The Effects of Postharvest Oils on Arthropod Pests of Citrus

by

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"...the action of white neutral oils may be ascribed almost entirely to suffocation."

de Ong, 1927.

"...even highly refined oil kills by virtue of its toxicity rather than suffocation.'

Ebeling, 1950.

"The mode of action of oils is mainly physical, but it may also be chemical. Suffocation is the most widely favored theory explaining how oils kill insects and mites."

Davidson et al, 1991

"I am confused!"

Taverner, 1996.

"I am less confused, but utterly amazed!

Taverner, 1999.

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Summary

Insect control using petroleum oils is historically based on the assumption that the mode of action is anoxia, ie. the oil blocks the tracheae and suffocates the insect. Many studies on petroleum spray oils against a range of pests indicate the greatest efficacy with higher molecular weight oils. Low molecular weight oils (< 300) are considered ineffective because they can be expelled from the tracheal system of some insects or are too volatile to persist for long enough to result in suffocation. Petroleum spray oils are predominantly narrow range oils with a 50% distillation temperature equivalent to a C21 to C24 alkane because they have demonstrated high insecticidal efficacy and are reasonably safe to plants.

This study investigated the effects of a number of petroleum and vegetable oils against citrus pests; the most interesting comparisons were between Ampol C15 CPD, a formulated C15 alkane, and Ampol C23 DC-Tron NR, a commercial petroleum spray oil. Importantly, CPD was shown to be much more efficacious in dips against citrus pests than DC-Tron. These products are quite different and their acute effects on the insects possibly relate to distinct physical characteristics. The former is a homogeneous C15 alkane with a molecular weight of 212. It is liquid at room temperature (alkanes with ≥16 carbon atoms are waxes at room temperature), and has a molecular volume of 278 ml $mole^{-1}$ at 15°C. In contrast, DC-Tron contains no alkanes and its C23 nomenclature refers to its mean equivalent *n*-paraffin carbon number; its 50% distillation temperature is the same as the boiling point of a C23 alkane. It has an average molecular weight of 350 and most molecules are paraffinic (% $C_P \geq 60\%$) C15 mono-cyclic molecules with side chains. Their average molecular volume is 417 ml $mole^{-1}$ at 15°C.

CPD was more efficacious than DC-Tron against the active stages of LBAM and mealybug. The alkane caused symptoms in treated insects that were inconsistent with anoxia. The oil dissolved the waxy coating of the mealybug exposing them to desiccation. LBAM larvae were rapidly immobilised and showed obvious signs of dehydration 2-4h after dipping. Additional symptoms, such as twitching of the prolegs and darking of the haemolymph suggested other systems might be disrupted due to contact with the oil.

Confocal microscopy shows that CPD can rapidly move down into the nerve ganglion via the tracheal system. Measurement of the spontaneous electrophysiological activity of the peripheral nerves of larvae indicated that oil directly affects the nervous system through contact, inducing a rapid onset of multiple nerve firing in peripheral nerves. The effect is likely to be due to the non-specific absorption of the oils on lipoprotein membranes resulting in the disruption of a highly specific process, such as selective membrane permeability to ion flow.

Petroleum spray oils are generally considered to produce a lethal effect on eggs by forming a physical barrier over the chorion to prevent the normal gaseous exchange. Oils with a molecular weight >320 volatilise little over a 24h period and are considered the best ovicides. CPD, with a molecular weight of 212, should be an inefficient ovicide due to its high volatility. However, despite high oil residue losses and a residue of < 3μg/cm^2 after 24h exposure, CPD is able to effectively kill LBAM eggs. This suggests that the mode of action of CPD on eggs may not be a physical barrier inhibiting gaseous exchange across the egg surface.

Petroleum spray oils are assumed to have no residual effect. However, LBAM larvae surviving oil dips showed reduced fecundity and fertility as adult females. DC-Tron significantly reduced fecundity whereas larvae treated with CPD produced egg numbers similar to controls. Treatment of larvae with either oil reduced the hatch of eggs they produced as adults. The rate of dissipation of DC-Tron from larvae is probably slower than the C15 alkane and this may have contributed to its increased effects.

The effectiveness of CPD can not be explained by the traditional paradigm that oils kill by suffocation and higher molecular weight hydrocarbons are more efficacious. Different emulsifiers are used in each product, which may have had some influence on the results. Emulsifiers applied either at high concentrations in emulsion with water or in formulation with neat oil, significantly increased insect mortality. However, the concentration, and thereby, the effect of emulsifiers alone would be highly diminished when applied as water/formulated oil emulsions. For this reason, and the chemical similarity of the emulsifiers generally used in oil formulation (ie., non-ionic surfactants) it is unlikely that emulsifiers have been primarily responsible for the different effects of each product on insects. Other factors must be contributing to the efficacy of the alkane and the chronic effects of both oils on insects. The application technique of dipping rather than spraying, the oil depositing characteristics of the formulations and/or an alternative mode of action may all influence the relative efficacy of the oils.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in my university or any other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Candidate's signature Date

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Publications

The following is a list of the journal and conference publications arising from the thesis:

Taverner P.D., Bailey P.T. and Roush R.T. 1998. Old myths and new oils: insecticidal oils in action. In: Pest Management – Future challenges. Proceedings of the 6th Australasian Applied Entomological Research Conference, Brisbane, Australia. (eds Zalucki M.P., Drew R.A.I. and White G.G.) Vol 2. Pp 186-193. The University of Queensland Printery.

Taverner P.D., Bailey P.T., Hodgkinson M. and Beattie G.A.C. 1999. Postharvest disinfestation of lightbrown apple moth, *Epiphyas postvittana*, with an alkane. Pesticide Science **55**: 1159-1166.

Taverner P.D. 1999. Drowning or Just Waving?- A Perspective on the Modes of Action of Petroleum Derived Oils against Arthropod Pests of Plants. Proceedings of Spray Oils Beyond 2000 – Sustainable Pest and Disease Management (in press)

Please note that some of the chapters are written in a 'stand alone' format for submission as manuscripts.

Chapter 1

Literature Review.

1.1 Composition of Crude Petroleum Oils

Crude oil is in the main a mixture of numerous hydrocarbons. The 4 hydrocarbon groups in petroleum oil are:

(1) Paraffins - A class characterised by the aliphatic (non-cyclic) chain structure and the type formula of C_nH_{2n+2}. An enormous number of isomers are possible, the number increasing with the size of the molecule. The higher the molecular weight, the higher the boiling point, specific gravity and viscosity.

(2) Naphthenes - Saturated ring hydrocarbons. In low boiling fractions, they are monocyclic and in high boiling fractions, they are polycyclic.

(3) Aromatics - Ring hydrocarbons with conjugated double bonds. The aromatics occur principally as benzene and its derivatives. Aromatic rings may be combined with naphalenic rings, but even one aromatic ring imparts aromatic properties to that compound. Cyclic hydrocarbons may have paraffinic side chains.

(4) Unsaturates - All hydrocarbons, aliphatic or cyclic, which have one or more active double or triple bonds. They are characterised by their reactiveness with halogens and their tendency towards oxidation.

In addition to the above groups, crude oil contains resinous compounds. These compounds remain in the higher boiling fractions and may contain oxygen, sulphur and nitrogen. Tar oils, containing toxic phenols, are more effective as insect ovicides than more refined oils.

Crude oils are usually designated as paraffin-based or asphaltic (naphthenic), depending on the content of paraffinic and naphthenic compounds. The majority of crudes do not belong to any distinct group and have intermediate properties.

