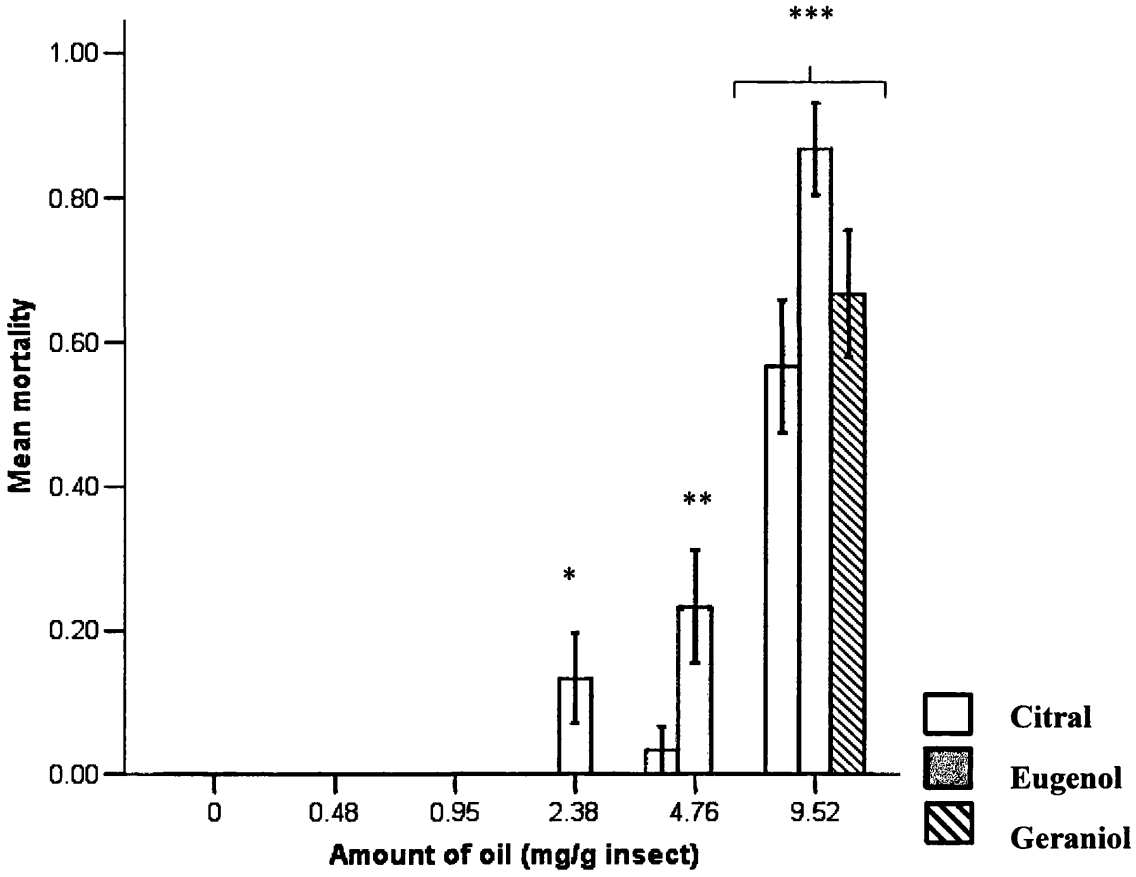
[a]Estimates of 95% confidence limits could not be calculated reliably due to limitations of the dose range.

Figure 7. Bar charts of mean number (± S.E) of cockroach deaths (1.00 equals 100% mortality) caused by citral, geraniol and eugenol administered by 3 different routes: topical application to the dorsal thorax (pronotum), topical application to the ventral abdomen, or intra-abdominal injection: A,B) pronotum; C,D) abdomen; E,F) injection. Absolute amounts added to each species were the same (i.e. 0.5, 1.0, 2.5, 5 and 10mg). Asterisks indicate significant difference from untreated, methanol- and olive oil-treated, and saline-injected animals (all zero mortality).* $P<0.05$; ** $P<0.01$; *** $P<0.001$. N=30 for each treatment. Charts represent data recorded 168 h after treatment.

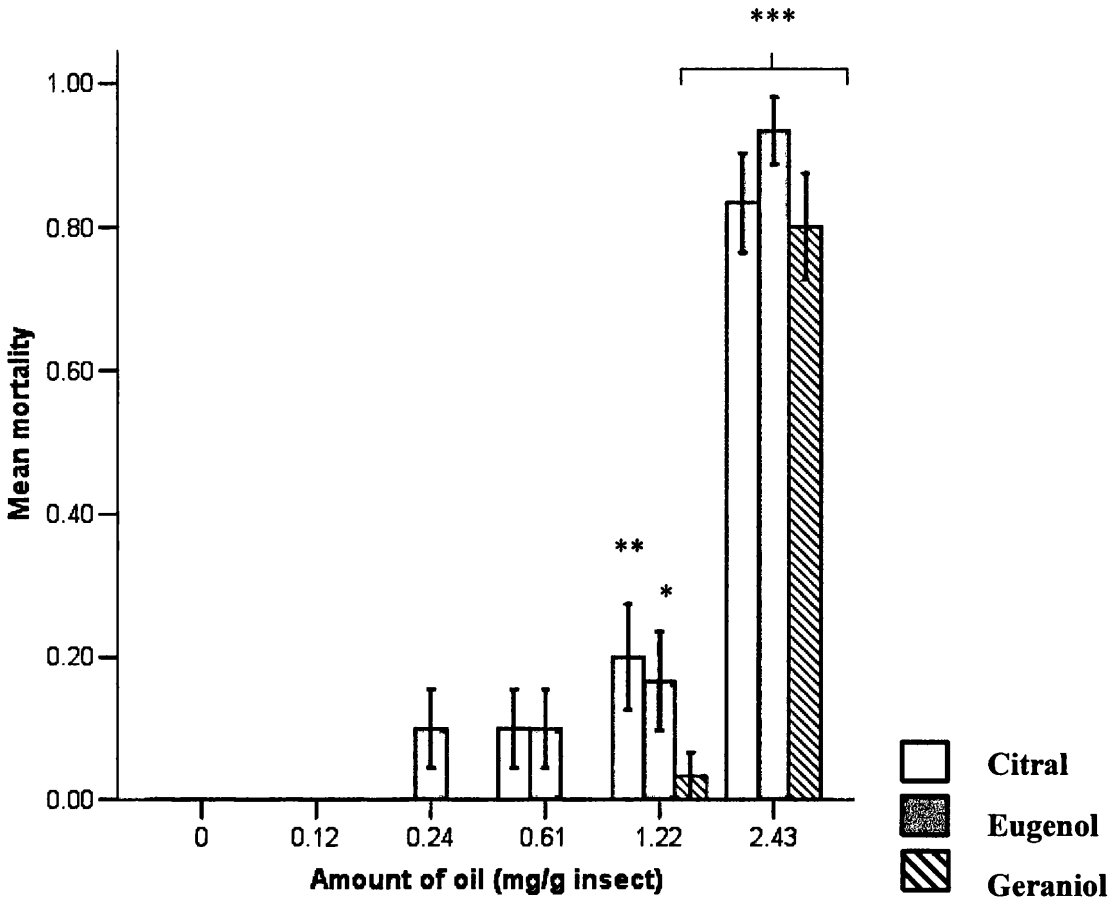
A. *B. discoidalis* pronotum



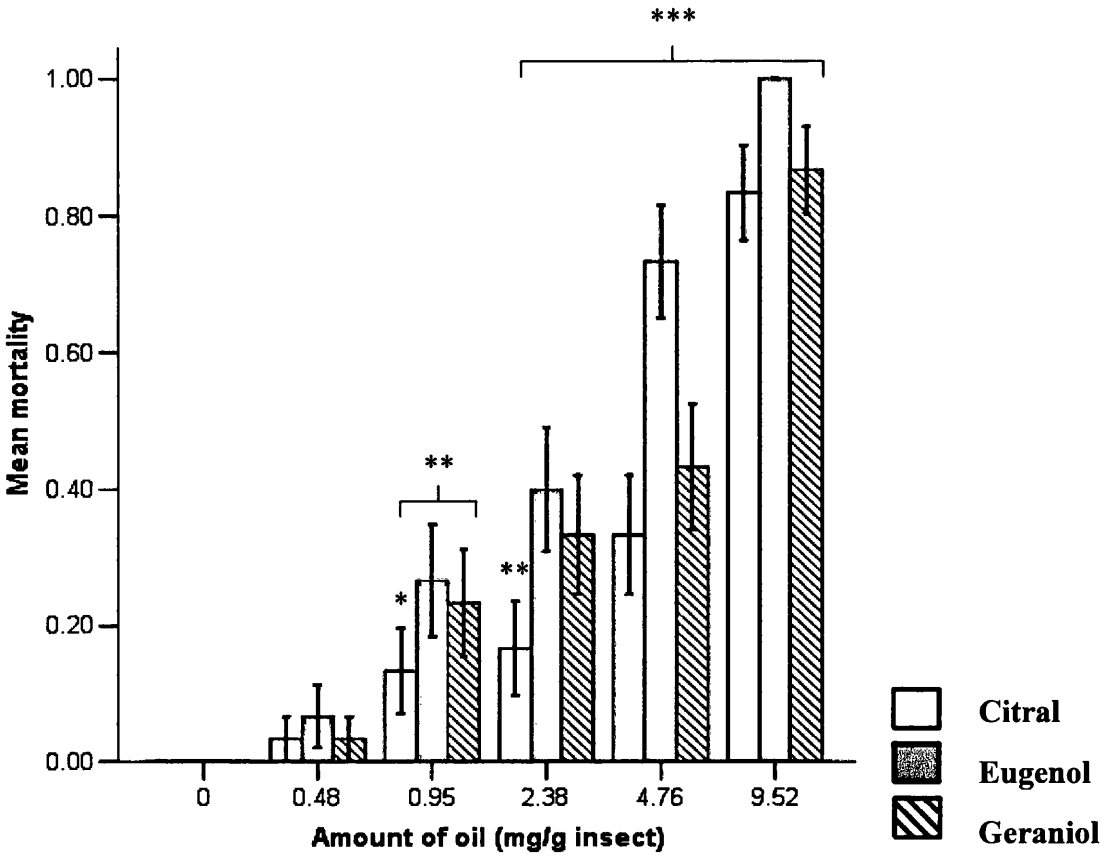
B. *P. americana* pronotum



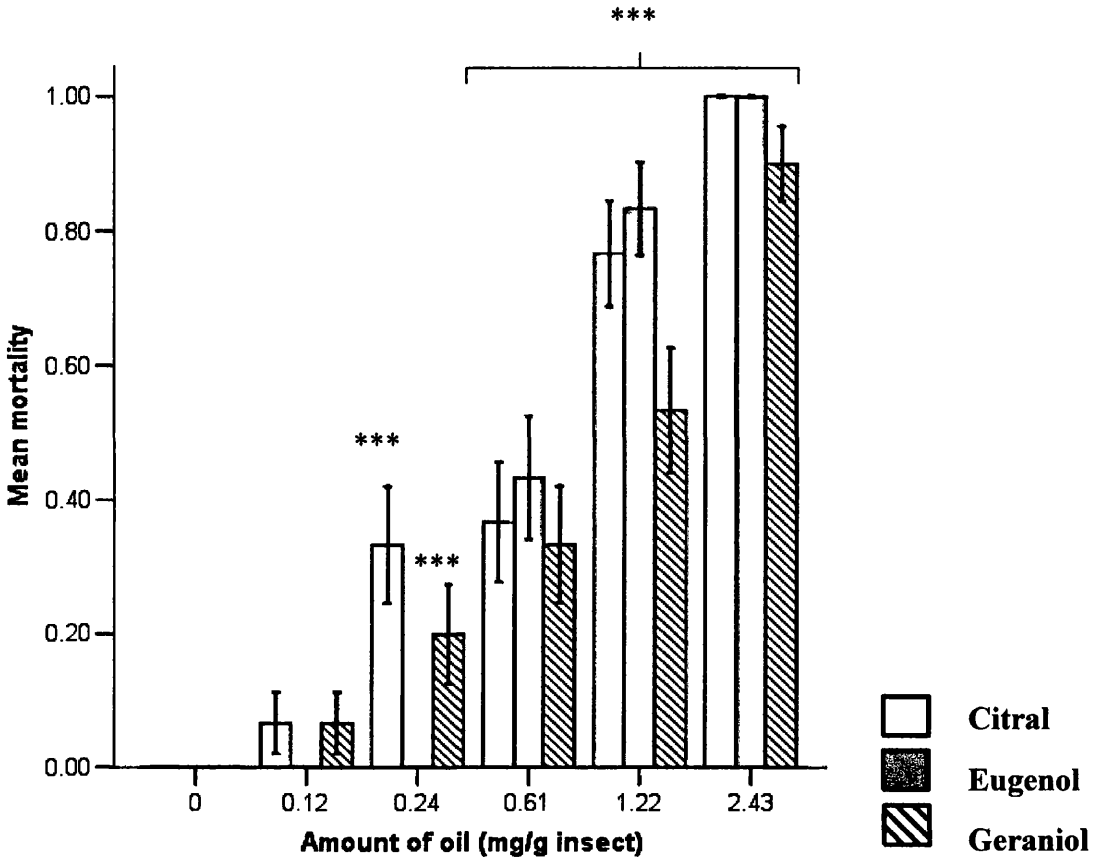
C. *B. discoidalis* abdomen



D. *P. americana* abdomen



E. *B. discoidalis* injection



F. *P. americana* injection

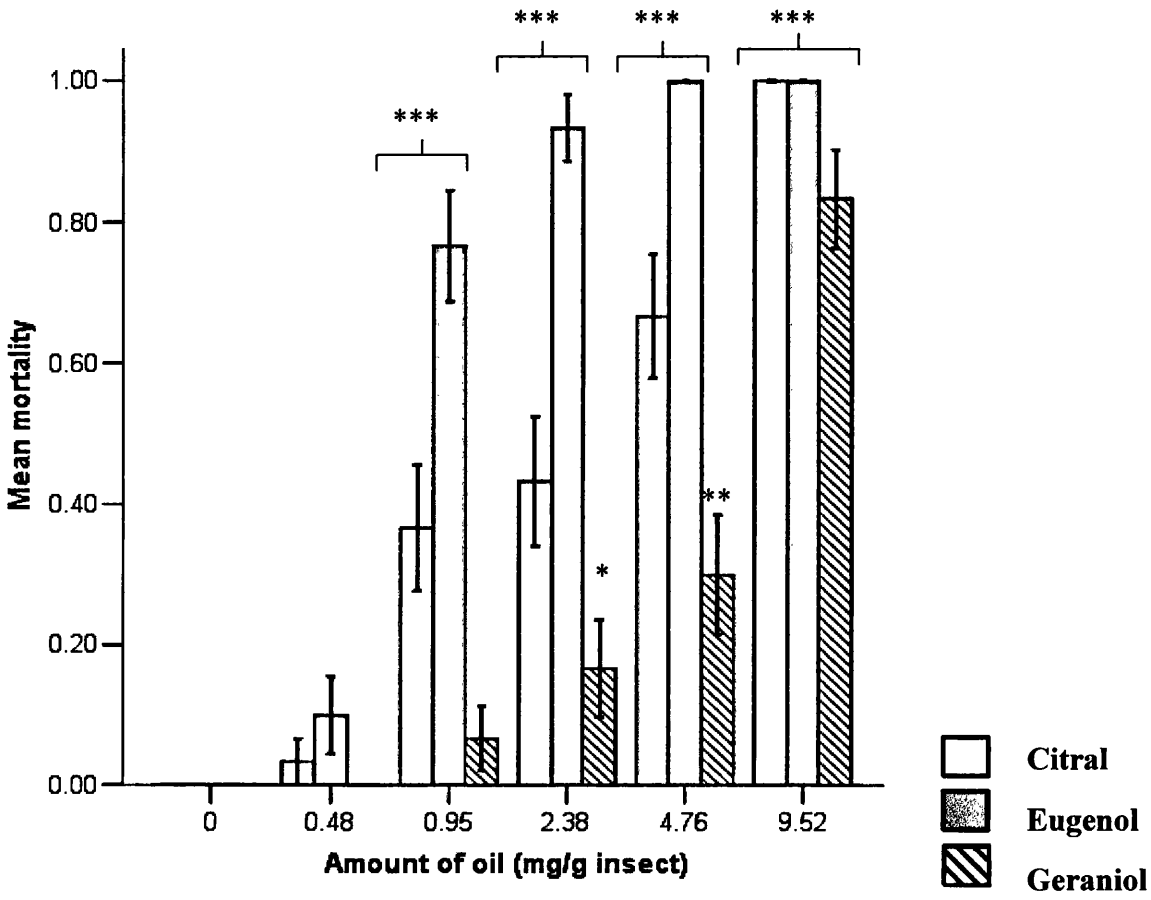


Table 3. LT_{50} (h) for the two highest doses (1.22 and 2.43mg/g insect, i.e. 5 and 10mg/animal) of citral, geraniol and eugenol applied topically to the dorsal thorax (pronotum) or ventral abdomen, or by intra-abdominal injection to *B. discoidalis*.

Chemical and application site (mg/g)	LT_{50} (95% confidence limits) (h)
CITRAL	
Pronotum	
1.22	[b]
2.43	410.00[a]
Abdomen	
1.22	318.88 (213.03-1347.56)
2.43	41.97 (-119.39-83.94)
Injection	
1.22	71.18 (19.46-107.18)
2.43	35.25 (18.92-48.26)
EUGENOL	
Pronotum	
1.22	[b]
2.43	682.72[a]
Abdomen	
1.22	248.65 (195.07-427.71)
2.43	76.40 (58.80-92.86)
Injection	
1.22	70.74 (38.42-96.37)
2.43	36.04 (28.85-42.68)
GERANIOL	
Pronotum	
1.22	[b]
2.43	482.63[a]
Abdomen	
1.22	308.43a
2.43	56.13 (-17.36-92.04)
Injection	
1.22	124.61 (86.61-235.10)
2.43	29.38 (-188.06-71.17)

[a]Estimates of 95% confidence limits could not be calculated reliably due to limitations of the time range.

[b]No mortality occurred.

Table 4. LT_{50} (h) for the two highest doses (4.76 and 9.52mg/g insect, i.e. 5 and 10mg/animal) of citral, geraniol and eugenol applied topically to the dorsal thorax (pronotum) or ventral abdomen, or by intra-abdominal injection to *P.americana.*

Chemical and application site (mg/g)	LT_{50} (95% confidence limits) (h)
CITRAL	
Pronotum	
4.76	308.43[a]
9.52	109.10 (63.71-218.29)
Abdomen	
4.76	177.94 (140.95-306.72)
9.52	71.68 (38.68-97.91)
Injection	
4.76	85.53 (-22.76-180.33)
9.52	21.57 (16.63-26.13)
EUGENOL	
Pronotum	
4.76	239.10 (185.30-413.71)
9.52	56.69 (15.10-83.33)
Abdomen	
4.76	65.71 (-49.53-116.22)
9.52	19.42 (14.45-23.90)
Injection	
4.76	32.65 (28.26-37.16)
9.52	22.28 (17.37-26.86)
GERANIOL	
Pronotum	
4.76	[b]
9.52	77.16 (-15.17-138.67)
Abdomen	
4.76	151.69 (115.33-275.89)
9.52	63.57 (21.79-92.49)
Injection	
4.76	217.05[a]
9.52	46.76 (-56.08-84.88)

[a]Estimates of 95% confidence limits could not be calculated reliably due to limitations of the time range.

[b]No mortality occurred.

Comparison of application sites

It was anticipated that injection of oils would be more effective than topical application because there was a direct introduction into the body cavity without the barrier of the integument. This was indeed the case for *B. discoidalis* in terms of LC_{50} (Table 1, Page 28); the pronotum was the most resistant site, with little mortality at the doses tested. This is also clear when comparing the mean mortality charts for the three application sites (Figure 7A, C, E, Pages 31, 33, 35). Unlike the topical abdominal applications and the injections, no significant mortality was caused at any dose when the oils were applied to the pronotum.

In *P. americana*, the oils were also least effective when applied to the pronotum, with LC_{50} values beyond 72 h again higher than those for the other sites, but the pattern was less clear before this time (Table 2, Page 29). Beyond 24 h, the LC_{50} values for eugenol and citral were lowest when injected, whereas for geraniol the topical abdominal application had the lowest eventual LC_{50} value. Mean mortality showed similar patterns, with fewer deaths occurring over the dose range when applied to the pronotum; however, significant levels did occur at certain doses, e.g. 2.38mg/g eugenol ($P<0.05$)(Figure 7B, Page 32). At the highest dose, there was no significant difference in the mortality caused by topical abdominal applications and injections in either species.

Comparison of oils

In *P. americana*, eugenol was more effective at killing than the other two oils in all cases, having lowest values of LC_{50}, and generally higher mean mortality (for example, at 2.38mg/g, eugenol caused significantly more deaths than citral or geraniol when applied to the pronotum; $P<0.05$). Effects of citral were not significantly

different from those of geraniol following topical application to the pronotum or abdomen, but citral generally caused significantly higher mortality than geraniol when injected ($P<0.05$), and at 168 h its LC_{50} value was approximately half that of geraniol (3.20 compared to 6.32; Table 2, Page 29).

In *B. discoidalis*, there was more variation between the oils. For example, eugenol had the lowest LC_{50} value after 24 h when applied to the pronotum, but was equal to citral and lower than geraniol beyond 72 h. Similar variation occurred with the abdominal applications, with citral having the lowest values at 24-96 h, and eugenol having the lowest after this period. For injections, citral generally had the lowest LC_{50}.

Comparison of species

LC_{50} values tended to be higher for *P. americana* than *B. discoidalis* when expressed per unit weight, suggesting that *P. americana* is less sensitive to the oils. Expressed in absolute terms of weight, however, the LC_{50} values were similar.

Internal concentrations required to cause mortality

No significant mortality was caused when an absolute dose of less than 1mg (0.24mg/g body weight *B. discoidalis*; 0.95mg/g body weight *P. americana*) of any of the oils was injected into either species. If the maximum body fluid estimation for both species is assumed (550µl for *B. discoidalis*; 150µl for *P. americana*), the minimum concentration required to cause a significant level of mortality of any of the oils was at least 10^{-2}M. This dose is probably above the solubility of the oils in water, suggesting that the body fluids were saturated. Of course, this is a very rough estimation, but it does suggest that high doses are required to kill the insects.

Time

Generally, the LC_{50} values decreased with time (Tables 1 and 2, Pages 28 and 29), particularly between the 24 and 96 h assessment periods. In most cases the LT_{50} values followed the order: injection<abdomen<pronotum (shown for the two highest doses in Tables 3 and 4, Pages 37 and 38); however there were some exceptions (e.g. *P. americana* exposed to eugenol). The LT_{50} was always above 20 h. There was much variation in terms of which oil acted fastest.

Dye injection

On most occasions, the dye could be seen through the integument spreading within the animal to more anterior locations such as the thorax and head (Figure 8A, Page 42). Despite this, it did not always distribute evenly and tended to form 'pockets' within certain regions of the body (Figure 8B, Page 42). Leakage from the injection site (or other regions) did not occur.

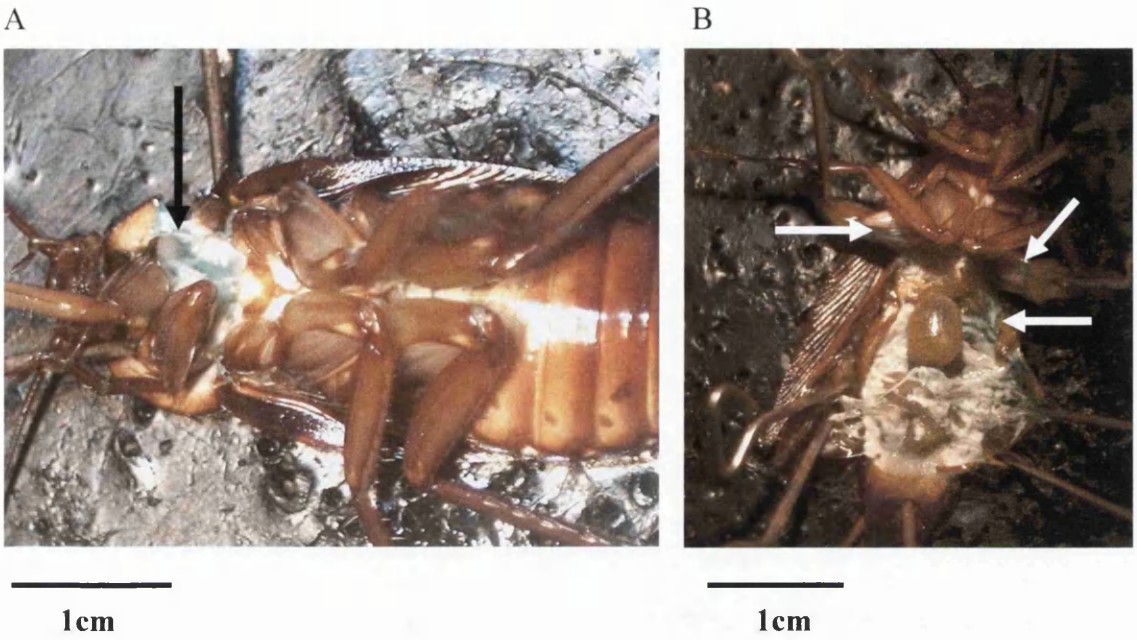


Figure 8. Images of Basilen Blue dye 1 h after it was injected into the abdomen of *P. americana*. A) The dye often spread to more anterior positions of the body such as the thorax. B) A ventral dissection of the animal revealed that the dye tended to form pockets in certain regions of the body cavity. Areas of dye accumulation are indicated by arrows.

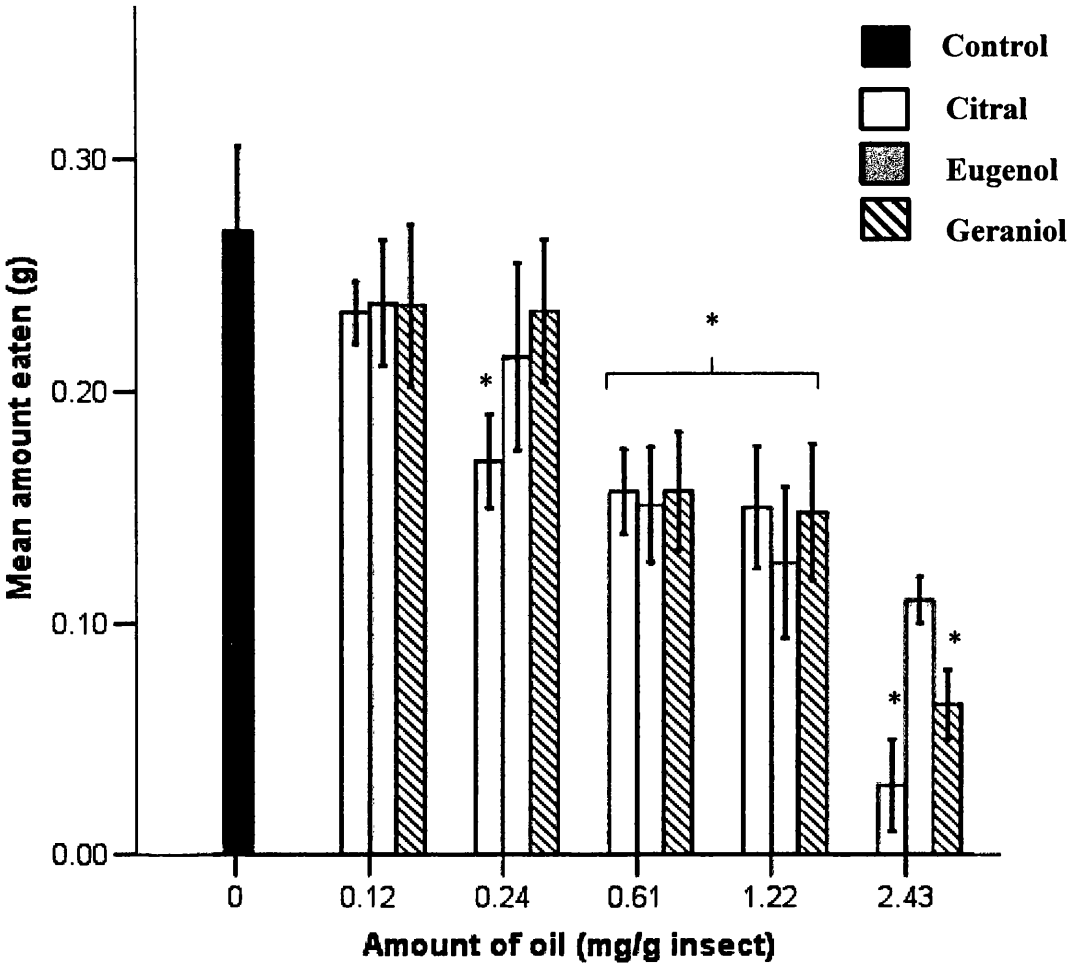
2.32 Food consumption

Figure 9 (Page 44-45) shows effects on the amount of food consumed in 168 h by *B. discoidalis* when the oils were topically applied to the abdomen. Only individuals appearing healthy at the experimental end-point were included in the analysis. This caused numbers of animals to vary between treatments (due to mortality) and the statistics for the higher doses need to be treated with caution, but it provided useful information.

When compared to the methanol control, doses of 0.24mg/g citral and above caused a significant decrease in feeding ($P<0.05$). Similar effects were seen for doses of 0.61mg/g geraniol and above; 0.61 and 1.22mg/g eugenol also significantly decreased feeding. There was no significant difference in food consumption between the untreated, methanol- or olive oil-treated insects.

Interestingly, the oils did not cause a significant decrease in food consumption at any dose when applied to the pronotum or when injected (not shown). Neither methanol nor olive oil had a significant effect on feeding when compared to untreated animals.

Figure 9. Effect of citral, eugenol and geraniol applied topically to the ventral abdominal surface, on the mean amount of food (g) consumed in 168 h by *B. discoidalis* (error bars represent SE). N values are displayed in the table below the chart; the numbers of animals varied because of some deaths. *Significantly different from methanol- treated individuals ($P<0.05$). Doses represent mg/g body weight.



N values for the bar chart above.

Amount of oil (mg/g)	N values		
	Citral	Eugenol	Geraniol
0	30	30	30
0.12	30	30	30
0.24	30	27	30
0.61	27	27	30
1.22	24	25	29
2.43	5	2	6

2.33 Repellent effects

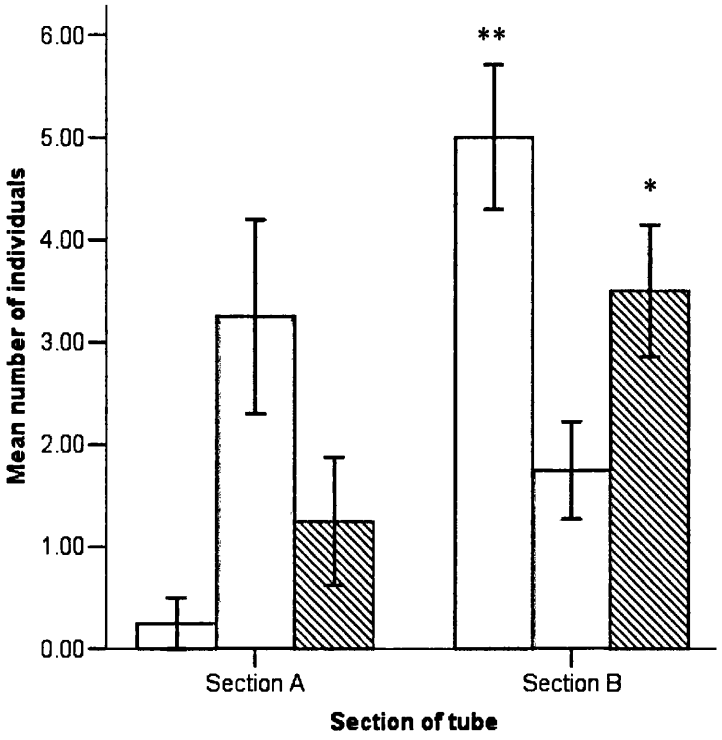
Oils were considered to be repellent if significantly fewer animals were in the half of the olfactometer containing the oil compared to the other half. The mean number of cockroaches in each half was calculated from the data recorded in each assessment period (15 min, 1, 2, 6, 12 and 24 h) for each replicate (i.e. one overall value was obtained for each replicate, incorporating all assessment periods). The mean of the five replicates was then calculated and is displayed in Figure 10 (Pages 47-50).

B. discoidalis was repelled by citral ($P<0.01$) and geraniol ($P<0.05$) at 10mg (approx. 1mg/cm^2 filter paper) (Figure 10A, Page 48), but not at 1mg (0.1mg/cm^2) (Figure 10B, Page 48). Eugenol showed no repellent effect at any of the doses. All three oils were repellent to *P. americana* at 10mg ($P<0.001$ for citral and geraniol; $P<0.01$ for eugenol) (Figure 10C, Page 49). The pattern was similar for 1mg citral and geraniol ($P<0.001$ for citral; $P<0.01$ for geraniol) but eugenol had a weaker (non-significant) effect at this dose (Figure 10D, Page 49). Interestingly, repelled individuals were normally found at the furthest point possible from the treated paper.

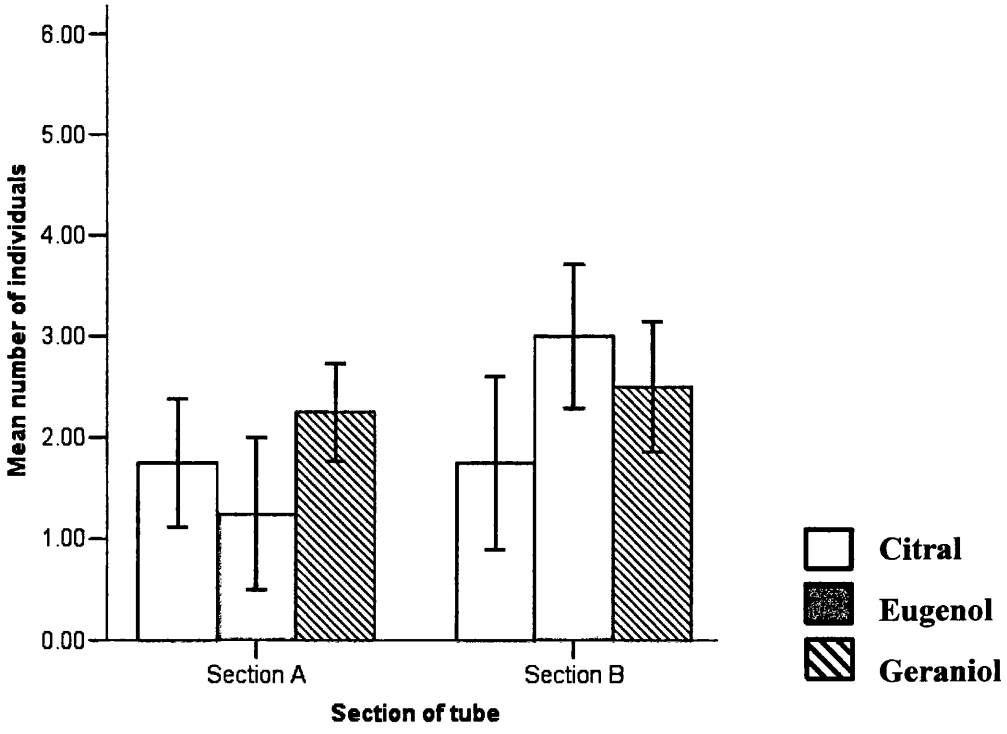
No evidence of attractant effects was seen at these or lower (0.1mg) doses. Methanol showed no repellent or attractant properties of its own when compared to an untreated piece of filter paper (Figure 10E, Page 50).

Figure 10. Repellent effects of citral, eugenol and geraniol on *P. americana* and *B. discoidalis*. The mean number of individuals was calculated from five batches of six cockroaches (i.e. 30 animals for each treatment). Section A of the olfactometer contained the oil soaked filter paper. A) *B. discoidalis* 10mg oil (1mg/cm^2 filter paper). B) *B. discoidalis* 1mg oil (0.1mg/cm^2). C) *P. americana* 10mg oil (1mg/cm^2). D) *P. americana* 1mg oil (0.1mg/cm^2). E) Solvent control (0.1ml methanol alone, in section A) for both species. *P<0.05, **P<0.01, ***P<0.001, significantly fewer animals in the section of the olfactometer containing the oil.