The composition of oils has practical significance in the manufacture of spray oils. The presence of unsaturates in spray oils makes them more reactive. Oxidation of unsaturated hydrocarbons forms asphaltogenic acids, which injure plant foliage (Tucker 1936). Paraffinic spray oils have a much greater insecticidal efficacy than naphthenic oil on some insects, including San Jose scale, *Quadraspidiotus pernicious* (Chapman, Avens and Pearce 1944), red scale, *Aonidiella aurantii* (Maskell), (Riehl and Carman 1953) and citrus red mite, *Panonychus citri* (McGregor), (Riehl and Jeppsen 1953) and greater efficacy on the eggs of the oriental fruit moth, *Grapholita molesta* (Busck) and codling moth, *Cydia pomonella* L., (Chapman, Pearce and Avens 1943). Pearce and Chapman (1952) suggested that paraffinic oils have a better spreading coefficient on the older bark of deciduous trees, improving control of European red mite (*Panonychus ulmi* (Kock)) eggs.

1.2 Composition and Properties of Insecticidal Oils

1.2.1 The Manufacture of Spray Oils

Ebeling (1950) describes in detail the methods and features of distillation in the commercial production of spray oil. The principle of oil refining is that by heating petroleum crude to successively higher temperatures, vapours are given off which condense to form a series of products. These products differ in volatility, specific gravity, viscosity and molecular composition. In practice, crude oil is separated into different fractions by heating at high temperatures and allowing the vapours to ascend the fractioning tower. The higher the vapours ascend the lower their boiling temperature, with the gasolines the lightest fraction drawn off as

liquids. The next lightest fractions are kerosenes, followed by the fuel oils and gas oils, which have limited applications for spray oils. Most spray oils are derived from the next fraction, the lighter lubricating oils, which distil at 315^{0}C to 482^{0}C (at atmospheric pressure) or 191^{0}C to 274^{0}C at 10mm mercury (Hg).

After distillation, a number of undesirable compounds are removed from spray oils by chemical refining. Sulphur and nitrogen compounds, along with aromatics, are removed by adding an extraction solvent, such as phenol. Next, the fraction is hydrogenated to remove any remaining unsaturated bonds and prevent oxidisation into phytotoxic compounds (Tucker 1936).

The remaining fraction contains mostly paraffinic compounds, including straight chained molecules of 16 or more carbon atoms. If left in the oil they can congeal in storage or spraying during cold weather. The oils are dewaxed by adding a solvent and refrigerating. Straight chain molecules above C_{12} precipitate and are removed by filtering. Higher carbon numbers than C_{13} are still found in paraffinic spray oils because paraffin molecules may have a highly branched, isoparaffinic structure, which remain liquid at up to 19 or 20 carbon atoms. Other paraffinic forms have a nucleus of 5 carbon atoms, a cycloparaffin ring, and a long carbon side chain. In highly paraffinic spray oils, it is likely that there is a mixture of these forms.

1.2.2 Unsulphonated Residues

Gray and de Ong (1926) demonstrated that the unsaturated hydrocarbons were injurious to plants and that their removal makes oils relatively harmless to plants. Sulphuric acid was used to refine oils and remove phytotoxic compounds, and out of this practice a sulphonation test was developed which measures the safety of an oil to plant tissue. The purity of oils is defined by the percentage of oil, which does not react with sulphuric acid, the unsulphonated residue (U.R.). A

minimum of 92% U.R. was recognised as a necessary property for safe application on fruit trees (Riehl 1981).

1.2.3 Viscosity

Viscosity has been used prominently as a physical criterion of insecticidal effectiveness. Viscosity measures the flow rate, and is an indication of the molecular weight of the oil. However, while relative differences in oil heaviness can be shown by viscosity, this property can only be applied to compare insecticidal effectiveness among oils with the same basic structure. Paraffinic oils tend to have faster flow rates (ie., lower viscosities) than naphthenic oils of the same molecular weight (Riehl 1981).

An early apparatus for measuring viscosity was the Saybolt Universal Viscometer. Viscosity is expressed as the number of seconds for 60 ml of oil at 100^0F (37^0C) to flow through an orifice in the apparatus, or 'seconds Saybolt Universal' (SSU). The viscosity of most spray oils is 60 to 100 seconds.

1.2.4 Distillation Point

The distillation specifications are now the most used criteria for classifying spray oils. Modern specifications include a 50% distillation point, which indicates the temperature at which 50% of the oil distils, and the 10 to 90% distillation range, which gives the range in temperature (degrees^0) required to distil 10 to 90% of the oil. The wider the distillation range the less uniformity in molecular size. This is significant because hydrocarbon molecules boiling below 199^0C are thought to have relatively poor pesticidal activity; with those above 200^0C to 212^0C being more effective, although phytotoxicity increases as the boiling point approaches 235^0C to 238^0C (Davidson *et al.* 1991).

This approach is the basis of the manufacture of narrow range spray oils, with a 50% distillation point between 200^{0}C to 238^{0}C. Highly paraffinic oil with a narrow distillation range around those temperatures combines good pesticidal action and a high degree of plant safety. Despite a relatively narrow distillation range, these spray oils are not homogenous. They contain a complex mixture of hydrocarbons and are given a mean carbon number rating equivalent to a C19 to C24 reference.

1.2.5 Molecular Weight

The molecular weight indicates the size of the hydrocarbon molecule and is directly related to the distillation range. Larger molecules distil at higher temperatures and a wide distillation range indicates a wide range of molecular sizes. The size of molecules in spray oils ranges from 16 carbon atoms with a molecular weight of 226 to 32 carbon atoms with a molecular weight of 400. Molecules with carbon numbers between 20 to 26 show high pesticidal action with minimal plant toxicity. Gas chromatography is a convenient method to analyse the mean size and range of molecular sizes (Furness *et al.* 1987).

The carbon number is required as a specification on spray oils in Australia. For example, Ampol's DC-Tron Plus is marketed as C-24NR, indicating a narrow range oil with a median carbon number of 24.

1.3 Classification of Spray Oils

Before the introduction of narrow range oils in the mid-1960's, spray oils were divided into two broad groups: dormant oils and summer oils. The dormant oils were the heaviest of the spray oils. They extended over a wide range of viscosity, from 70 to 360 SSU (Brown 1951). They were not applied during the growing season because they would burn foliage due to their low unsulphonated residue of less than 94%. Summer oils (40-85 SSU) were applied to the foliage

of orchard trees during the growing season to control mites and scale insects (Diaspididae). The lighter summer oils (40-65 SSU) were used as adhesives in sprays and dusts, or as solvents that served as carriers for other toxic chemicals. Summer oils required refinement to greater than 94% UR.

In addition, during the 1940's California listed 5 grades of oil based on the range of temperatures at which they distilled. The Grade 1 had a boiling point where approximately 70% of its weight distilled at 200^0 C. It was the lightest oil and tended to be unsatisfactory against mites and scale (Diaspididae). A heavy grade 5 distilled only approximately 20% of its weight at 200^0 C. Although more efficacious, a Grade 5 oil caused adverse tree and fruit reaction (Ebeling 1950).

The smaller molecular sizes reduced the efficacy, while the larger sizes caused phytotoxicity. The term "narrow range" oil was used to define oil with a narrow range of molecular sizes suited to spray oils. They are not classified under the old Californian grades system. Narrow range oils are generally safe for use in summer and winter.

1.4 Spray Supplements and Emulsions

As early as 1865, kerosene was used for the control of scale insects (Diaspididae) by applying directly to the infested part of the tree without dilution. Later, oils were diluted with water before application, but, with mechanical agitation alone the solution was prone to separation into oil and water layers, leading to variable results. The addition of supplements, such as whale oil soap, enabled the solution to be kept thoroughly mixed during mechanical agitation and circulation.

Two main supplements, emulsifiers and spreaders, have been added to spray oils to improve their efficacy and safety. Both of these supplements have surfactant properties. Emulsifiers operate in the tank and allow two heterogeneous liquids to be kept thoroughly mixed. The spreading or wetting agent, operates at the moment of impact by reducing the surface tension and so increasing the contact between spray droplets and spray surface. It is important to note that a water soluble spreader will reduce the surface tension of the water only. An oil soluble spreader would be required to reduce the surface tension of the oil.

The spreader and the emulsifier perform the same function in controlling oil deposit. As the amount of emulsifier is increased, the solution "tightens", ie. the emulsion becomes more stable, leading to poor oil deposit on the target. High levels of spreader also produce excessive run-off of oil. Most spray oils are formulated so that the oil and water phase quickly separate, or 'break', on contact with the foliage. A rapid separation of the oil and water phase, or 'quick-break', produces a high deposit of oil over the leaf, with water quickly shedding off the foliage. However, a reduction in emulsion stability has it limits, as too little emulsifier can lead to an excessive oil deposit.

At present, citrus spray oils are formulated with a non-ionic surfactant. They are more soluble in hydrocarbons than most ionic detergents making it possible to make a single phase emulsifiable concentrate. Different surfactants can be mixed together and their field performance is often superior to either separately. A system called the hydrophilic-lipophilic balance (HLB) allows some level of selection of an appropriate surfactant. However, the optimal amounts for each application have to be found by trial and error.

1.5 Mechanisms of Toxicity

The most widely held theory on the mode of action of petroleum spray oils is that they act physically, by blocking the spiracles. Early evidence of this theory originates from work in California during the 1920's. At the time, there was considerable interest in the use of oils as an insecticide due to the evolution of hydrocyanic acid (HCN) resistant scale (Diaspididae) across increasing areas (Quayle 1922). In 1926, de Ong, looking for a substitute for HCN fumigation, found that highly refined (saturated) lubricating oils penetrated a short distance into the main tracheal trunk of scales and remained there indefinitely, apparently killing by suffocation. In contrast, light oils containing a large amount of unsaturated hydrocarbons passed through the body cavity, dissolving the fat bodies and eventually the internal cellular structure. The unsaturated components in insecticidal oils was removed from spray oils as they were also toxic to plants (Gray and de Ong 1926). The following year, work by de Ong *et al.* (1927), suggested that saturated hydrocarbons were essentially "non-toxic" to insects. In these experiments, Coleman's mealybug (*Phenacoccus colemani* Ehr.) were immersed in Oronite Crystal oil, a highly refined lubricating oil (106 SSU, 98% UR), and were found to survive longer in oil than when immersed in water or in an atmosphere of pure hydrogen. The mode of action of saturated hydrocarbons was seen as physical, whereas the unsaturated components of the oil were considered chemically toxic. Shepard (1939) proposed three theories on how oils kill insects; the saturated components of the oil block the spiracles resulting in suffocation, the liquid unsaturated components penetrate the tissues "corroding" them, and volatile components act as fumigants. The use of oils as insecticides was dominated at an early stage by saturated petroleum oils to mitigate the deleterious effects of unsaturated hydrocarbons on plant foliage. As such, the mode of action of insecticidal oils has become synonymous with suffocation.

Further work on saturated oils showed that the insecticidal efficacy and phytotoxicity were closely related to the molecular weight of the hydrocarbons, as indicated by the distillation range (Knight *et al.* 1929, Smith 1932). Comparisons of naphthenic-based and paraffin-based oil stocks concluded that paraffins were more effective against a range of insects and their eggs (Chapman *et al.* 1943; Chapman *et al.* 1944; Riehl and Carman 1953; Riehl and Jeppsen 1953). These authors did not explain why paraffins are more efficacious against certain insects. Ebeling (1945) suggested that the relative persistence or oxygen permeability of the oil films may be factors. Pearce and Chapman (1952) suggested that paraffins have a better spreading coefficient on the older bark of deciduous trees, improving control of European red mite (*Panonychus ulmi*) eggs.

The ability to produce different distillation cuts of the same stock oils allowed comparisons of oil with a narrow range of hydrocarbon molecules. Considerable field work has shown that light petroleum oils, with a 50% distillation temperature less than the boiling point of a C19 alkane, gives poor control for a range of pests in the field (Chapman *et al.* 1944; Riehl and Carmen 1953; Riehl and Jeppson 1953, Trammel 1965). These results have been used to improve the selection of insecticidal oils based on structural character and molecular size. They also provide support of suffocation as the primary mode of action of insecticidal oils. Increasing the molecular weight of the oil increased its persistence and was thought to account for its higher efficacy. Light oil fractions were considered unsuitable in the field because they dissipated before suffocation could occur (de Ong 1926).

Modern petroleum spray oils are refined to have a narrow boiling range of molecular weight hydrocarbons, with a high saturated paraffinic content (~70%). These oils are far less damaging than unsaturated oils to a range of plants (Furness and Maelzer 1981; Baxendale and Johnson 1988), but still retain their insecticidal efficacy (Chapman *et al.* 1962; Trammel 1965).

The efficacy of spray oils against insects improves by increasing the molecular weight of the oil molecules. However, the risk of phytotoxicity also increases with molecular weight (Riehl 1969). Most modern narrow range spray oils have a median carbon number per molecule equivalent (Furness *et al.* 1987) of between C20 to C26 (Davidson *et al.* 1991).

The mode of action of oils was investigated in the beginning of this century and only sporadically over the last 50 years. Over that period the properties and components of spray oils have changed. Importantly, modern narrow range oils are formulated with oil soluble and water soluble non-ionic surfactants, rather than only water soluble surfactants, such as blood albumin. Alternative uses for oils, such as reducing transmission of viral diseases by clogging insect vector's stylets (Gibson and Cayley 1984), as oviposition repellents (Shultz *et al.* 1983) and postharvest dips to remove pests from citrus (Taverner and Bailey 1995), have produced very different oils. There have been few studies looking at the mode of action of modern insecticidal oils despite substantial changes to their properties, formulation techniques and application methods. Studies of the various modes of action of oils should be considered in the context of their specific properties. A wider historical perspective of the modes of action of petroleum oils against arthropods is discussed in the following sections.

1.5.1 Fumigant Action

Shafer (1911) found that kerosene vapours had an effect on the nervous system of insects. Sen (1914) killed mosquito larvae in a vial of water by wetting the cotton plug with kerosene. Freeborn and Atsatt (1918), as a result of their experiments, believed that blocking of the tracheae was not an important factor, but, the vapours of kerosene entered the tracheae with lethal results. This is contrary to the belief, even today, that the kerosene kills primarily by suffocation. Petroleum oils applied as contact insecticides may actually kill by the toxicity of vapours entering the respiratory system. However, the theory applies only to relatively volatile

oils, as the higher boiling fractions have so little volatility they could show no apparent fumigant action (Moore and Graham 1918a). The lower range in the paraffin series, up to decane, show moderate fumigant action and are considered to be "physically toxic", ie., they act as a narcotic (Ferguson and Pirie 1948). The higher analogues, such as the lubricating oils and modern spray oils, are not sufficiently volatile to show fumigant action, and so it is likely that other factors become the prime influence of mortality.

1.5.2 Physical Action - Narcosis

Narcosis means sleep or unconsciousness, which is the action of oils on insects. The symptoms are described by the word “knockdown”, denoting a paralysis of the insect. Many narcotics are not the direct cause of death to the extent that the narcosis is completely reversible, unless the narcotic has been applied in high concentrations for prolonged periods. Hydrocarbons, such as kerosene, are physical narcotics, being apolar organic compounds which are soluble in lipids (Brown 1951). Once volatile oils have penetrated into the tracheal system they are not necessarily confined, and may diffuse into the haemolymph through the walls of the tracheae. Dyes have been used to observe the diffusion of oils through tracheal walls and into the haemolymph in a number of insects (Roy *et al.* 1943; Moore and Graham 1918b). Oil entering the haemolymph would preferentially lodge in lipid containing tissues in close connection with the tracheoles, including the nerve sheaths and the lipoproteins of the brain. The pharmacological effect of the absorption of hydrocarbons into phospholipid membranes is not clear. Hassall (1982) suggested that physical toxicity caused by inert substances, such as petroleum oils, may be due to their non-specific absorption on lipoprotein membranes resulting in the disruption of a highly specific event. This would not involve the formation of specific chemical attachments to receptors or active site of enzymes.