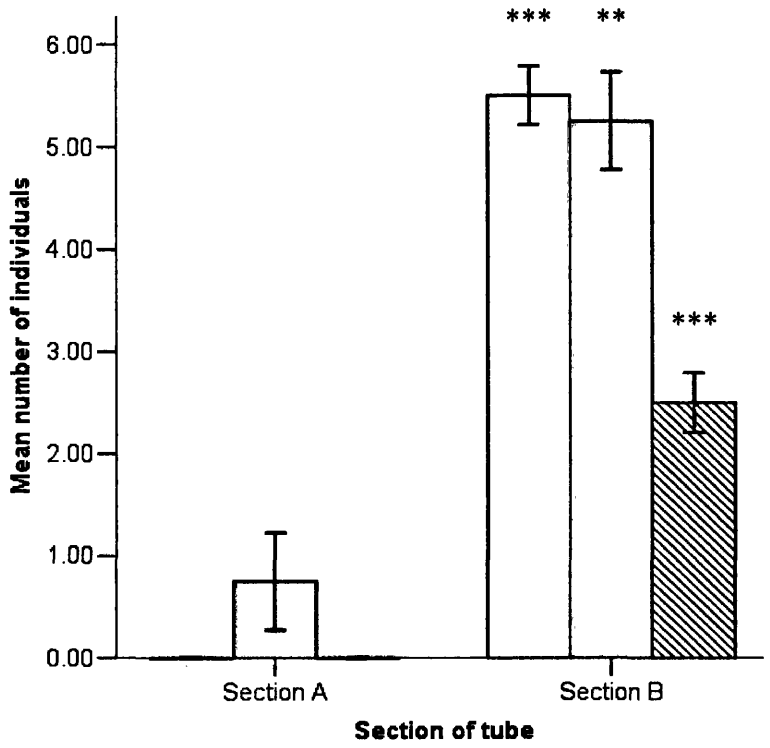
A. *B. discoidalis* 10mg oil



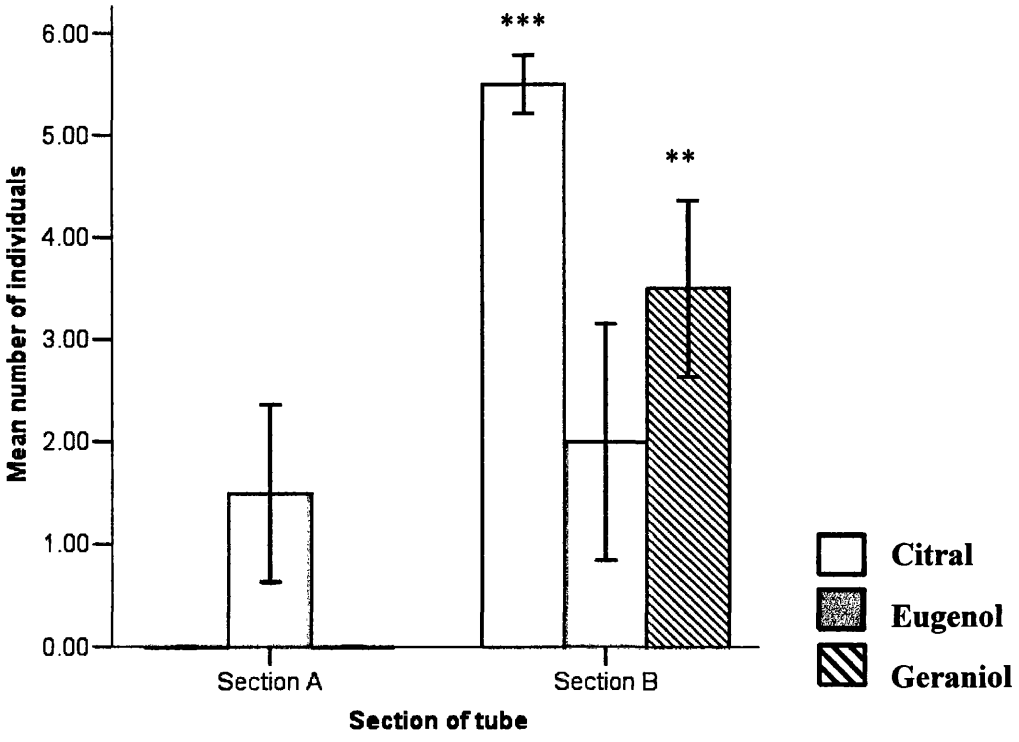
B. *B. discoidalis* 1mg oil



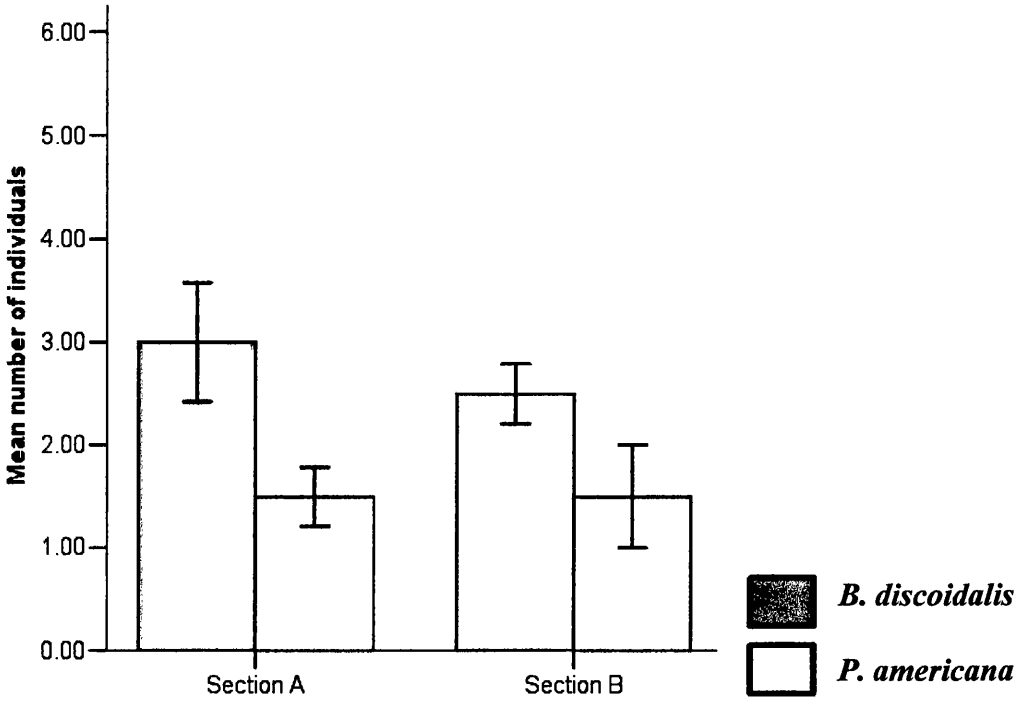
C. *P. americana* 10mg oil



D. *P. americana* 1mg oil



E. Control


6.00
5.00
4.00
3.00
2.00
1.00
0.00
Mean number of individuals
Section A
Section B
B. discoidalis
P. americana

2.34 Discussion

All three oils had insecticidal effects on both species of cockroach. Eugenol was the most effective on *P. americana* but there was more variation in *B. discoidalis*. The efficacy depended on the site of application: mortality was generally lower when the oils were topically applied to the pronotum rather than topically applied to the abdomen or injected into the animal. More oil can probably pass through the thinner integument of the abdomen compared to that of the pronotum, and the oils might have a higher probability of accessing laterally positioned spiracles and possibly enter the body *via* this route. Also, the proximity of the oil to the ventral nerve cord in abdominal applications might be a factor, since essential oils may have a neurotoxic mode of action (Coats et al, 1991; Kostyukovsky et al, 2002). The symptoms of the toxic effects observed in this study (hyperactivity and twitching) have been reported for other essential oils (e.g. Enan, 2001) and it has been suggested that they are characteristic of neuroexcitation, with mortality possibly caused by energy depletion and neuromuscular fatigue (Scharf, 2003).

At the experimental end point, the LC_{50} values were lowest for all oils when injected into *B. discoidalis* and for eugenol and citral injected into *P. americana*; surprisingly the eventual LC_{50} for the topical abdominal application of geraniol was actually lower than that for injection. There was no significant difference in mortality at the highest doses between the topical abdomen application and injection for any of the oils. This suggests that the abdominal integument provides little protection against high volumes of oils, whose lipophilic properties probably enable them to penetrate the waxy insect cuticle (Sampson et al, 2005) and enter the body cavity at relatively high concentrations. This lipophilic character has been suggested to be responsible for the broad spectrum of biological activities of essential oils (Lahlou, 2004). The

injections did not cause the highest mean mortality at lower doses of geraniol in *P. americana*. For example, at 0.95mg/g geraniol caused significantly more deaths when applied to the abdomen than when injected ($P<0.05$). This was unexpected if the oil kills by a neurotoxic action but there are several possible explanations. For example, the vapour of the oil might enhance direct contact toxicity (many essential oil constituents have fumigant toxicity; Lee et al, 2001), though the vapour trials alone caused no mortality. The oil might damage the integrity of the cuticle, perhaps causing desiccation. Furthermore, the dye injection experiments showed that injected material did not always distribute evenly, so the oils may not reach their target site at a sufficiently high concentration to exert their effects. Although the dye is physically very different from the oils, its use provided information on the possible spread of injected compounds (at least initially) and it indicated that there was no escape of injected material from the body cavity. The oils are relatively insoluble in water, so haemolymph concentrations might readily approach saturation and therefore show little variation with different routes of application, especially if their lipophilic nature enables them to penetrate the cuticle quickly. No mortality was recorded with olive oil, suggesting that it is not the physical properties of the oils that are the direct cause of mortality, though they may aid integument penetration.

Direct comparisons of LC_{50} values with previous studies such as that by Ngoh et al (1998) are difficult because of the different methods of application used (e.g. contact *via* impregnated filter paper). Huang et al (2002) however, performed direct contact toxicity tests of eugenol (applied to the thorax) on two species of stored-product beetles, *Sitophilus zeamais* and *Tribolium castaneum*. The LC_{50} was 31.0mg/g for *S. zeamais* and 30.7mg/g for *T. castaneum* after 7 days, higher than any of the LC_{50} values in the present experiments and indicating lower sensitivity to

eugenol by these species. Enan (2001) showed *P. americana* to be less sensitive to eugenol than *Blattela germanica* and *Camponotus pennsylvanicus*. The current study shows that all three oils are more effective, per unit weight, at killing *B. discoidalis* than *P. americana* (though the absolute values were similar).

The oils always took over 20 h to kill 50% of the insects even at the highest dose, and LT_{50} values showed a similar site-dependent pattern to the LC_{50}, being highest for application to the pronotum. Most mortality occurred in the first 96 h post-treatment. Prior to death, cockroaches always had a period of knockdown; recovery from this state did not occur, making it a good predictor of eventual death.

Experiments with surviving individuals of *B. discoidalis* showed that the oils (particularly citral) caused a decrease in feeding over 168 h when applied to the abdominal integument. For example, low doses of citral (0.24mg) caused no mortality or knockdown, but significantly decreased feeding, suggesting that the antifeedant effect is separate to the insecticidal effect, at least over 168 h. No oil had a significant effect on feeding when injected, possibly for some of the reasons mentioned previously. For example, vapour effects might have enhanced the efficacy of topical application even though they had no effect on their own. Huang et al (2002) also found that some essential oil constituents (e.g. eugenol) significantly reduced food consumption of *S. zeamis,* though they added the oil to the food rather than the animal. It is unlikely that reduction in feeding contributed to mortality in the current study because preliminary tests showed that *B. discoidalis* could survive for more than 168 h without food; in the longer term, however, the "antifeedant" effects may well have a role in killing. The decrease in feeding may be a result of stress (caused by oil exposure) and not a specific effect.

Eugenol, in contrast to citral and geraniol, was not repellent to *B. discoidalis* at any of the doses tested. All three oils had significant repellent effects against *P. americana*, though only citral and geraniol caused significant repellence at the lower doses. The current data contrast with Ngoh et al (1998) who found that benzene derivatives such as eugenol were more repellent to *P. americana* than other terpenes, although citral and geraniol were not tested in their study. These authors also suggested that essential oils may show a biphasic effect, acting as attractants at low doses. Low doses (0.1mg) in the current study, however, had no effect (results not shown).

All three oils have potential in pest management programmes, and have the advantage of being rapidly biodegradable (Sampson et al, 2005) and relatively safe (Isman, 2000). As mentioned earlier, one factor that may contribute to their safety is if their targeting of insect octopamine receptors at micromolar concentrations, and disruption of normal octopaminergic signalling, actually produces their insecticidal effects (Enan, 2001). This suggests that those oils (such as eugenol) that bind to octopamine receptors should kill insects at low haemolymph concentrations.

The behavioural symptoms seen in the current study (e.g. hyperactivity, tremors and feeding inhibition) appear to be similar to those for octopamine agonists, adding to the evidence that essential oils target octopamine receptors. For example, the commercially used formamidine acaricide/insecticide chlordimeform activates octopamine receptors and increases locomotor activity, and causes a loss of coordination in treated locusts (Evans and Gee, 1980). Hiripi et al (1999) showed that, in addition to toxic effects, the formamidines demethylchlordimeform and didemethylchlordimeform inhibit feeding in locusts, and they suggested that this is mediated through octopamine receptors. Furthermore, Nathanson et al (1985) showed

that some phenyliminoimidazolidines (octopamine agonists) had similar effects to formamidines on the Tobacco hornworm (*Manduca sexta*). Octopamine itself also disrupted motor and feeding activity.

Despite the similarity of acute symptoms of oil application to those of octopamine agonists, other evidence from this current study suggests that activation of octopamine receptors is unlikely to be solely responsible for the knockdown and deaths caused. The estimated body fluid concentrations that produced significant levels of mortality (10^{-2}M) were much higher than those in the biochemical receptor studies of Enan (2001; 2005b) and Kostyukovsky et al (2002) (ca. 10^{-6}M). It must be noted, however, that the estimated concentrations are probably higher than the solubility of the oils in water, possibly limiting the concentration of oil within the body fluid. Other factors that may complicate the estimation of the internal concentration of injected compounds include excretion, physiological breakdown (possible detoxification) and partitioning of the lipophilic oils into fatty tissue (injection of Basilen Blue clearly demonstrated the uneven distribution of injected material); any oil that is not in solution is more likely to pool than spread rapidly. Therefore, it may be that much smaller amounts of the compounds than calculated were actually in solution in the haemolymph. Nevertheless, the oils were injected close to the ventral nerve cord (which contains octopaminergic cells and receptors within its ganglia), and they might be expected to reach relatively high concentrations in the haemolymph or tissues in that area (it was possible to obtain concentrations in saline in excess of 2mM; see chapter 5). Even if the estimated value is inaccurate by a factor of 100, the approximate dose required to cause mortality would still be at least 100 times those suggested to affect octopamine receptors. Direct measurements are necessary to determine concentrations, especially in the nervous system, but if they

are indeed higher than micromolar, this would probably include a much larger range of effects than those on octopamine receptors that could contribute to knockdown and death (various effects on the cockroach ventral nerve cord are discussed in the next chapter). Furthermore, disruption at sites outside the nervous system may contribute to the insecticidal actions of essential oil constituents.

Chapter 3

Comparison of effects of insecticidal essential oils and octopamine on activity in the nerve cord, foregut, and dorsal unpaired median neurons of cockroaches

3.10 Introduction

The acute response of cockroaches to essential oil constituents (hyperactivity and tremors) was similar to that seen by other workers, who suggested that these symptoms signify a neurotoxic mode of action (Coats et al, 1991; Kostyukovsky et al, 2002; Scharf, 2003). Despite this, there have been hardly any studies on the neurophysiological effects of essential oil constituents in insects.

Karr et al (1990) and Coats et al (1991) performed non-invasive neurophysiological studies on the earthworm *Eisenia fetida*. Both groups found that the monoterpenoids limonene, pulegone, myrcene, α-terpineol and linalool caused a reduced conduction velocity and a block of activity in the medial and lateral giant fibres. They also showed that the amplitude of the muscle electrical response associated with the medial giant fibre spikes was decreased. The same authors made attempts to study the neurotoxic actions of the monoterpenoid *d*-limonene in the cockroach nervous system, but they had many difficulties with the volatility and poor solubility of the compound.

Szabadics and Erdélyi (2000) showed that eugenol blocked synaptic transmission and calcium currents, inhibited spike generation, decreased membrane potential, increased inward resistance and decreased the response of neurons to gamma-aminobutyric acid (GABA), ACh and glutamate in the snail *Helix pomatia*. Such neurophysiological effects of essential oil constituents do not appear to have been examined in insects.

Enan (2001) showed that low doses of eugenol (10^{-6}M) caused a significant increase in adenosine 3',5'-cyclic monophosphate (cAMP) in the nervous system of *P. americana*, a similar effect to that of 10^{-6}M octopamine. Higher doses of eugenol (10^{-5}M) caused a decrease in cAMP production. The increase in cAMP by

octopamine (10^{-4}M) was blocked by a mixture of three essential oil constituents: eugenol, α-terpineol and cinnamic alcohol (total concentration 10^{-3}M). It was also demonstrated that low doses of eugenol (2 x 10^{-6}M) significantly decreased octopamine receptor binding. Both octopamine and eugenol increased cockroach heart rate.

It was subsequently shown by Enan (2005b) that eugenol (2.5 x 10^{-5}M) mimicked octopamine (10^{-5}M) in increasing intracellular calcium levels in cloned cells from the brain of *P. americana* and *Drosophila melanogaster*, and this was also found to be mediated *via* octopamine receptors. Furthermore, the toxicity of eugenol was increased in mutant *D. melanogaster* which were deficient in octopamine synthesis, and the author suggested that this is evidence that the toxicity is mediated through the octopaminergic system (this was not the case for geraniol). It was concluded that these cellular changes induced by eugenol are responsible for its insecticidal properties. Kostyukovsky et al (2002) reached a similar conclusion, suggesting possible competitive activation of octopaminergic receptors by essential oil constituents; they found significant effects at concentrations as low as 10^{-8}M in abdominal epidermal tissue of *Helicoverpa armigera*.

3.10 Aims

Despite the apparent neurotoxic symptoms caused by various essential oil constituents in insects, there has been very little work performed to confirm this. The current study aimed to analyze the effects of essential oils on aspects of the neurophysiology of cockroaches. Also, in relation to the biochemical studies on octopamine receptors by Enan (2001; 2005b) and Kostyukovsky et al (2002), the effects of the oils were compared with those of octopamine to determine any relationship between the two in their actions on the nervous system. For example, particular attention was paid to the dorsal unpaired median (DUM) neurons in the ventral nerve cord because these are known to release and respond to octopamine (Washio and Tanaka, 1992; Achenbach, 1997; Roeder, 1999). Effects on nerve cord activity and on foregut contractions (mediated through the ingluvial ganglia) were also examined. Some of the effects of the oils were similar to those of octopamine, but the effective doses were higher than those found by Enan (2001, 2005b) and Kostyukovsky et al (2002). Interestingly, of the three oils tested, eugenol was the least like octopamine in its effects. The octopaminergic effects reported by other workers suggest a specificity of eugenol for particular sub-types of octopamine receptor.

3.20 Materials and methods

3.21 Experimental animals

P. americana were purchased from Blades Biological Supplies (UK) and *B. discoidalis* were from the departmental breeding colony. Both species were maintained as described in the previous chapter.

3.22 Chemicals

Eugenol, citral, geraniol and octopamine were purchased from Sigma-Aldrich (UK).

3.23 Experimental preparations

Extracellular electrophysiological recordings were made from the ventral nerve cord (isolated or *in situ*), and intracellular recordings were made from DUM neurons in the isolated terminal abdominal ganglion (TAG); measurements were also made of foregut contractions. In each case the preparations were bathed in physiological saline containing 160mM NaCl, 3.1mM KCl, 5mM $CaCl_2$, 4mM $MgCl_2.6H_2O$, 5mM HEPES (pH 7.2) at room temperature (18-22^{0}C).

Solubility of oils

The preparations were exposed to citral, geraniol or eugenol by dissolving the oils directly in physiological saline. Care was taken to ensure that the oils, which are relatively insoluble, were actually in solution. The chemicals were not dissolved first in an organic solvent such as ethanol because of possible effects of the solvent on the actions of the oils and the need for additional controls. It proved unnecessary because the oils appeared to dissolve completely in saline at the concentrations used in the experiments, without use of organic solvent, if they were subjected to 1 h of vigorous

stirring on a magnetic stirrer. After this time, the tiny droplets of oil on the surface of the saline did not reappear, and there was no sign of them even when left unstirred for several days in a sealed container. The fact that there were dose-related effects up to the maximum concentration used (2 x 10^{-3}M) indicated that the solutions were not saturated.

Attention was also paid to possible loss of the volatile oils through evaporation, though this actually occurs very slowly in the pure compound (for example, a 15µl drop of undiluted eugenol on a coverslip, open to the air, lost less than 15% of its volume in 24 h). The experimental solutions were in sealed vessels, without aeration, and only exposed when they were in the tissue bath, through which they flowed continuously. As a further check, solutions were left overnight in unsealed flasks and no loss of potency was found. Possible adsorption of oils by the glassware, which might have reduced the apparent concentrations, was not examined.

Extracellular electrophysiological recordings

Isolated abdominal nerve cords (Figure 11, Page 64) were placed in a Perspex experimental bath (volume 1 ml) containing physiological saline. The connectives between the 4th and 5th abdominal ganglia were drawn into a glass suction electrode and impulse activity was amplified with a home-made, differential a.c. amplifier and displayed on a storage oscilloscope (Tektronix 5111). The recordings were further displayed and stored on a computer using a micro1401 ADC interface (Cambridge Electronic Design; sampling rate 60 kHz) and subsequently analysed using Spike2 software (Cambridge Electronic Design). Another suction electrode was used to stimulate the nerve cord between the 5th and 6th abdominal ganglia using an isolated stimulator (Digitimer DS3). The preparation was superfused with physiological saline

at 3ml min^{-1} using a peristaltic pump (Gilson Minipulse). In semi-intact preparations the legs and wings were removed and the cockroach pinned dorsal surface uppermost in a 30 ml, wax-lined dish. The cuticle was cut and pinned back, and the internal organs removed to reveal the nerve cord (Figure 12, Page 65). The anal cerci were raised above the level of the saline with a piece of Perspex to keep them dry and responsive to stimulation by air currents (Figure 13, Page 66), which initiated a burst of activity in the giant fibres of the nerve cord (part of the 'escape response'). The recording electrode was again positioned between the 4^{th} and 5^{th} abdominal ganglia. Figure 14 (Pages 67-68) shows examples of spontaneous and evoked extracellular activity.

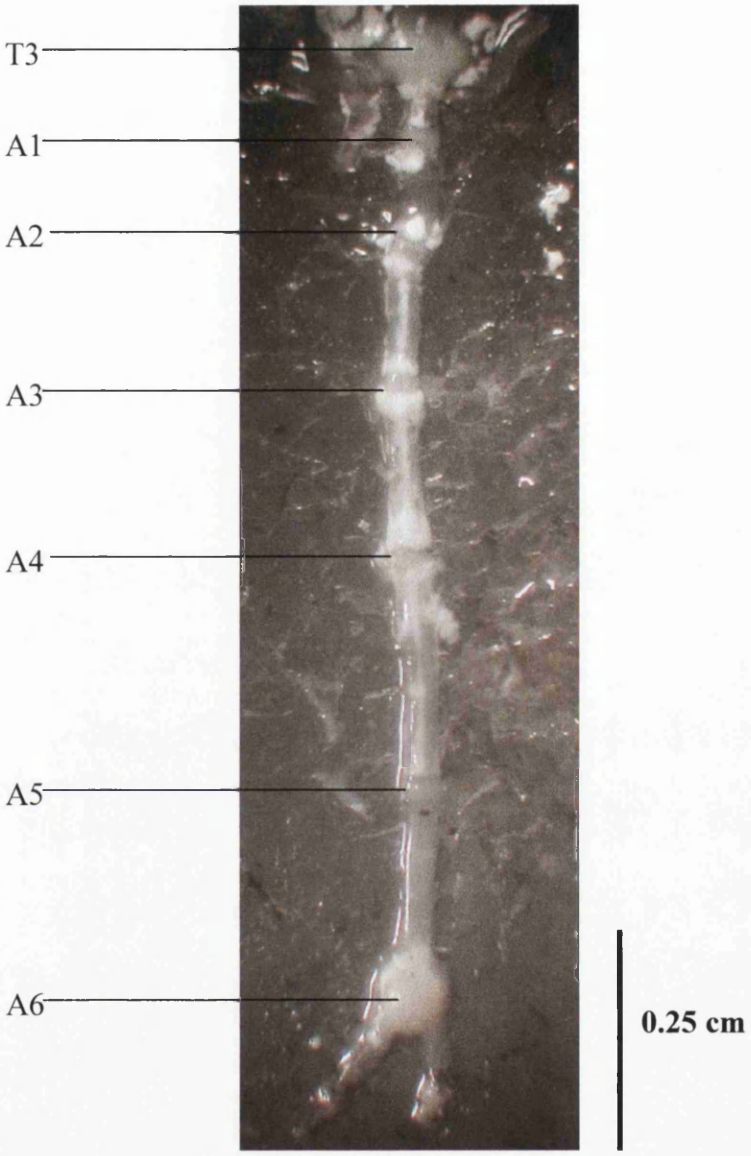


Figure 11. Dorsal view of the isolated abdominal (and part of the thoracic) portion of the ventral nerve cord of *P. americana*. T3-metathoracic ganglion; A1-A6–abdominal ganglia. A6 is the terminal abdominal ganglion (TAG) used for intracellular recordings.

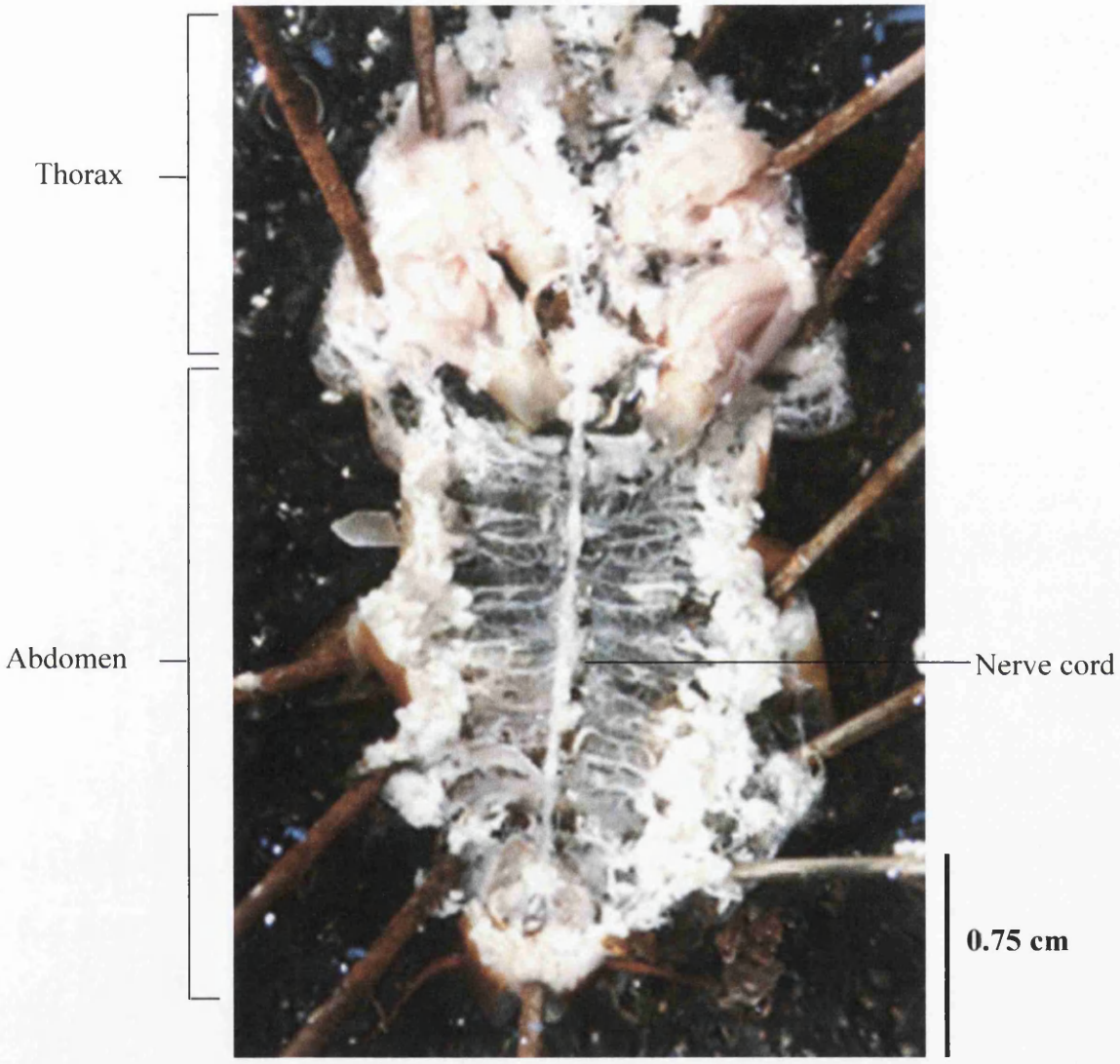


Figure 12. Dorsal view of a dissected *P. americana* showing the exposed nerve cord *in situ*.

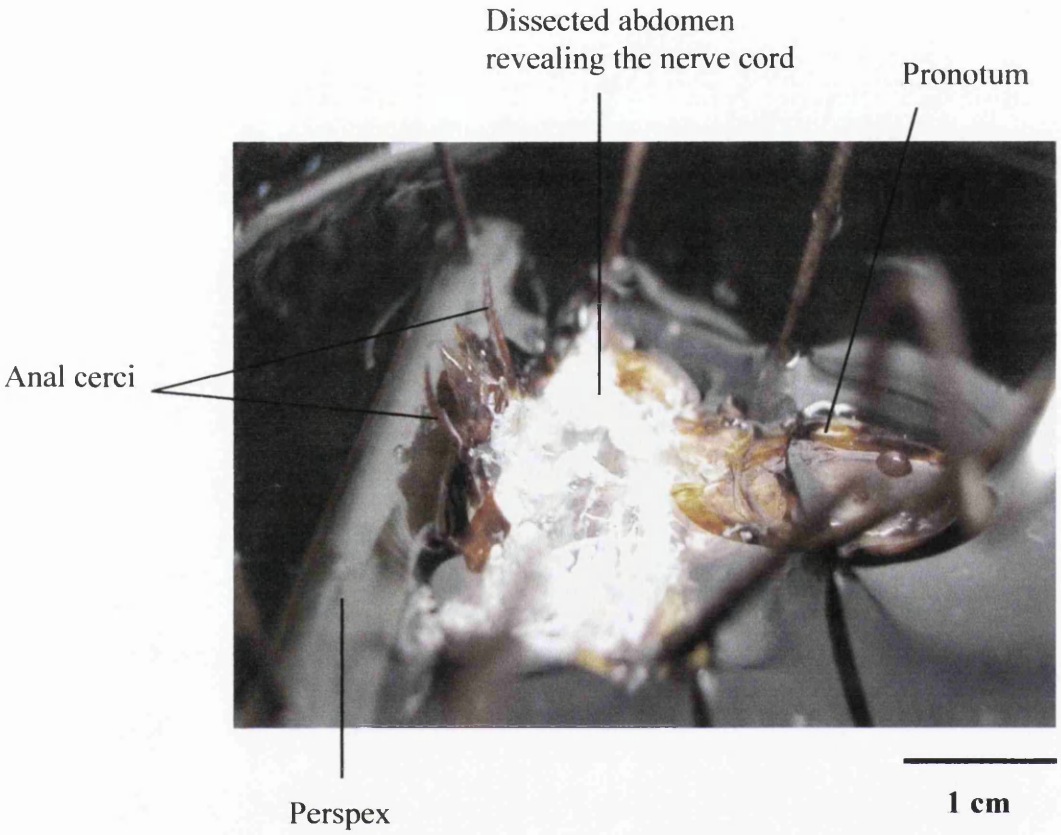
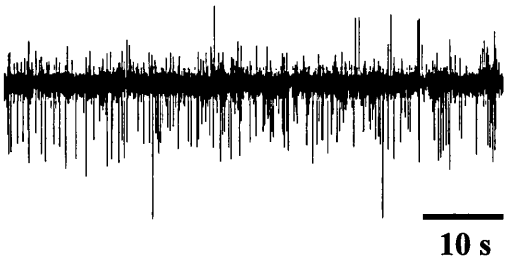


Figure 13. Semi-intact preparation of *P. americana*. The anal cerci were raised above the saline level (by Perspex) to keep them dry and responsive to stimulation by air currents.

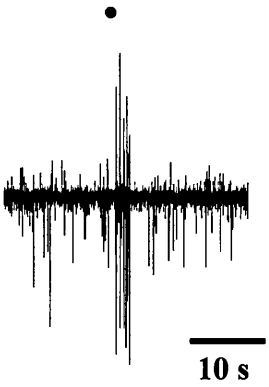
Figure 14. Examples of spontaneous and evoked extracellular activity recorded from the nerve cord of *P. americana* between abdominal ganglia 4 and 5. A) Spontaneous impulses in a semi-intact preparation. B) Spontaneous impulses in an isolated preparation. C) Response of a semi-intact preparation to a brief (ca. 2 s) air puff applied to the cerci. D) Response of an isolated preparation to a single, supramaximal 1 ms stimulus to connectives between abdominal ganglia 5 and 6. Responses indicated by dots in C and D (initial 'spike' in D is the stimulus artefact).

A. Spontaneous activity in semi-intact

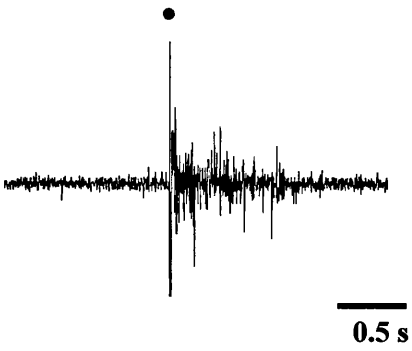


B. Spontaneous activity in isolated

C. Response to cercal stimulation



D. Response to electrical stimulation



Intracellular recordings

Recordings were made from cell bodies on the dorsal surface of the TAG of *P. americana*. Recordings were also made from cells on the dorsal surface of the metathoracic ganglion (T3), but in agreement with Crossman et al (1971), these cells were more difficult to penetrate despite their larger size, and so were not used in the experiments. Recordings were made from DUM neurons as these are octopaminergic (Evans and O'Shea, 1977; Orchard and Lange, 1985; Stephenson and Spörhase-Eichmann, 1995) and respond to octopamine in a dose-dependent manner (Washio and Tanaka, 1992; Achenbach, 1997). The cells were identified by their large somata (30-60μm) and location in the posterior midline area of the ganglion.

The TAG was removed and pinned with its dorsal surface uppermost to the Sylgard base of a Perspex experimental bath (volume 1ml) (Figure 15, Page 71). The ganglion was de-sheathed with electrolytically-sharpened tungsten needles and fine forceps to allow recording of the DUM neurons on its surface. Neutral Red (500μg/ml) was used in some of the earlier experiments to facilitate removal of the sheath and visualisation of neurons (Washio and Tanaka, 1992). As soon as light staining had occurred the dye was washed from the preparation, and almost all colour had disappeared from the ganglion before starting experiments. Although an effect of Neutral Red has been noted on DUM neurons (small reduction in spike amplitude; Lundquist and Nässel, 1997), no effect on recordings was found in the current study. Although Neutral Red is taken up by DUM cells, it is probably non-specific (Dymond and Evans, 1979; Evans, 1985).

Conventional intracellular recording and stimulating techniques were used. Individual DUM neurons were impaled with a glass microelectrode containing 3M KCl (resistance 30-35 MΩ) which was connected to an Axoclamp 2A amplifier

(Axon Instruments) *via* a chlorided silver wire. The recording electrode was also used for current injection. Recordings were displayed on a storage oscilloscope (Tektronix 5111) and connection to a loudspeaker was used to facilitate penetration. The intracellular recordings were displayed and stored on a computer using the same software as in the extracellular experiments. The preparation was superfused with physiological saline at 3ml min^{-1} using a peristaltic pump. The cell was left to stabilise for 30 min after impalement and was considered suitable for experimentation if spikes were generally above 25mV after this period. Figure 16 (Page 72) shows examples of spontaneous and evoked action potentials recorded from the TAG.

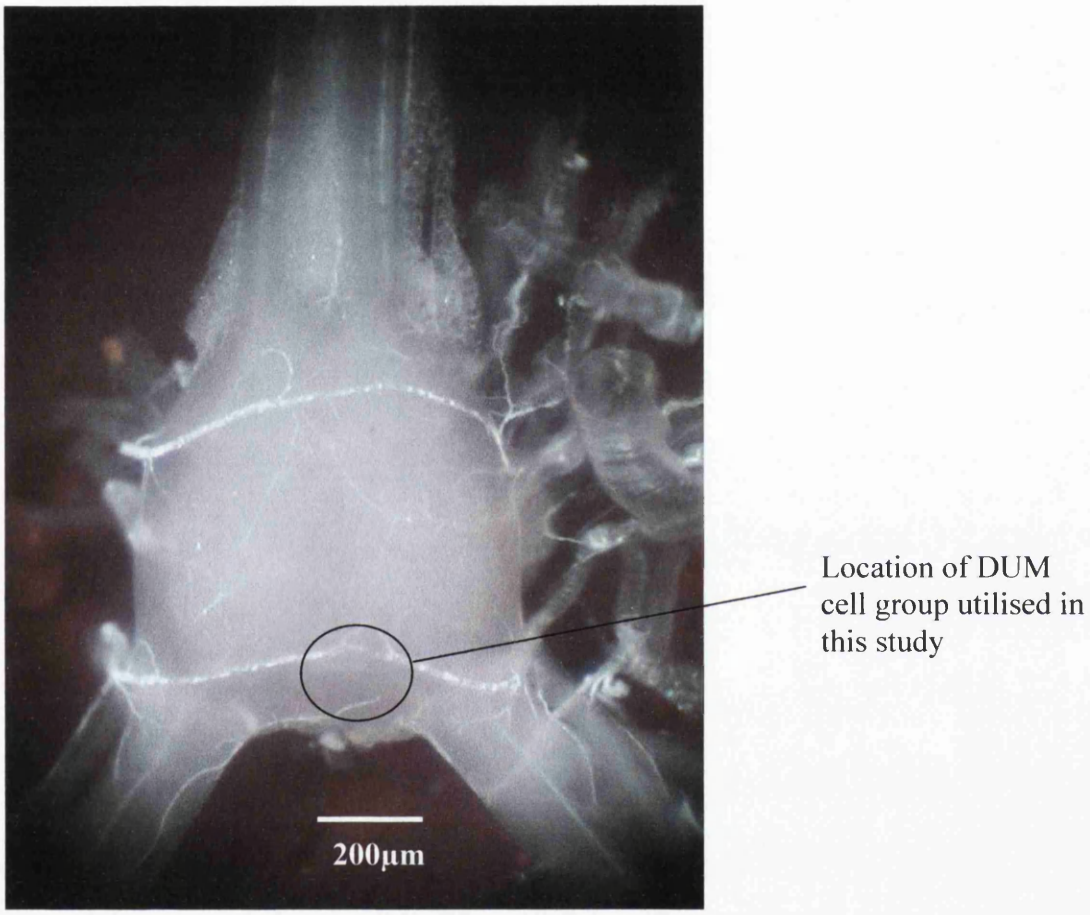
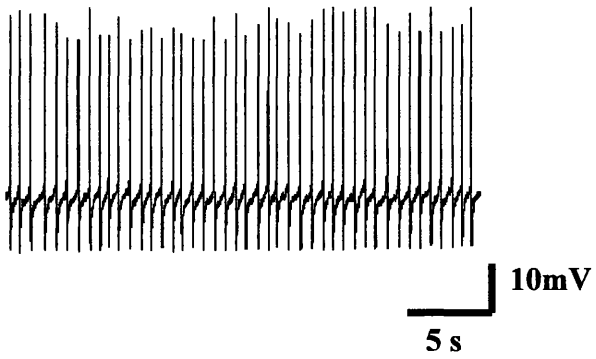


Figure 15. An isolated terminal abdominal ganglion (TAG) from *P. americana* before desheathing. Recordings were restricted to cells in the posterior midline of the ganglion in an attempt to minimise variation.

A. Spontaneous activity



B. Response to depolarizing current

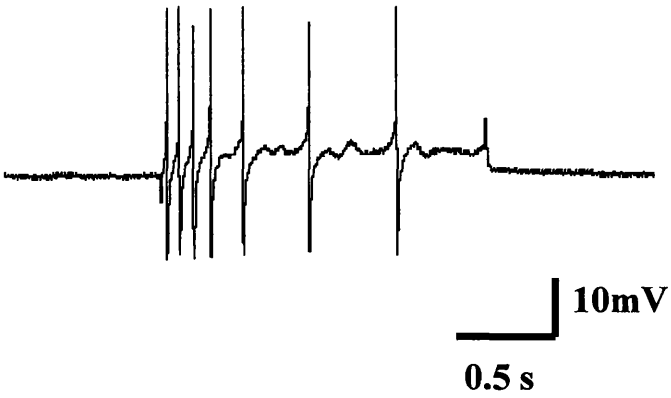


Figure 16. Examples of action potentials recorded from DUM neurons of the isolated TAG of *P. americana*. A) Spontaneous impulses. B) Impulses evoked by injection of depolarizing current.

Measurement of foregut contractions

The foregut was isolated and separate pieces of thread were tied to each end. It was then suspended vertically in a glass flask (85 ml) by anchoring it posteriorly to a weight at the base of the flask and tying it anteriorly to a transducer (SRI) (Figure 17, Page 75) to record isotonic contractions, which were monitored on a pen recorder (Brush 2200S). Figure 18A (Page 76) shows typical spontaneous activity recorded in these experiments. The preparation was maintained in saline and the oils were added (dissolved in saline at their final concentration) from the bottom of the flask and overflowed at the top. Use of dyes in separate experiments showed that almost full substitution occurred within 1 min. This procedure in itself did not affect spontaneous contractions; initial attempts to remove some or all of the saline and then replace it with saline containing an essential oil showed that foregut contractions could change irrespective of the presence of the oil, and therefore this method was not used. Most experiments were performed on *B. discoidalis*, and the foregut tension was adjusted to about 7.5×10^{-3}N.

In addition to spontaneous movements, evoked contractions (Figure 18B, Page 76) were recorded in response to stimulation *via* two stainless steel flank electrodes that were positioned on either side of the foregut without touching it. Stimuli were provided by a constant-current stimulator (Digitimer DS7) controlled by a Master-8 pulse generator (A.M.P.I.). Generally, 1 ms pulses were applied at 75 Hz for 2 s.

3.24 Statistics

Where effects on spike frequency were studied, the results are expressed as mean number of spikes per second ± standard error. Significance of effects is evaluated by two-tailed Student's *t* tests.