1.5.3 Physical Action - Suffocation

The most favoured theory explaining how oil kills is that the spiracles of insects become flooded with oil leading to asphyxiation (Davidson *et al.* 1991). de Ong (1926) showed that lubricating oil entering the spiracles penetrated a short distance into the tracheae of red scale, *Aonidiella aurantii*, apparently resulting in suffocation. The viscosity of the oil affects penetration, with oils of intermediate viscosity found to penetrate slowly, whereas light oils penetrate quickly (Hoskins 1933). Most oils of low viscosity have no difficulty in spreading over an insect cuticle. Mineral oils and aqueous solutions of wetting agents, with a surface tension to about 50% of water are able to enter tracheae (Brown 1951). Emulsions can penetrate tracheae, either as the oil fraction after 'breaking' or as the intact emulsion (Moore and Graham 1918b). de Ong (1926) found that immersing Coleman's mealybug, *Phenacoccus colemani,* in highly refined kerosene (98% UR) resulted in increased toxicity relative to the unsaturated lubricating oils (70-106 SSU, 98% UR). Another experiment showed that kerosene was expelled from the trachea of red scale (de Ong *et al.* 1927). Ebeling (1950) suggested that this made kerosene less effective than petroleum spray oils in the control of red scale.

Investigations looking at the mortality of various insects by immersion in "non-toxic" liquids has shown that suffocation is an extremely slow process (de Ong *et al.* 1927; Ebeling 1945). Insects show a high degree of resistance to anoxia and can recover from a deep oxygen debt. The extended period taken to kill insects immersed in unsaturated lubrication oil is consistent with the effects of suffocation. Ebeling (1945) found that Potato tuber moth, *Phthorimaea operculella* (Zeller), larvae became motionless much more rapidly in either nitrogen or water than in lubricating oil. The oil treated larvae, however, became discoloured if left in the oil, or dried and shrivelled if exposed to the air, in much less time than is required for death in nitrogen or water. In addition, the viscosity of the oil influenced the time to death, but only in the presence of air. Completely immersed larvae lived as long in kerosene (97% UR) as lubricating oils (60-

90 SSU, 92-94% UR). However, when the larvae were dipped in kerosene and exposed to air, death was more rapid. Ebeling (1945) inferred that even the most highly refined oil kills by virtue of its toxicity many hours before death by suffocation. Apparently, desiccation, oxidation, vaporisation, or some other influence of the air increases the insecticidal properties of the oil. He argued that if air is kept from the bodies of insects, their period of survival was greatly increased.

1.5.4 Physical Action - "Corrosion" and Cell Disruption

de Ong (1926) discovered that kerosene low in unsaturated hydrocarbons (2-3%) showed poor efficacy against scale insects. He proposed that the unsaturates were responsible for the toxic action of petroleum oils, by dissolving cellular structures. Under these circumstances, as oils became more refined (saturated) it would be expected that cell disruption would be removed. However, oils low in unsaturates may be affecting cellular structures in insects over a prolonged period.

Saturated oils can disrupt plant processes at the cellular level. Studies by van Overbeek and Blondeau (1954), suggest that oils disrupt cellular membranes, probably by solubilising membrane lipids. Oranges dipped in Ampol's Citrus Postharvest Dip (CPD) recorded increased levels of ethylene production (Mark Hodgkinson pers. comm.), which is consistent with increased cell disruption (Knoche and Noga 1991). It is plausible that the effect of oils on plant cell membranes would extend to insect lipids, such as cuticular waxes and membranes.

In addition, modern spray oils are formulated with emulsifiers, which can have profound effects on biological tissues, particularly plant waxes and cuticles. Gaskin (1995) reviewed techniques for determining surfactant-induced phytotoxicity, and concluded that "the primary mechanism of surfactant phytotoxicity appears to be disruption and solubilisation of biological membranes".

Some surfactants can penetrate very rapidly into plant cuticles, depending on their chemical structure and the nature of the plant cuticle (Silcox and Holloway 1989). Spray oil formulations are becoming more complex with the inclusion of more surfactant blends. Given the similarity of insect cuticle structure, particularly the epicuticle, with plant cuticles (Hurst 1941) surfactants probably affect insect membranes as well plant cuticles. Earlier studies show that various insects when exposed to surface-active solutes, such as glyceryl monooleate, glycol oleate and glyceryl monolaurate, rapidly desiccate (Wigglesworth 1941, 1945; Ebeling 1950; Ebeling and Wagner 1959). Wigglesworth (1941, 1945) believed that saturated oils dissolved the lipoid surface layer covering the cuticle allowing polar substances to draw out moisture. Insects exposed to the higher molecular weight oils required a longer interval to desiccate, presumably due to a decrease in mutual solubility of the oil and wax as the molecular volume of the oil increases.

Slow solubilisation of insect membranes may explain some residual effects on insects after treatment with petroleum spray oils. Ebeling (1936) found that approximately 30% of adult red scale die after exposure with a Grade 5 (Heavy) oil spray without tracheal penetration. Red scale treated with a dose of oil insufficient for immediate death continued to die over several weeks, presumably due to prolonged impairment of their physiological processes. Surviving females had reduced reproductive capacity, with a large number of dead crawlers grouped about the female pygidium. Baxendale and Johnson (1990), showed similar residual control using narrow range oils on sycamore lacewings, *Corythuca cillata L.*, and observed incomplete pupation in the larvae of European pine sawfly, *Neodiprion sertifer* (Geoffroy). They concluded that a mode of action other than respiratory interference may be involved.

1.5.5 Physical Action - Desiccation

Mortality through desiccation is a significant threat to arthropods and there are several areas where an arthropod can lose water. It can occur from the cuticle itself, the respiratory system

via the spiracles, from the excretory system and the mouth. The cuticle is considered an import barrier to water loss and has been extensively studied. A comprehensive study of its structure can be found in Hepburn (1985) and only a brief account of it will be given here. The insect cuticle is secreted by the epithelial cells and comprises an inner procuticle and outer epicuticle. The epicuticle is of considerable importance as a major function is to exclude water and hydrophilic substances while admitting lipophilic substances (Brown 1951).

The outer layer of the cuticle, the outer epicuticle, is perforated by many channels which connect with the procuticle beneath and potentially make it a major site for water loss (Locke 1965; Machin and Lampert 1985). However, the cuticle is covered by a layer of free lipid, which blocks the epicuticular channels and waterproofs the cuticle. The main function of the cuticular lipids is to minimise water transpiration. Water permeability of the insect cuticle and the role of cuticular lipids have been extensively studied and periodically reviewed (Ebeling 1974, Edney 1977, Lockley 1988, Hadley 1994).

Early work on the effect of petroleum oils on the permeability of cuticles was related to the use of oil solvents as carriers for chemical insecticides into insects. It has long been known that the addition of oils to suspensions of many insecticides increases the toxicity of the solution (Hurst 1940; Burtt 1945). Generally, the effectiveness of the oil as a carrier varies with the molecular weight. In sprays for the housefly, a heavy kerosene gives quicker knockdown and higher kill than a light grade (Richardson 1932). Among the paraffins, the best carriers are found in the range of hexane and dodecane, falling off with medicinal paraffin because it is too viscous (Hurst 1943). Oil solvents were considered to act as carriers by allowing the insecticide to pass through the normally impermeable lipid layer of the epicuticle.

Ebeling (1945) showed that desiccation induced by oils was contributing to mortality. He found potato tuber moth, *Phthorimaea operculella*, larvae dipped in oil followed by exposure to air became "shrivelled" and died much more quickly than when immersed in oil. Ebeling (1950) found the more rapid the appearance of water on the surface of the cuticle, the shorter the survival time of potato tuber moth larvae when exposed to straight oil and emulsive oils with various surface-active solutes, such as glyceryl monooleate, glycol oleate and glyceryl trioleate.

Various theories have been proposed to explain water loss induced by petroleum oils. Wigglesworth (1945) and Beament (1945) proposed that the insect lipid layer consisted of an inner monolayer of tightly packed polar lipid molecules, which when orientated at an angle of 60^0 reduces water permeability of the cuticle. Wigglesworth (1941) found that when insects were immersed in oil alone, water droplets appeared on the cuticle surface and the addition of strongly polar substances to the solution accelerated the process. Wigglesworth thought that the oil disrupted the order of polar substances in the lipid layer of the epicuticle, leading to increased transpiration. However, later work by Gilby and Cox (1963) showed that the cuticular lipid was not highly polar and consisted of mainly non-polar hydrocarbons. Molecular orientation is unlikely to be responsible for the efficiency of a cuticular lipid as a water barrier.

Hurst (1940) suggested that an oil solvent enters the lipoid layer of the cuticle and becomes incorporated in it, leading to the lipoprotein layer becoming more permeable due to an "increase in free volume and decrease in functional viscosity". Petroleum oils have not been shown to become incorporated into insect cuticular lipids. However, a change in the composition of the solid lipids has been shown to alter water permeability. Ebeling (1974), when reviewing work using artificial lipid films as models of insect cuticles, stated that "the water permeability of solid lipids is greatly increased by the addition of liquid hydrocarbons".

Locke (1965) proposed the waterproofing abilities of cuticular lipids depend largely on their phase behaviour. The composition of lipid mixtures alters their melting range (Gibbs 1995) and subsequently phase behaviour. Hadley (1977) and Toolson (1982) have shown that changes in the hydrocarbon content of desert arthropod cuticles affect their water loss. The hydrocarbon chain length, particularly of methylalkanes, have an important role in waterproofing, although the role is not clearly understood. Hadley's (1977) and Toolson's (1982) studies support Ebeling's (1974) inference that the introduction of liquid hydrocarbons into the insect cuticle would increase the permeability of the insect cuticle to water. However, the phase behaviour of cuticular lipids are very complex, with over 100 different compounds identified from the cuticular lipids of insect species (Lockley 1988). The interaction between different lipid components and how they affect biophysical properties is poorly understood.

An alternative hypothesis is supported by the work of Croghan and Noble-Nesbett (1990), following earlier work by Treherne and Willmer (1975a,b). Croghan and Noble-Nesbett (1990) proposed that the epithelial cells actively transport solute across their apical cell membranes. The negative hydrostatic pressure created in the procuticle acts as a compressive force on the epicuticle reducing the molecular free-space and reducing water permeability. The hydrostatic pressures would be a function of the transport processes in the epithelial cells which, in many cases, are under endocrine control. This model predicts a relationship between cuticular water loss and endrocrine control. The mechanism for the increase of water permeability of the cuticle from chemical insecticides is attributed to the neurotoxic action on the secretory activity of the epithelial cells (Ebeling 1974). However, any possible action of petroleum oil on this control mechanism was not considered by Ebeling, but perhaps water loss is an effect of toxicity at a site other than the insect cuticle.

1.5.6 Mode of Action as Ovicides

Petroleum oils have been used for many years as ovicides and several theories have been proposed as to the mode of action. O'Kane and Baker (1934) developed a technique to demonstrate the penetration of insect eggs immersed in oil, based on the Rohrbaugh (1934) method for differentiating natural plant oils from spray oils. They found that after prolonged immersion oils can penetrate the chorion, the outer covering of the egg, and caused death by coagulation of protoplasm, or interference with hormone or enzyme activity. In a review by Hoskins (1943), oils were considered to be able to harden the egg membrane to prevent hatching, interfere with the egg's water balance or ability to exchange gasses.

Smith and Pearce (1948) believed that although penetration of the egg chorion can occur it is not essential to kill eggs. They found that oils markedly reduce the respiration rate of eggs, but washing eggs to remove the oil film from the surface of the egg restored viability. The duration of oil exposure and the quantity of oil on the egg were critical to cause high mortality. They proposed that oil produces a lethal effect on eggs by mechanical interference with normal gaseous exchange. Observations on untreated eggs in their respirator led them to hypothesise that an accumulation of toxic metabolites rather than a restriction of oxygen was the direct lethal agent.

As noted in Section 1.1, paraffin-based oils show greater efficacy than naphthenic-based oils on the eggs of the oriental fruit moth and codling moth (Chapman *et al.* 1943). Pearce and Chapman (1952) suggested that paraffins have a better spreading coefficient on the older bark of deciduous trees, improving control of European red mite eggs. Overall, the heavier oils, with a molecular weight >320, which volatilise little over a 24h period are better ovicides (Pearce and Chapman, 1952; Fiori *et al.*, 1963).

1.6 Conclusion

The vast majority of work on the mode of action of petroleum oil was done on the lubricating oils, prior to the development of narrow range oils. It was found that insects immersed in heavy saturated oils survived for long periods. Insects show a high degree of resistance to anoxia and slow death by immersion in oil was consistent with suffocation. The idea of smothering is supported by the field efficacy of petroleum spray oils on many insects. Lubricating oils, with a high viscosity, result in higher efficacy, while less viscous oils show poor efficacy. Generally, the heavier the oil, the greater the oil deposit on the insect's surface, and, consequently, the greater the effectiveness of smothering.

However, there are limitations to the theory of suffocation for oil sprays. Paraffinic oils show greater efficacy than naphthenic oils, despite a lower viscosity (Chapman *et al.* 1943; Chapman *et al.* 1944; Riehl and Carman 1953; Riehl and Jeppsen 1953). Much of the theory of suffocation is based on the results of immersion experiments (Shafer 1911; de Ong 1926; de Ong *et al.* 1927; Ebeling 1936, Ebeling 1945; Ebeling 1950), which do not necessarily relate to field spraying. In the field, oil is sprayed to run-off, but target surfaces are left with a thin film of oil. Ebeling (1945) found that thin films of oil kill much more rapidly than would be indicated by suffocation. Some influence of the air increases the insecticidal properties of oils when deposited as a thin film.

Ebeling (1936) showed that oil sprayed on orange trees killed about 30% of red scale without tracheal penetration. Therefore, being smothered is not necessary for mortality to occur. Insect mortality has been shown to continue for several weeks after an oil application and can affect the reproductive capacity of survivors (Ebeling 1936; Baxendale and Johnson 1990).

The effect of petroleum oils on insects is complex and progressive. An insect exposed to oil probably undergoes a range of responses, depending on the properties of the oils. Very light fractions, up to decane, have fumigant properties. Light liquid fractions are highly penetrative and may diffuse through the tracheoles to effect the lipoproteins of the nervous system, inducing a narcosis.

Exposure to oil films leads to increased water loss in insects, which may contribute to mortality. Increased water loss could be due to the oil dissolving the protective cuticular hydrocarbons (Wigglesworth, 1941). An increase in cuticle permeability may be due to the incorporation of liquid hydrocarbons into the cuticle altering the phase behaviour (Lockley 1965; Ebeling 1974).

Hassall (1982) suggested that physical toxicity caused by inert substances, such as petroleum oils, may be due to non-specific absorption on lipoprotein membranes, resulting in the disruption of a highly specific event. Oils lodged in lipoproteins, within the nerve sheath, may disrupt spiracular closing, endocrine control of cuticular permeability (Croghan and Noble-Nesbett 1990) or some other essential process.

Heavier fractions are thought to kill by suffocation. However, before this occurs their smothering action may also lead to an accumulation of CO_2 within the insect, resulting in a narcosis. If there is insufficient oil to smother the pest, the heavy fraction may adhere to the insect, and, in time, penetrate to solubilise cell membranes.

An understanding of the mode of action of different fractions of petroleum oils could lead to a more appropriate formulation of oils for specific purposes. It would be particularly useful if factors, such as the influence of air on a thin film of oil, were considered in formulating oils. Currently, spray oils are formulated by trial and error to optimise wetting and 'break' to

smother pests. As such, the heaviest oil possible is preferred, with phytotoxicity being the limiting factor. However, there are applications, such as in dipping, when lighter oils may be more efficacious if formulated to accentuate their distinct physio-chemical properties.