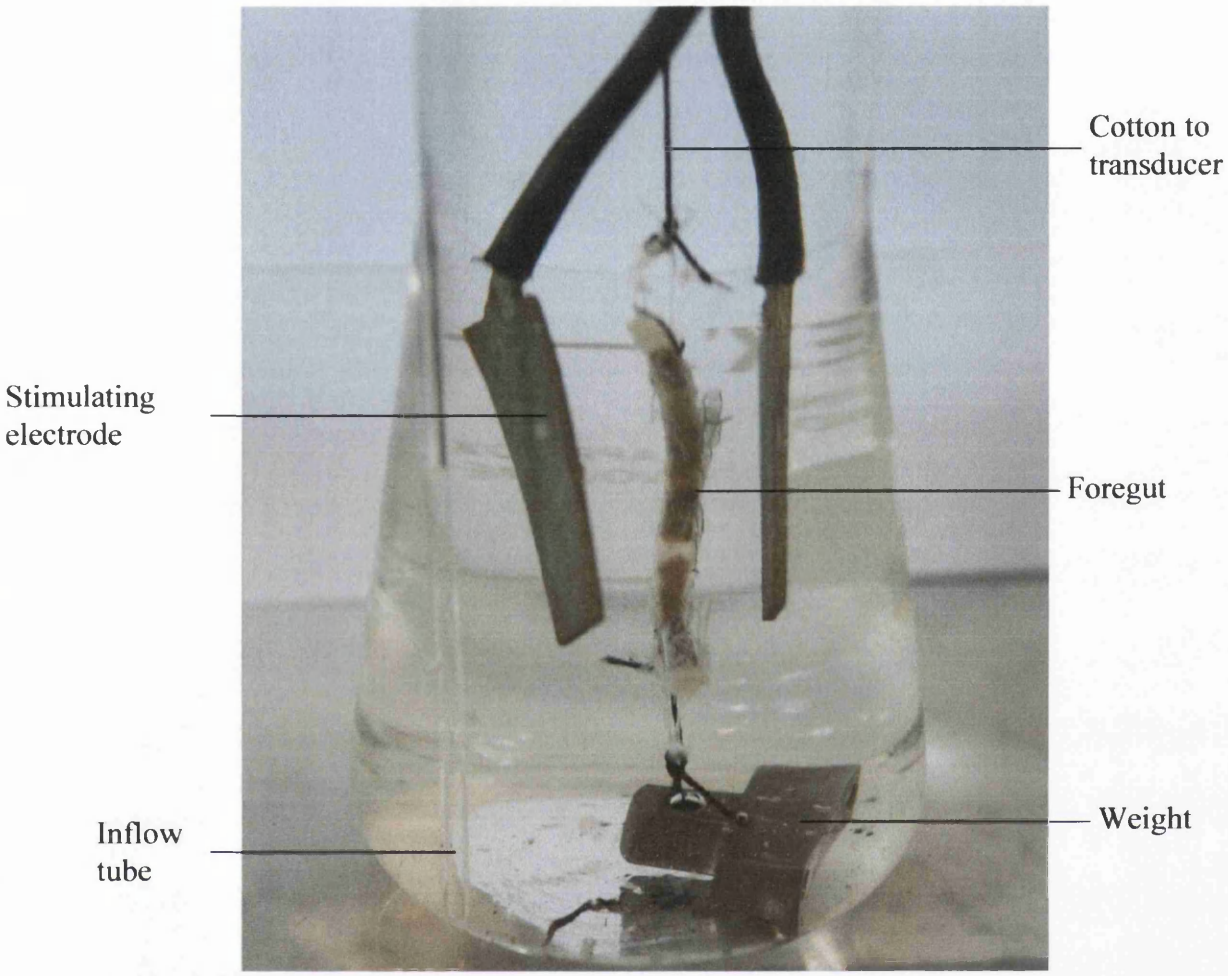
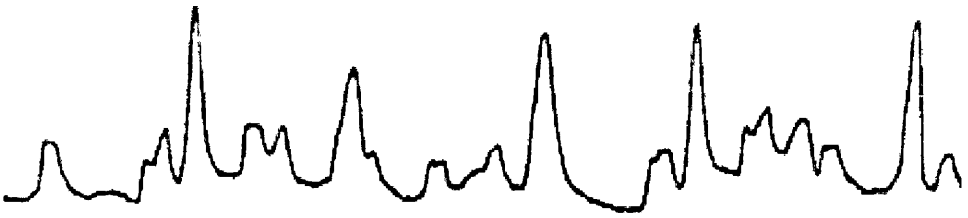


Figure 17. Experimental setup for recording isotonic contractions of the cockroach foregut (*B. discoidalis*, in this case).

A. Spontaneous activity



B. Response to stimulation

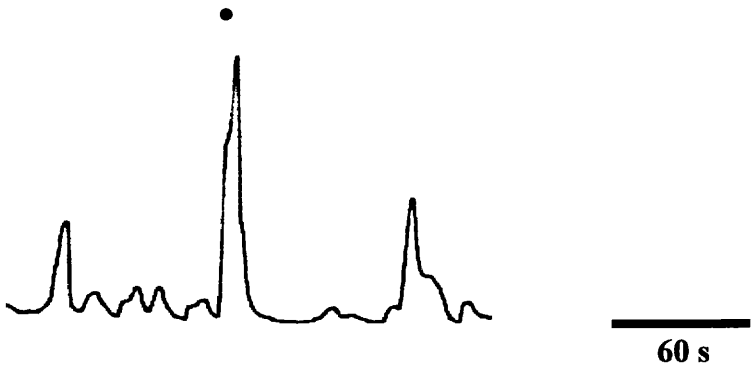


Figure 18. Examples of isotonic foregut contractions of *B. discoidalis*. A) Spontaneous activity. B) Response to flank stimulation (1 ms stimuli at 75Hz for 2 s). The response is indicated by the dot; the stimuli were sub-maximal to avoid loss of part of the evoked contraction off scale.

3.30 Results

3.31 Effects of essential oils on electrical activity recorded extracellularly from the ventral nerve cord

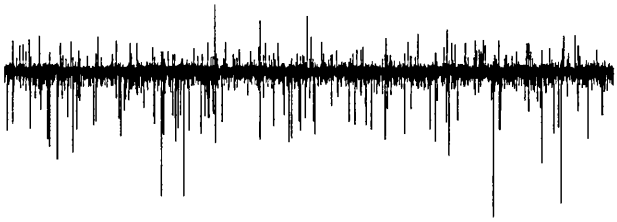
Effects of eugenol

Spontaneous impulse activity, recorded from the connectives between abdominal ganglia 4 and 5 in semi-intact and isolated preparations, was decreased in amplitude and frequency by eugenol in both species of cockroach and both types of preparation (shown for a semi-intact preparation of *B. discoidalis* in Figure 19, Pages 78-79). The threshold dose was around 5 x 10^{-4}M, and a significant decrease in spike frequency occurred at 10^{-3}M in isolated preparations and 2 x 10^{-3}M in semi-intact preparations ($P<0.05$) (Figure 20, Pages 80-81). Similar depressant effects were found for: (i) responses to cercal stimulation by a brief air current applied from a Pasteur pipette (Figure 21, Page 82), i.e. a burst of large spikes that were progressively reduced in amplitude and frequency with increasing concentration, and abolished at approximately 10^{-3}M (N=5), and (ii) responses to a single, supramaximal 1 ms stimulus applied to the connectives between abdominal ganglia 5 and 6 (Figure 22, Page 83), i.e. a train of spikes lasting about 0.5 s that showed a decrease in spike amplitude and frequency, and a reduced duration of train (N=5). All effects were dose-dependent and reversed with a saline wash.

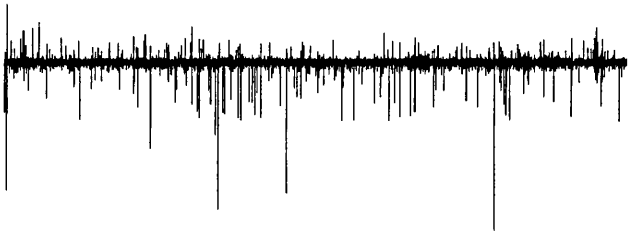
Figure 19. Eugenol produces a dose-dependent reduction in amplitude and frequency of spontaneous impulses in the ventral nerve cord of *B. discoidalis* between abdominal ganglia 4 and 5 (semi-intact preparation). A) Control. B) 5 x 10^{-4}M eugenol. C) 10^{-3}M eugenol. D) 2 x 10^{-3}M eugenol. Recordings B-D were made after 10 min exposure to the oil. Partial recovery occurred after a 10 min washout of eugenol (E).

A. Control

B. 5 x 10^{-4}M eugenol



C. 10^{-3}M eugenol

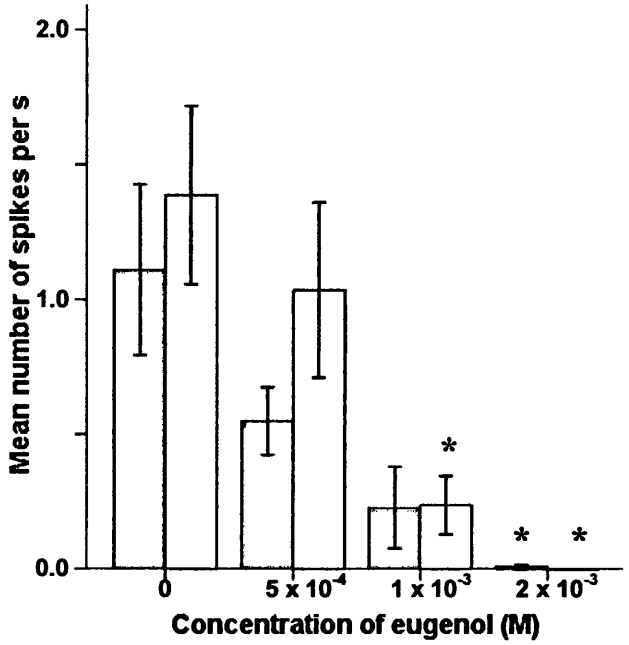


D. 2 x 10^{-3}M eugenol

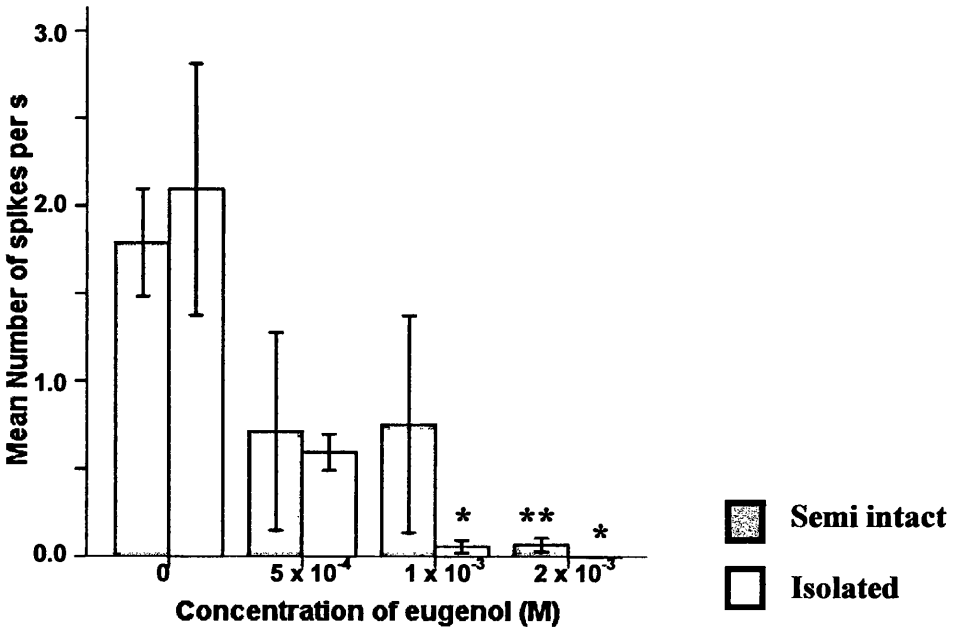
E. Partial recovery

Figure 20. Bar charts showing effects of eugenol on mean (±SE) spontaneous impulse frequency in the ventral nerve cord for isolated and semi-intact preparations from *B. discoidalis* (A) and *P. americana* (B) respectively (N = 5 for each preparation). *Significantly different from control, $P<0.05$, **$P<0.01$. All data was recorded 600 s after oil application.

A. *B. discoidalis* mean spike frequency



B. *P. americana* mean spike frequency



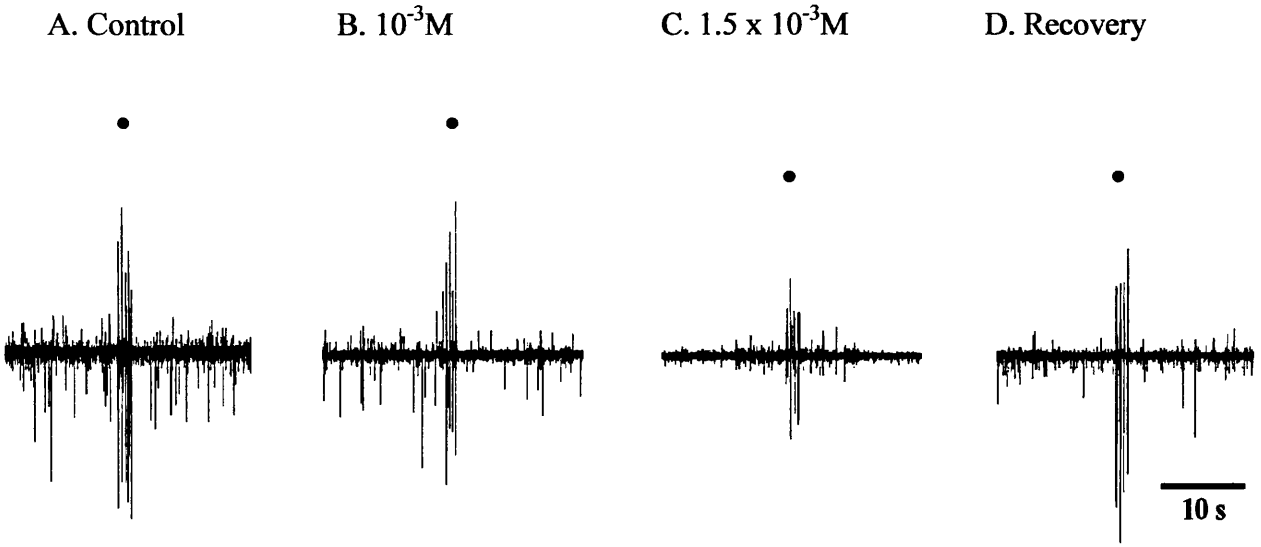


Figure 21. Effect of eugenol on the cercal blow response (indicated by dots) in a semi-intact preparation of *B. discoidalis*. Recordings were made from the abdominal nerve cord between ganglia 4 and 5, and stimuli were puffs of air (ca. 2 s) from a Pasteur pipette. A) Control. B) 10^{-3}M eugenol. C) 1.5 x 10^{-3}M eugenol. D) After 10 min saline wash. Recordings B and C were made after 10 min exposure to the oil.

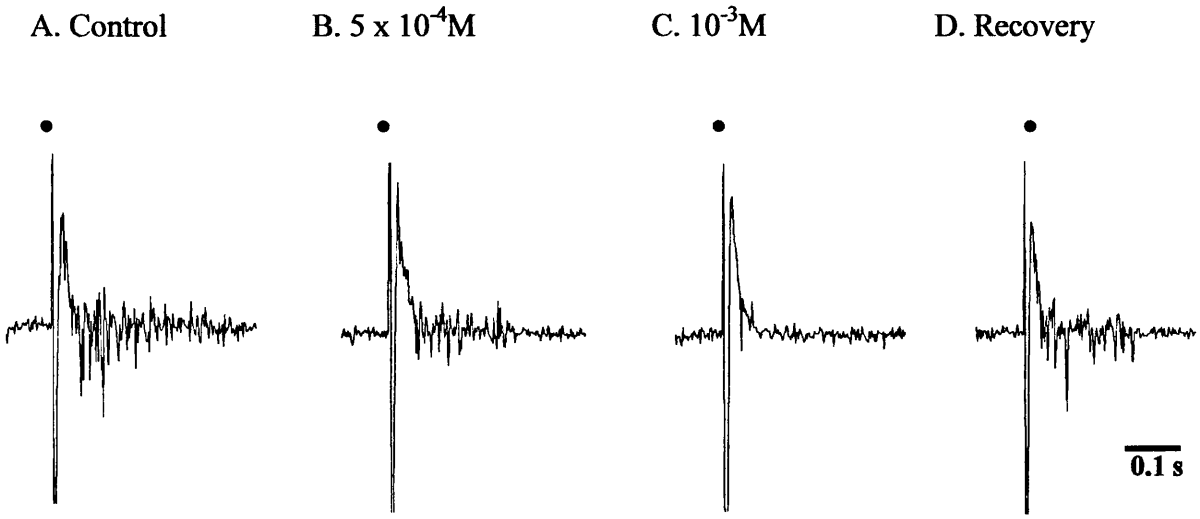


Figure 22. Effect of eugenol on the response of the isolated ventral nerve cord of *B. discoidalis* to a single, supramaximal 1 ms stimulus. The recording electrode was between abdominal ganglia 4 and 5, and the stimulating electrode between ganglia 5 and 6. Dots above the recordings indicate the stimulus artefact. A. Control. B. 5 x 10^{-4}M eugenol. C. 10^{-3}M eugenol. D. After 10 min saline wash. Recordings B and C were made after 10 min exposure to the oil.

Effect of geraniol and citral

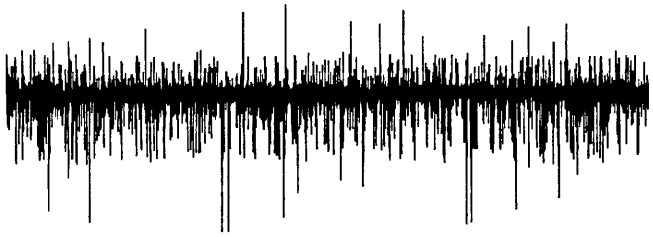
In contrast to eugenol, geraniol produced a biphasic effect in *P. americana*, with lower doses (ca. 2.5 x 10^{-4}M) significantly increasing spontaneous activity ($P<0.01$ for semi-intact preparations; $P<0.05$ for isolated preparations) and higher doses (ca. 2 x 10^{-3}M) causing a significant decrease in spike frequency ($P<0.05$) (Figures 23 and 24, Pages 85-88). A similar biphasic effect was seen in the isolated nerve cord of *B. discoidalis*, with 10^{-3}M causing a significant increase in spike frequency ($P<0.05$) (Figure 24B, Pages 87-88). Only two out of five semi-intact preparations of *B. discoidalis* showed this biphasic response.

Citral showed a similar trend to geraniol in both species, but with a weaker excitatory effect (N=5; not shown). Octopamine (10^{-6} to 10^{-2}M) had no effect on spike frequency in either preparation.

Figure 23. Geraniol produces a biphasic dose effect on spontaneous impulse activity in the ventral nerve cord of *P. americana*, recorded between abdominal ganglia 4 and 5. A) Control. B) 2.5 x 10^{-4}M geraniol. C) After 10 min wash. D) 2 x 10^{-3}M geraniol. E) After 10 min wash. Recordings in the presence of the oil were made 10 min after exposure to each dose.

A. Control

B. 2.5 x 10^{-4}M geraniol



C. 10 min saline wash

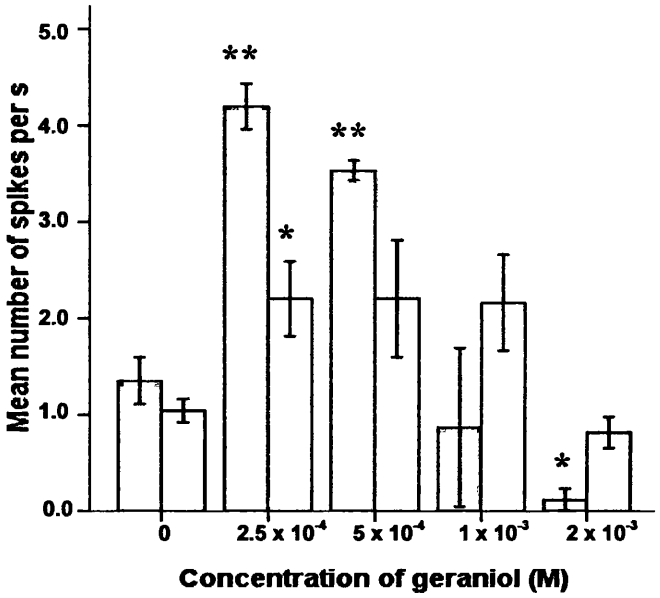
D. 2 x 10^{-3}M geraniol

E. 10 min saline wash

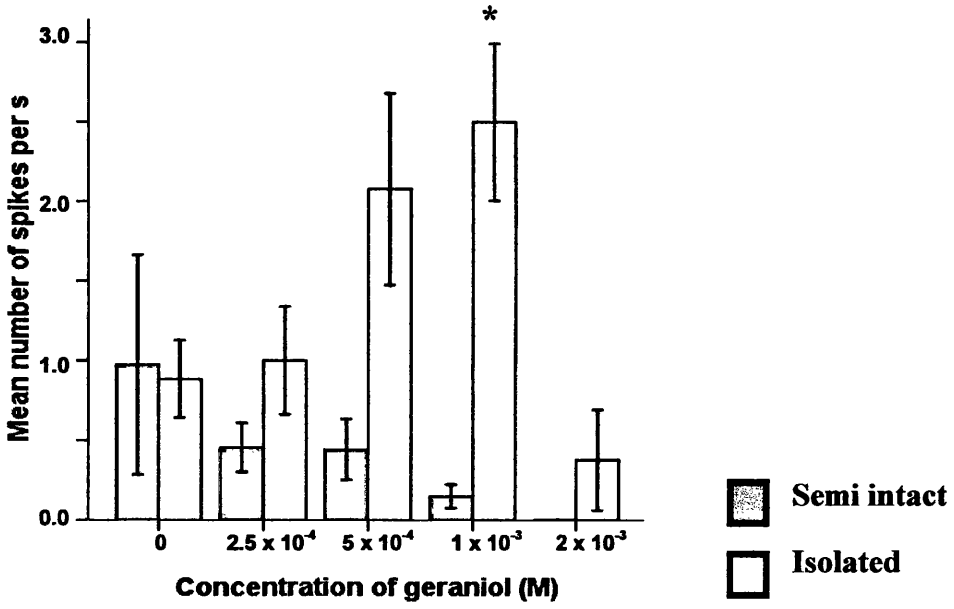
10 s

Figure 24. Bar charts showing effect of geraniol on mean (±SE) spontaneous impulse frequency in the ventral nerve cord in isolated and semi-intact preparations from *P. americana* (A) and *B. discoidalis* (B) respectively (N = 5 for each preparation). *Significantly different from control, $P<0.05$; **$P<0.01$. All data was recorded 10 min after application of oil.

A. *P. americana* mean spike frequency



B. *B. discoidalis* mean spike frequency



3.32 Effects of essential oil constituents and octopamine on DUM neurons recorded intracellularly in the terminal abdominal ganglion of *P. americana*

The TAG contains many DUM neurons, but the recordings described here were restricted to cells in the posterior midline section of the ganglion. The effects of the oils on spontaneous firing in the ventral nerve cord were mirrored in effects on DUM neurons.

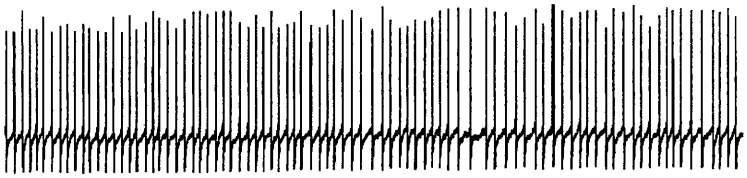
Effects of eugenol on DUM neurons

Figure 25 (Pages 90-91) shows a typical effect of eugenol on a spontaneously firing DUM neuron. There was a significant decrease in spike frequency at 10^{-3}M ($P<0.05$; Figure 26, Page 92) and a progressive decline in amplitude and undershoot (Figure 27, Page 93). Effects were at least partially reversible after a saline wash (Figure 25E, Pages 90-91).

A further effect of eugenol occurred during depolarization of silent DUM neurons *via* current applied through the recording microelectrode. Figure 28 A,B (Pages 94-95) shows that spike frequency during applied depolarization decreased in the presence of 5 x 10^{-4}M eugenol. At 10^{-3}M, however, the firing pattern changed from regular firing to bursts driven by plateau potentials (Figure 28 C,D, Pages 94-95). Recovery of original firing occurred after washing (Figure 28E, Pages 94-95).

Figure 25. Eugenol reduces impulse frequency, spike amplitude and undershoot in spontaneously firing DUM neurons recorded intracellularly from the terminal abdominal ganglion of *P. americana*. A-E show an example of recordings from a single neuron. A) Control. B) 10^{-4}M eugenol. C) 5 x 10^{-4}M eugenol. D) 10^{-3}M eugenol. Recordings in the presence of the drug were made after 10 min exposure. E) After 10 min saline wash.

A. Control

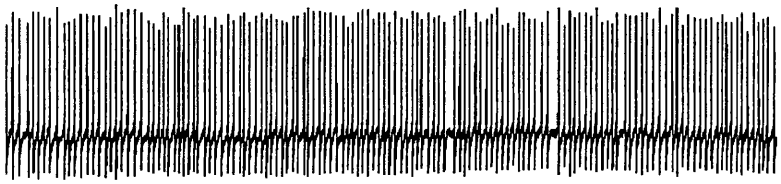


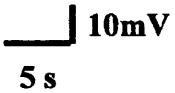
B. 10^{-4}M eugenol

C. 5 x 10^{-4} M eugenol

D. 10^{-3}M eugenol

E. 10 min wash





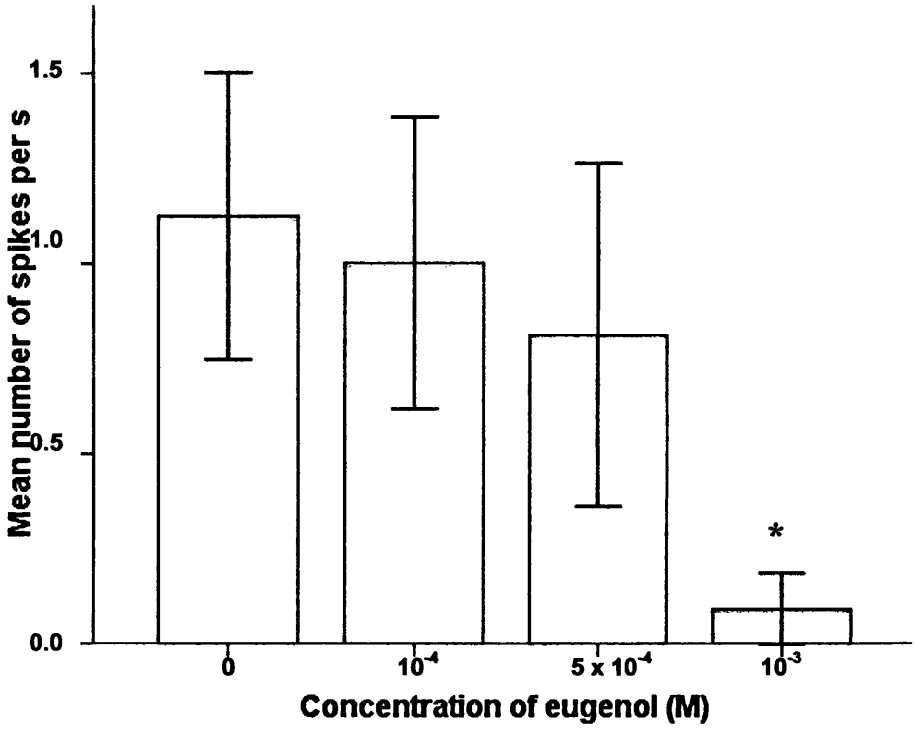


Figure 26. Bar chart showing effects of eugenol on mean (±SE) spontaneous impulse frequency of DUM neurons in the TAG (N = 5). *Significantly different from control, $P<0.05$. Data was recorded after 10 min exposure to the oil.

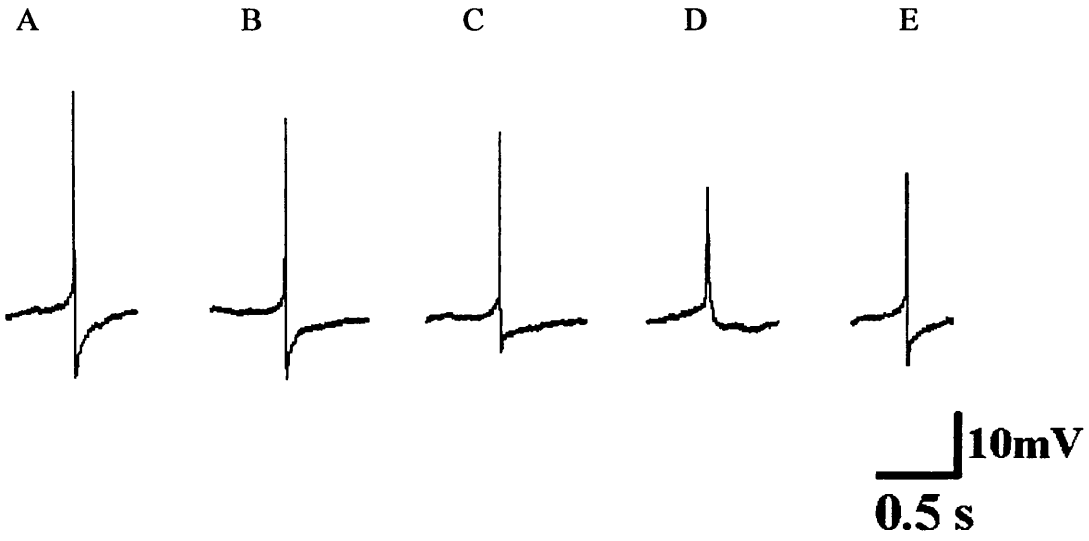
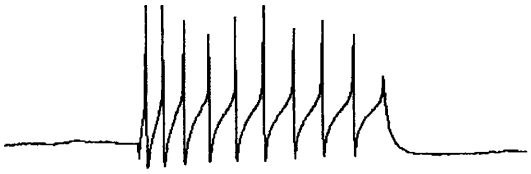


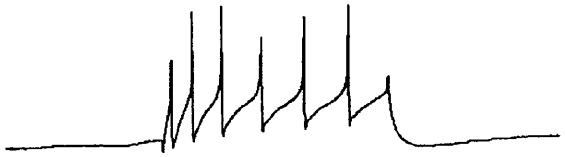
Figure 27. Effect of eugenol on spike undershoot. Spontaneously occurring impulses were recorded from the same DUM neuron after exposure to increasing concentrations of eugenol. A) Control. B) 10^{-4}M eugenol. C) 5 x 10^{-4}M eugenol. D) 10^{-3}M eugenol. E) After 10 min saline wash. Recordings in the presence of the oil were made after 10 min exposure.

Figure 28. Eugenol induces burst firing in a depolarized DUM neuron recorded intracellularly from the terminal abdominal ganglion of *P. americana.* Depolarizing current pulses (ca. 1 s), applied through the recording microelectrode, were the same amplitude in each case. A) Control. B) 5 x 10^{-4}M eugenol; 200 s exposure. C,D) 10^{-3}M eugenol; exposure for 100 s and 200 s respectively. E) After 200 s wash. Note the production of plateau potentials and burst firing in 10^{-3}M eugenol.

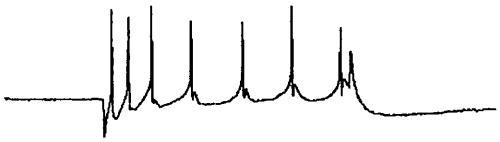
A. Control



B. 200 s after 5×10^{-4}M eugenol

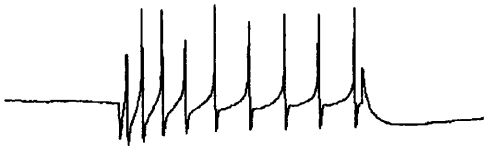


C. 100 s after 10^{-3}M eugenol



D. 200 s after 10^{-3}M eugenol

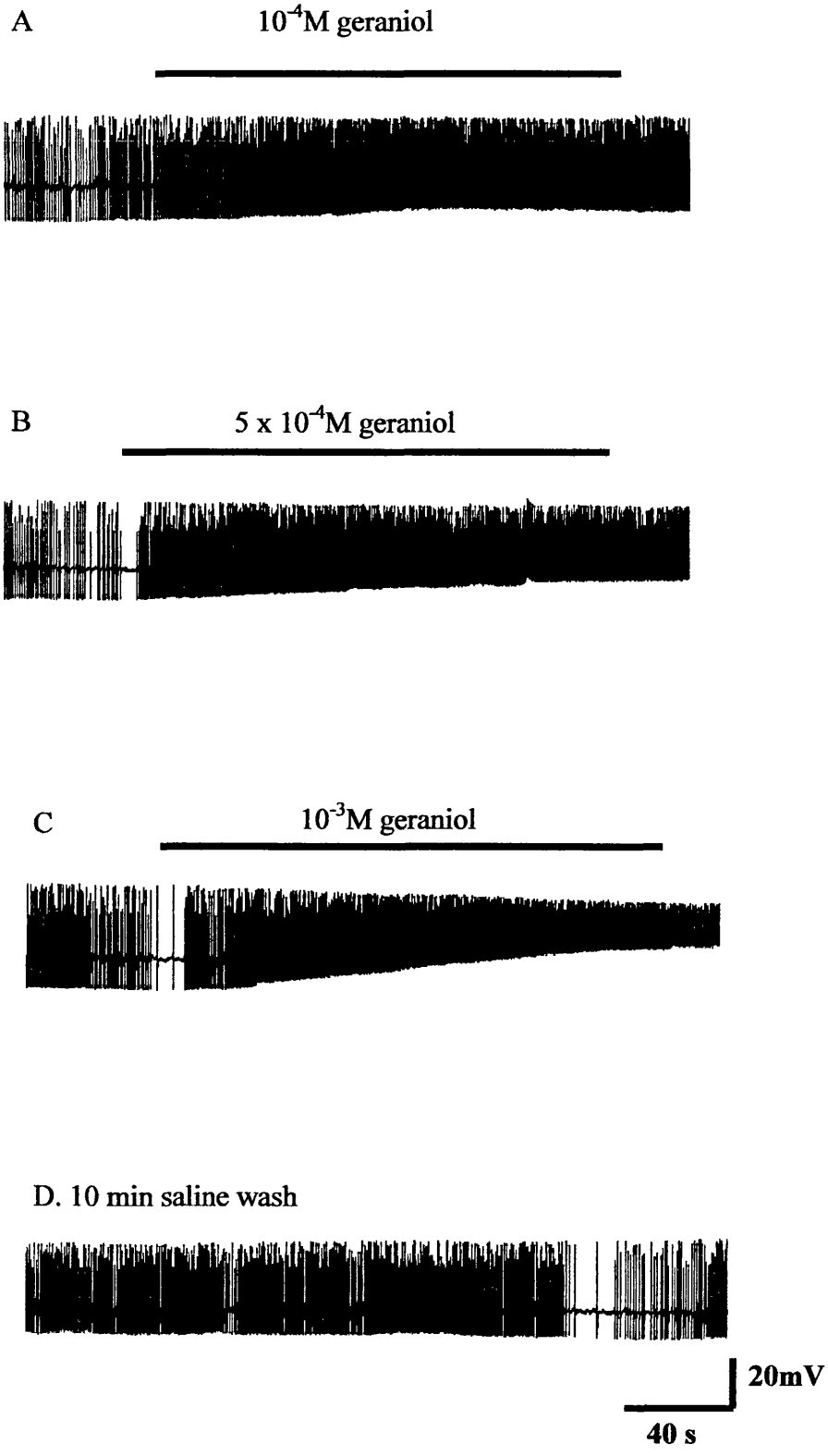
E. 200 s wash



Biphasic action of geraniol on spontaneously firing DUM neurons

Geraniol had no stimulatory effect on silent DUM neurons (N=5) but it depolarized and increased the firing rate in spontaneously active neurons at relatively low doses (Figure 29, Pages 97-98). The threshold for this increase was ca. 5×10^{-5}M, with 10^{-4}M causing a significant increase in spike frequency ($P<0.05$) (Figure 30, Page 99). Spike amplitude and undershoot were considerably reduced by 10^{-3}M (Figure 31, Page 100), and at 2×10^{-3}M the effect on spike frequency was reversed (frequency decreased; $P<0.05$). As with the extracellularly recorded activity in the nerve cord, citral showed a similar pattern to geraniol, but with a weaker excitatory effect (N=5, not shown). In silent DUM neurons, geraniol and citral decreased the excitability of cells that were depolarized by current applied through the recording microelectrode (threshold ca. 10^{-4}M; Figure 32, Pages 101-102), but no plateau potentials were produced.

Figure 29. Geraniol has an excitatory effect on spontaneously active DUM neurons recorded intracellularly from the terminal abdominal ganglion of *P. americana* A-C) show an example of excitatory effects on a single DUM neuron. Period of application is indicated by the line above the recordings. A) 10^{-4}M geraniol. B) 5 x 10^{-4}M geraniol. C) 10^{-3}M geraniol increases spike frequency, but decreases amplitude and undershoot. Recordings B and C were made after a 10 min wash to allow recovery from the previous dose. D) Recovery after 10 min saline wash.


A
10^{-4}M geraniol
B
5 x 10^{-4}M geraniol
C
10^{-3}M geraniol
D. 10 min saline wash
20mV
40 s

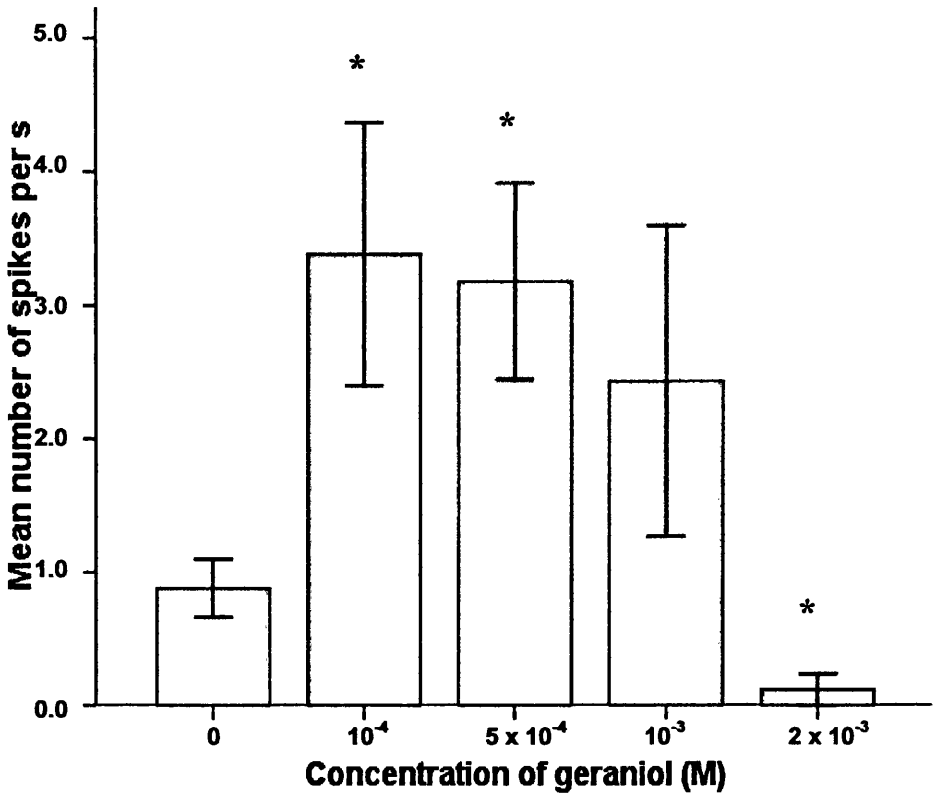


Figure 30. Bar chart showing biphasic effects of geraniol on mean (±SE) spontaneous firing frequency of DUM neurons (N = 5). *Significantly different from control, $P<0.05$. Data was recorded 10 min after exposure to the oil.