Chapter 2

The Toxicity of Selected Petroleum and Vegetable Oil Dips to Exposed Mealybug.

2.1 Introduction

Citrophilus mealybug (*Pseudococcus calceolariae* (Maskell)) and the Longtail mealybug (*Pseudococcus longispinus* (Targioni Tozzetti)) are the major mealybug species associated with the citrus growing districts of inland Australia. Citrophilus mealybugs first appeared in Australian citrus orchards in the mid 80's, rapidly spreading to become the most common mealybug species in most areas. Both species are regarded as serious field pests in citrus. Mealybugs produce a sticky honeydew which is a good growth medium for sooty mould. The resultant sticky, black scum gives the fruit an unsightly appearance, resulting in the loss of that fruit for export.

Mealybugs can also survive the packing line process by sheltering in the calyx and navel end of oranges. During the sea voyage, the mealybug continue to develop and move onto the surface of the oranges. Both of these species are common in many overseas destinations, including the USA, however, interception of live mealybugs on export oranges arriving in the USA have caused lengthy delays pending identification. In some seasons, mealybug levels have been high enough to require re-packing for cosmetic reasons alone. Delays to market access and re-packing of oranges are costly to the industry.

Ampol's Citrus Postharvest Oil, CPD, gave high efficacy against lightbrown apple moth (*Epiphyas postvittana* (Walker)) larvae in a dip (Taverner and Bailey 1995a). Commercial trials using a 3% rate of postharvest oil in a fungicide dip resulted in high mortality of oranges naturally infested by mealybug (Taverner and Bailey 1995b). The removal of mealybug from

fruit is highly desirable and oil formulations may provide an effective surface 'clean-up' before export.

This study aims to provide dose mortality curves for selected petroleum and vegetable oils against adult mealybug, and thereby compare their relative toxicity when applied as dips.

2.2 Materials and Methods

2.2.1 Mealybug and Oil Details

Citrophilus and Longtail mealybug adults were collected from cultures maintained on butternut pumpkins, *Cucurbita pepo* L., over several years in separate rearing rooms at the South Australian Research and Development Institute (SARDI), Adelaide.

Ampol Research and Development Laboratories, Brisbane Qld, supplied a Citrus Postharvest Dip, Ampol C15 CPD, (an alkane with a carbon number of 15, paraffin content ie. %Cp>99%) and a commercial spray oil, Ampol DC-Tron NR (narrow range oil with mean equivalent *n*-paraffin carbon number of a C23 alkane (Furness *et al.* 1987); %Cp<70%). General specifications of these oils can be found in Appendix 1. Total Solvents, Paris, France, supplied two highly paraffinic oils, C19 Citrole NR and C22 NHC5 NR. The specifications of Total Citrole, a commercial petroleum spray oil, are given in Boutuorlinsky *et al.* (1996) and Herron *et al.* (1998). An emulsifiable vegetable oil-based product, Eco-oil, used in agricultural spraying, was supplied by Organic Crop Protectants Pty Ltd, Lilyfield NSW, and an experimental vegetable oil-based postharvest dip, AWH-96-04, was supplied by the Victorian Chemical Company, Richmond Vic.

2.2.2 Bioassays

Initially, adult citrophilus mealybugs were dipped in oil at one dose (2,500ppm). The relative efficacy of each oil was determined by this preliminary comparison. The procedure involved using Adelaide tap water (pH 6.5) because it approximated Riverland Irrigation/Domestic water (pH6.0) used in packingsheds more closely than de-ionised water (pH8.4). Water quality can affect the emulsion stability of oil formulations (Mark Hodgkinson, personal communication). Forty adult mealybug were dipped in each oil emulsion. The control treatment consisting of 20 mealybugs, which were dipped in tap water.

Ten adult mealybugs were collected per phial using fine camel hair brushes. The mealybug were transferred into a fine wiremesh spherical cage ("tea-infuser") 400mm in diameter. The closed cage containing the mealybugs was suspended in a 500ml beaker of emulsion for 30 seconds. The emulsion was well agitated prior to dipping. This procedure was repeated until 40 mealybug per treatment were dipped. The treated larvae were removed and placed onto the upper surface of mature Navel orange leaves within a plastic container. The container was sealed and placed in a rearing room (20±2°C, 65% RH, and natural lighting of 14h day:10h night). Mortality was assessed at 24h after dipping. Mealybugs were counted as dead if they did not move after repeated prodding with a needle. Mealybugs were recovered and placed in 80% alcohol for 12-24h. The length(L) and width(W) of the mealybug body was measured, using a graticule under a microscope, on a representative sample of mealybugs (15/treatment). The mealybugs were considered to be two dimensional, with the surface area(SA) calculated as:

$$\mathbf{SA = 2xLxW}$$

Further dip bioassays were conducted to compare the postharvest oil, CPD, against a commercial narrow range spray oil, DC-Tron, used against mealybugs in the field. The

bioassay procedure was as previously described, with 40 mealybug tested per dose. Six doses were selected for each oil to give responses of between 5% to 100% mortality. Most doses for CPD were selected to cause greater than 75% mortality, as the lethal doses of interest were LC_{90} or LC_{95}. In addition, the efficacy of CPD against Long-tail mealybug was compared to Citrophilus mealybug.

Citrole (C19) and NHC5(C22) were compared with CPD(C15) and DC-Tron(C23), respectively, by testing adult Citrophilus mealybugs at rates to give responses between 5% and 95% mortality. The dipping procedure was as described above.

2.2.3 Analysis

Probit analysis fitted a regression model to the quantal assay data from oil dips using LeOra Software, Polo-PC. In this package, the data for each preparation is analysed separately using maximum likelihood (ML) procedures. Heterogeneity factors and likelihood ratio testing of equality and parallelism of dose mortality curves were calculated using this program.

The surface areas of the two mealybug species were compared for each treatment by ANOVA, using Statistix v4.1 (Analytical software, 1994). Comparison of the means for the same dose group in each species was made by the least significant difference method, LSD (T), where T is the Student's t-statistic from the degrees of freedom (df) associated with the mean square of error.

2.3 Results

2.3.1 Preliminary Screening of Oils

Initial bioassay testing of oils against adult mealybugs at a single rate (2,500ppm) showed 2 distinct efficacy groupings of petroleum oils according to molecular weight (Table 2.1). The

lighter, highly paraffinic oils, CPD (C15) and Citrole (C19), demonstrated the highest efficacy, with 100% mortality at 2,500ppm. The higher molecular weight paraffinic oils, NHC5 (C22) and DC-Tron (C23) recorded similar levels of mortality, but, were significantly lower than either CPD or Citrole. Dipping in vegetable oils resulted in the lowest mortality at this rate.

2.3.2 Visual Effects of Oil Treatment

Observations of oil treated mealybugs showed that light paraffin oils acted as a solvent, dissolving the white waxy cuticle covering the mealybugs (Figure 2.1). DC-Tron showed no comparable solvent action on mealybug wax at the same concentrations (10,000ppm).

Table 2.1 Mortality of adult Citrophilus mealybug dipped at 2,5000ppm for 6 oil formulations, and held at 20°C.

Formulation	*%Paraffin content*	*Mean Carbon number*	*Oil Type#*	*%Mortality(SEM)**
CPD	98+	15	p	100 (0.00)a
Citrole	90+	19	p	100 (0.00)a
NHC5	90+	22	p	57.5 (11.1)b
DC-Tron	70	23	p	49.0 (5.77)b
AWH-96-04	-	-	v	17.5 (4.88)c
Eco-oil	-	-	v	0.0 (0.00)d

petroleum oil (p) and vegetable based oil (v).
* Means within a column followed by the same letter are not significantly different ($F = 55.47$, $df=5$, $p>0.05$; Critical value for comparison=16.99, LSD(T)). SEM=standard error of the mean.