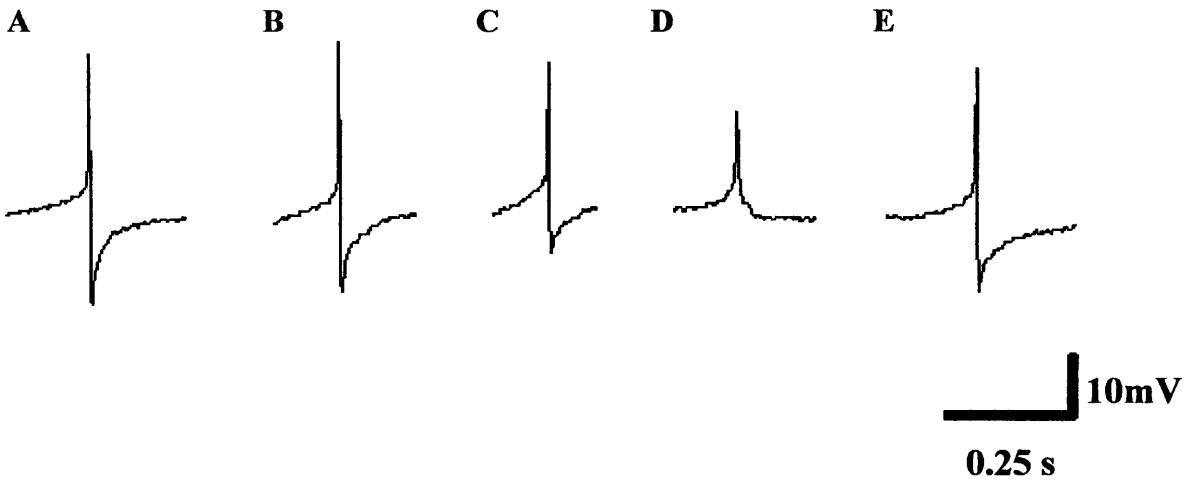
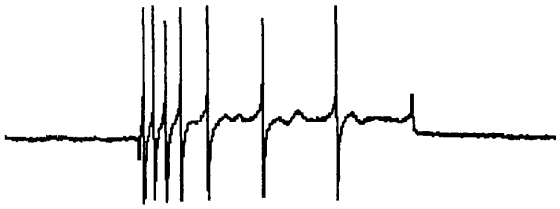


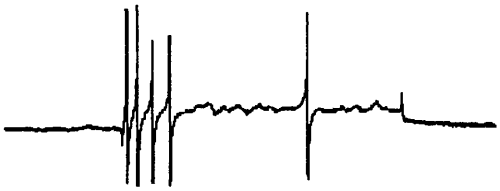
Figure 31. Effect of geraniol on spike undershoot. Spontaneously occurring action potentials were recorded from the same DUM neuron after exposure to increasing concentrations of geraniol. A. Control. B. 10^{-4}M geraniol. C. 5 x 10^{-4}M geraniol. D. 10^{-3}M geraniol. E. After 10 min saline wash. B-D were recorded 10 min after oil application.

Figure 32. Geraniol decreases excitability in a silent DUM neuron recorded intracellularly from the terminal abdominal ganglion of *P. americana.* A) Control response to depolarizing current. B) and C) show decreased responses to the same current in the presence of 10^{-4}M geraniol and 5 x 10^{-4}M geraniol respectively. D) In 5 x 10^{-4}M geraniol, a repetitive discharge was produced by increasing the current. E) Recovery of response to initial current after 10 min saline wash.

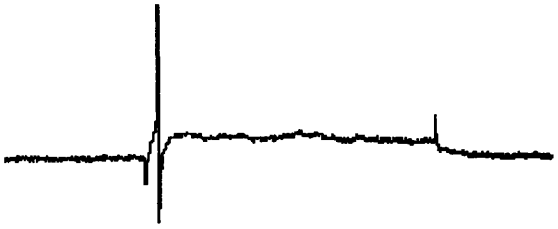
A. Control



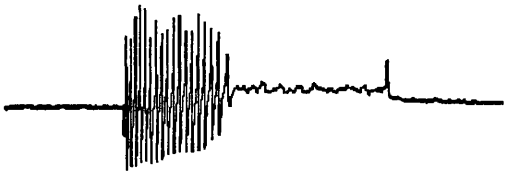
B. 10^{-4}M geraniol



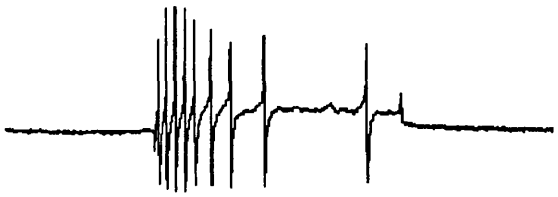
C. 5 x 10^{-4}M geraniol

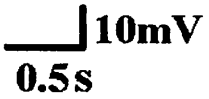


D. 5 x 10^{-4}M geraniol; raised current



E. 10 min wash





Effects of octopamine on DUM neurons

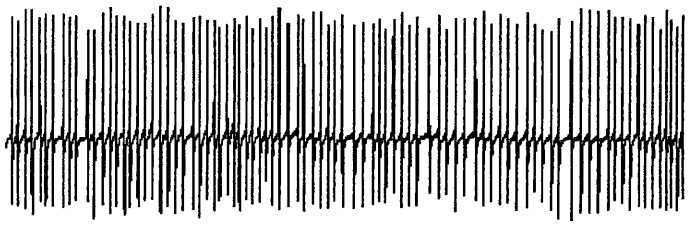
Below 10^{-5}M, octopamine had no effect on the spontaneous firing rate of DUM neurons in the TAG, but at higher concentrations it produced a dose-dependent increase in firing frequency (Figure 33, Pages 104-105) and it activated silent neurons (N=5, not shown). Little or no effect on spike undershoot or amplitude was recorded.

Effect of eugenol on the octopamine response

The excitatory response to octopamine (10^{-4}M) was unchanged when tested in the presence of eugenol (10^{-4}M), which alone had little effect on firing (Figure 34, Page 106). The response of DUM cells to depolarizing current was unaffected by octopamine (10^{-7} to 10^{-4}M) (N=5, not shown). Higher doses of octopamine were not tested in order to avoid non-specific effects; previous studies on DUM neurons have used this range, and indeed Achenbach et al (1997) found a change from excitation to inhibition in DUM cells in the 5th abdominal ganglion when the octopamine dose was increased to around 10^{-5}M.

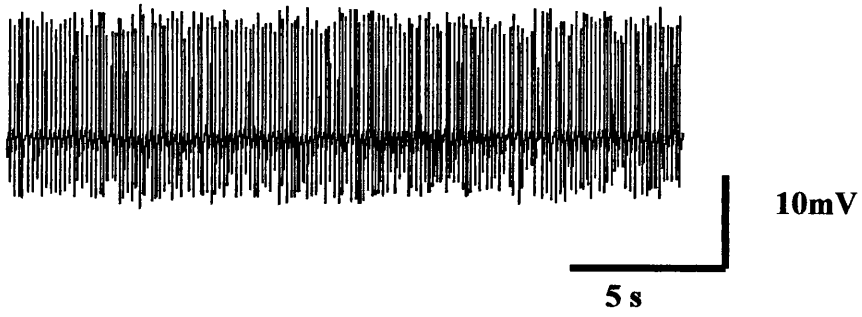
Figure 33. Example of excitatory effect of octopamine on a single DUM neuron from the terminal abdominal ganglion of *P. americana*. A) Control. B) Increased firing rate after 1 min application of 10^{-5}M octopamine. C) Further increase in firing rate after 1 min application of 10^{-4}M octopamine. Recovery occurred after a 10 min saline wash (not shown).

A. Control



B. 10^{-5}M octopamine

C. 10^{-4}M octopamine



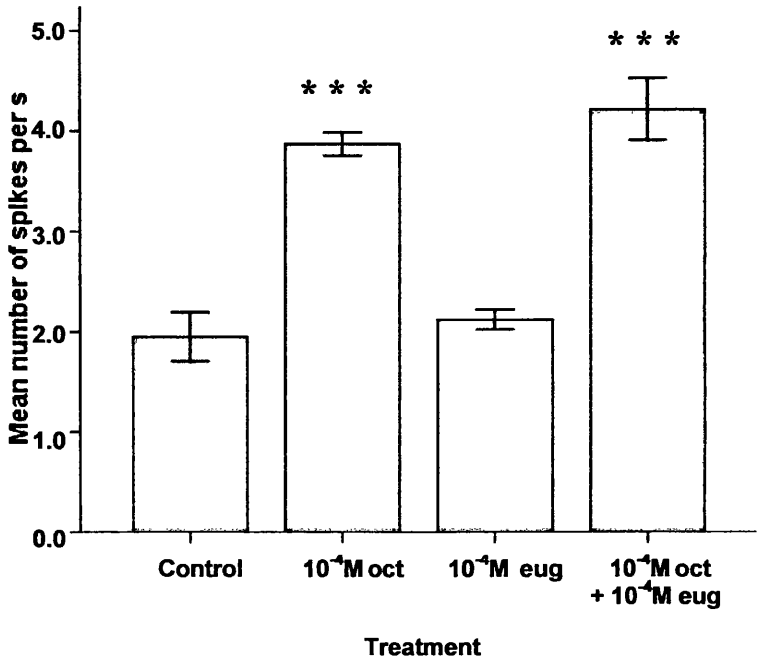


Figure 34. Bar chart showing effects of 10^{-4}M octopamine (oct), 10^{-4}M eugenol (eug), and a mixture of 10^{-4}M octopamine/10^{-4}M eugenol on mean (±SE) firing rate of DUM neurons (N = 5). ***Significantly different from control, $P<0.001$. There was no significant difference between mean firing rates in octopamine and eugenol/octopamine mixture. Neurons were exposed to 10^{-4}M eugenol for 5 min before the mixture was added.

3.33 Effects of essential oils and octopamine on foregut contraction

Experiments were performed mainly on the isolated foregut of *B. discoidalis*, and the results below refer specifically to this species (each experiment was also done at least twice on *P. americana*, with similar results). The gut generally showed rhythmic, spontaneous contractions, although these were not always regular.

Citral and geraniol, at concentrations above about 10^{-4}M, reversibly increased the frequency of the spontaneous activity and tended to make it more regular. Both initiated contractions in quiescent preparations (Figures 35 A and B, Pages 108-109), an effect that was not usually reversed by washing. At 2 x 10^{-3}M these oils blocked all spontaneous activity. In contrast, eugenol reversibly reduced spontaneous activity at 10^{-4}M and above (Fig. 35D, Pages 108-109). Foregut contractions are mainly controlled by two ingluvial ganglia, which are fused in cockroaches (Bullock and Horridge, 1965). To obtain more information on the strength of the blocking effect of eugenol, the preparation was stimulated by 1 ms pulses *via* metal flank electrodes. In order to elicit a response, the stimuli had to be given repetitively, and maximal contraction was produced by 800 mA pulses at 75 Hz for about 2 s. Figure 36 (Page 110) shows that this contraction was almost abolished by 5 x 10^{-4}M eugenol, with reversal on washing.

The effect of octopamine was again different from that of eugenol (but similar to that of geraniol and citral) in increasing the frequency of spontaneous contractions and making them more regular (Figure 35C, Page 108-109). It had, however, no effect on the evoked response (N=3, not shown).

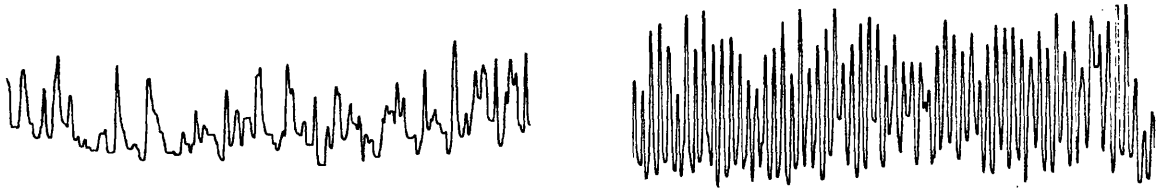
Figure 35. Examples of the effects of essential oils and octopamine on foregut contractions in *B. discoidalis*. The recordings are from different preparations. A1) Control, showing little spontaneous activity. A2) 2.5 x 10^{-4}M citral initiated regular contractions (the gain in B was halved). B1) Control. B2) 10^{-4}M geraniol increased the frequency and regularity of contractions. C1) Control. C2) 10^{-4}M octopamine increased the frequency and regularity of contractions. D1) Control. D2) 5 x 10^{-4}M eugenol abolished activity (lower doses did not cause excitation). The effect was reversible (not shown). Note that citral and geraniol resemble octopamine in their effects, whereas eugenol does not. All recordings taken 10 min after oil application.

A1. Control

A2. 2.5 x 10^{-4}M citral

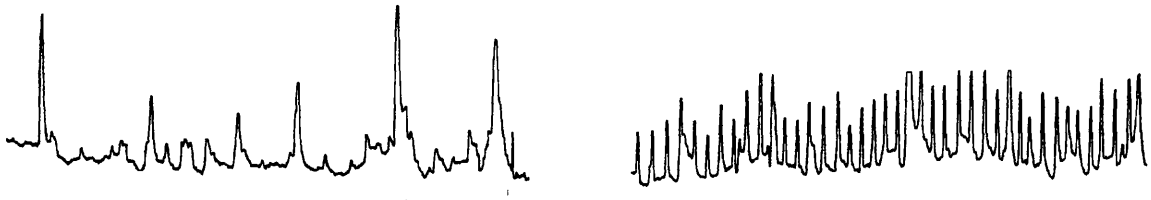
B1. Control

B2. 10^{-4}M geraniol



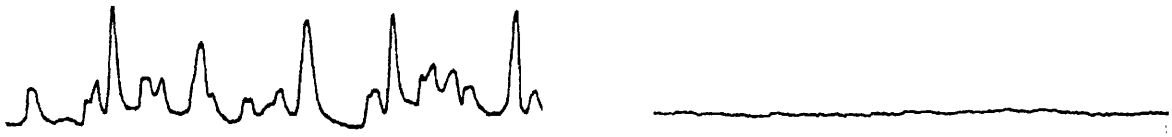
C1. Control

C2. 10^{-4}M octopamine



D1. Control

D2. 5 x 10^{-4}M eugenol



60 s

A. Control B. 5 x 10^{-4}M eugenol C. 10 min wash

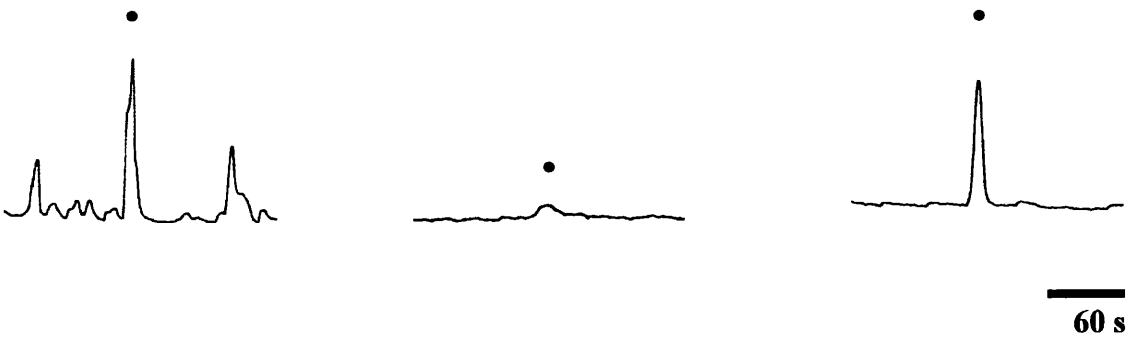


Figure 36. Effect of eugenol on the response of the foregut to flank stimulation (1 ms stimuli at 75Hz for 2 s). A) Control. B) 5 x 10^{-4}M eugenol almost abolished the response. C) Partial recovery after a 10 min saline wash (responses are indicated by dots above the traces).

3.40 Discussion

Eugenol (5 x 10^{-4}M) decreased spontaneous nerve activity in all preparations, and produced an almost complete block of spikes at about 2 x 10^{-3}M. Geraniol (and to a lesser extent, citral) had a biphasic effect on most preparations: low concentrations (ca. 10^{-4}M) increased spontaneous activity (though did not activate silent DUM neurons) while higher doses (ca. 2 x 10^{-3}M) decreased or abolished spikes (apparently by depolarization inactivation in DUM neurons). The low-dose increase in spontaneous nervous activity may explain some of the acute symptoms seen in the intact animal (e.g. hyperactivity). A similar pattern was observed on spontaneous contractions of the foregut, with eugenol decreasing movements at 10^{-4}M and above, and citral and geraniol producing dose-related biphasic effects.

Eugenol has been claimed to exert its insecticidal effects by binding to octopamine receptors (Enan, 2001; 2005b). In the current studies, however, eugenol did not mimic octopamine in its effects on DUM neurons, producing inhibition rather than excitation of spiking. Octopamine showed more similarities to citral and geraniol in its effects on DUM neuron activity (excitation of spontaneously firing cells); indeed, essential oil constituents other than eugenol have been shown to interact with octopamine receptors (Enan, 2001; 2005b; Kostyukovsky et al, 2002). In contrast to citral and geraniol, octopamine activated quiescent neurons, produced only excitatory effects and had no effect on spike undershoot or amplitude. Excitation of DUM cell activity by octopamine has been shown previously in *P. americana* for thoracic ganglia (Washio and Tanaka, 1992) and the 5th abdominal ganglion (Achenbach et al, 1997). The latter paper describes excitation occurring at 10^{-6}M and depression at around 10^{-5}M, whereas no biphasic effect of octopamine (10^{-7} - 10^{-4}M) was found in the experiments on the 6th abdominal ganglion in the current study.

Octopamine also contrasted with eugenol in its excitation of foregut contractions. In this respect it resembled low-dose citral and geraniol, though it did not produce a biphasic effect at the concentrations tested. Similarly, Luffy and Dorn (1992) showed that octopamine increased the frequency of the spontaneous contractions of the midgut of the stick insect *Carausius morosus*. Octopamine had no effect on electrically-evoked contractions whereas they were abolished by all of the oils. The oils also reduced the response of the nerve cord to cercal and electrical stimulation, in contrast to octopamine. Thus these results suggest that eugenol, at least, was not acting as an octopaminergic agonist (unless, perhaps, as an inverse agonist). Absence of antagonistic effects is indicated by the finding that eugenol did not influence the excitatory action of octopamine on DUM neurons, although it should be noted that octopamine only produced a significant effect at 10^{-4}M, which might have been too high a concentration for a blocking effect to be seen (the possibility cannot be ruled out that these particular DUM neurons were not a primary target for octopamine). Furthermore, preliminary experiments showed that even the octopamine antagonist phentolamine (10^{-6} to 10^{-4}M) did not block the octopamine response.

Interestingly, eugenol changed the firing pattern from regularly spaced impulses to bursts driven by plateau potentials when DUM neurons were depolarized by applied current. Induction of plateau potentials by octopamine in locust thoracic neurons has been reported by Ramirez and Pearson (1991), and although this did not occur in the present experiments, it is conceivable that a specific concentration (which was not tested) might have produced the same effect.

Octopamine also failed to mimic the oils by showing no effect (up to 10^{-2}M) on extracellularly recorded spike activity in the nerve cord. This may, however, have

been caused by the neuroglial sheath, which provides an effective barrier between neurons and haemolymph and prevents access to receptors (Grolleau and Lapied, 2000). For example, Hoyle (1952) found that locust nerves could withstand 1.4 x 10^{-1}M potassium ions for several hours before a conduction block occurred, but there was an immediate block when the nerves were desheathed. Insect ganglia are relatively insensitive to acetylcholine because of the nerve sheath barrier (see Pichon, 1974). Essential oils are lipophilic and therefore likely to gain access more easily to underlying neurons. When the sheath was removed for intracellular recording of DUM neurons, octopamine produced effects at concentrations of 10^{-5}M and above. The sheath may also account for the lack of effect of octopamine on the response to stimulating the cerci. Goldstein and Camhi (1991), however, report an enhanced response of intracellularly recorded and desheathed interneurons to cercal stimulation upon topical application of octopamine to the TAG, again contrasting with the current findings of reduced responses in the presence of the oils

Although the results in this study appear to differ from those of Enan (2001, 2005b) in showing no clear action of eugenol on octopamine receptors, the receptors in his experiments were from insect brain and might have had different agonist/antagonist specificity (there are several different octopamine receptor classes in insects, and they often differ in their localisation – see Evans, 1981; Evans and Robb, 1993; Roeder, 1999; Evans and Maqueira, 2005 for reviews). It would be interesting to examine effects on other cells apart from DUM neurons and also on related neurotransmitters such as dopamine. Enan (2005a) provides evidence that some oils bind to tyramine receptors in *Drosophila* brain, so there may be general actions on monoamine neurotransmission or neuromodulation.

There is, however, a further difference between the current results and those of Enan (2001; 2005b) and also Kostyukovsky et al (2002). Their effective doses were typically lower than those in this study (ca. 10^{-6}M compared to 10^{-4}M), although these workers studied receptor binding and biochemistry rather than electrophysiology (affinity of receptors and intrinsic activity are independent). Similar high-dose effects of eugenol (9×10^{-4}M) were reported on a variety of pre- and post-synaptic properties in molluscs (Szabadics and Erdélyi, 2000), which included block of responses to several neurotransmitters, but unfortunately did not include investigation of monoamines.

A potential problem with high doses is the possibility of non-specific effects that could perhaps mask any receptor-mediated actions. For example, Enan (2005b) found that the essential oil, trans-anethole, appeared not to target octopamine receptors because it produced no reduction in receptor binding of the agonist ^{3}H-yohimbine, yet it mimicked octopamine by increasing levels of cAMP. Enan (op. cit.) considered the possibility that it bypassed the OA receptor to produce this effect (e.g. activating G-proteins or acting directly on adenylyl cyclase). Unlike the natural ligand, the lipophilic oils are able to enter neurons and produce direct intracellular effects.

It was shown in the previous chapter that relatively high concentrations of the oils are needed for mortality, suggesting that the reported low-dose effects on octopamine receptors are not the cause of knockdown and death. The doses of essential oils needed to kill insect pests, and their mechanism of action, are potentially important for the safety of humans and other vertebrates. It was previously mentioned that Roeder (1999) and Enan (2001) draw attention to the fact that vertebrates possess very few octopamine receptors, and that specific octopamine

receptor binding might not produce any serious effects in vertebrates exposed to the oils. Higher doses are, however, far from specific (probably related to their lipophilic nature); for example, in addition to the effects outlined above, they have anti-cholinesterase activity, which may also contribute to their insecticidal actions (Mills et al, 2004). Essential oils affect the nervous system of rats (Brodin and Røed, 1984), and have cytotoxic (Prashar et al, 2004), genotoxic (Lazutka et al, 2001) and oestrogenic (Howes et al, 2002) activity in humans. Thus they are potentially unsafe, in spite of their widespread use in food and perfumes.

In summary, the effects of eugenol on DUM neuron and foregut activity provided little indication that eugenol acted as an octopamine mimetic or antagonist. Since other workers have found agonist and antagonist effects of eugenol in insects (Enan, 2001; 2005b; Kostyukovsky et al, 2002), this suggests that the oil targets specific sub-types of octopamine receptor. Geraniol and citral showed some similarities to octopamine and possibly warrant further study in this respect. A low-dose interaction with octopaminergic or other monoaminergic receptors might well account for the insecticidal actions of some essential oils, but more evidence is needed to confirm this. If, as the results suggest, high and relatively non-specific concentrations are needed to disrupt nervous activity and to kill insects, then there could be a range of contributing factors (including effects on non-nervous tissues), and precise mechanisms could be difficult to elucidate.

Chapter 4

Toxic and antifeedant effects of essential oil constituents on *Lymnaea stagnalis*.

4.10 Introduction

The molluscicidal effects of various plant extracts on aquatic snails have been reviewed by Bergeron et al (1996), Clark and Appleton (1997), Bezerra et al (2002) and Sukumaran et al (2002), though these authors did not focus specifically on essential oil constituents. De Souza et al (1991) reviewed the toxic effects of eugenol and derivatives (e.g. O-methyleugenol) against *B. glabrata* and showed that it had potent ovicidal and molluscicidal activity against this species. Singh et al (1997) found that the essential oil constituents cineole, oleoresin and citral from ginger rhizome (*Zingiber officanale*), and thymol from fruits of *Trachyspermum ammi* caused significant mortality in *L. acuminata* and *I. exustus*. Lahlou and Berrada (2001) analysed the effects of twenty-eight essential oils isolated from Moroccan aromatic plants against *Bulinus truncatus*, the intermediate host of urinary schistosomiasis, and showed that extracts of bitter orange (*Citrus aurantium*) and oregano (*Origanum compactum*) had significant molluscicidal activity; they studied the chemical composition of the oils and found that thymol and geraniol were the most potent constituents against this species of snail.

Due to the toxic effects in molluscs and apparent 'safety' of these compounds to humans and other vertebrates, it has been suggested that they may be used as molluscicidal agents in the environment (Singh et al, 1997); for example, the potential of using essential oils in schistosomiasis control was analysed by Lahlou and Berrada (2001). Clark and Appleton (1997) stated that molluscicides are the most important way of controlling schistosomiasis where the volume of water is relatively small; plant molluscicides are much cheaper and environmentally acceptable than their synthetic counterparts, making them useful alternatives.

4.11 Aims

The aim of this study was to assess the toxic effects of citral, geraniol and eugenol on *L. stagnalis*. Although these compounds have been shown to have molluscicidal activity against other species of aquatic snail, it was thought necessary to confirm the effects and determine lethal concentrations as a precursor to analyzing possible mode(s) of action of these compounds in molluscs (Chapter 5), as there may be species-specific differences in susceptibility (as shown for insects in Chapter 2). Antifeedant properties of these compounds were also studied, apparently for the first time in molluscs, to determine whether these could contribute to the toxic actions.

In addition to the effects of essential oils, the effect of a thiosulphinate, allicin (Figure 37, Page 119) was assessed. This compound can be extracted from crushed or cut garlic *Allium sativum*, which has significant molluscicidal activity against *Lymnaea acuminata* (Singh and Singh, 1993) and some terrestrial molluscs (Schüder et al, 2003). Allicin is chemically distinct from the essential oils but is reported to be the active molluscicidal component of garlic (Singh and Singh, 1995), and it was included in the study for comparison.

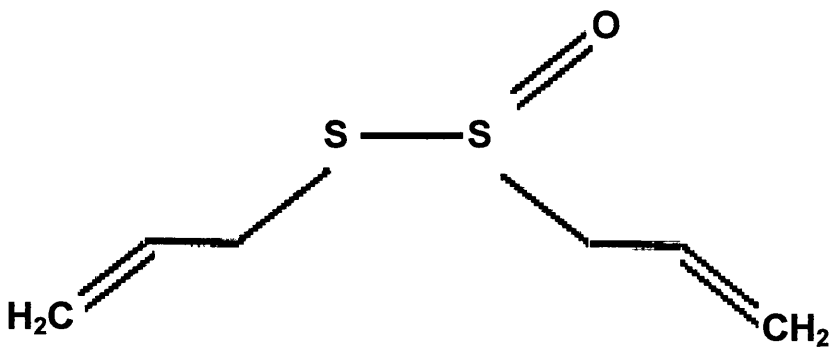


Figure 37. Chemical structure of the thiosulphinate, allicin.

4.20 Materials and Methods

4.21 Experimental animals

Freshwater whelks (*L. stagnalis*) were collected from a local pond. They were maintained in freshwater aquaria at room temperature (18-22°C) under a 12L:12D photoperiod and fed on lettuce leaves. Snails with a shell length of 20-25mm were chosen for experiments.

4.22 Chemicals

Eugenol, citral and geraniol were obtained from Sigma-Aldrich, and allicin [allyl 2-propenethiosulphinate] was purchased from Allimax International (UK). Olive oil and sunflower oil (standard supermarket products), which were presumed to be inactive, were also tested to determine whether the physical properties of oils were responsible for any effects.

The chemicals were dissolved in dechlorinated tap water, taking care to ensure that the oils were actually in solution (see section 3.23 for details). Unfortunately, olive- and sunflower oil were even less soluble than their essential oil counterparts and so were applied as emulsions (spun vigorously on a magnetic stirrer for 4 h prior to experimentation). Although unsatisfactory, at least it gave an indication of whether the general physical properties of oils had any effects on the snails. Allicin was in powder form and was readily soluble.

4.23 Feeding experiments

A range of concentrations of the test substances were made, and 40ml of each dose was poured into a glass Petri dish (88mm diameter). The same volume of water alone was used for controls. A single snail was placed in each Petri dish, initially in dechlorinated tap water without food for 24 h, before experimentation. Solutions were changed every 24 h to prevent the build up of waste products and to avoid any possible loss of potency of the compounds that could occur through separation, degradation or evaporation.

At the start of experiments, a $4cm^2$ square piece of washed lettuce was placed in the centre of each Petri dish and one snail was placed directly onto the lettuce (Figure 38, Page 122). The lids of the dishes prevented escape. After 24 h the area of lettuce that had been consumed was estimated by drawing its outline onto graph paper. A new piece of lettuce was then added to the Petri dish. This procedure was repeated for 7 days. All experiments were carried out at room temperature (18-22°C).

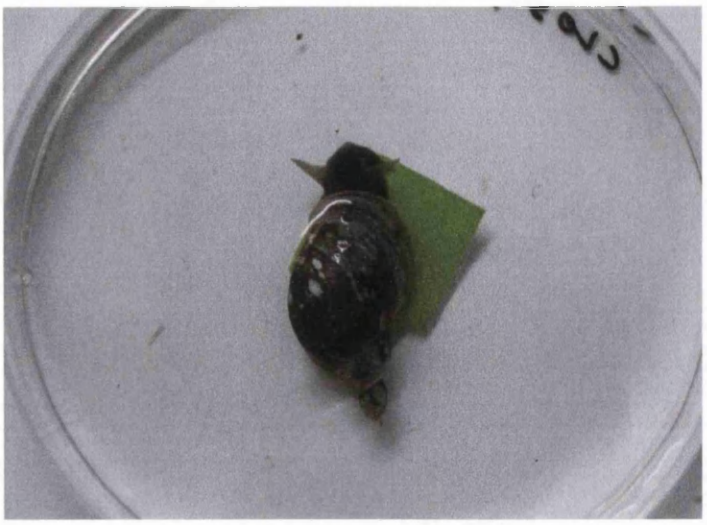


Figure 38. Experimental set up for antifeedant experiments. One snail was placed on a $4cm^2$ piece of lettuce in a glass Petri dish, and the amount of food consumed over seven days was recorded. Solutions were changed and fresh food was added every 24 h.

4.24 Assessment of animals

The condition of the animals was assessed every 15 min for the first two h of exposure, then every 24 h by touching the tentacles with a pair of forceps. A healthy individual responded to the stimuli by rapidly retracting the tentacle or its whole body. Dead snails were unresponsive to stimuli, withdrawn into their shell and often had a shrivelled appearance. Thirty animals per treatment were used.

4.25 Statistical analysis

LC_{50} and LT_{50} values were calculated using probit analysis (Finney, 1952). Mean ($\pm$ SE) number of deaths and amount of food consumed were also calculated and evaluated by two-tailed Student's *t* tests to determine significance between means.

4.30 Results

4.31 Mortality

Symptoms of toxicity

All three essential oil constituents were toxic to the snails. During the first two h of exposure to lethal doses of the oils, the snails were generally inactive compared to control animals, the tentacles were limp, the response to tactile stimulation was reduced and the snails appeared swollen, causing the mouthparts (radula) to protrude (Figure 39B, Page 125); these symptoms were labelled as 'knockdown'. Separate experiments showed that if snails displaying these symptoms were placed into fresh water, they always recovered; however when kept in the experimental solutions, they were always dead by the end-point of the experiment (168 h), making these symptoms good indicators of eventual mortality. All dead individuals displayed these symptoms prior to death. Dead snails tended to have a shrivelled rather than swollen appearance (Figure 39C, Page 125). Interestingly, at least 20% of knocked down individuals had everted genitalia after exposure to the oils for 24 h (Figure 40, Page 126).

Analysis of mortality

Despite these symptoms, none of the oils (maximum dose tested 10^{-3}M) caused significant mortality after 24 h, and this is reflected in the high values of LC_{50} (too high to calculate reliably for citral and geraniol; Table 5, Page 128). At the end point of the experiment however, significant mortality was produced by 10^{-4}M eugenol, 2.5 x 10^{-4}M citral, and 5 x 10^{-4}M geraniol (all $P<0.001$) (Figure 41, Page 127). Thus the order of potency was eugenol > citral > geraniol. No deaths occurred in untreated or olive/sunflower-oil treated snails; high concentrations (10^{-2}M) of allicin caused excessive mucus production but no deaths (Figure 42, Page 129).

The LC_{50} values decreased with time and the steepest decline occurred within the first 96 h, showing that most deaths occurred within this period. The LT_{50} values displayed in Table 6 (Page 128) show that all doses of the oils took over 24 h to kill 50% of the experimental animals. As expected, higher doses generally took less time to kill the snails, but there was some variation between the oils in speed of effect. For example, at 5 x 10^{-4}M, geraniol had the lowest LT value, but at 10^{-3}M, the LT_{50} values suggest that citral acted more quickly.

A. Untreated

B. 2 h exposure to 5 x 10^{-4}M eugenol

C. 48 h exposure to 5 x 10^{-4}M eugenol

Figure 39. Changes in appearance of snails exposed to eugenol (similar effects were seen for citral and geraniol). A) Untreated animal. B) Individual that had been exposed to 5 x 10^{-4}M eugenol for 2 h. Note the swollen appearance of the animal, causing the mouthparts to protrude (indicated by arrow). Also note that the tentacles are no longer erect; it remained in this state for at least 24 h, before dying. C) Dead individual (exposed to 5 x 10^{-4}M eugenol for 48 h). Note the shrivelled appearance.

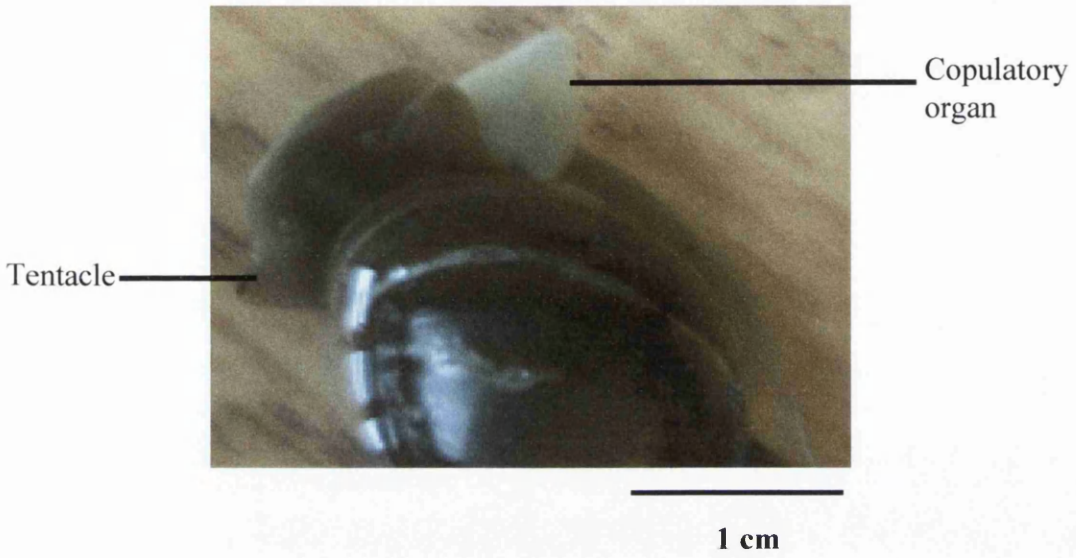


Figure 40. Symptoms of toxicity were sometimes accompanied by the protrusion of genitalia, occurring in about 40% of individuals exposed to 5 x 10^{-4}M citral.

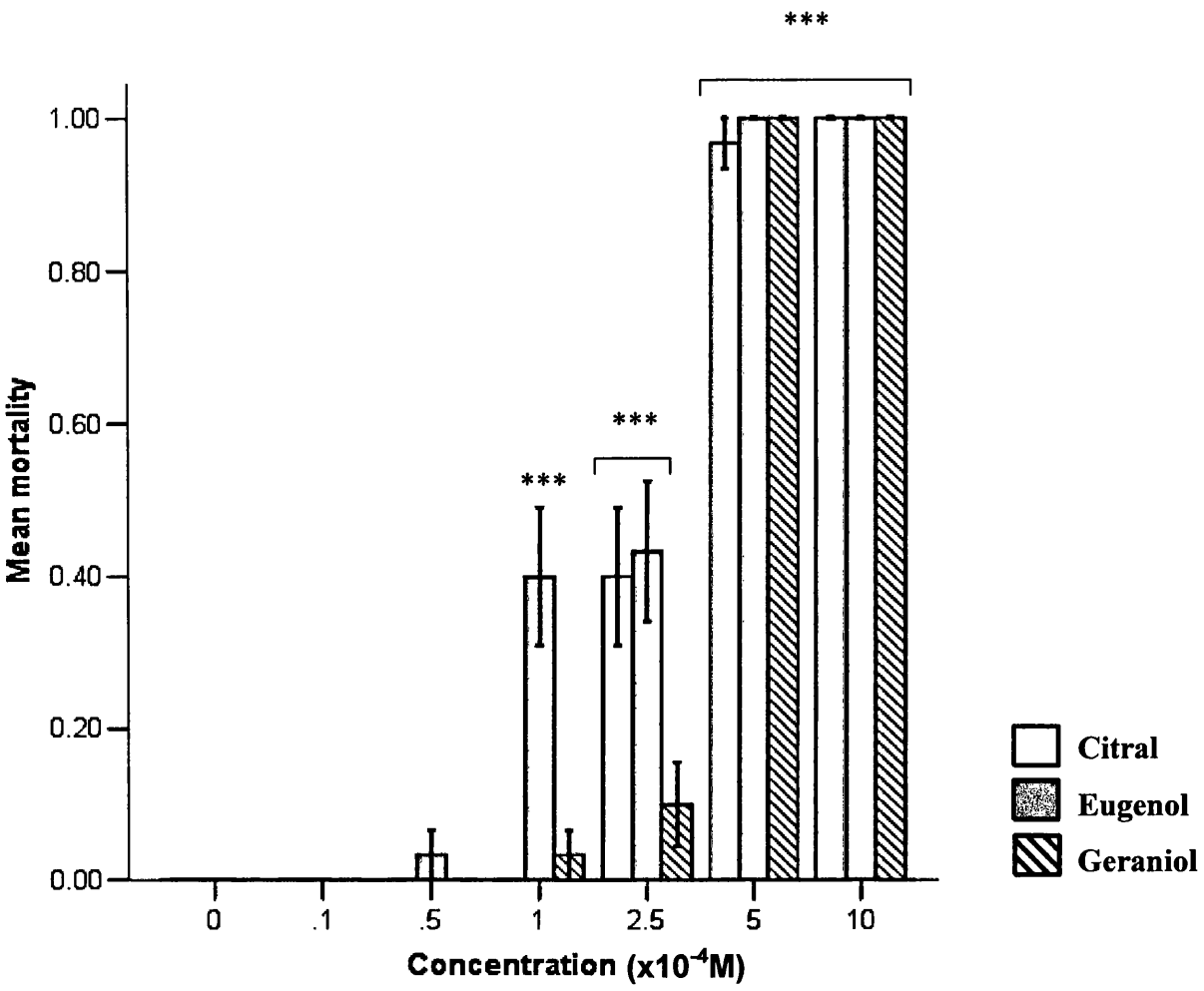


Figure 41. Bar chart of mean (± S.E) mortality caused by citral, geraniol and eugenol (1.00 equals 100% mortality) after 168 h (N=30 for each oil and concentration). ***significantly different from untreated snails and those treated with olive oil or sunflower oil ($P<0.001$).

Table 5. LC_{50} (x 10^{-4}M) for *L. stagnalis* exposed to three essential oil constituents.

	LC_{50} (95% confidence limits)						
Oil	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**168 h**
Citral	[b]	5.64 (4.94-6.64)	4.17 (3.69-4.77)	3.24 (2.85-3.70)	3.11 (2.74-3.57)	2.99 (2.62-3.45)	2.93 (2.57-3.38)
Eugenol	17.07 (12.31-39.30)	13.75[a]	7.23 (4.45-19.15)	2.61 (1.67-5.28)	2.55 (1.61-5.17)	2.41 (1.51-4.95)	2.26 (1.41-4.78)
Geraniol	[b]	7.37 (4.64-16.79)	4.03 (3.59-4.51)	3.96 (3.50-4.50)	3.28 (2.57-4.61)	3.28 (2.57-4.61)	3.28 (2.57-4.61)

[a]Estimates of 95% confidence limits could not be calculated reliably due to limitations of the dose range.

[b]No mortality.

Table 6. LT_{50} (h) for *L. stagnalis* exposed to three essential oil constituents. The two lowest doses are not included in the table because there were few deaths.

	LT_{50} (95% confidence limits)			
Oil	**10^{-4}M**	**2.5 x 10^{-4}M**	**5 x 10^{-4}M**	**10^{-3}M**
Citral	[b]	173.29 (150.43-215.62)	64.99[a]	38.21[a]
Eugenol	172.01 (146.24-222.56)	169.23 (145.31-214.14)	63.02 (56.69-69.56)	60.07 (53.81-66.49)
Geraniol	365.62[a]	403.92[a]	56.50 (37.04-73.58)	46.00 (23.51-49.80)

[a]Estimates of 95% confidence limits could not be calculated reliably due to limitations of the time range.

[b]No mortality.

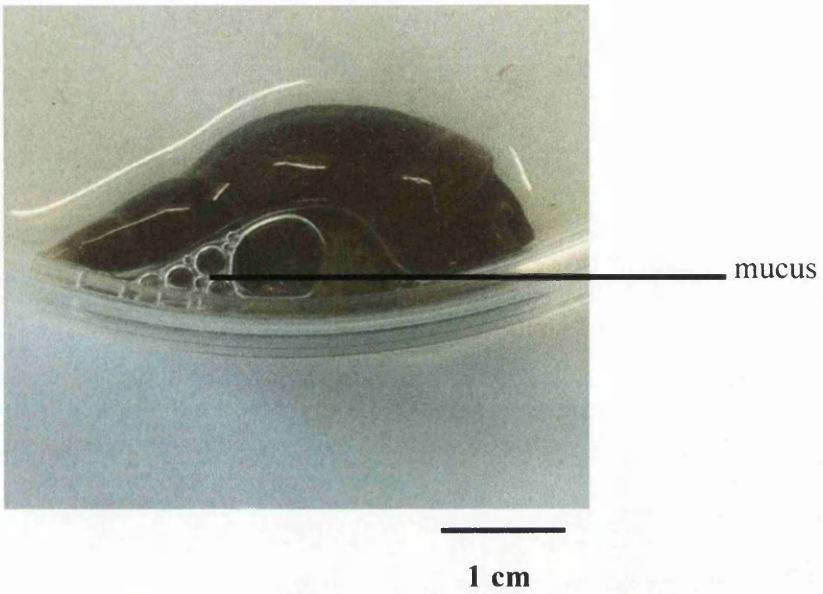


Figure 42. High doses of allicin (ca. 10^{-2}M) caused excessive mucus production, but no deaths.

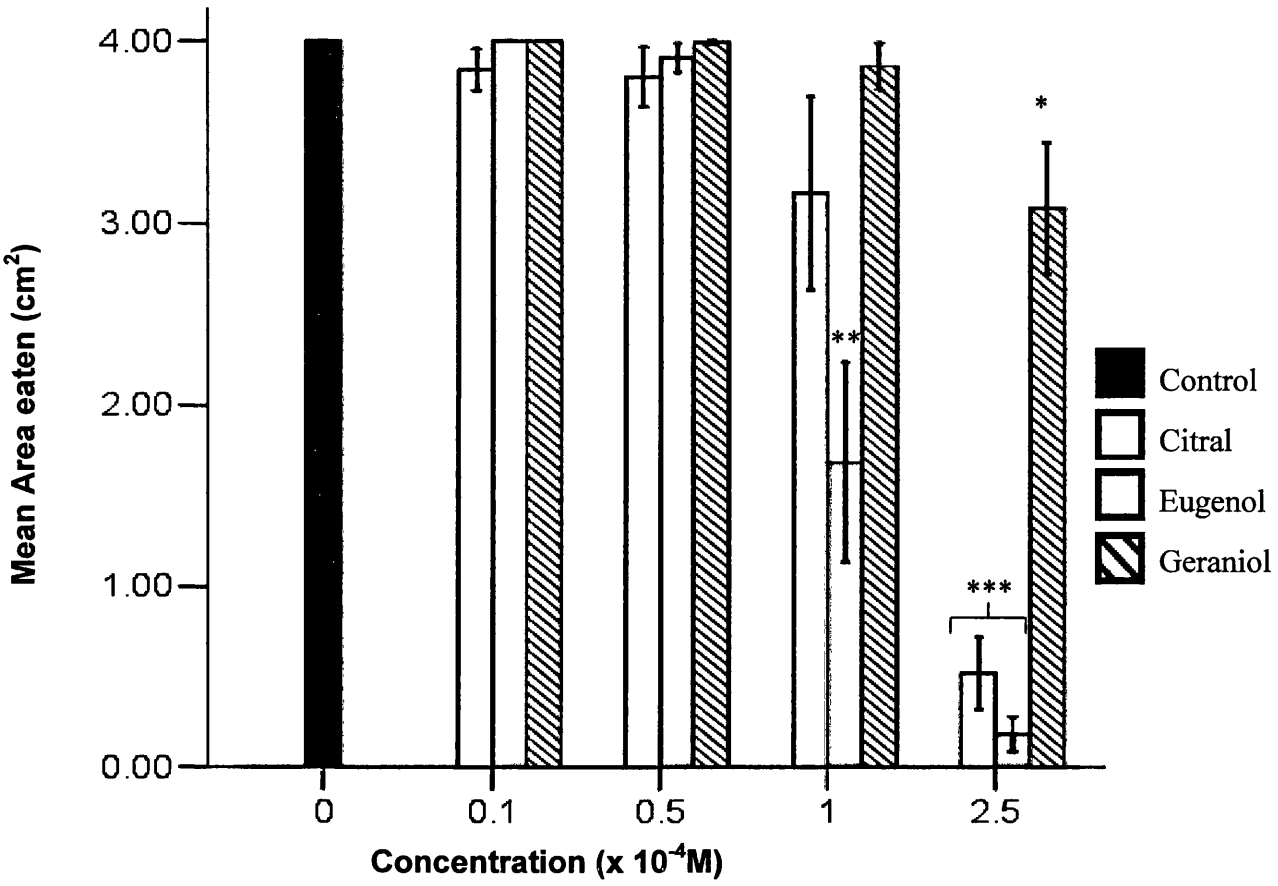
4.32 Feeding

The amount of lettuce eaten was recorded for all individuals that were considered healthy at the time of assessment. Figure 43 (Pages 131-133) displays the mean area of lettuce consumed after 24 h (A) and at the end point of the experiment (168 h; B). Dead individuals, or those displaying symptoms of toxicity were omitted from the analysis, so values of N vary for the higher doses, reducing the reliability of the statistical analysis.

After 24 h, eugenol caused a significant reduction in food consumption at concentrations of 10^{-4}M ($P<0.01$) and 2.5×10^{-4}M ($P<0.001$). Citral and geraniol decreased food consumption at 2.5×10^{-4}M ($P<0.001$ and $P<0.05$ respectively) but were not significantly effective at the lower concentration. The two highest doses (5×10^{-4}M and 10^{-3}M) were not included in the analysis because of the high levels of knockdown that occurred after 24 h. After 168 h the area of lettuce consumed by snails exposed to 10^{-4}M citral and eugenol was still reduced significantly ($P<0.05$), while geraniol no longer had a significant effect. The two higher doses were not included in the analysis because 100% mortality occurred in most cases. Olive oil, sunflower oil and allicin (up to 10^{-2}M) had no significant effect on feeding.

Figure 43. Effect of citral, eugenol and geraniol on feeding in *L. stagnalis*. A) After 24 h. B) After 168 h. Error bars represent SE. *significantly different from control $P<0.05$; **$P<0.01$. The tables under the bar charts show the values of N (reduced at the higher doses because of knockdown or death of some snails).

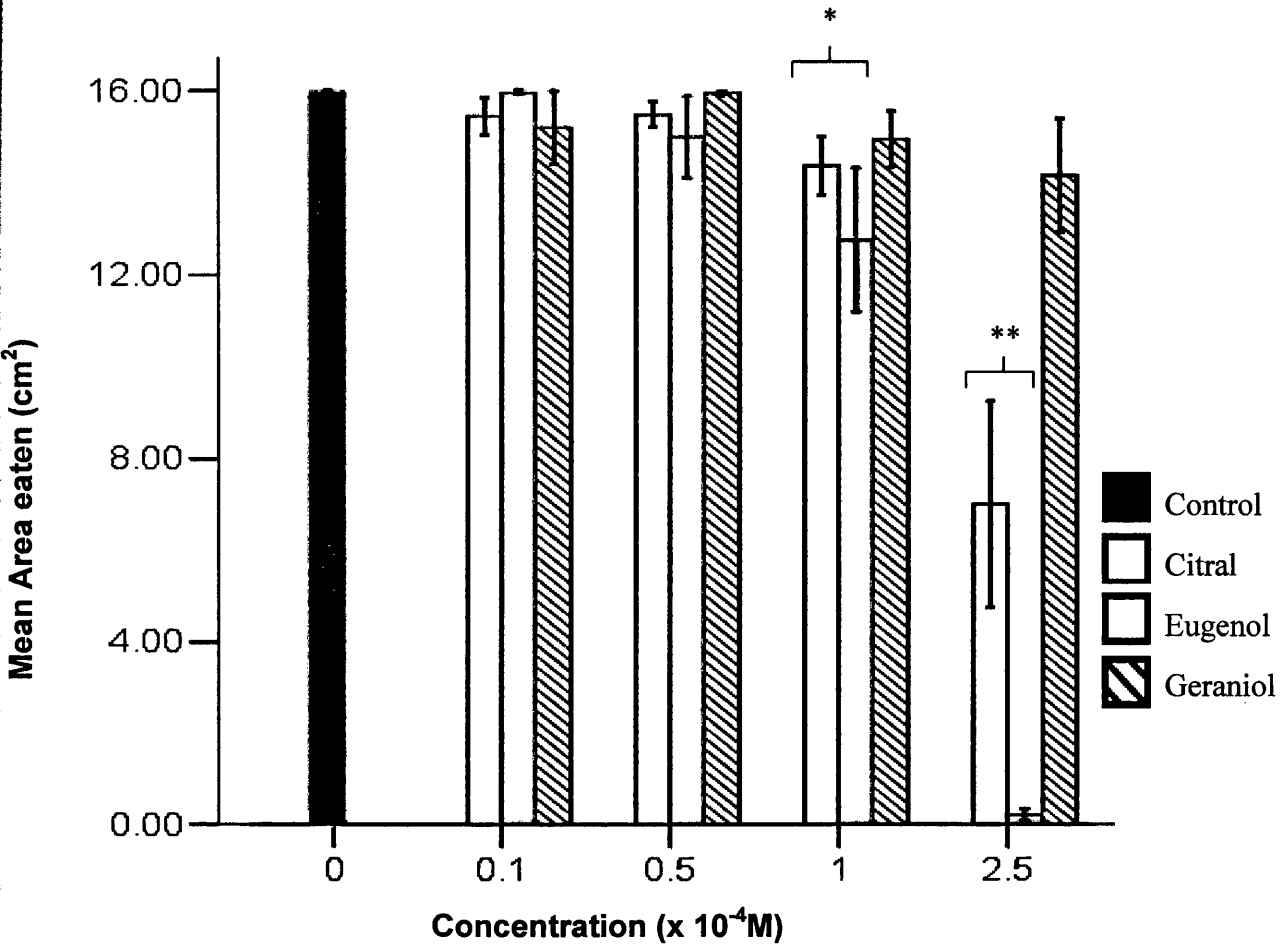
A. Food consumed in 24 h



Values of N for the above chart

Oil concentration ($x10^{-4}M$)	N values		
	Citral	Eugenol	Geraniol
0	30	30	30
0.1	30	30	30
0.5	30	30	30
1.0	30	30	30
2.5	27	27	29

B. Food consumed in 168 h



Values of N for the above chart

Oil concentration (x10^{-4}M)	N values		
	Citral	Eugenol	Geraniol
0	30	30	30
0.1	30	30	30
0.5	30	29	30
1.0	30	18	29
2.5	18	17	27

4.40 Discussion

All three essential oil constituents were toxic to *L. stagnalis* in a dose- and time-dependent manner. Acute symptoms of exposure to lethal doses of the oils included a lack of response to tactile stimulation of the tentacles, with recovery of individuals if placed into untreated water, suggesting that the effect is a type of anaesthesia. Nevertheless, if the snails were kept in the treated water for the duration of the experiment (168 h), these symptoms were good indicators of mortality as these individuals always died. In addition to the apparent anaesthetic effects of the oils, lethal effects were often characterised by swelling of the body and sometimes by protrusion of the mouthparts and genitalia. De Souza et al (1991) found a similar phenomenon in *B. glabrata*, with O-methyleugenol having an anaesthetic effect and causing the copulatory and urethral organs to project from the body. Lahlou and Berrada (2001) showed that some essential oil constituents caused *B. truncatus* to 'inflate and extend out of the shell'.

Eugenol required the lowest concentration to produce significant mortality (10^{-4}M) and had slightly lower LC_{50} values than citral or geraniol at all assessment periods. This contrasts with work on other aquatic molluscs; for example, Lahlou and Berrada (2001) found that eugenol had a much weaker molluscicidal action against *Bulinus truncatus* than geraniol. Variability in susceptibility of different species is not uncommon, and also occurred in insects (Chapter 2). Despite causing significant mortality at lower doses than citral and geraniol, the LT_{50} values for eugenol suggest that it acted more slowly than the other two compounds at high doses.

Allicin caused no knockdown or deaths even at concentrations as high as 3 x 10^{-2}M. The only visible effect of allicin was an increased mucus production which may suggest an irritant effect. These results are not in agreement with those of Singh

and Singh (1995) who identified allicin as the active molluscicidal component of garlic, causing death in *L. acuminata* and *I. exustus*. Deaths might have occurred if the tests had been run for longer than 168 h, although Singh and Singh (op. cit.) found significant mortality within 96 h. Different allicin sources might account for the discrepancy, or differences in species susceptibility. In preliminary experiments, large amounts of dried, powdered garlic were also ineffective, suggesting that it is not toxic to *L. stagnalis*.

High concentrations of olive oil or sunflower oil emulsions caused no deaths and had no effect on feeding in the snails. The very low solubility of these compounds made a direct comparison with the essential oils difficult, but the results suggest that it is not the physical properties of the oils that are responsible for the toxic effects seen (apart from their lipophilic nature possibly aiding entry into the animal and target tissues).

Mortality at high doses hindered statistical analysis of feeding because it reduced the number of animals that could be tested. Significant effects on food consumption were seen, however, in cases where there were no obvious toxic symptoms, suggesting that the killing and antifeedant effects are separate. All three essential oil constituents significantly reduced the amount of food consumed within 24 h, though the effects were less pronounced for geraniol. After 168 h, only citral and eugenol had a significant effect, and it appears that the weaker antifeedant effects of geraniol were transient. Preliminary experiments showed that *Planorbis corneus* responded to the compounds in a similar way (data not shown).

It is often considered that defence against consumers (predators or herbivores) is the primary function of the high concentrations of secondary metabolites in organisms such as higher plants and marine invertebrates (Burkholder, 1973;

Dyrynda, 1985), and 'antifeedant' effects may be a contributory factor. Indeed, many compounds from marine invertebrates (e.g. sponges, cnidarians, molluscs and bryozoans) inhibit feeding in predators (Pawlik, 1993). They may simply make the organisms distasteful or there may be other deterrent effects such as stressing the consumer. It has been suggested that defensive potential is a function of concentration and duration of exposure (Dyrynda, 1986), a claim substantiated in the current study. At very low doses, a chemical may have a signalling role, while at intermediate doses the same chemical may have noxious roles (unpleasant, but causing no damage), and at high doses it may be lethally toxic (Dyrynda, op. cit.). As with the cockroaches, it is unlikely that the apparent antifeedant effects were responsible for mortality within the time period of the experiment, as the snails could survive without feeding for at least ten days.

It has been suggested that attempts to assess the effects of secondary metabolites by dissolving them into water holding the consumer has minimal ecological relevance because such methods "may be no more realistic than suffocating humans in chocolate syrup and then concluding that chocolate is toxic" (Hay, 1996). If this is the case, however, it does not readily explain why high doses of allicin (or olive/sunflower oil) had no effect on the snails in the current study. The essential oil constituents all had killing effects at lower doses than the other compounds, suggesting that they have specific characteristics that make them toxic.

Despite the molluscicidal and antifeedant nature of these compounds to aquatic molluscs in the laboratory, their use in a large-scale mollusc control programme, as suggested by some authors (Singh et al, 1997), may be hindered by their low solubility in water. Lahlou and Berrada (2001) showed that their solubility could be improved through the use of Tween 80, a 'tensio-active' solvent, and stated

that it had an important influence on their biological activity by ensuring a better contact between oil and snail. Some organic solvents (e.g. methanol) were tested in the current study, but only relatively high doses increased the solubility of the oils, and might have had effects of their own. For the concentrations of oil that were needed, it was unnecessary to dissolve them first in an organic solvent.

As with insects, the mechanism of the molluscicidal action of essential oil constituents is unknown. One suggested mode of action is to disrupt the ionic and water balance of the snail, due to a change in the permeability of cell membranes (Appleton, 1985; Lahlou and Berrada, 2001); this would account for the swelling effect in the current study. Alternatively, there may be breakdown of osmotic equilibrium resulting from disruption of neurohormonal control which has been shown to cause molluscs to inflate and extend out of the shell (Lahlou and Berrada, op. cit.). A reduction in heart rate has also been suggested to contribute to mortality (Lahlou, 2004).

Essential oil constituents have been suggested to have a neurophysiological mode of action on a variety of invertebrates (e.g. Szabadics and Erdélyi, 2000), and effects on the nervous system might contribute to the antifeedant, knockdown and lethal actions seen in the current study. For example, effects on the sensory apparatus (lips and tentacles) and/or the peripheral components of the nervous system, possibly affect the animal's ability to sense the presence of appetitive signals. The compounds may also act centrally on the buccal ganglion feeding network, which coordinates the rasping and swallowing movements of the snails (Vehovszky and Elliott, 2002).

The next chapter studies the effects of these compounds on the nervous system of *L. stagnalis* and *P. corneus* in a further attempt to elucidate possible mode(s) of action. The octopaminergic system (suggested to be a target in insects) was again a

focus of attention because octopamine has a major role as a transmitter and modulator in the circuitry that controls feeding (Elliott and Vehovszky, 2001), thus providing a useful starting point for investigating possible mechanisms of action of essential oil constituents. Interestingly, Vehovszky et al (1998) showed that the octopamine antagonists phentolamine, DCDM and NC-7 inhibited the normal feeding response of *L. stagnalis* apparently through their actions on octopaminergic receptors in the feeding system. Furthermore, in the presence of these drugs, tactile stimulation of the head caused no withdrawal of the tentacles and the body tone was significantly altered, leading the authors to suggest that octopamine has roles in muscle function in addition to feeding regulation. Thus, some of the effects of these octopamine antagonists appear to be similar to those of the oils described in the current study, and further suggest a link between the oils and octopamine.

Chapter 5

Effects of essential oil constituents and octopamine on gut movements and electrophysiology of the buccal ganglia in freshwater pulmonate molluscs.

5.10 Introduction

Essential oil constituents may have a neurotoxic mode of action (Coats et al, 1991; Kostyukovsky et al, 2002) and, in chapter 3, the oils were all shown to affect the cockroach nervous system in ways that could contribute to their insecticidal properties. It was demonstrated in chapter 4 that the oils also had molluscicidal activity, with citral and eugenol having antifeedant properties. The current chapter seeks to understand any possible neurophysiological basis of these actions on molluscs.

The anatomical features of the molluscan central nervous system (such as large and identifiable neurons) make it an accessible model for neuronal studies of central organisation of behaviour (Vehovszky and Elliott, 2000). The nervous system of freshwater molluscs has been well studied, and many nerve cells have been identified and classified on the basis of their morphological and physiological characteristics and the roles that they play within the animal. For example, feeding in *L. stagnalis* (and other molluscs) is largely controlled by the buccal ganglia, which coordinate the rasping and swallowing movements of the buccal mass (Benjamin, 1983; Vehovszky and Elliott, 2002), together with the cerebral ganglia which receive inputs from the lips and tentacles (Elliott and Susswein, 2002).

Unlike the situation with insects, no link has been made between essential oils and octopamine receptors in molluscs. Octopamine does, however, play an important role as a neurotransmitter and neuromodulator in the control of molluscan feeding (Elliott and Vehovszky, 2001). Indeed, octopamine is found predominantly in the buccal ganglia (Hiripi et al, 1998) and therefore, provided a useful starting point for investigating possible mechanisms of action of the oils in molluscs.

In *L. stagnalis*, the feeding network in the buccal ganglia is composed of motoneurons, central pattern generator interneurons and higher order interneurons that act together with cerebral interneurons to produce a rhythmic pattern of motor output. This pattern is known as the 'fictive feeding rhythm' because it corresponds to the cycles of movements of the buccal mass and associated mouthparts such as the radula (protraction, rasping and swallowing) (Hiripi et al, 1998). Similarly, Arshavsky et al (1988a; b; c) identified cell types in another freshwater mollusc, *P. corneus*, according to their burst firing in relation to protraction and retraction of the radula.

In *L. stagnalis*, buccal ganglion neurons release octopamine at synapses with other members of the feeding network, producing excitatory or inhibitory postsynaptic potentials and initiating the fictive feeding rhythm (Hiripi et al, 1998; Vehovszky and Elliott, 2000; Elliott and Vehovszky, 2001). Perfusion of isolated ganglia with octopamine also elicits a patterned discharge. The buccal ganglion cells were therefore targets for the current study and enabled investigations into the effects of essential oil constituents on octopamine receptors.

5.11 Aims

The overall aim of this chapter was to analyse whether the molluscicidal effects of essential oil constituents, identified in the previous chapter, could be mediated *via* effects on electrical activity in the nervous system of freshwater molluscs, and attempt to discover possible mechanisms of action. The effects were compared to those of octopamine to determine evidence of any relationship between the compounds, as claimed for insects (Enan 2001; 2005b; Kostyukovsky et al, 2002).

Other possible targets were also investigated: acetylcholine receptors, dopaminergic transmission, electrical transmission and spike generation.

5.20 Materials and Methods

5.21 Experimental animals

Specimens of *L. stagnalis* were collected from a local pond and *P. corneus* were purchased from Blades Biological Supplies Ltd (UK). They were maintained as described in the previous chapter. Electrophysiological experiments were carried out on both species, yielding similar results, but most of the reported intracellular experiments were carried out on *P. corneus*. Although less well studied, its feeding rhythm generator is similar to that in *L. stagnalis* (Arshavsky et al, 1988), and at this stage it was general features that were of interest. One of the main reasons for using *P. corneus* was that a specific neuron that responded to octopamine could be readily identified. Detailed behavioural experiments were not carried out on *P. corneus* but preliminary experiments showed that it responded in the same way to the oils as *L. stagnalis*.

5.22 Chemicals

The test chemicals were the essential oil constituents citral, geraniol and eugenol, and the garlic extract, allicin. Phentolamine, metoclopramide and yohimbine were used as octopamine antagonists (Evans, 1981; Hiripi et al, 1998; Howell and Evans, 1998; Vehovszky et al, 1998). Neurotransmitters used were octopamine, ACh and dopamine. All chemicals were purchased from Sigma-Aldrich (UK), except allicin, which was from Allimax International (UK).

5.23 Electrophysiology

Buccal ganglia/buccal mass preparations (for extracellular recordings) (Figure 44, Page 147) or isolated buccal ganglia (for intracellular recordings) (Figure 45, Page 148) were pinned to the Sylgard base of a Perspex experimental bath (volume 1ml) and immersed in a continous flow (3ml min^{-1}) of physiological saline (NaCl 80mM, KCl 4mM, $CaCl_2$ 7mM, $MgCl_2$ 5mM, HEPES 20mM, pH 7.2). On some occasions, the cerebral ganglia remained attached to the buccal ganglia (*via* the cerebro-buccal connectives).

Extracellular recordings were made from the dorso-buccal nerves of a buccal ganglia/buccal mass preparation, with a glass-tipped suction electrode, and impulse activity was amplified with a home-made, differential a.c. amplifier and displayed on a storage oscilloscope as described earlier (chapter 3).

Intracellular recordings from isolated buccal ganglia were made with glass microelectrodes filled with 3M KCl (15-25MΩ). Examples of recordings of spontaneous and evoked activity are shown in Figures 46 and 47 respectively (Pages 149 and 150). Unlike the TAG of the cockroach, the snail ganglia did not need to be desheathed or stained because the orange cells were clearly visible and relatively easy to penetrate. On occasion, however, Protease (Type XIV, Sigma) was needed to weaken the sheath of *L. stagnalis*. Neurons on the dorsal surface of the ganglia were used. Hiripi et al (1998), Vehovszky et al (1998), Elliott and Vehovszky (2000), Vehovszky and Elliott (2000) and Vehovszky et al (2000) have identified many cell types and defined their properties in *L. stagnalis*. Similarly, Arshavsky et al. (1988a; b; c) described the various cell types in *P. corneus* (though they did not identify specific neurons). These studies provided useful reference guides; all of the current recordings from the buccal ganglia of *P. corneus* were from rhythmic neurons which,

from their positions and firing patterns, appeared to be from groups 3, 4, 5 and 7, which are not endogenously bursting but are driven by excitatory and inhibitory inputs. Exceptions were the two largest, easily identified cells (one in each ganglion), which were not studied by Arshavsky et al (op. cit.) because they showed little rhythmic activity. In the current experiments, however, although these cells were generally inactive or discharged regularly, they could fire rhythmically in phase with the other burst-firing cells, and they were also of interest because they appeared to be directly hyperpolarized by octopamine. They were labelled as BN1 by Arshavsky et al (op. cit.), but are referred to here as giant buccal cells (GBCs).

Conventional electrophysiological recording was used (see Chapter 3 for details). Recordings were further displayed and stored on a computer using a micro1401 ADC interface (Cambridge Electronic Design; sampling rate 60 KHz) and subsequently analysed using Spike2 software (Cambridge Electronic Design). After impalement, the cell was left to stabilise for 30 min and was considered suitable for experimentation if spikes were generally above 30mV after this period.

Iontophoresis

Octopamine, dopamine and ACh were applied locally by iontophoretic injection from a microelectrode filled with the neurotransmitter at a concentration of about 0.1M in distilled water. The electrode was manoeuvred so that it was directly above, but not touching the penetrated cell and a positive current pulse was applied to the electrode to eject the neurotransmitter. In some early experiments, a Digitimer (Neurolog) NL900A amplifier was used instead of the Axoclamp 2A amplifier, and this had no facility for iontophoresis. Therefore, iontophoretic current was passed through a 140MΩ resistor (to ensure constancy) and monitored in terms of the voltage across a

10 KΩ series resistor. In order to reduce the possibility of the electrode becoming blocked, the solutions were filtered through a 0.2μm millipore filter, and electrode resistance was lowered by gently pushing the tip against a piece of the snail's tissues (a few high voltage, positive and negative 'clearing pulses' also helped to avoid blockage). Retaining currents were found to be unnecessary and were, therefore, not used.

Sufficient time was allowed between iontophoretic applications to avoid receptor desensitization.

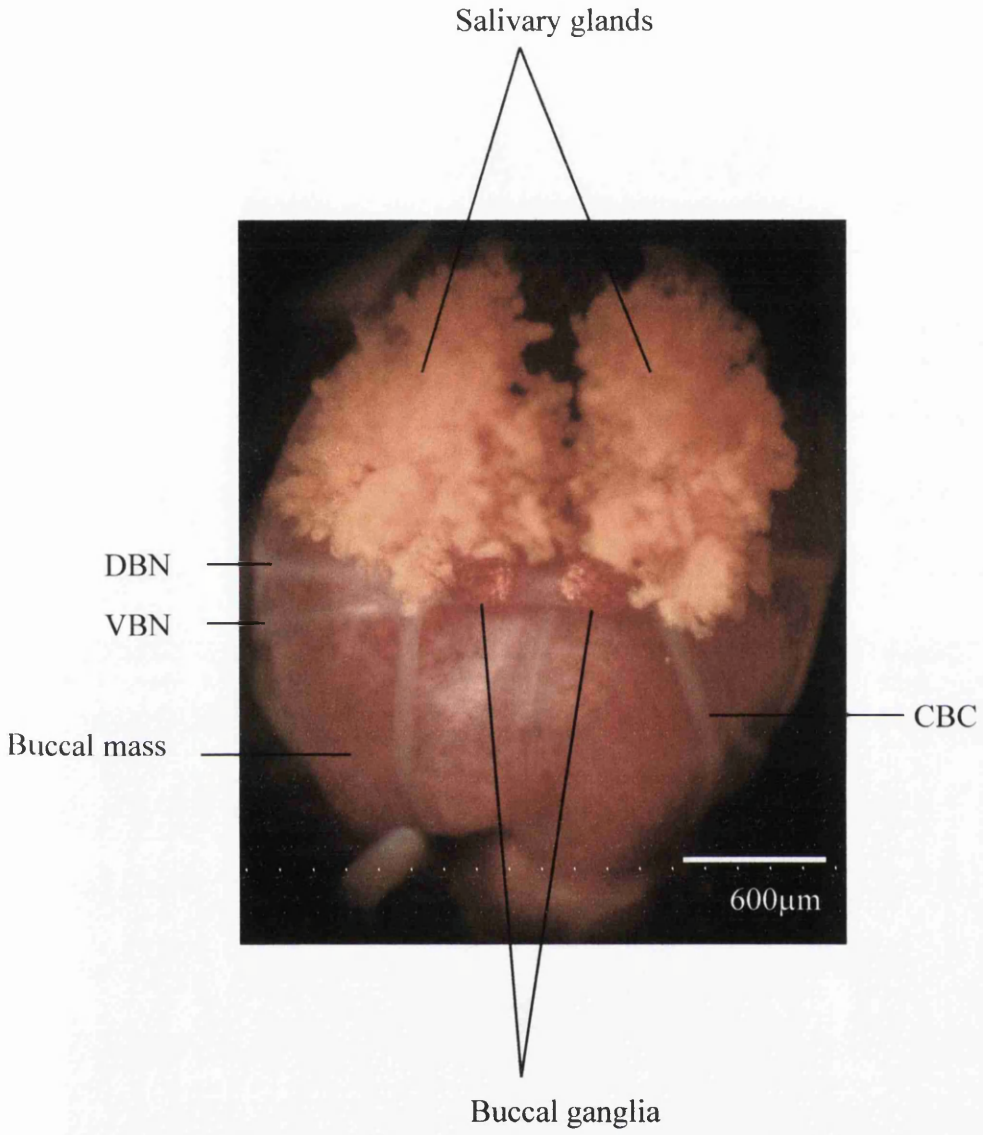


Figure 44. Buccal ganglia of *P. corneus in situ*, showing the buccal mass and associated nerves. DBN – dorso-buccal nerve; VBN – ventro-buccal nerve; CBC – cerebro-buccal connective.

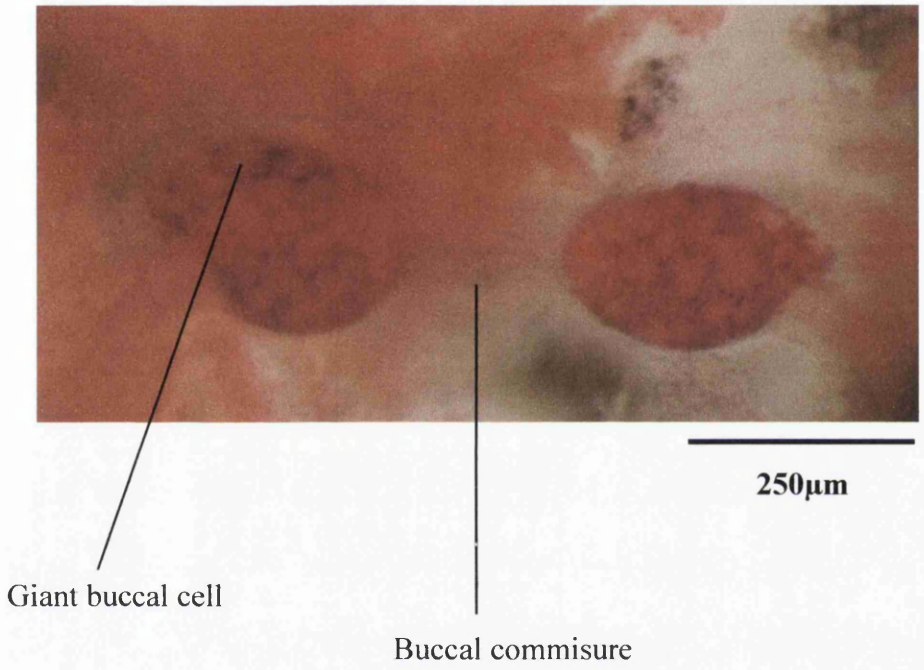
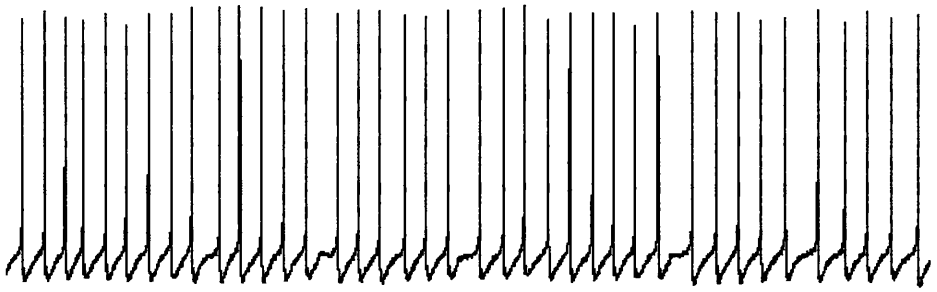


Figure 45. Isolated buccal ganglia showing position of the giant buccal cell.

A. Action potentials from a buccal ganglion cell



B. Burst activity from a buccal ganglion cell

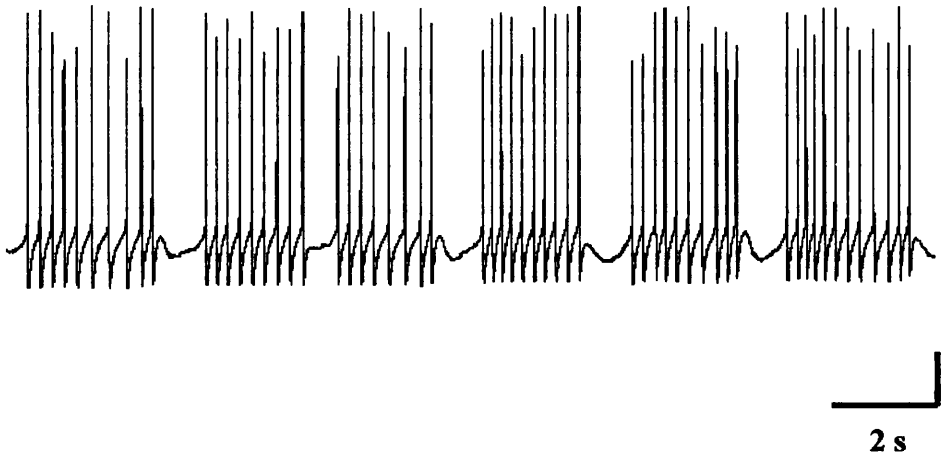
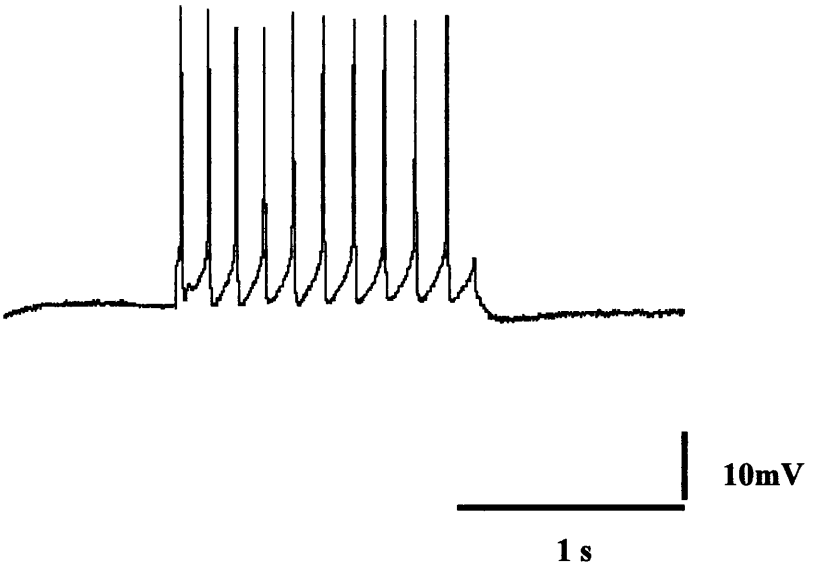


Figure 46. Spontaneous impulse activity from buccal ganglion cells of *P. corneus*. Some cells showed regular firing (A), and some fired in bursts (fictive feeding) (B).

A. Response to depolarizing current



B. Response to hyperpolarizing current

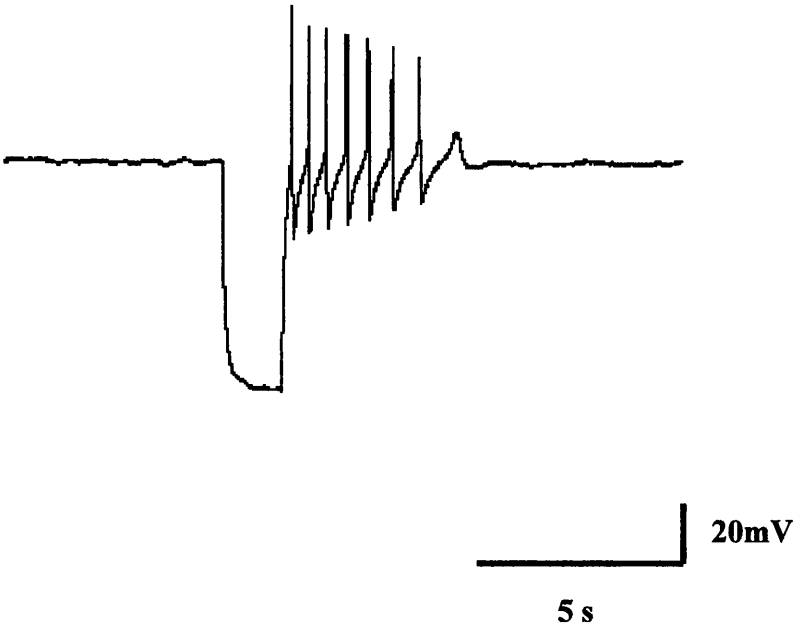


Figure 47. Evoked responses of a *P. corneus* buccal ganglion cell. A) The cell responded to a depolarizing current with a train of impulses. B) Rebound spikes were elicited in response to injection of a hyperpolarizing current.

5.24 Gut movements

Movements of the buccal mass and radula are too complex to study effectively with a transducer, but they were monitored visually during extracellular recordings from buccal nerves in order to relate nerve and muscle activity (rhythmic movements in isolated preparations of the buccal mass/buccal ganglia varied from tiny contractions to full extension and rasping of the radula). For intracellular recording, the buccal ganglia were isolated to avoid problems of movements dislodging the electrodes and to remove possible peripheral effects of the oils which might complicate the analysis. Isolating the buccal ganglia has been shown to have little effect on the patterned output (Arshavsky et al, 1988).

The buccal mass propels food into the oesophagus, which was isolated and its movements recorded independently. Contractions of the isolated oesophagus, which was suspended vertically in a Perspex container (volume 10ml), were recorded by tying one end to a weight placed at the base of the container and the other end to a transducer (SRI) to record isotonic contractions which were monitored on a pen recorder (Brush 2200S). The preparation was maintained in physiological saline and the chemicals were added (dissolved in saline at their final concentration) from the bottom of the flask and overflowed at the top. This procedure in itself did not affect spontaneous contractions. Tension on the foregut was adjusted to about 2.5×10^{-3}N.

5.25 Statistical analysis

Many of the results involved changes in firing pattern, where statistical analysis was inappropriate (and unnecessary). For effects on spike frequency, the frequency is expressed as mean ± standard error and significance of effects is evaluated by two-tailed Student's *t* tests.