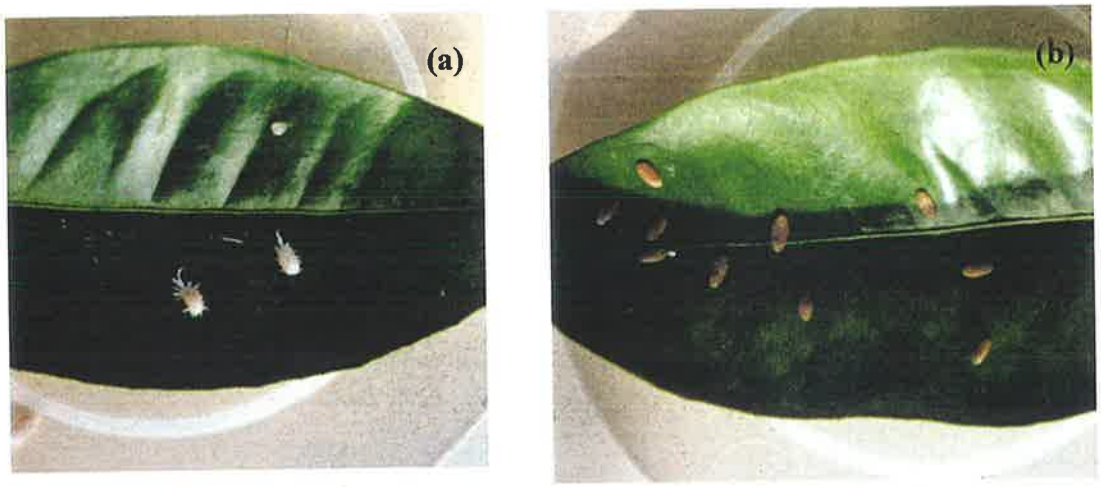

Figure 2.1 Appearance of mealybugs (a) untreated and (b) dipped in 10,000ppm CPD.

2.3.3 Dose Response

The dose response data showed that adult Citrophilus mealybugs tended to be more tolerant to dipping in DC-Tron than to CPD. Both species showed high susceptibility CPD, with mortality occurring at concentrations of 50ppm and above. The observed 24hr mortality of adult Citrophilus (DC-Tron and CPD) and Longtail mealybugs (CPD) is shown in Table 2.2, 2.3 and 2.4, respectively. As no controls died, the natural response was zero.

Table 2.2 Mortality of adult Citrophilus mealybugs dipped in 6 doses of DC-Tron, and held at 20°C.

Dose (ppm)	*Log10 (Dose)*	*Mortality (n)*	*Sample Size (10/replicate)*
0	0.000	0	20
250	2.398	0	40
500	2.699	2	80
1000	3.000	6	80
5000	3.699	28	40
10000	4.000	36	40
20000	4.301	40	40

Table 2.3 Mortality of adult Citrophilus mealybugs dipped in 6 doses of CPD, and held at 20°C.

Dose (ppm)	*Log10 (Dose)*	*Mortality (n)*	*Sample Size (10/replicate)*
0	0.000	0	20
50	1.699	2	40
100	2.000	16	40
250	2.398	29	40
500	2.699	38	40
1000	3.000	40	40
2500	3.398	40	40

Table 2.4 Mortality of adult Longtail mealybugs dipped in 6 doses of CPD, and held at 20°C.

Dose (ppm)	*Log10 (Dose)*	*Mortality (n)*	*Sample Size (10/replicate)*
0	0.000	0	20
50	1.699	11	40
100	2.000	19	40
250	2.398	33	40
500	2.699	37	40
1000	3.000	40	40
2500	3.398	40	40

Estimates of the intercept, the slope, and the standard error of each estimate are as follows:

Citrophilus

DC-Tron intercept=-9.531±0.816, slope=2.724±0.236 (Figure 2.2)

CPD intercept=-4.502±0.633, slope=2.256±0.291 (Figure 2.2)

Longtail

CPD intercept=-6.575±0.789, slope=3.052±0.356 (Figure 2.3)

The expected frequency of the mortality responses, the deviation from observed, and the probability of the responses are shown in Table 2.5, 2.6 and 2.7.

Table 2.5 Observed and expected mortality, deviation, and probability of response of adult Citrophilus mealybugs dipped in 6 doses of DC-Tron.

Dose (ppm)	*Observed Mortality*	*Expected Mortality*	*Deviation*	*Probability of Response*
250	0	0.05	-0.054	0.001
500	2	1.171	0.829	0.015
1000	6	6.955	-0.955	0.087
5000	28	28.270	-0.270	0.707
10000	36	36.548	-0.548	0.914
20000	40	39.420	0.580	0.986

χ^2=1.4854, df=4 heterogeneity factor = 0.37

Table 2.6 Observed and expected mortality, deviation, and probability of response of adult Citrophilus mealybugs dipped in 6 doses of CPD.

Dose (ppm)	*Observed Mortality*	*Expected Mortality*	*Deviation*	*Probability of Response*
50	2	3.291	-1.291	0.082
100	16	12.750	3.250	0.319
250	29	30.854	-1.854	0.771
500	38	38.070	-0.070	0.952
1000	40	39.803	0.197	0.995
2500	40	39.997	0.003	0.999

$\chi^2 = 2.4588$, df=4 heterogeneity factor = 0.61

Table 2.7 Observed and expected mortality, deviation, and probability of response of adult Longtail mealybugs dipped in 6 doses of CPD.

Dose (ppm)	*Observed Mortality*	*Expected Mortality*	*Deviation*	*Probability of Response*
50	11	10.065	0.935	0.252
100	19	20.154	-1.154	0.504
250	33	32.716	0.284	0.818
500	37	37.747	-0.747	0.944
1000	40	39.530	0.470	0.988
2500	40	39.969	0.031	0.999

$\chi^2 = 1.0321$, df=4 heterogeneity factor = 0.26

Table 2.8 Estimates of the lethal dose and their 95% confidence limits for 2 mealybug species dipped in DC-Tron or CPD.

Oil type	*Mealybug species*	*LC_{50} (ppm) (95%CL*)*	*LC_{90} (ppm) (95%CL*)*	*LC_{99} (ppm) (95%CL*)*
DC-Tron	Citrophilus	1,068.4 (808-1.294)	9,328.0 (7,196-13,040)	22,562 (15,720-36,810)
CPD	Citrophilus	142.69 (117.9-171.1)	375.24 (296.9-522.9)	825.38 (580.4-1,412.1)
CPD	Longtail	99.02 (75.4-124.2)	366.27 (275.7-555.6)	1,064.0 (673-2,222)

* Confidence limits

2.3.4 Likelihood Ratio Tests - Comparison of Oils

The likelihood ratio (LR) test for equality is to determine if the two regression lines are equal, ie. the slopes and intercepts are the same. The LR test statistic from this data (χ^2=355, df=1: p=0.00) shows that the lines are unequal, ie., the hypothesis of equality is rejected. Although the lines are not equal, the LR test for parallelism (χ^2=0.6, df=1; p=0.438) suggests that the slope of the lines are equal, ie., the lines are parallel. The different dose response and parallelism of the 2 regression lines can be seen in figure 2.2.