5.30 Results

The results from *L. stagnalis* and *P. corneus* were similar and therefore, to avoid repetition, the data are generally shown for one species. Allicin had no effect on spontaneous or evoked electrical activity in the buccal ganglia or on oesophageal movements at doses of 10^{-6}-10^{-2}M, and so is not discussed further in this section.

5.31 Extracellular recording in semi-intact preparations

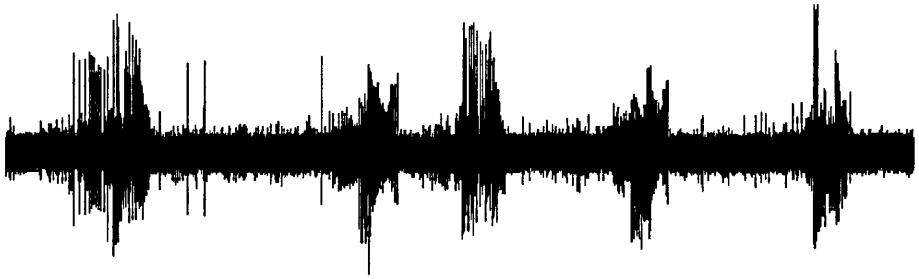
Preparations of the isolated buccal mass, with the buccal ganglia attached, generally showed little spontaneous movement but were activated into rhythmic activity by citral and geraniol. This was reflected in burst firing recorded from the dorso-buccal nerves (see Arshavsky et al, 1988a for nomenclature).

Citral (2.5 x 10^{-4}M) significantly increased spike frequency of the dorso-buccal nerves in both *P. corneus* and *L. stagnalis* ($P<0.05$; N=5) (Figures 48 and 49, Pages 153-155) by initiating burst firing. Geraniol had similar effects in both species at 5 x 10^{-4}M ($P<0.001$; N=5) (Figures 50 and 51, Pages 156-158). Eugenol (10^{-4} to 10^{-3}M) usually had no stimulatory effect (N=5) but on two occasions initiated transient burst activity (lasting a few min). Octopamine (5 x 10^{-5}M) initiated burst firing and significantly increased spike frequency ($P<0.01$ for *L. stagnalis*, $P<0.001$ for *P. corneus*; N=5) (Figure 52 and 53, Pages 159-161). The threshold dose for octopamine was about 10^{-6}M.

Figure 48. Citral induces burst firing in a dorso-buccal nerve. A-C show an example of recordings in an isolated buccal ganglia/buccal mass preparation from *L. stagnalis*. A) Control. B) 200 s after 5 x 10^{-4}M citral. C) After 5 min saline wash.

A. Control

B. 200 s after 5 x 10^{-4}M citral



C. After 5 min saline wash

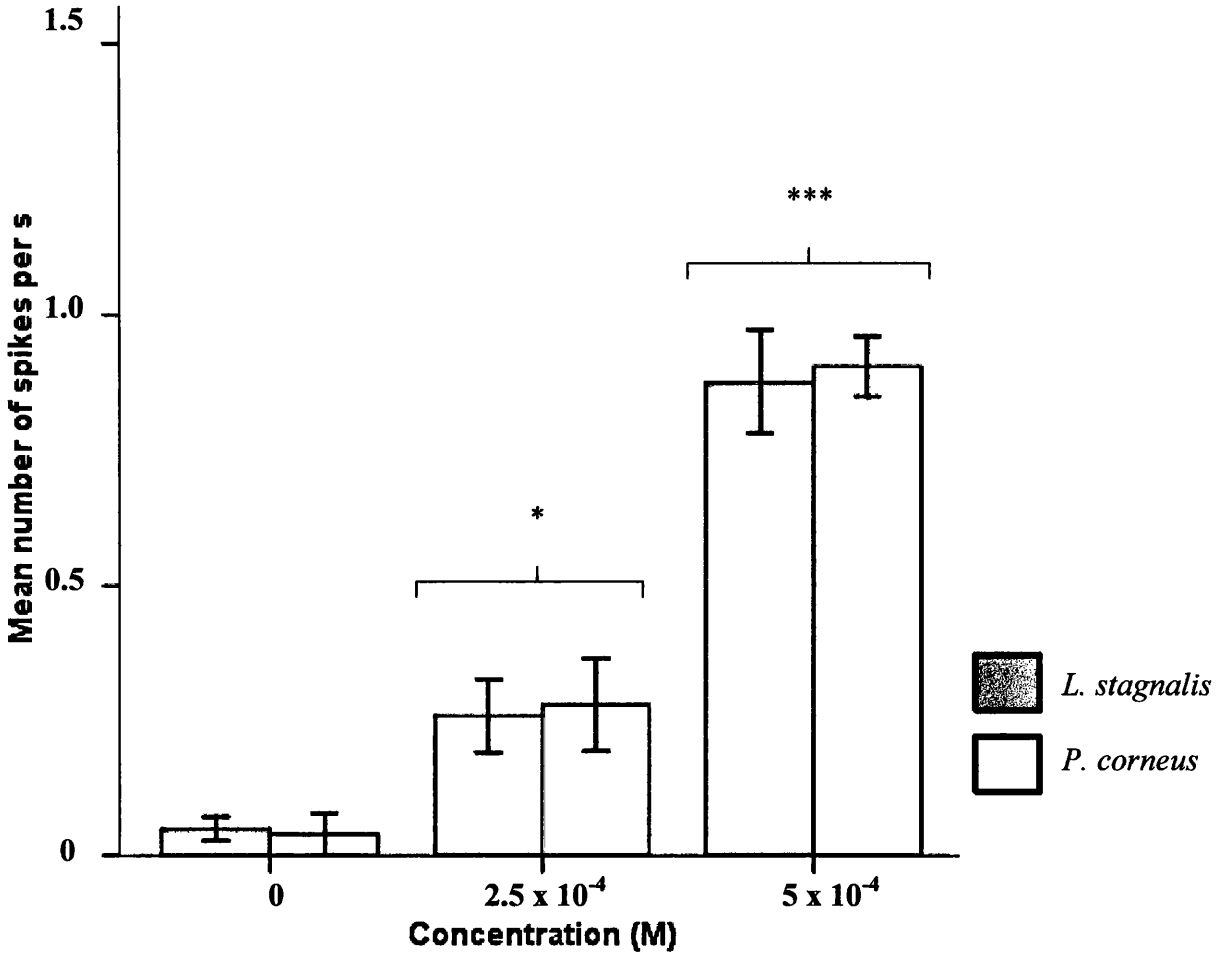
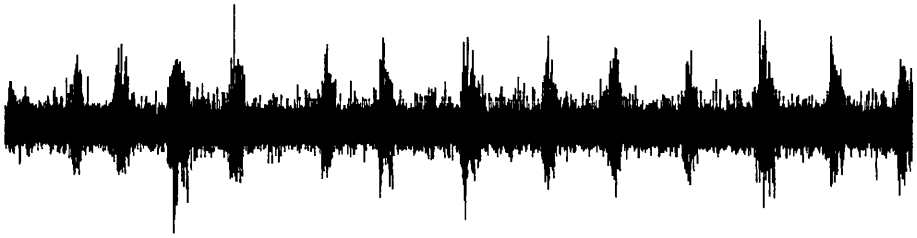


Figure 49. Bar chart showing effect of citral on mean (±SE) spontaneous impulse frequency in dorso-buccal nerves recorded from isolated buccal ganglia/buccal mass preparations of *L. stagnalis* and *P. corneus* (N = 5 for each preparation). *Significantly different from control, P<0.05; ***P<0.001. All data taken 200 s after oil application.

Figure 50. Geraniol induces burst firing in a dorso-buccal nerve. A-C show an example of recordings in an isolated buccal ganglia/buccal mass preparation from *L. stagnalis*. A) Control. B) 200 s after 5 x 10^{-4}M geraniol. C) After 5 min saline wash.

A. Control

B. 200 s after 5 x 10^{-4}M geraniol



C. After 5 min saline wash

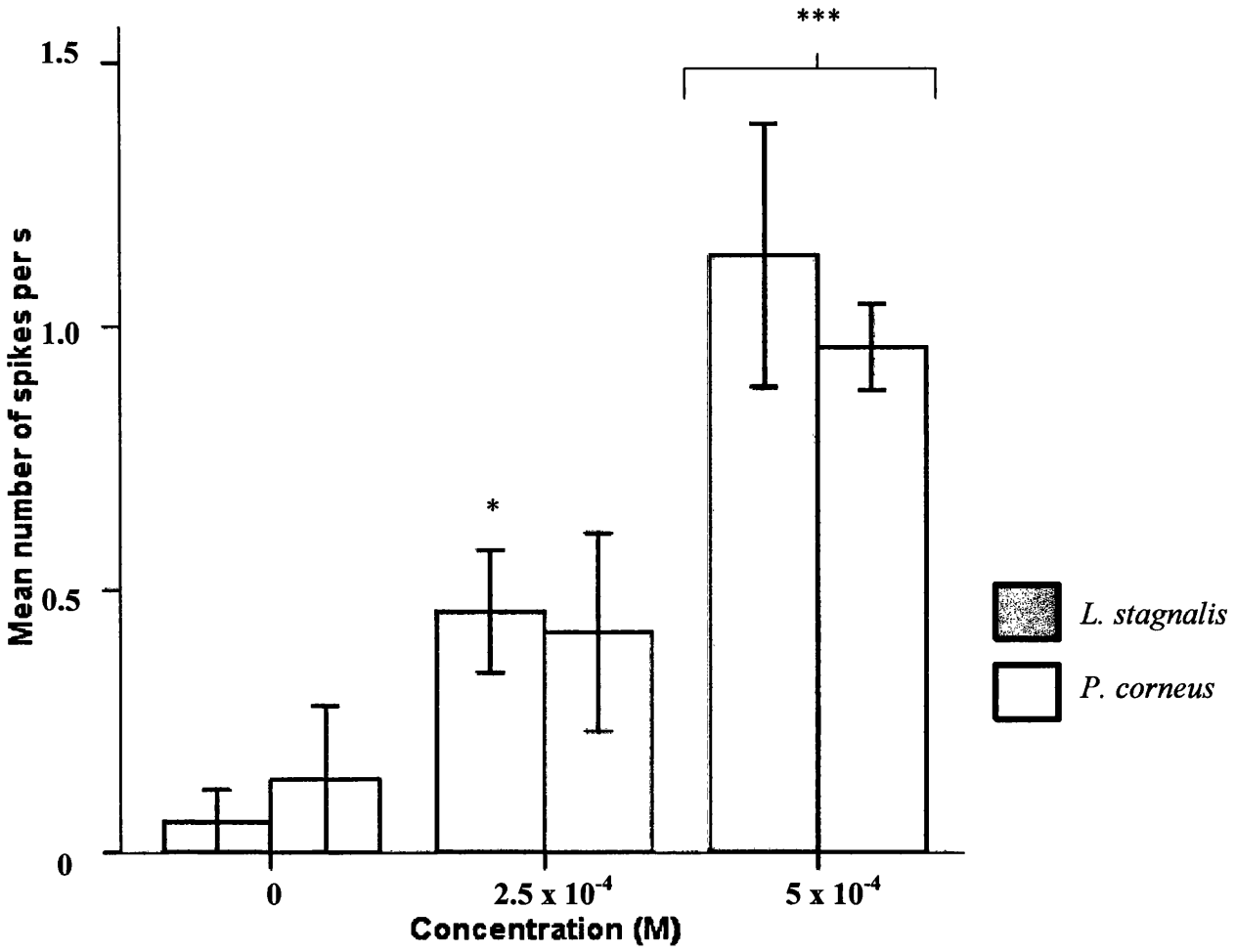
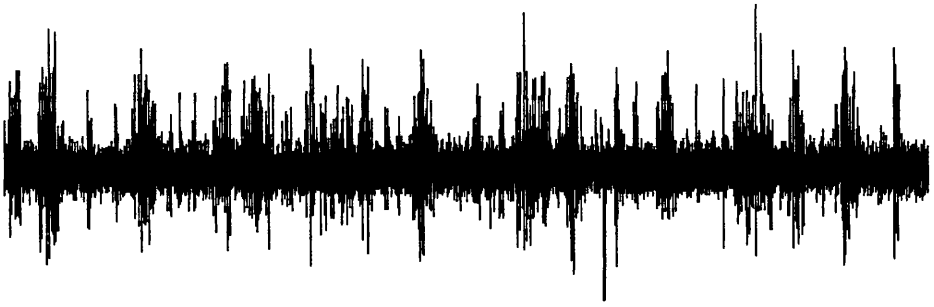


Figure 51. Bar chart showing effect of geraniol on mean (±SE) spontaneous impulse frequency in dorso-buccal nerves recorded from isolated buccal ganglia/buccal mass preparations of *L. stagnalis* and *P. corneus* (N = 5 for each preparation). *Significantly different from control, $P<0.05$; ***$P<0.001$. All data taken 200 s after oil application.

Figure 52. Octopamine induces burst firing in a dorso-buccal nerve. A-C show an example of recordings in an isolated buccal ganglia/buccal mass preparation from *L. stagnalis*. A) Control. B) 200 s after 10^{-4}M octopamine. C) After 5 min saline wash.

A. Control

B. 200 s after 10^{-4}M octopamine



C. After 5 min saline wash

10 s

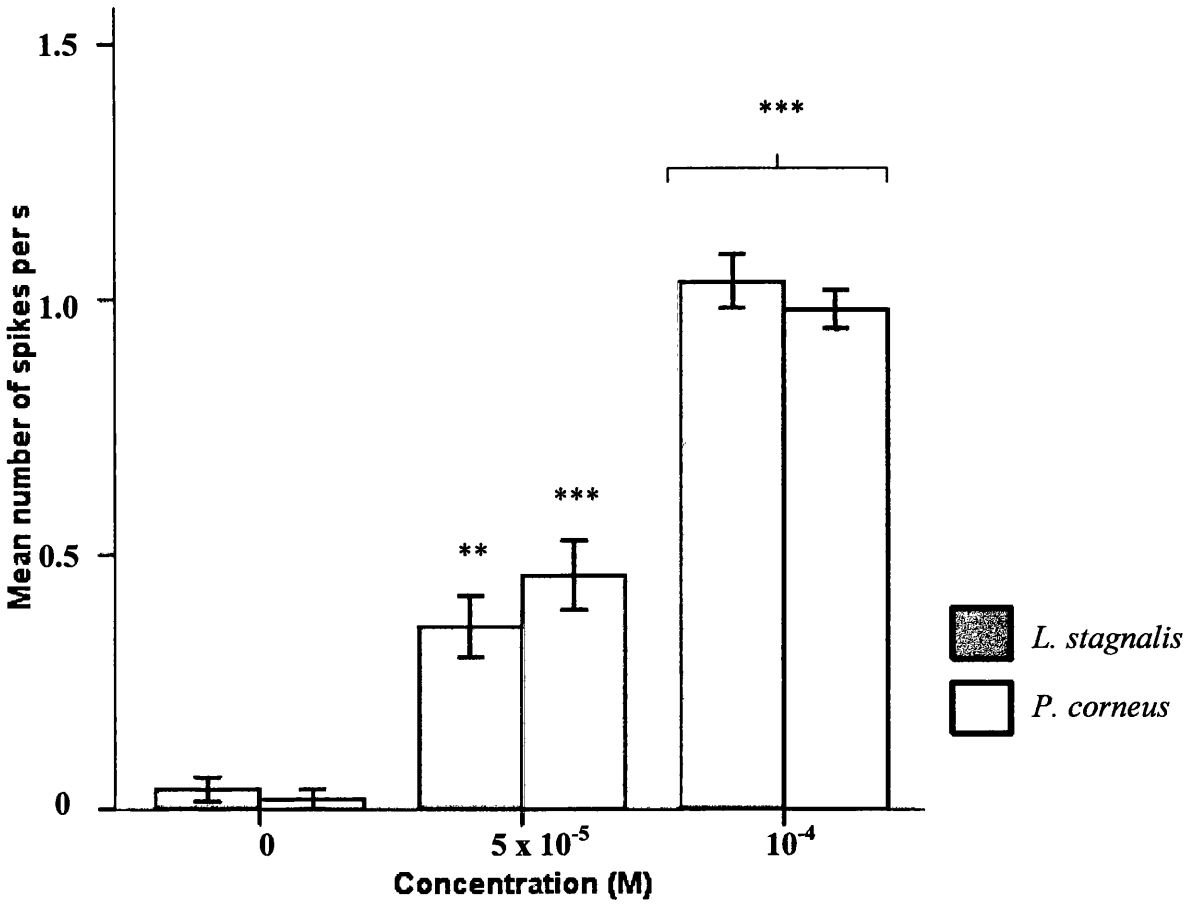


Figure 53. Bar chart showing effect of octopamine on mean (±SE) spontaneous impulse frequency in dorso-buccal nerves recorded from isolated buccal ganglia/buccal mass preparations of *L. stagnalis* and *P. corneus* (N = 5 for each preparation). **Significantly different from control, P<0.01; ***P<0.001. All data recorded 200 s after octopamine application.

5.32 Intracellular recording of neurons in isolated buccal ganglia

Unless stated otherwise, all recordings are from *P. corneus*.

Effect of eugenol on spontaneous and evoked impulse activity

Eugenol (10^{-3}M) significantly decreased or blocked spontaneous firing in unidentified buccal ganglion neurons of both *P. corneus* ($P<0.001$, N=5) and *L. stagnalis* ($P<0.01$, N=5) within 5 min of application (Figures 54 and 55, Pages 164-166). In the GBC, however, 2 out of 5 cells showed a transient excitation (Figure 56, Pages 167-168).

Eugenol (ca. 10^{-3}M) blocked the response to depolarizing current (Figure 57, Pages 169-170) and (in the GBC) the rebound spikes initiated by injection of a hyperpolarizing pulse (Figure 58, Page 171). All effects were reversed by washing off the eugenol.

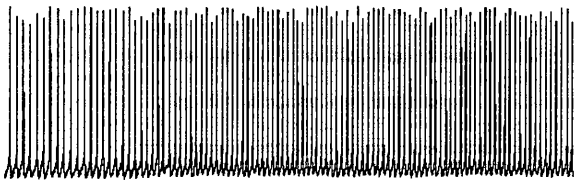
Effect of citral and geraniol on spontaneous and evoked impulse activity

Citral and geraniol had reversible excitatory effects on quiescent cells and changed the firing pattern of active preparations at a threshold dose of about 10^{-4}M. In all cells tested in the buccal ganglia and in the cerebral giant cells (CGCs) of the cerebral ganglia, citral and geraniol initiated burst firing (Figures 59-61, Pages 172-176), with bursts driven by excitatory and (usually) inhibitory inputs (when the cell was hyperpolarized, the frequency of the bursts remained the same). Spike bursts became shorter at higher doses of geraniol and citral (10^{-3}M), and at ca. 2×10^{-3}M there was a decrease in spike frequency and synaptic activity (Figure 61, Page 176). Analysis of mean spike frequency showed that significant increases in frequency were produced by geraniol and citral at 2.5×10^{-4}M in quiescent or slowly firing cells (less than 0.5 spikes per s) ($P<0.001$, N=5 for *L. stagnalis*; $P<0.01$, N=5 for *P. corneus*) (Figure 62,

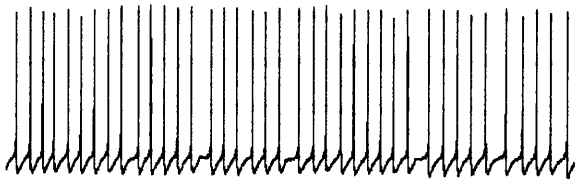
Pages 177-178). In active neurons (firing at a rate of more than one spike per s) the mode of firing changed, but not necessarily the overall spike frequency; at higher doses (ca. 2 x 10^{-3}M), there was, however, a significant decrease in spike frequency ($P<0.001$; N=5) (Figure 63, Pages 179-180). The response to depolarizing current was decreased at high doses (Figure 64, Page 181).

Figure 54. Eugenol decreases spike frequency of a spontaneously firing GBC. A) Control. B) 200s after 5 x 10^{-4}M eugenol. C) 200s after 10^{-3}M eugenol. D) Some synaptic activity returned after a 10 min saline wash. E) Spikes returned after 30 min in saline.

A. Control

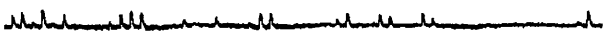


B. 200 s after 5 x 10^{-4}M eugenol

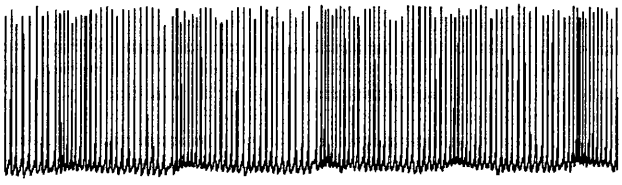


C. 200 s after 10^{-3}M eugenol

D. After 10 min saline wash



E. After 30 min saline wash



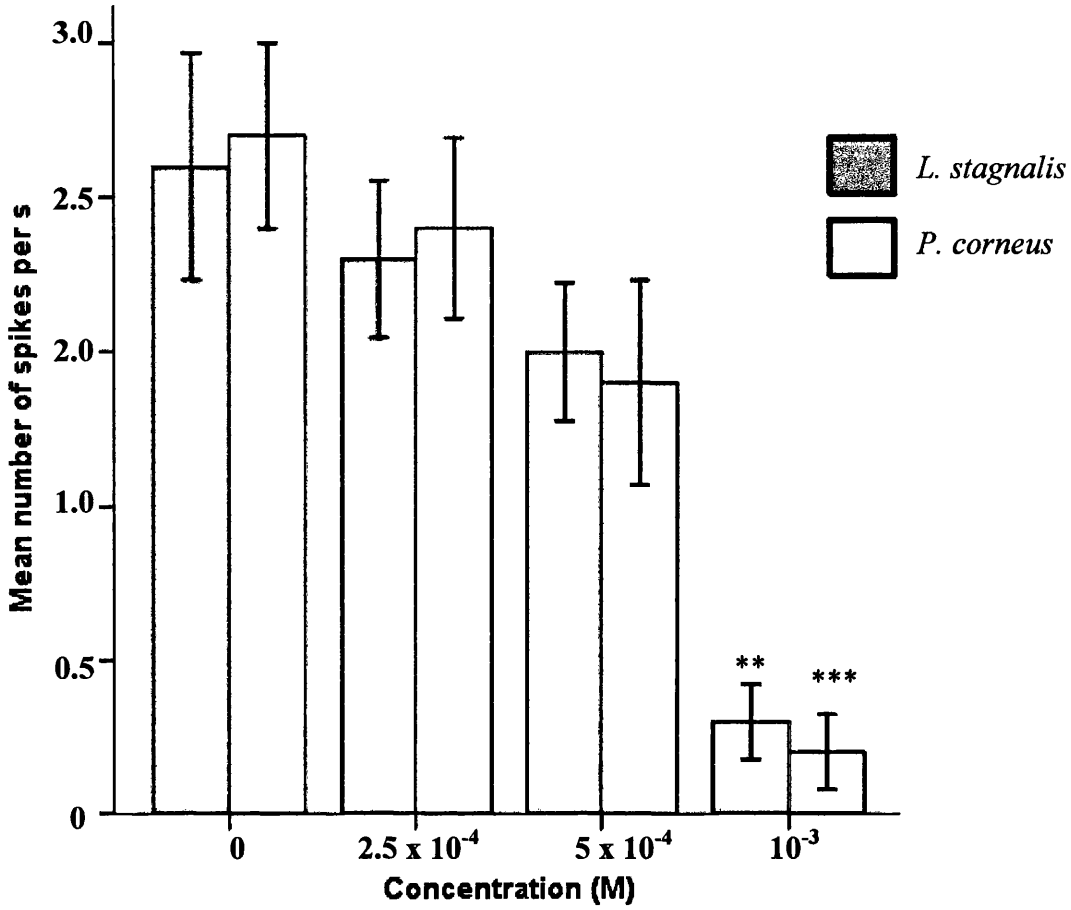
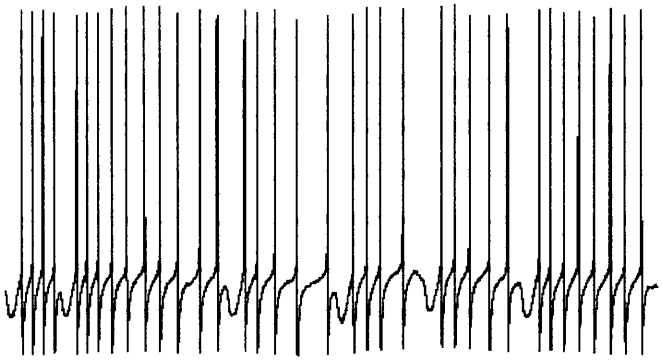


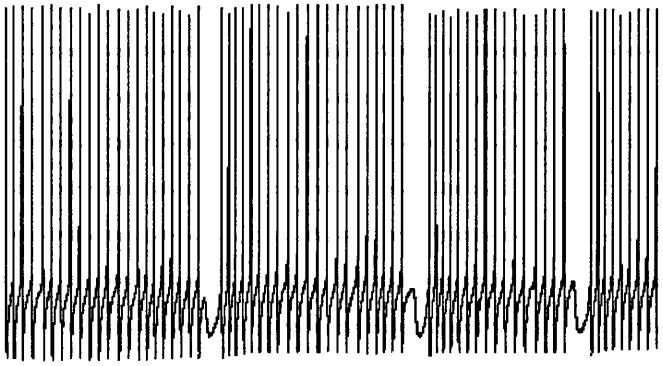
Figure 55. Bar chart showing effect of eugenol on mean (±SE) spontaneous impulse frequency for spontaneously firing buccal ganglion neurons of *L. stagnalis* and *P. corneus* (N = 5 for each preparation). **Significantly different from control, P<0.01; ***P<0.001. All data recorded 200 s after oil application.

Figure 56. Biphasic effect of eugenol on an active GBC: transient excitation followed by inhibition. A) Control. B) After 100 s exposure to 5 x 10^{-4}M eugenol, spike frequency increased. C) After 5 min exposure, spikes were abolished. The preparation recovered 10 min after washout of eugenol (not shown).

A. Control



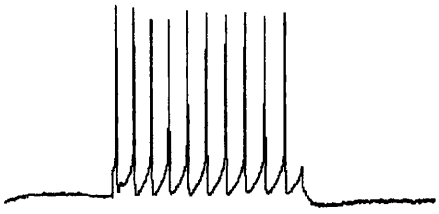
B. 100 s exposure to 5 x 10^{-4}M eugenol



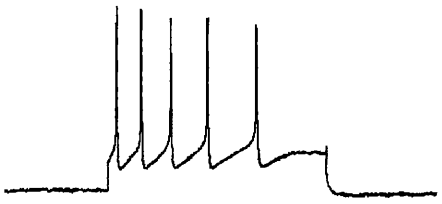
C. 5 min exposure to 5 x 10^{-4}M eugenol

Figure 57. Eugenol reversibly inhibits spikes elicited by depolarizing current in a GBC. A) Control. B,C,D) The cell became progressively less excitable after the application of 5 x 10^{-4}M eugenol. E) Recovery after 10 min saline wash. The amplitude of the current was the same in each recording but the bridge balance changed in E.

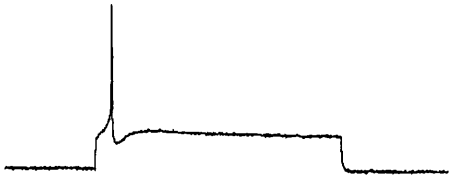
A. Control



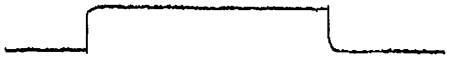
B. 100 s after 5 x 10^{-4}M eugenol



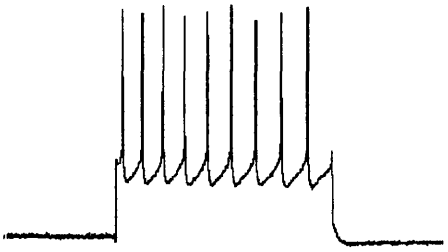
C. 200 s after 5 x 10^{-4}M eugenol



D. 5 min after 5 x 10^{-4} M eugenol



E. After 10 min saline wash



1 s

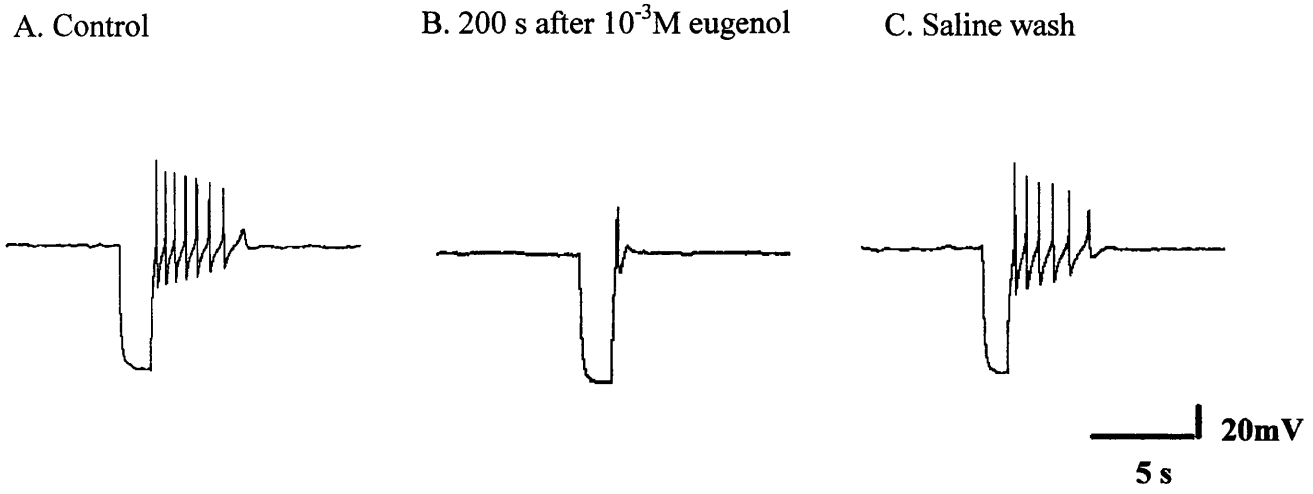
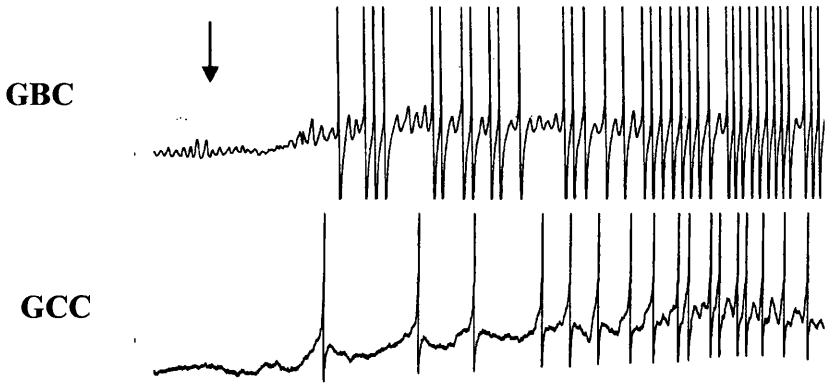


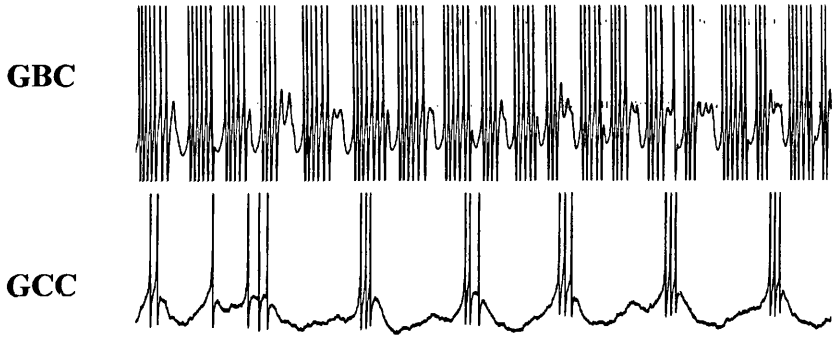
Figure 58. Eugenol reversibly inhibits rebound spikes elicited after a hyperpolarizing current was applied to a GBC. A. Control. B. After 10^{-3}M eugenol. C. After 10 min saline wash.

Figure 59. Geraniol initiates burst firing in a GBC and a cerebral giant cell (CGC) (recorded simultaneously). A) 2.5 x 10^{-4}M geraniol caused depolarization and initiated firing in both cells. B) The cells began to burst fire 100-200 s after initial application. C) Burst firing stopped 10 min after removal of geraniol. The arrow represents the beginning of geraniol application. Spikes are truncated. Note the IPSPs in the GBC which were typical of the cell's response to geraniol. Spikes are clipped as this trace was taken from a pen recorder (Brush 2200S).

A. 2.5 x 10^{-4}M geraniol



B. 200 s after 2.5 x 10^{-4}M geraniol



C. After 10 min saline wash

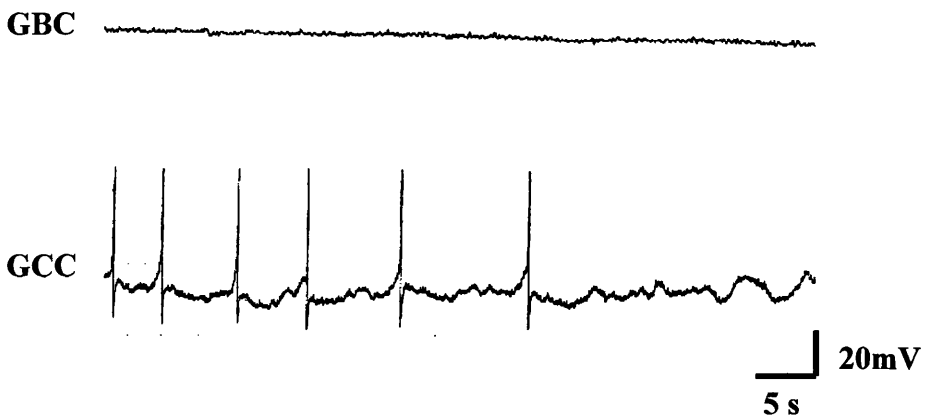
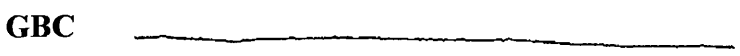
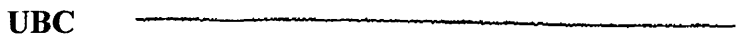


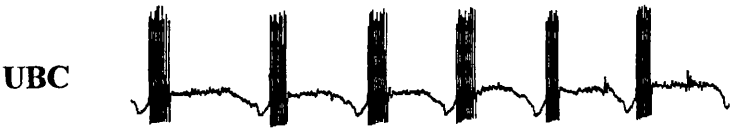
Figure 60. Citral initiates burst firing in a GBC and an unidentified cell (UBC) in the buccal ganglia (recorded simultaneously). A) Control. B) 100 s after 5 x 10^{-4}M citral C) After 10 min saline wash.

A. Control

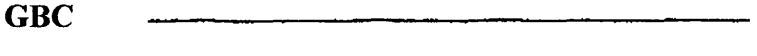


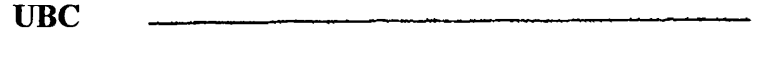


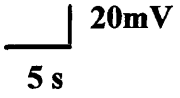
B. 100 s after 5 x 10^{-4}M citral



C. After 10 min saline wash







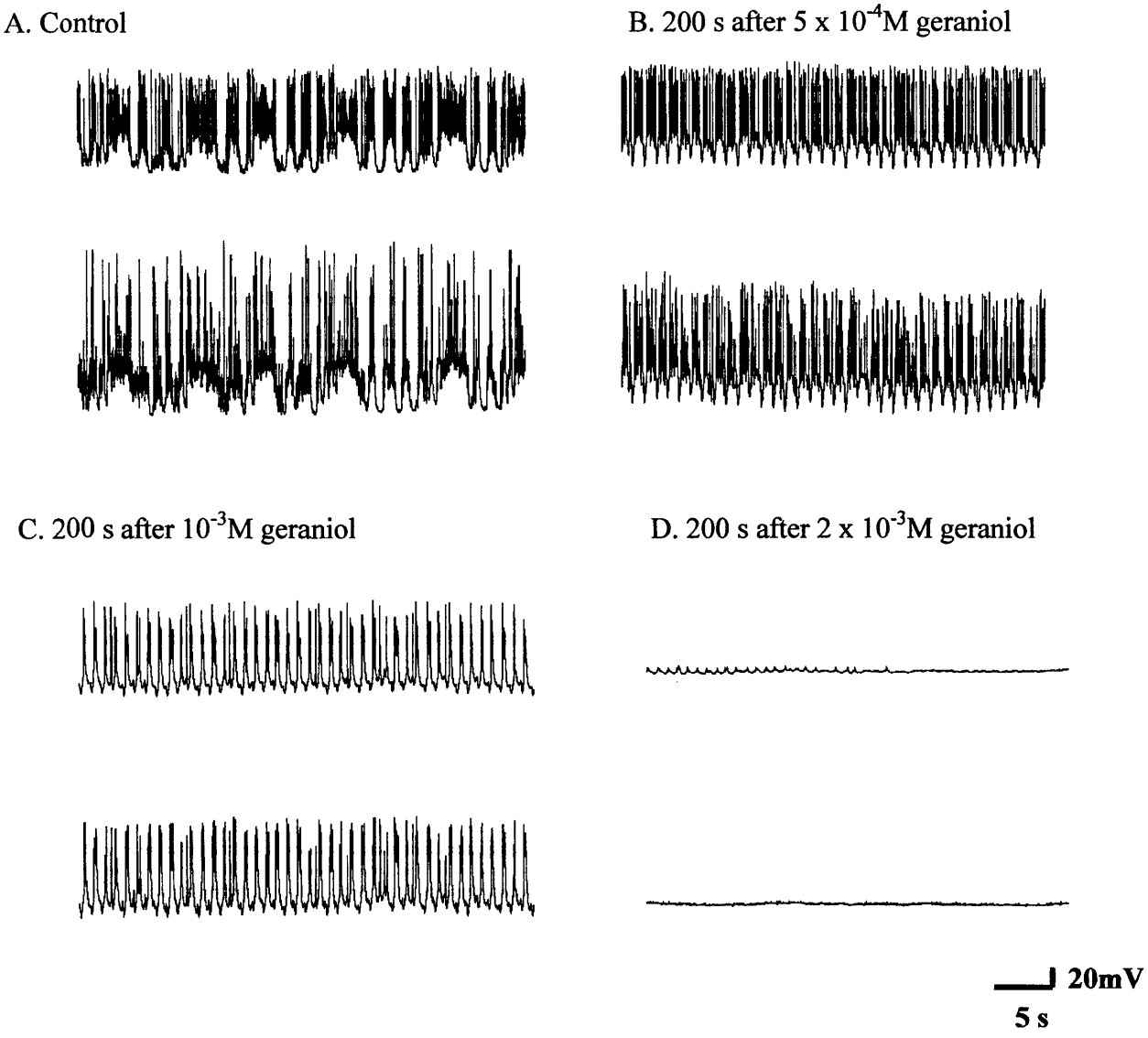
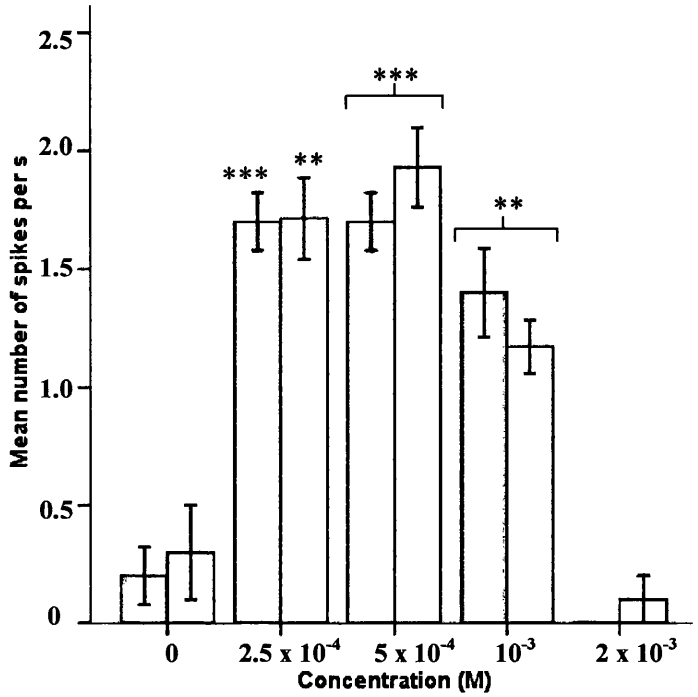


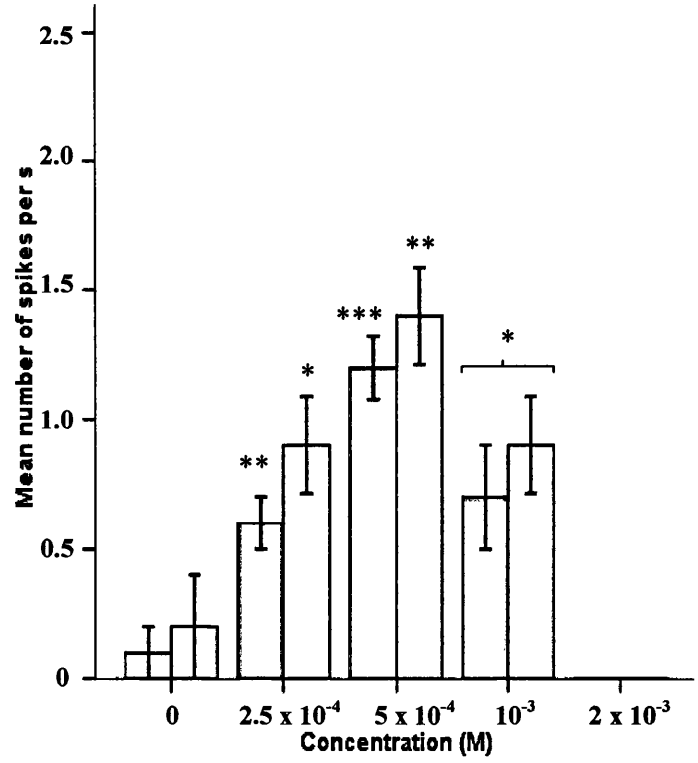
Figure 61. Response to geraniol of two unidentified buccal ganglion cells (recorded simultaneously). A) Control. B) 5 x 10^{-4}M geraniol increased burst frequency in both cells. C) Spike bursts became shorter in response to 10^{-3}M geraniol. D) 2 x 10^{-3}M abolished spikes, although small membrane potential fluctuations remained. Variation in spike amplitude was common.

Figure 62. Bar charts showing effect of geraniol (A) and citral (B) on mean (±SE) spontaneous impulse frequency for quiescent or slowly firing (less than 0.5 spikes per s) buccal ganglion cells of *L. stagnalis* and *P. corneus* (N = 5 for each preparation). *Significantly different from control, $P<0.05$; **$P<0.01$; ***$P<0.001$. All data recorded 200 s after oil application.

A. Geraniol



B. Citral



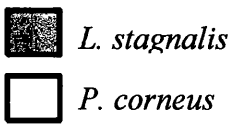
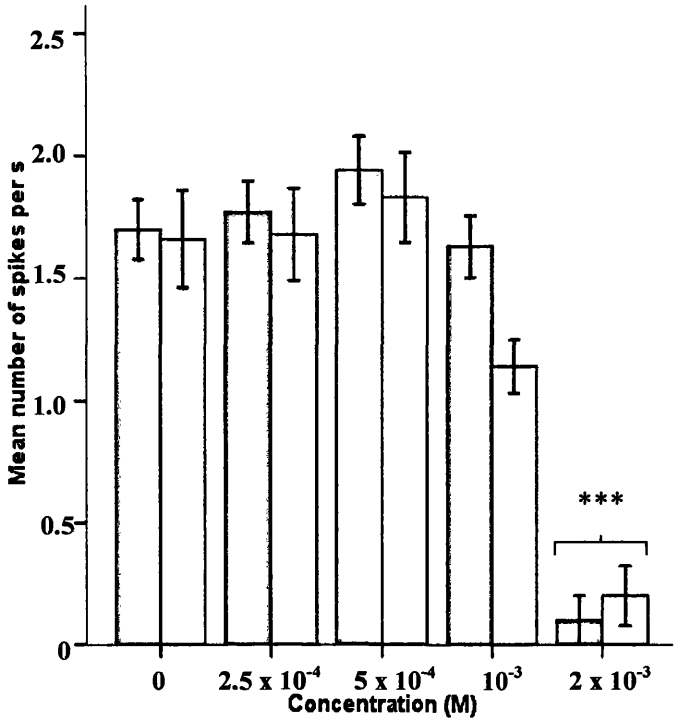
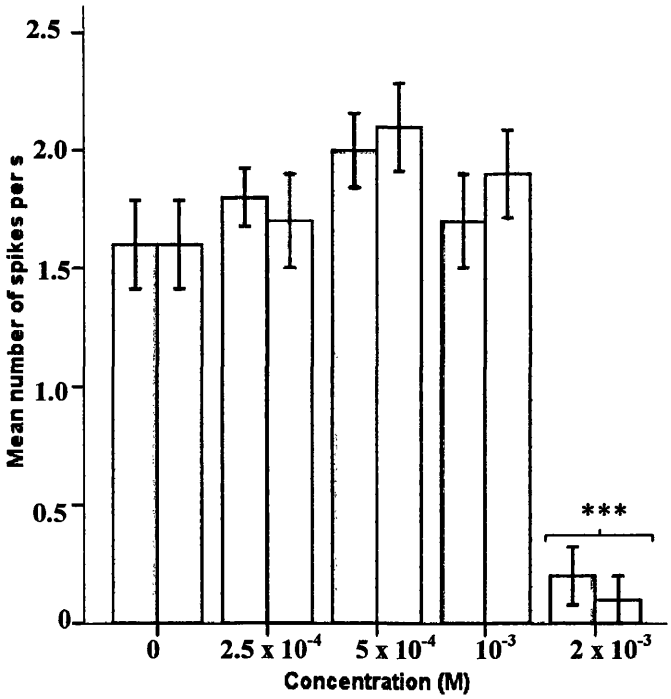


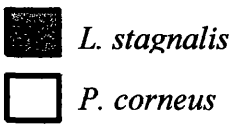
Figure 63. Bar charts showing effect of geraniol (A) and citral (B) on mean (±SE) spontaneous impulse frequency for buccal ganglion neurons firing in excess of 1 per s (N = 5 for each preparation). ***Significantly different from control, $P<0.001$. All data recorded 200 s after oil application. Although doses below 2×10^{-3}M did not significantly alter mean spike frequency, in every case the firing pattern changed from a fairly regular discharge to burst firing.

A. Geraniol

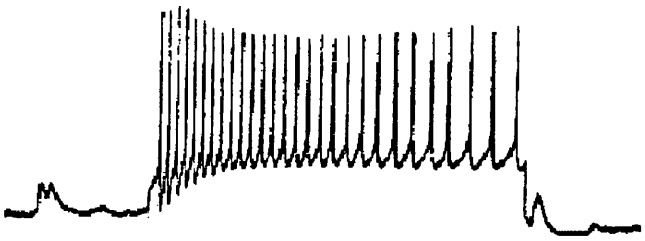


B. Citral

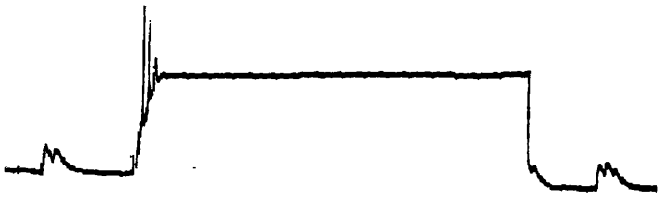




A. Control



B. 2 x 10^{-3}M citral



C. After 10 min saline wash

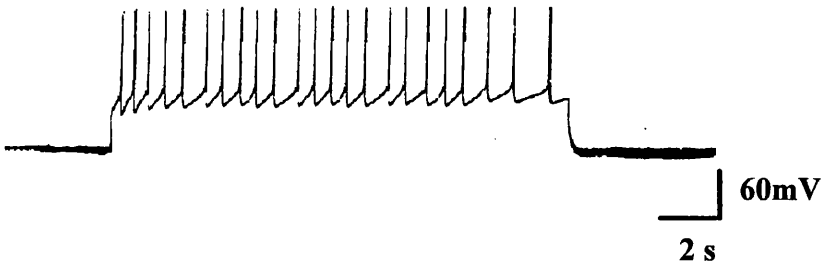


Figure 64. Citral reversibly inhibits spikes elicited by depolarizing current in a buccal ganglion cell of *L. stagnalis*. A) Control. B) After 2 x 10^{-3}M citral C) After a 10 min saline wash.

Effect of octopamine on spontaneous and evoked neuronal activity in buccal ganglion neurons

Perfusion of octopamine (threshold ca. 10^{-6}M) depolarized and activated most neurons that were recorded in the buccal ganglia. Octopamine generally produced burst firing in quiescent (Figure 65, Page 183) and spontaneously active cells (Figure 66, Page 184), similar to that produced by citral and geraniol. As with citral and geraniol, a significant increase in overall spike frequency only occurred in quiescent or slowly firing cells ($P<0.01$) (Figure 67, Pages 185-186).

In the GBC, however, octopamine produced a hyperpolarization of 10-15mV (Figure 65, Page 183). If not already present, large IPSP's were produced, progressively decreasing in amplitude with the hyperpolarization; these were usually in phase with IPSPs in other buccal ganglion neurons that were induced to burst fire. Octopamine (threshold ca. 10^{-6}M) decreased spike frequency in spontaneously firing GBCs and reversibly inhibited spikes elicited by a depolarizing current (the latter is shown in Figure 68, Page 187).

Application of octopamine to GBCs by iontophoresis also hyperpolarized them (Figure 69, Page 188), suggesting a direct effect. In any one buccal ganglion as many as 4 cells (in addition to the GBC) were found to hyperpolarize in response to iontophoresed octopamine, but no depolarizing responses could be found, suggesting that the excitatory effects of perfused octopamine were indirect.

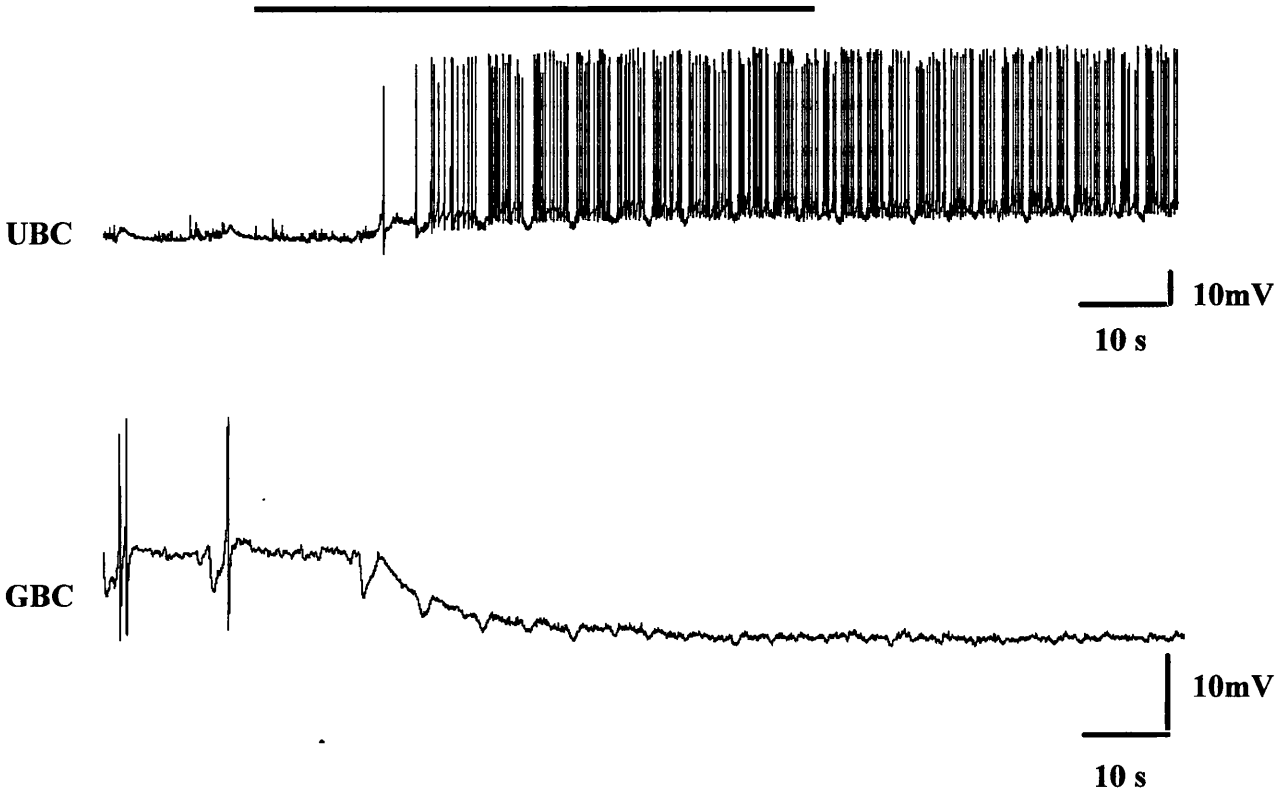


Figure 65. Octopamine activates a quiescent unidentified buccal ganglion cell, inducing burst firing (UBC, top trace), and inhibits a GBC (bottom trace) recorded simultaneously. The bar above the recordings represents the period of application. Note that the IPSPs in the GBC are in phase with those in the UBC.

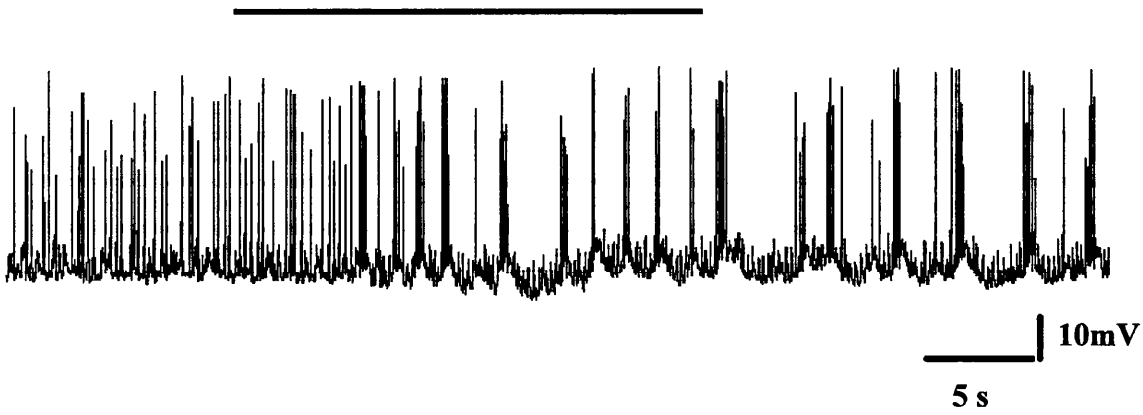
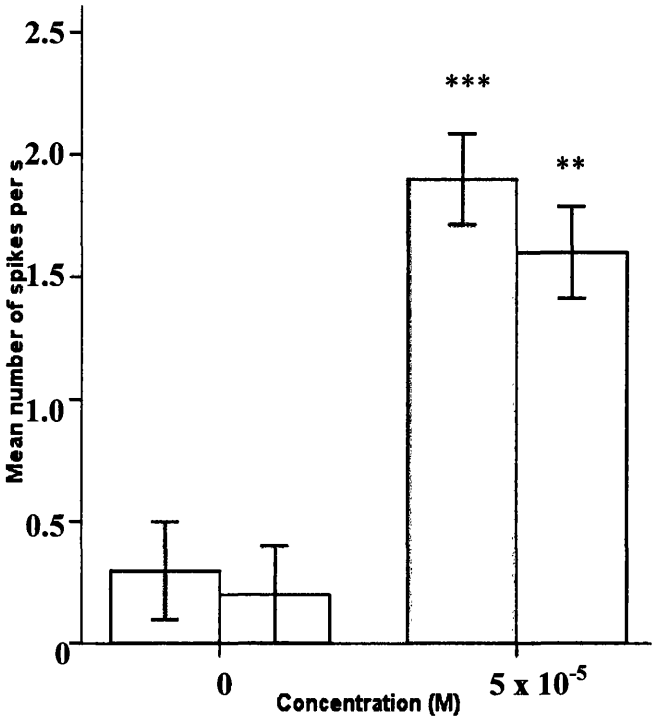


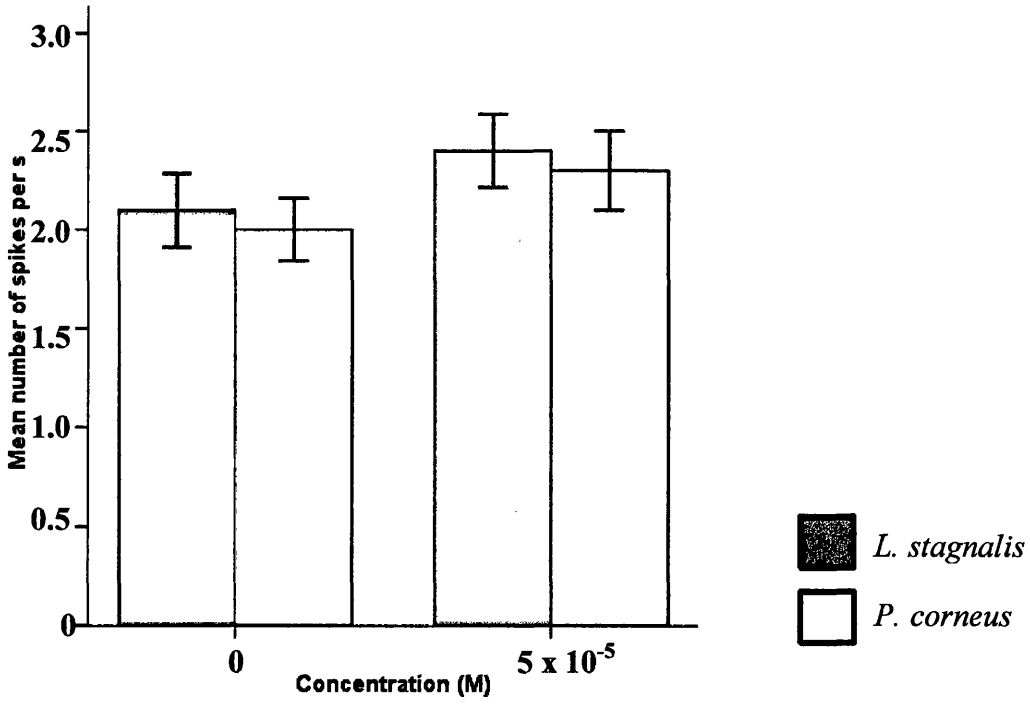
Figure 66. Octopamine (5 x 10^{-5}M) initiates burst firing in an active, unidentified buccal ganglion cell (period of application represented by bar). Variation in size of action potentials is typical for some buccal ganglion neurons; see Arshavsky et al (1988).

Figure 67. Bar charts showing effect of octopamine on mean (±SE) spontaneous impulse frequency for quiescent (A) and active (B) unidentified buccal ganglion neurons of *L. stagnalis* and *P. corneus* (N = 5 for each preparation). **Significantly different from control, P<0.01; ***P<0.001. All data recorded 10 s after octopamine application.

A. Quiescent



B. Active



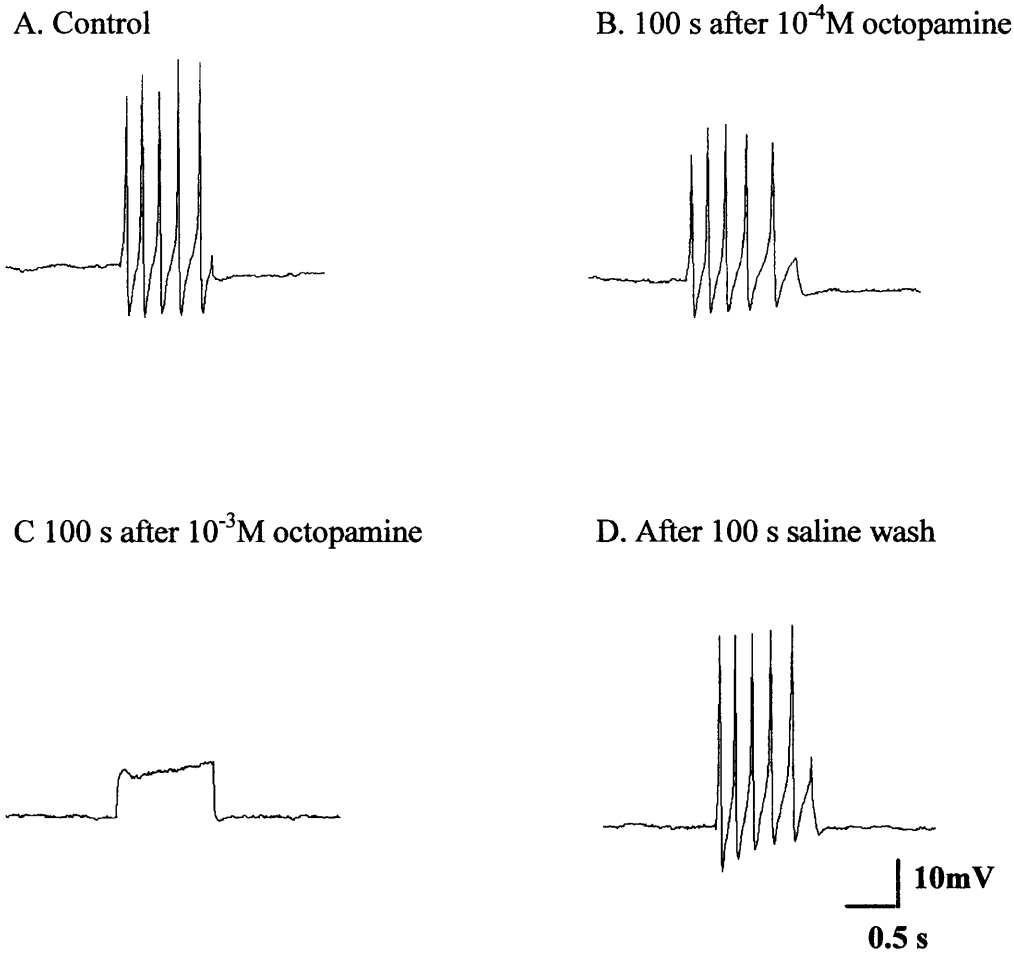


Figure 68. High doses of octopamine reversibly inhibit spikes elicited by depolarizing current in a GBC. A) Control. B) 100 s after 10^{-4}M octopamine. C) 100 s after 10^{-3}M octopamine D) After a 100 s saline wash. The membrane potential was reset to the original level by applied current in B and C.

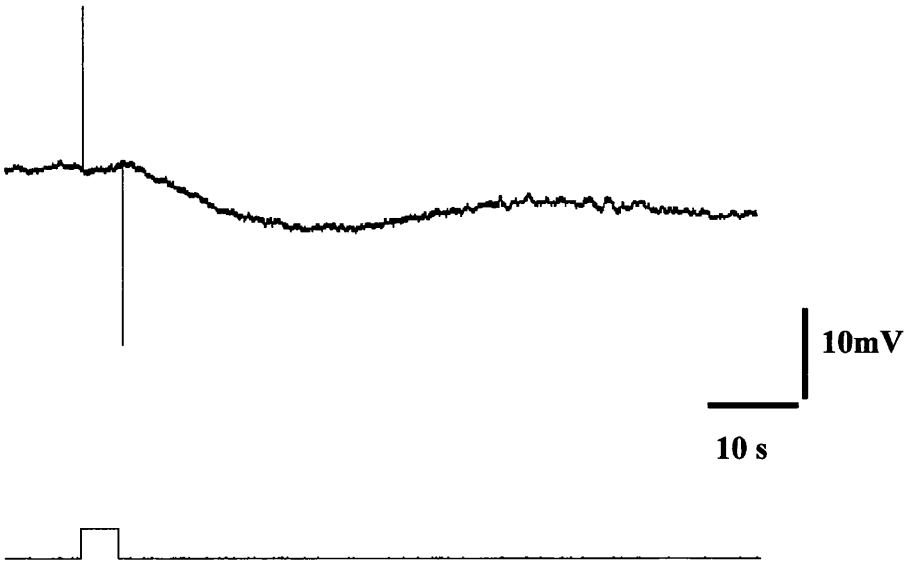


Figure 69. Iontophoresis of octopamine causes a GBC to hyperpolarize (upper trace; lower trace shows current pulse).

Effect of the oils on responses of buccal ganglion neurons to octopamine

None of the oils mimicked octopamine in hyperpolarizing the GBCs, indicating a lack of agonist activity. The oils were tested for octopamine receptor antagonism by observing their effects on the response of the GBCs to octopamine.

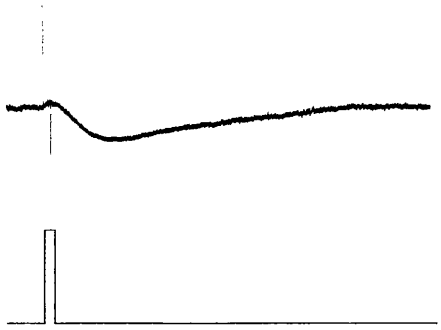
Submillimolar doses of eugenol had no effect on the hyperpolarizing response of GBCs to octopamine applied *via* perfusion or iontophoresis. Higher doses (ca. 10^{-3}M) reduced the response (shown for iontophoretically applied octopamine, Figure 70, Pages 190-191), though these doses also reduced spike frequency. Total block occurred at 2×10^{-3}M (not shown).

Similarly for citral and geraniol, doses below 10^{-3}M had no effect on the response of GBCs to octopamine, even when the cell was burst firing as a result of oil application (Figure 71, Pages 192-193). High doses (10^{-3} - 2×10^{-3}M) of geraniol and citral reduced the response in a similar way to eugenol (results not shown).

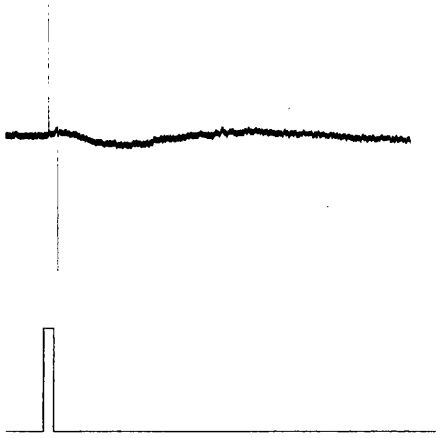
In other buccal ganglion cells, excitatory responses to octopamine were reduced by doses around 5×10^{-4}M and totally blocked by 10^{-3}M eugenol (shown for octopamine applied *via* perfusion, Figure 72, Pages 194-195; depolarizing responses to iontophoretically applied octopamine were not found). In more active buccal ganglion cells, 5×10^{-4}M eugenol had no effect on the octopamine-induced excitation; a block of excitation occurred with 10^{-3}M, but spontaneous spike frequency was also reduced or blocked.

Figure 70. High doses of eugenol reduce the hyperpolarizing response of a GBC to iontophoretically applied octopamine. A) Control. B) Response in the presence of 10^{-3}M eugenol. C) Response after washout of eugenol. Lower trace in each case shows iontophoretic current.

A. Control



B. Response in 10^{-3}M eugenol



C. After 30 min saline wash

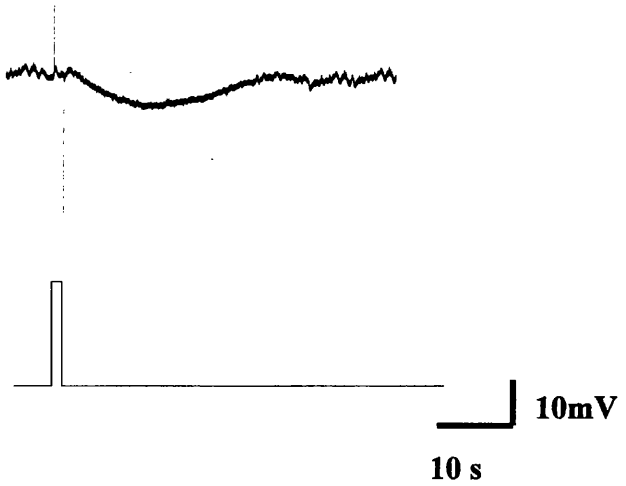


Figure 71. The response of a GBC to iontophoretically applied octopamine remains in the presence of doses of geraniol that elicit 'burst-firing'. A) Control. B) Response in the presence of 5 x 10^{-4}M geraniol. Lower trace in each case shows iontophoretic current.

A. Iontophoretically applied octopamine

B. Iontophoretically applied octopamine in the presence of 5×10^{-4}M geraniol

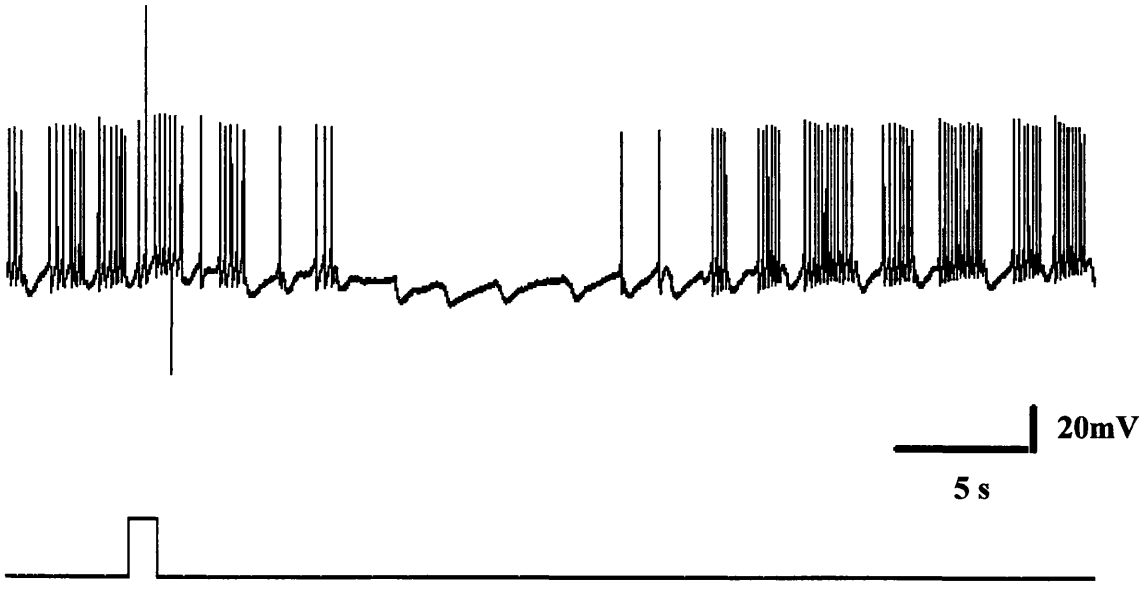
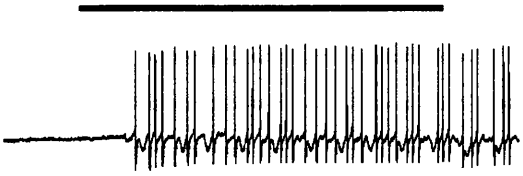


Figure 72. High doses of eugenol block the excitatory response of a buccal ganglion cell to 5 x 10^{-5}M octopamine applied *via* perfusion. A) 5 x 10^{-5}M octopamine. B) After a 10 min saline wash. C) 5 x 10^{-4}M eugenol. D) 5 x 10^{-5}M octopamine plus 5 x 10^{-4}M eugenol (E) 5 x 10^{-5}M octopamine plus 10^{-3}M eugenol. Horizontal bar represents the period of octopamine application.

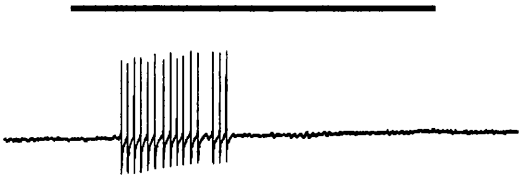
A. 5 x 10^{-5}M octopamine



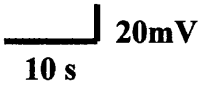
B. After 10 min saline wash

C. 5 x 10^{-4}M eugenol

D. 5 x 10^{-4}M eugenol plus 5 x 10^{-5}M octopamine



E. 10^{-3}M eugenol plus 5 x 10^{-5}M octopamine



Effect of octopamine antagonists on the response of buccal ganglion cells to octopamine

As the responses of the buccal ganglion cells to some of the oils were similar to those of octopamine (e.g. citral, geraniol and octopamine all produced a depolarization and initiated burst firing in buccal ganglion cells other than the GBC), the effects of octopamine antagonists, phentolamine, metoclopramide and yohimbine were examined to determine whether the oils target octopamine receptors. These drugs were tested first on responses to octopamine, to confirm their actions and to try to differentiate subtypes of octopamine receptor in the buccal ganglia.

Phentolamine

Phentolamine (threshold dose approximately 10^{-7}M) blocked the hyperpolarizing response of GBCs to octopamine (Figure 73, Page 198), usually converting it to an excitatory response (Figure 74, Page 199). This occurred whether octopamine was applied by perfusion or iontophoresis (Figures 73-75, Pages 198-201). Recovery was very slow, beginning at least 2 h after washout of phentolamine. Phentolamine (10^{-6}-10^{-3}M) had no effect on excitatory responses to octopamine in other cells tested (Figure 76, Page 202).

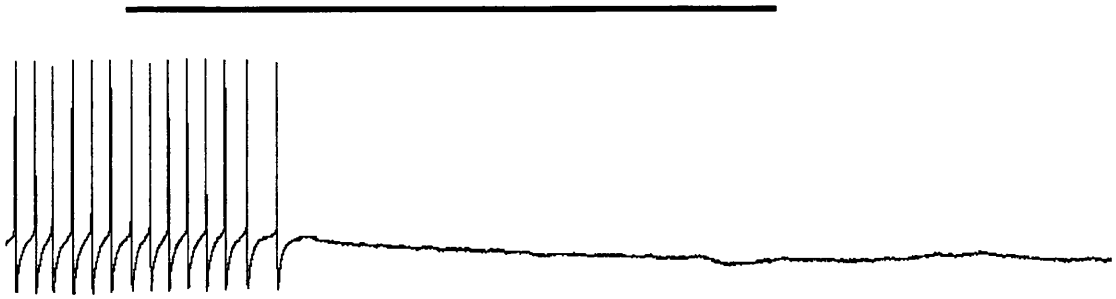
Yohimbine

Yohimbine had similar effects to phentolamine on octopamine responses but higher doses were required (ca. 5 x 10^{-4}M) (results not shown).

Metoclopramide

Metoclopramide (10^{-4}M) had no effect on hyperpolarizing octopamine responses in GBCs (N=3) but it blocked the initiation of burst firing normally caused by octopamine in other cells tested (N=3) (Figure 77, Pages 203-204). In the GBCs, metoclopramide (10^{-4}M) reduced or blocked the excitatory response to octopamine that occurred after administration of phentolamine (N=3) (Figure 78, Pages 205-206).

A. 5 x 10^{-5} M octopamine



B 5 x 10^{-5}M octopamine in the presence of 10^{-5}M phentolamine

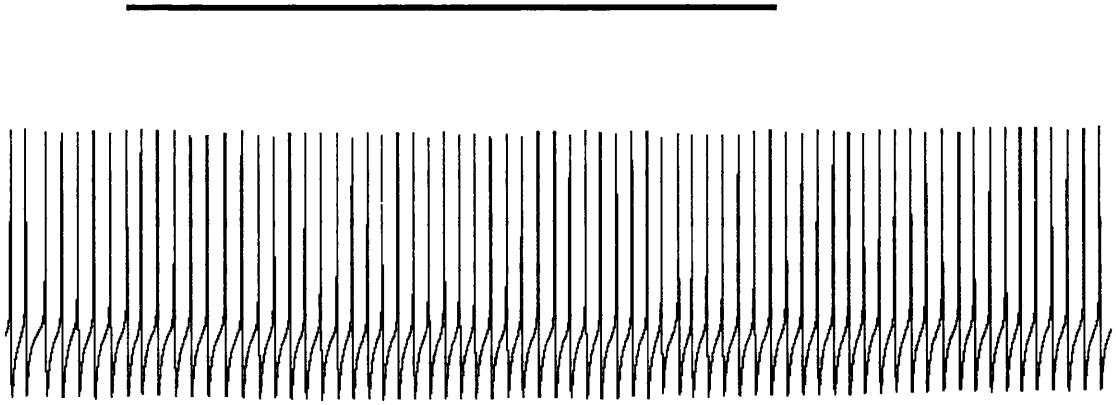
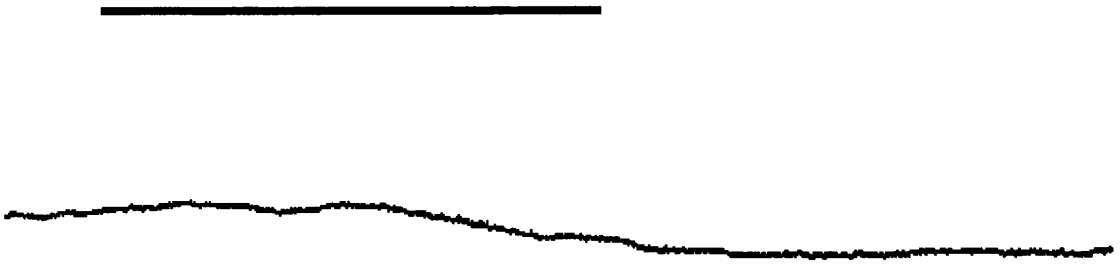


Figure 73. Phentolamine blocks the inhibitory response of a spontaneously firing GBC to octopamine. A) 5 x 10^{-5}M octopamine. B) 5 x 10^{-5}M octopamine in the presence of 10^{-5}M phentolamine (phentolamine was added 10 min before the introduction of octopamine). Perfusion of octopamine is shown by the horizontal bars above the recordings. There was no recovery of the response to octopamine 2 h after washout of phentolamine (not shown).

A. 10^{-4}M octopamine



B. 10^{-4}M octopamine in the presence of 10^{-5}M phentolamine

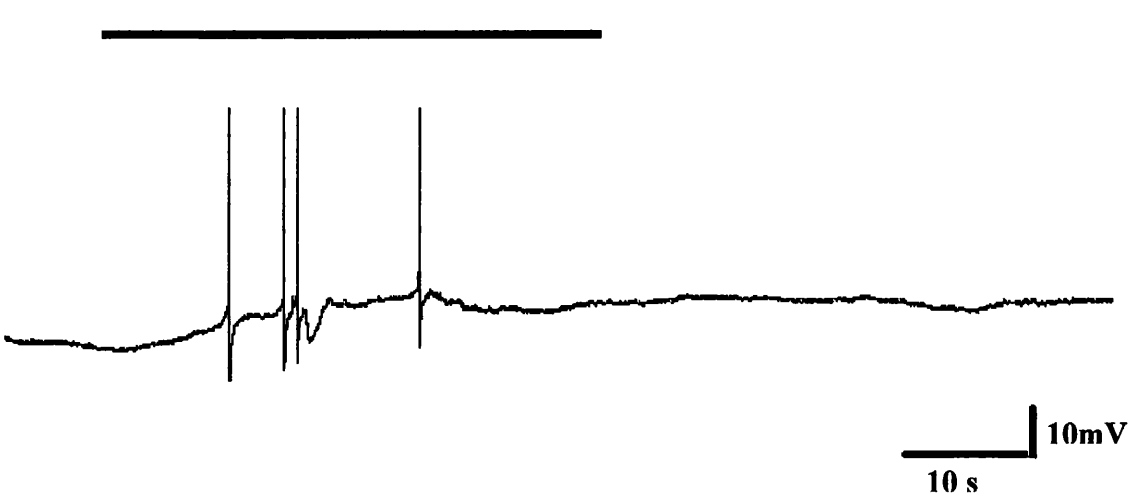
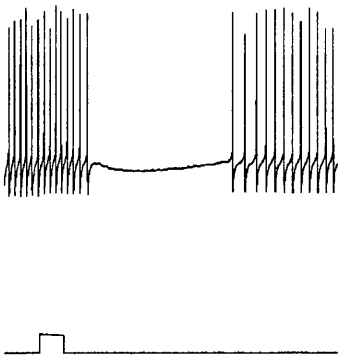


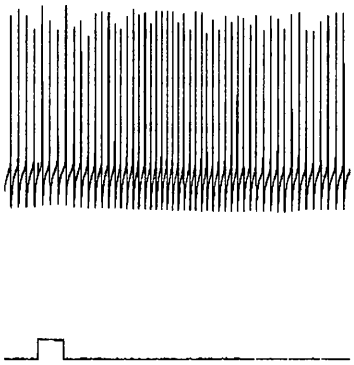
Figure 74. Phentolamine reverses the response of a GBC to octopamine applied by perfusion. A) 10^{-4}M octopamine. B) 10^{-4}M octopamine in the presence of 10^{-5}M phentolamine (introduced 10 min earlier). Horizontal bars represent period of octopamine application.

Figure 75. Phentolamine blocks the hyperpolarizing response of a GBC to iontophoretically applied octopamine. A) Control. B) Response in the presence of 10^{-5}M phentolamine. C) Response in the presence of 10^{-5}M phentolamine; injection current raised. Note the change from inhibition to excitation in the presence of phentolamine.

A. Iontophoretically applied octopamine

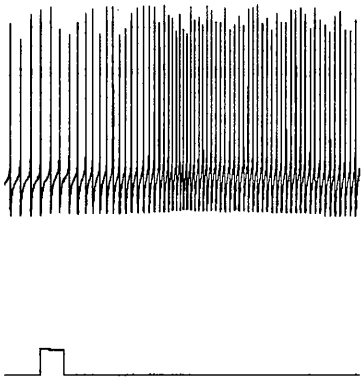


B. Iontophoretically applied octopamine in the presence of 10^{-5}M phentolamine

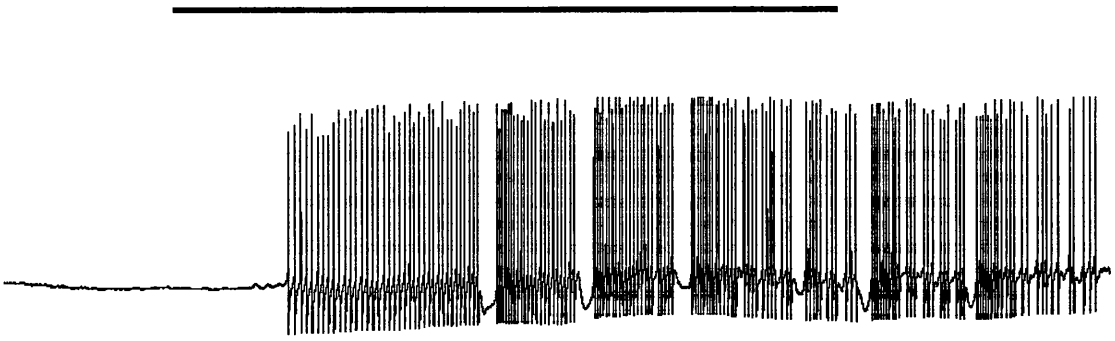


C. Iontophoretically applied octopamine in the presence of 10^{-5}M phentolamine;

current raised



A. 5 x 10^{-5}M octopamine



B. 5 x 10^{-5}M octopamine in the presence of 10^{-5}M phentolamine

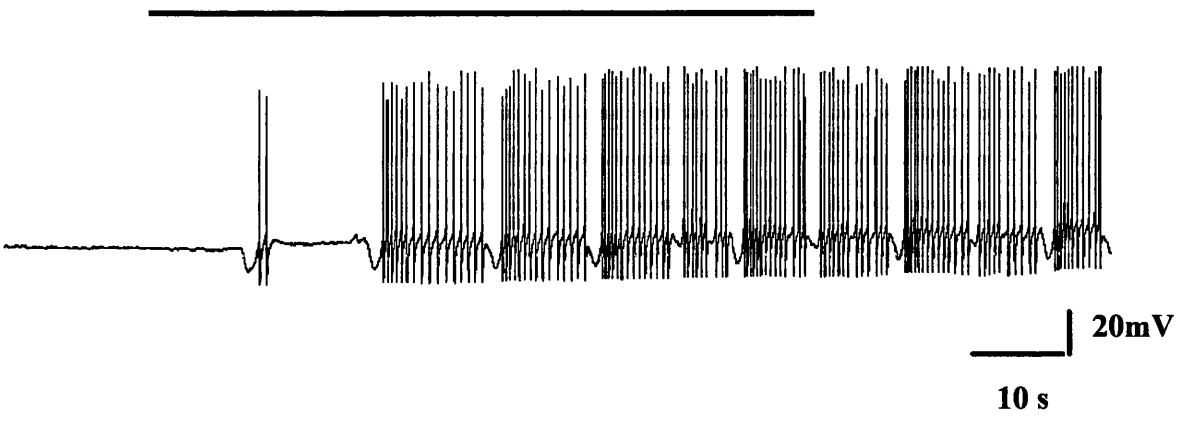
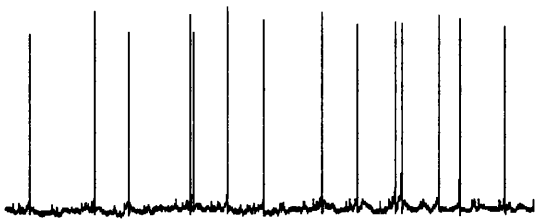


Figure 76. Phentolamine has no effect on the excitatory response of buccal ganglion cells to octopamine. A) Response of an unidentified neuron to 5 x 10^{-5}M octopamine. B) 5 x 10^{-5}M octopamine in the presence of 10^{-5}M phentolamine (introduced 20 min earlier). Application of octopamine is shown by the horizontal line above the recordings.

Figure 77. Metoclopramide blocks the response to octopamine in an unidentified buccal ganglion cell. A) Control. B) 60 s after 10^{-4}M octopamine. C) After a 60 s saline wash. D) 60 s after 10^{-4}M octopamine plus 10^{-4}M metoclopramide. The cell had been exposed to metoclopramide for 20 min before the s application of octopamine (to allow time for any blocking action to develop and to avoid desensitization to octopamine).

A. Control



B. 60 s after 10^{-4}M octopamine

C. After 60 s saline wash

D. 60 s after 10^{-4}M octopamine in the presence of 10^{-4}M metoclopramide

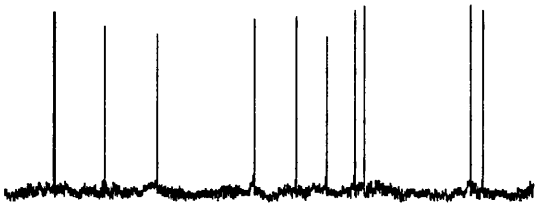
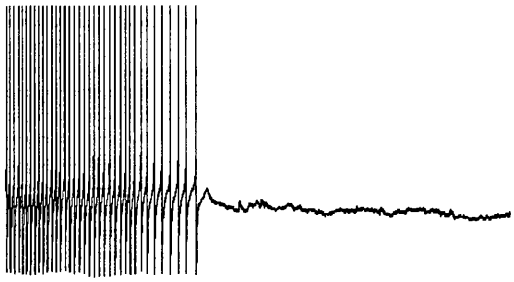
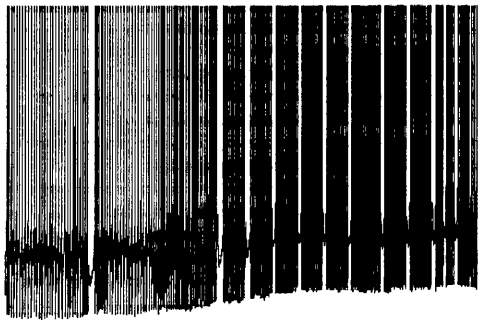


Figure 78. Metoclopramide reduces the excitatory response of a GBC to perfusion of octopamine in the presence of phentolamine. A) 5 x 10^{-5}M octopamine hyperpolarizes the GBC. B) 15 min after addition of 10^{-5}M phentolamine, 5 x 10^{-5}M octopamine produces excitation. C) 10^{-4}M metoclopramide (plus 10^{-5}M phentolamine) reduces the response to 5 x 10^{-5}M octopamine. Horizontal bars represent period of application of octopamine.

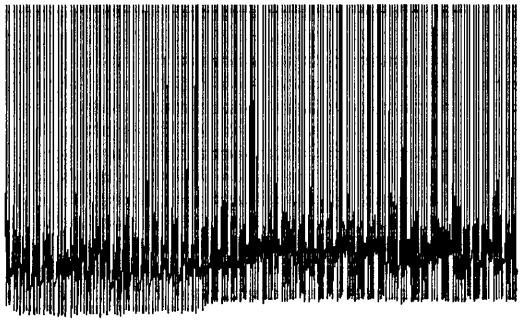
A. 5 x 10^{-5}M octopamine



B. 5 x 10^{-5}M octopamine in the presence of 10^{-5}M phentolamine



C. 5 x 10^{-5}M octopamine in the presence of 10^{-5}M phentolamine plus 10^{-4}M metoclopramide



Effect of octopamine antagonists on the response of buccal ganglion cells to the oils

Phentolamine

Phentolamine had no effect on the response to eugenol, citral or geraniol (shown for eugenol and geraniol; Figures 79 and 80, Pages 208-210). The threshold dose for blocking hyperpolarizing responses to octopamine was about 10^{-7}M, while concentrations as high as 10^{-3}M were ineffective on responses to the oils.

Yohimbine

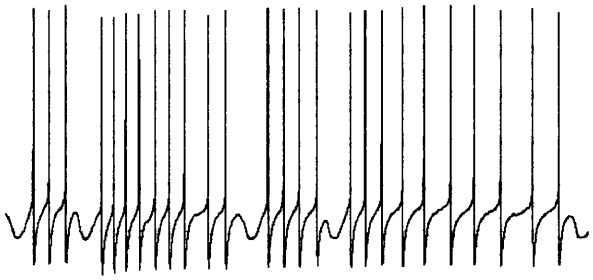
Yohimbine (10^{-6} to 10^{-3}M) also had no effect on the response to eugenol, citral or geraniol (results not shown).

Metoclopramide

Metoclopramide, which blocked depolarizing rather than hyperpolarizing responses to octopamine, had no effect on any of the responses to the oils (shown for geraniol, Figure 81, Pages 211-212) up to doses of 10^{-3}M (N=3 for each oil).

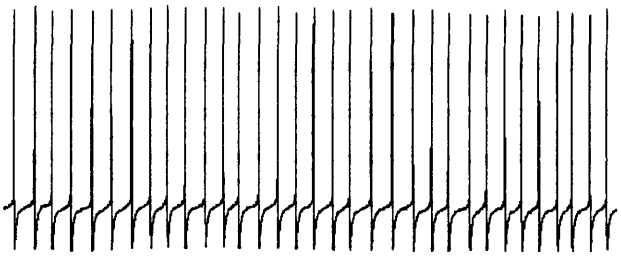
Figure 79. Phentolamine has no effect on the response of a GBC to eugenol. A) Control. B) 10^{-3}M eugenol. C) After 10 min washout of eugenol. D) 10^{-3}M eugenol in the presence of 10^{-5}M phentolamine. The phentolamine was added 20 min before the second application of eugenol.

A. Control



B. 200 s after 10^{-3}M eugenol

C. After 10 min saline wash



D. 200 s after 10^{-3}M eugenol in the presence of 10^{-5}M phentolamine

10mV

2 s

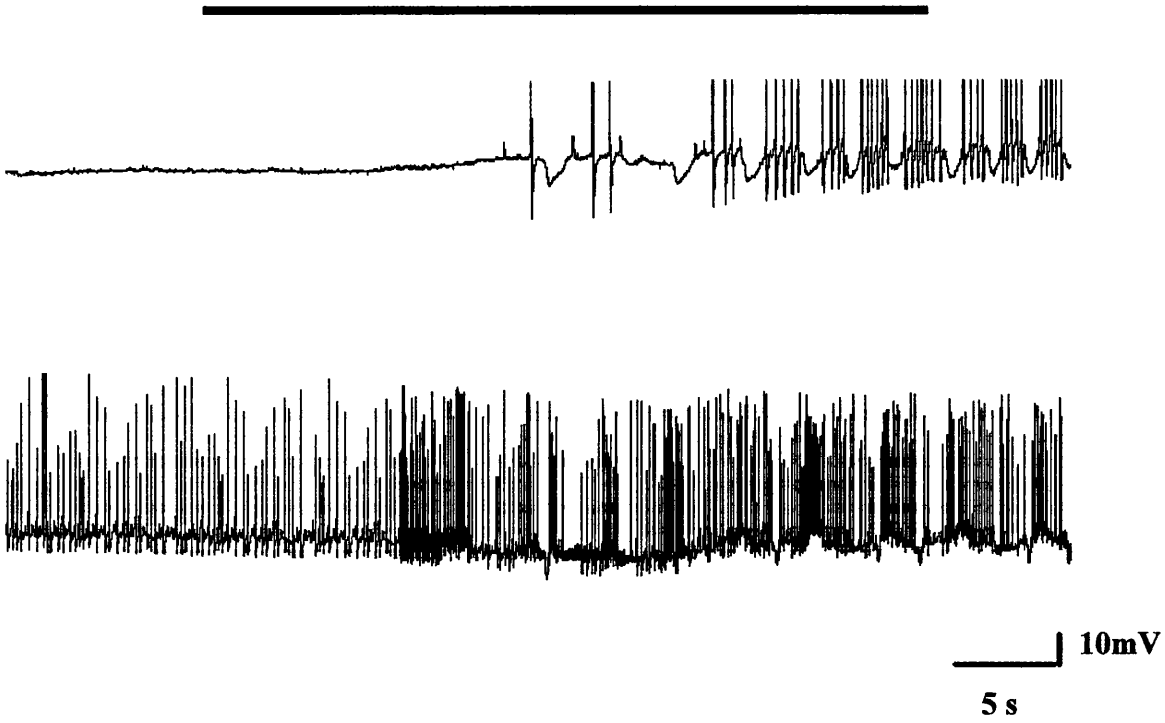
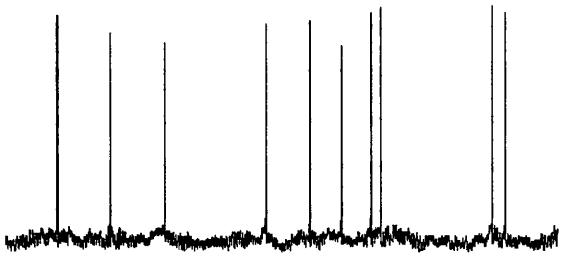


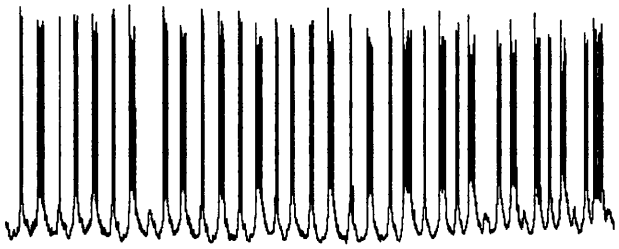
Figure 80. Phentolamine (10^{-5}M) does not block the response to 5 x 10^{-4}M geraniol (initiation of burst firing) in a GBC (top trace) or an unidentified buccal ganglion cell (recorded simultaneously). Horizontal bar at top of trace indicates the period of geraniol application. Phentolamine was added 20 min before the introduction of geraniol.

Figure 81. Metoclopramide (10^{-4}M) does not block burst firing initiated by geraniol. A) Control. B) Burst firing in response to perfusion of 5 x 10^{-4}M geraniol. C) Recovery after removal of geraniol. D) Geraniol continues to elicit burst firing in the presence of 10^{-4}M metoclopramide (metoclopramide was added 30 min before the second perfusion of geraniol).

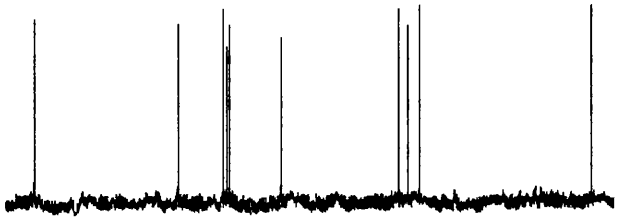
A. Control



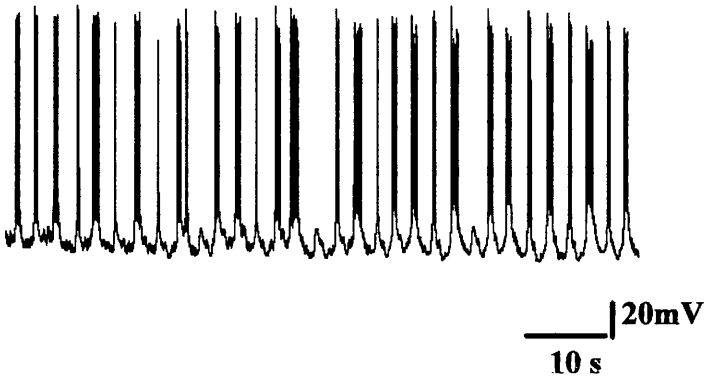
B. 60 s after 5 x 10^{-4}M geraniol



C. After 10 min saline wash



D. 100 s after 5 x 10^{-4}M geraniol and 10^{-4}M metoclopramide



Effect of the oils on the response of buccal ganglion neurons to ACh

Depolarizing responses to iontophoretically applied ACh were reversibly reduced or abolished by all three oils at 5 x 10^{-4}M (N=3) (Figure 82, Pages 214-215).

Effect of the oils on the response of visceral ganglion neurons to dopamine

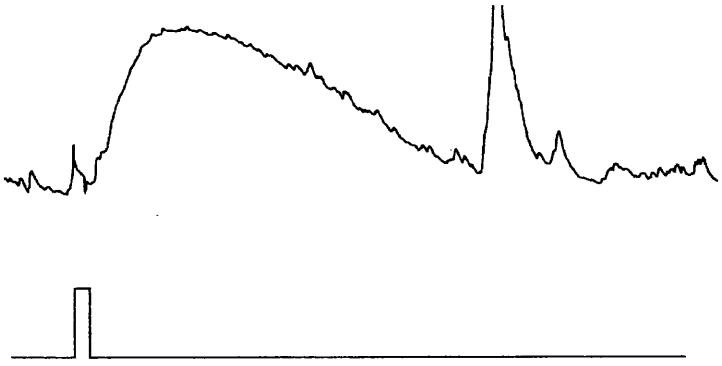
Depolarizing responses of visceral ganglion neurons to iontophoresed dopamine were reduced by the oils at doses around 10^{-3}M (Figure 83, Pages 216-217).

Effects of the oils on electrical coupling

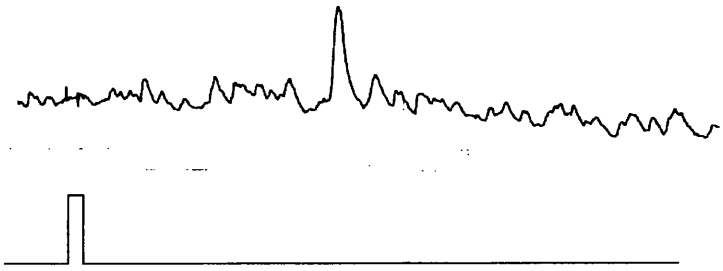
Millimolar concentrations of the oils had no effect on electrical coupling between visceral ganglion neurons (N=3) (Figure 84, Page 218).

Figure 82. The oils block the depolarizing response to iontophoretically applied ACh. A) Control response (iontophoretic current is shown in the lower trace). B) Response is abolished in the presence of 5 x 10^{-4}M eugenol. C) Partial recovery 10 min after removal of citral.

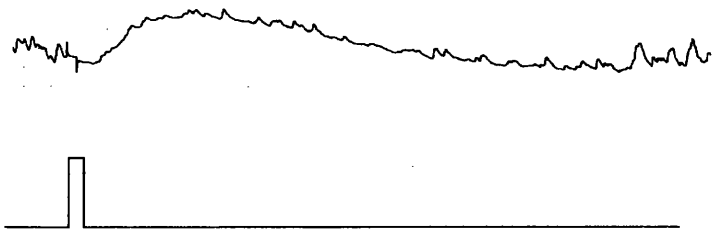
A. Control



B. After 5 x 10^{-4}M eugenol



C. After 10 min saline wash



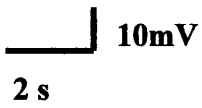
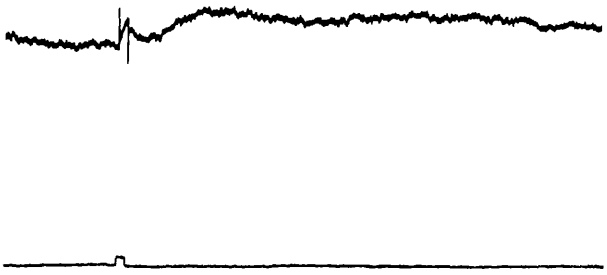


Figure 83. High doses of the oils reduce the depolarizing response of cells in the visceral ganglia to iontophoretically applied dopamine. A) Control response. B) Response in the presence of 10^{-3}M eugenol. C) Partial recovery after a 10 min saline wash.

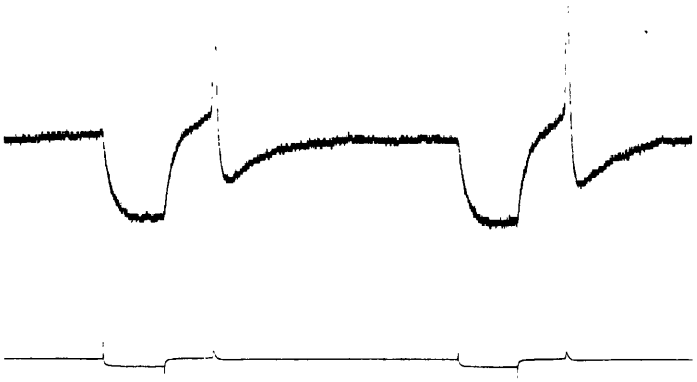
A. Control

B. Response in 10^{-3}M eugenol



C. Partial recovery

A. Control



B. 200 s after 2 x 10^{-3}M eugenol

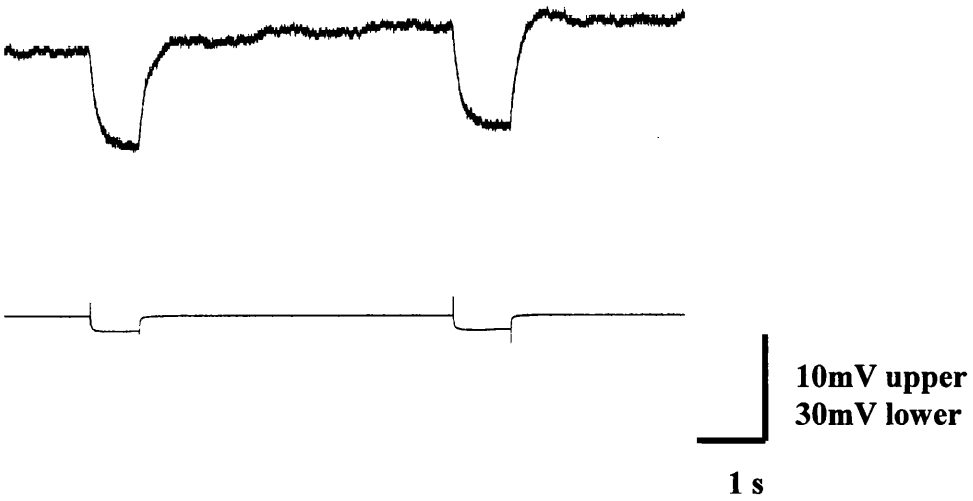


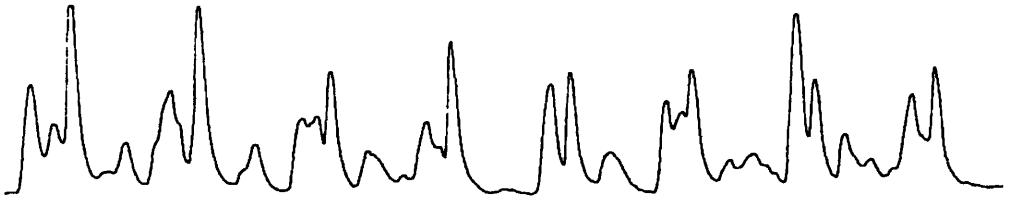
Figure 84. The oils do not block electrical coupling between neurons in the visceral ganglia. A) Hyperpolarizing pulses applied to one neuron (lower) are transmitted to another (upper). B) 2 x 10^{-3}M eugenol did not block the coupling but blocked rebound spike production. The pulses were slightly larger in B to try to elicit rebound spikes; the transmitted pulses increased proportionately in size.

5.33 Effects of the oils on oesophageal contractions and movements of the buccal mass

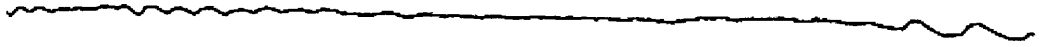
Experiments on semi-intact buccal mass-buccal ganglia preparations showed that geraniol and citral initiated dose dependent contractions of the buccal mass in quiescent preparations and increased the rate of contractions in spontaneously active preparations (threshold ca. 10^{-4}M). This effect lasted for several hours and was reversible on washing with saline. Eugenol tended to suppress any spontaneous activity.

In contrast to their stimulatory effects on the buccal mass and buccal ganglia, geraniol, citral and octopamine reduced spontaneous movements in the isolated oesophagus (shown for geraniol and octopamine; Figures 85 and 86, Pages 220 and 221 respectively). Eugenol had a similar effect (not shown). The threshold concentration was about 10^{-4}M for the oils and 10^{-5}M for octopamine (N = 5 in each case). The figures refer to experiments on *L. stagnalis*. The spontaneous contractions were variable between preparations and often complex; they continued practically unchanged for at least 3 h.

A. Control



B. 100 s after 5 x 10^{-4}M geraniol



C. After 5 min saline wash

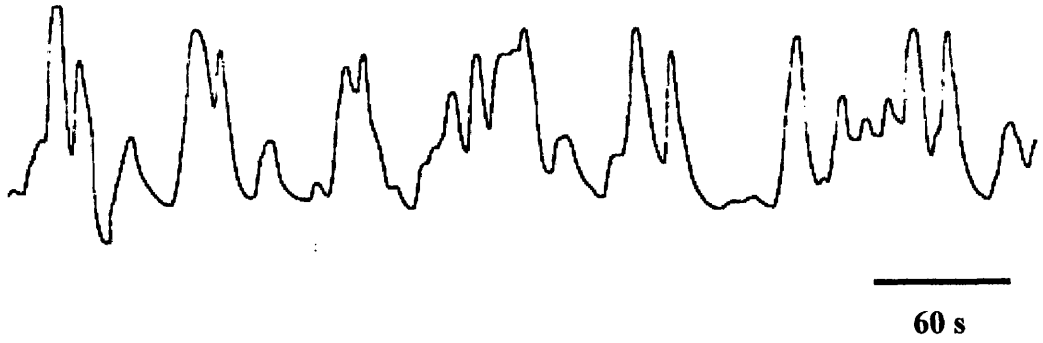


Figure 85. Geraniol reduces spontaneous oesophageal contractions. A) Control. B) 5 x 10^{-4}M geraniol. C) After a 5 min saline wash.

A. Control

B. 100 s after 10^{-5}M octopamine

C. After 5 min saline wash

Figure 86. Octopamine reduces spontaneous oesophageal contractions. A) Control. B) 10^{-5}M octopamine. C) After a 5 min saline wash.

5.40 Discussion

The buccal ganglia seemed a possible target for essential oil constituents if these compounds are indeed octopamine agonists, as suggested by Enan (2001; 2005b) and Kostyukovsky et al (2002). The buccal ganglia contain most of the octopamine in the molluscan nervous system (Hiripi et al, 1998), and are involved in the generation and modulation of the feeding rhythm. It was not surprising, therefore, to find that geraniol and citral (threshold ca. 10^{-4}M) both mimicked octopamine in initiating rhythmic movements of the buccal mass.

Extracellular recordings from the dorso-buccal nerves showed that citral and geraniol increased spike frequency and initiated regular bursts of impulse activity. These effects were similar to those of octopamine and contrasted with those of eugenol which did not usually initiate any activity apart from an occasional transient excitation.

Eugenol decreased spike frequency in most spontaneously firing buccal ganglion cells, and blocked or reduced the response to depolarizing current and rebound spikes following a hyperpolarizing pulse. This decrease in neuronal excitability is a possible cause of some of the characteristics of knockdown seen in intact snails in the previous chapter. Szabadics and Erdélyi (2000) also found that eugenol reversibly blocked synaptically evoked spikes in the terrestrial mollusc *Helix pomatia* at similar concentrations (9×10^{-4}M) to those in the current studies (5×10^{-4}). Some root canal sealers that are used in clinical dentistry contain eugenol and were shown by Asgari et al (2003) to reduce the duration and amplitude of action potentials, and the amplitude of after-hyperpolarizing potentials of neurons in the suboesophageal ganglia of *Helix aspersa*. These effects could be caused by blockage of sodium channel proteins which are targets for some local anaesthetics in

mammalian neurones (Scholz, 2002). They may also be the result of effects on calcium currents as described by Szabadics and Erdélyi (2000). In the current study, eugenol occasionally caused a transient increase in spike frequency, suggesting that it might have an excitatory effect at specific doses. No consistent excitatory response, however, was found with the range of doses used (10^{-8} to 2 x 10^{-3}M). An apparent biphasic pattern was found for some of the root canal sealers studied by Asgari et al (2003).

Geraniol and citral had similar effects on all cells tested in the buccal ganglia and also on the identified giant cell of the cerebral ganglia. In both silent and active preparations, rhythmic burst firing was initiated, characterized by a high frequency burst of spikes lasting for 1 to 5 s, usually followed by a large IPSP. Octopamine initiated a similar firing pattern to geraniol and citral in all cells apart from the GBC. Similar burst patterns have also been shown in various cells (e.g. B7, SO, B5 and N1M) by Vehovszky et al (1998) when octopamine was applied to the isolated buccal ganglia of *L. stagnalis*; they described it as the 'rhythmic pattern of fictive feeding'. Elliott and Vehovszky (2001) stated that approximately one third of isolated *L. stagnalis* CNS preparations produced this rhythmic pattern spontaneously and that it is similar to the sequence of activity seen during feeding in an intact snail. They showed that the pattern was modulated by stimulating the octopamine-producing OC interneuron (Vehovszky and Elliott, 2000; Elliott and Vehovszky, 2001). Rhythmic outputs caused by chemical application can result from alterations of intrinsic cell membrane properties or through effects on synaptic interactions (Hancox and Pitman, 1995).

The modulation of activity in the feeding network by the essential oil constituents may contribute to their antifeedant effects. In experiments to assess the

effects of the oils on rasping movements in intact animals, citral and geraniol clearly increased the movements in some animals (as they did in semi-intact buccal mass/buccal ganglia preparations), but contractions of the body wall musculature made observation of radula movements impossible in most animals. The oils may increase the frequency of movements, but they may change the normal pattern in a way that reduces food consumption. The commercially used molluscicide metaldehyde, has also been shown to induce bursting activity in motoneurons of the buccal ganglia of *L. stagnalis* (Mills et al, 1992). Perhaps such burst activity only superficially resembles normal activity, and disrupts the normal patterning.

Other actions of these oils (such as on the sensory system) might also contribute to their antifeedant properties, and it would be interesting to analyse their effects on the lips, tentacles and cerebral ganglia. Application of food substances to the lips causes burst activity (i.e. the feeding rhythm) in certain cells of the cerebral ganglia (Davis and Gillette, 1978; Kemenes et al., 2001; Whelan and McCrohan, 1996) and buccal ganglia (Vehovszky et al, 2004). It should be possible to determine whether the oils affect the ability of the snails to detect appetitive signals. It cannot be ruled out, however, that antifeedant effects are a consequence of stress caused by oil exposure.

Surprisingly, no depolarizing response of buccal ganglion neurons to iontophoresed octopamine was observed, though there seem to be few examples in the literature. Vehovsky et al (1998) studied effects of octopaminergic antagonists on hyperpolarizing responses to iontophoresed octopamine, but made no mention of depolarizing responses. Vehovszky and Elliott (2000a) later found that octopamine applied *via* iontophoresis caused a small depolarization (less than 5mV) in the B1 cell of *L. stagnalis.* The results of the current study suggest that most of the depolarizing

effects of octopamine that resulted in burst firing (when octopamine was applied by perfusion) were indirect, *via* activation of feeding interneurons that were not recorded. The effects of geraniol and citral appeared to be similarly indirect because if a cell firing in bursts (as a result of oil application) was hyperpolarized, the frequency of bursts remained the same, suggesting that it is being driven by other cells.

Octopamine hyperpolarized the GBCs, preventing burst firing (though spontaneous IPSP's were usually produced, in phase with those in burst-firing buccal ganglion neurons). In contrast, none of the oils hyperpolarized the GBCs, suggesting that they do not activate these hyperpolarizing octopamine receptors. The oils mimicked octopamine by blocking spontaneous firing of active GBCs and increasing spike threshold, but at high doses that are likely to be non-specific.

The effects of octopamine and relatively low doses of essential oils on other buccal ganglion neurons were similar to their effects on the nervous system of cockroaches, described in Chapter 3: Geraniol and citral (but not eugenol) mimicked octopamine in their excitatory effects and might therefore, activate excitatory octopamine receptors. This is further supported by the initiation of burst firing by octopamine, geraniol and citral, together with generation of rhythmic contractions of the buccal mass in semi-intact preparations. Also, octopamine and the oils all reduced contractions of the isolated oesophagus.

Phentolamine, which blocked hyperpolarizing responses to octopamine in the GBCs, did not affect the excitatory and burst-producing effects of octopamine or the two oils. Since concentrations as high as 10^{-3}M were ineffective on excitation, this shows a high specificity of the drug for octopamine receptors that mediate inhibition. In contrast to the situation regarding inhibition, no specific blocking drugs for octopamine-mediated excitation seem to have been described for molluscs.

Metoclopramide was tried as a potential antagonist for the excitatory octopamine receptors because in insects it blocks octopamine$_2$ receptors (Evans, 1981) whereas phentolamine is more specific for octopamine$_1$ receptors (Baines and Downer, 1994) (this receptor classification has now been revised - see Evans and Maqueira, 2005 – but does not affect the reason for choosing the two octopamine receptor antagonists). Metoclopramide did not block the excitatory responses to geraniol and citral at doses that were at least ten times higher than those that blocked the same responses to octopamine. This suggests that the oils do not produce their effects by activation of excitatory octopamine receptors.

With the exception of the GBCs, the excitatory effects of the oils or octopamine appeared to be indirect on the cells that were recorded, presumably acting *via* neurons of groups 1 and 2, which largely generate the feeding rhythm (Arshavsky et al, 1988). Phentolamine, however, seemed to offer the possibility of studying direct excitatory effects on the GBCs. The drug reversed the hyperpolarizing effect of octopamine, producing depolarization and excitation, which appeared to be direct responses because they were obtained by local, iontophoretic application. This suggests that hyperpolarizing and depolarizing receptor types co-exist on the GBCs, but the latter is normally masked by the former (signs of biphasic responses were occasionally seen, and in two GBCs the response to octopamine was purely excitatory). Again, depolarizing responses to citral and geraniol were unaffected by much higher doses of metoclopramide than those needed to block the depolarizing response to octopamine. This suggests that the oils do not activate either of the two types of octopamine receptor, though it does not rule out activation of other octopamine receptor subtypes; octopamine receptors in molluscs have not been studied in the same detail as those in insects, where four receptor subtypes have been

identified (Gerhardt et al, 1997). Effects on the oesophagus warrant further study in view of the similar inhibitory actions of octopamine and the oils.

Alternatively, the oils might target molluscan octopamine receptors as antagonists rather than agonists. They did block hyperpolarizing responses to octopamine, but also to dopamine, and only at doses above 10^{-3}M (when they started to block impulse generation); they were actually more effective at blocking responses to ACh. Any action on octopamine receptors is thus likely to be non-specific. Antagonism of depolarizing responses to octopamine was difficult to detect because geraniol and citral mimicked octopamine. Eugenol, however, did not block octopamine receptors mediating excitation at concentrations below 10^{-3}M.

The conclusions regarding mechanisms of (molluscicidal) action of essential oils on snails are similar to those for the cockroach. The relatively high concentrations of the oils needed to produce any effects indicate that a variety of non-specific actions could contribute to their molluscicidal properties, and that specific targeting of octopamine receptors is unlikely. Specific effects on particular octopamine receptors cannot be ruled out, but two octopamine receptor types were identified that were not influenced by the oils, either as agonists or antagonists. The sensitivity of the feeding network may contribute to the antifeedant and lethal effects of the oils, which would be expected to enter the animals from the water (irrespective of feeding) because of their lipophilic nature.

Garlic has molluscicidal properties (Singh and Singh, 1993), and the presumed active ingredient, allicin, was tested for comparison. In contrast to the oils (and metaldehyde) it had no electrophysiological effect on buccal ganglion neurons, even at doses as high as 3×10^{-2}M. Similar concentrations in the water had little effect on intact snails, apart from increasing mucus production.

In summary, geraniol and citral excite the buccal mass into a feeding-like rhythm of muscle contractions by direct activation of the buccal ganglia (since they initiate the associated rhythmic burst activity in isolated ganglia). Although this resembles fictive feeding initiated by octopamine, it might not represent the normal feeding pattern, and the oils do not appear to act on octopamine receptors.

Chapter 6

General conclusions

The essential oil constituents eugenol, citral and geraniol had insecticidal and molluscicidal effects, which were dose- and time dependent. They also had antifeedant and repellent activity in some cases, and may have potential for use in pest control programmes, probably to a greater extent in insects than aquatic molluscs owing to solubility limitations. Large scale trials are needed to assess the efficacy of these compounds in the field to determine if they have any real prospect of use as control agents or if they are only useful as relatively small-scale homeowner products.

The results were not in agreement with those of Enan (2001; 2005b) and Kostyukovsky et al (2002) in that there was little or no evidence that the oils were octopamine receptor agonists (or antagonists) in insects or molluscs. In insects, the discrepancy seems likely to result from different sub-types of octopamine receptor under investigation in the various studies. Nevertheless, the results do not support the claim by these authors (op. cit.) that essential oils cause death by a specific action on octopamine receptors, which in their experiments were activated by micromolar concentrations of oils. In the current study, near-millimolar concentrations were needed to kill insects and snails, and indeed to produce any electrophysiological effects, indicative of non-specific mechanisms of action that possibly involve multiple targets.

The neurophysiological experiments showed for the first time that essential oil constituents have electrophysiological effects on the nervous system of insects and on the buccal ganglia of molluscs, and these effects may explain some of the toxic symptoms seen in the intact animals. The effects of the monoterpenoids, geraniol and citral, were similar in cockroaches and snails (excitatory at low doses, inhibitory at high doses). Eugenol, a phenylpropenoid, tended to lack excitatory effects, probably a consequence of its different structure.

In both the insects and snails, low-dose excitatory effects of citral and geraniol were similar to those of octopamine, in agreement with an action as octopamine agonists, as shown for some essential oil constituents in biochemical studies (Enan, 2001; 2005b; Kostyukovsky et al, 2002). Experiments with octopamine antagonists were unsuccessful in cockroaches, but in snails they demonstrated that the two monoterpenoids were not agonists for excitatory or inhibitory octopamine receptors. Eugenol showed little similarity to octopamine in its effects in snails or cockroaches.

There were no antagonistic effects of the oils on the responses to octopamine in the nervous system of snails or cockroaches, except at high, non-specific doses. The results suggest that even if the oils do target a sub-type of octopamine receptor at micromolar concentrations, this is unlikely to be a major contributor to insecticidal or molluscicidal action.

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