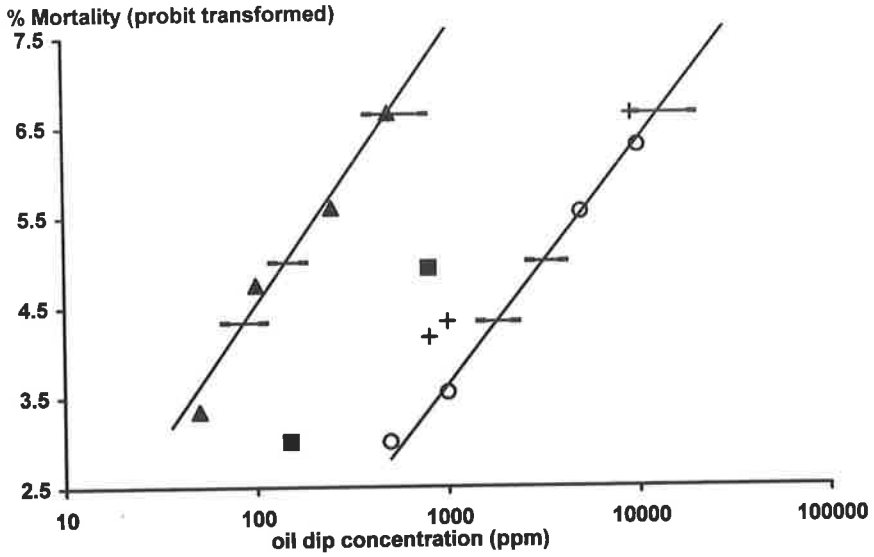

Figure 2.2 Dose response regression and 95% confidence limits at LC_{25}, LC_{50} and LC_{75} for adult Citrophilus mealybugs exposed to postharvest oil dip, CPD (▲), and DC-Tron (○), plus selected doses of Citrole (■) and NHC5(+).

Further comparisons using selected doses of NHC5 and Citrole on adult mealybug indicated that these oils were less efficacious than CPD (Figure 2.2). The order of efficacy from lowest to highest tended to increase as molecular weight decreased:

DC-Tron (C23)⇒ NHC5(C22)⇒ Citrole(C19)⇒ CPD(C15).

2.3.5 Likelihood Ratio Tests - Comparison of Species

The LR test statistic from this data (χ^2=7.56, df=2; p=0.02) shows that the lines are unequal, ie., the hypothesis of equality is rejected. Although the lines are not equal, the LR test for parallelism (χ^2=3.06, df=1; p=0.08) suggests that the slope of the lines are equal, ie., the lines are parallel. The close relationship of the 2 regression lines can be seen in figure 2.3.

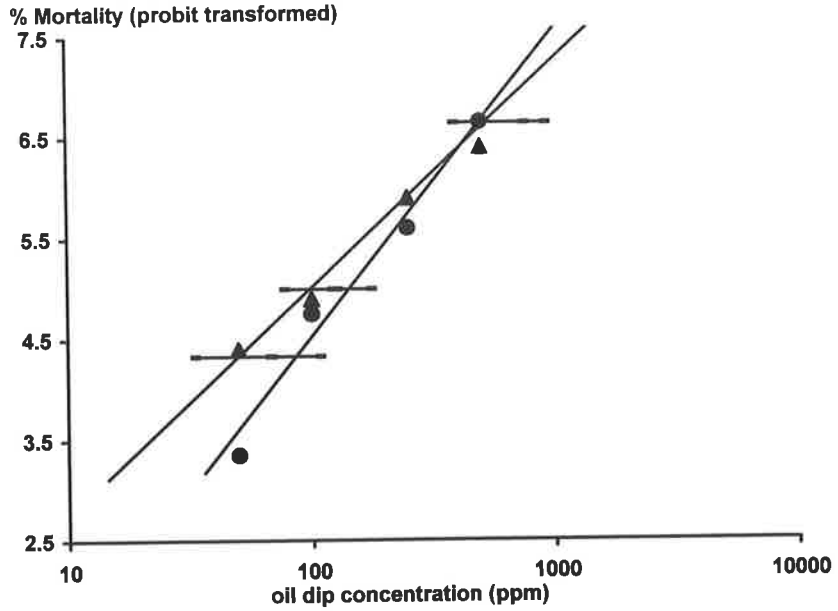

Figure 2.3 Dose response regression and 95% confidence limits at LC_{25}, LC_{50} and LC_{75} for adult Citrophilus (•) and Longtail (▲) mealybug to postharvest oil dip, CPD.

2.3.6 Surface Area of Mealybugs

Both species of mealybug were reared on pumpkins. However, the Longtail mealybug did not develop as well as Citrophilus on this medium. Analysis of mealybug surface area (SA) showed significant differences in size between the two species (Citrophilus mean $SA = 10.2mm^2$, Longtail mean $SA = 8.1mm^2$; $F = 150.6$, $df = 1$, $p < 0.01$). A comparison of the mean surface area of paired dose groups shows significant differences between the species in each dose category, as seen in table 2.9. However, a comparison of the LC_{50}'s, which is the estimate with the greatest statistical reliability, for each species (Table 2.8) shows that the two species are not significantly different, as indicated by the overlapping 95% confidence limits. Thus, the small difference in surface area were not demonstrably related to susceptibility.

Table 2.9 Comparison of the mean surface area (mm^2) of adult Longtail and Citrophilus mealybugs in the 6 dose categories.

Dose (ppm)	*Citrophilus*	*Longtail*	*Critical Value LSD 5%*
0	10.78	7.87	0.594
50	10.69	8.18	1.070
100	9.48	7.97	1.029
250	9.78	8.12	1.138
500	10.31	7.87	0.737
1000	10.51	8.32	0.769
2500	9.65	8.43	1.129

ANOVA: Difference in surface area of mealybug between dose groups of same species was not significant (Citrophilus: $F= 1.77$, $df=6$, $p>0.05$; Longtail: $F= 0.62$, $df=6$, $p>0.05$). Difference in surface area of mealybug between species was significant for dose groups 0 ($F=57.3$, $df=1$, $p<0.05$), 50 ($F=31.6$, $df=1$, $p<0.05$), 100 ($F=10.4$, $df=1$, $p<0.05$), 250 ($F=11.2$, $df=1$, $p<0.05$), 500 ($F=45.6$, $df=1$, $p<0.05$), 1000 ($F=27.8$, $df=1$, $p<0.05$), 2500 ($F=6.9$, $df=1$, $p<0.05$).

2.4 Discussion

2.4.1 Comparison in Efficacy of Oils

This study suggests that the efficacy of light petroleum oils, CPD (a C15 alkane), applied as a dip to mealybug are superior to the heavier molecular weight petroleum spray oils (DC-Tron and NHC5). This is contrary to earlier work on petroleum spray oils against a range of pests which showed greatest efficacy with higher molecular weight oils and poor insecticidal activity with hydrocarbon molecules below C19 (Chapman *et al.* 1944; Reihl and Carmen 1953; Reihl and Jeppson 1953, Trammel 1965). As a consequence of this work, spray oils are formulated using narrow range oils, with a median carbon number of between C21 to C24,

because they have demonstrated high pest efficacy and are reasonably safe to plants. The increased efficacy of light petroleum oils in this study may be due to the effect of dipping rather than spraying the oil emulsions. A comparison of oils by dose alone is difficult as the different formulations of the oils may affect the oil deposit characteristics, ie. one formulation may deposit more oil on the subject than another at the same emulsion concentration (Campbell 1972). An alternative mode of action may also influence the relative efficacy of the oils. Any possible effects on efficacy needs to be investigated.

Insect control using petroleum spray oils is based on the theory that the mode of action is anoxia, ie. the oil blocks the tracheae and suffocates the insect (Davidson *et al.* 1991). Light oils can be expelled from the tracheal system of some insects making them less likely to cause suffocation (de Ong *et al.* 1927). Insects can survive for long periods without air and because light oils are volatile, they may not persist for long enough to result in suffocation. However, this study shows high efficacy with relatively volatile paraffins. The increased efficacy of light petroleum oils applied as dips may be due to an alternative mode of action. Light paraffins were observed to dissolve the protective waxy coating on mealybug exposing them to a high risk of rapid desiccation (Figure 2.1).

The two vegetable oils selected showed the lowest efficacy. However, formulation changes may increase oil deposits and, therefore, pest efficacy. Phytotoxicity caused by these vegetable oils would also need to be investigated.

2.4.2 Comparison of Mealybug Species

Bioassay results show that mealybug adults of both species are susceptible to dipping in postharvest oil, CPD, at relatively low rates. The LC values established for mealybug adults (LC_{90}=375ppm for exposed Citrophilus mealybug) are much lower than corresponding LC

values established for exposed lightbrown apple moth (LC_{90}=1,723ppm for exposed 3rd instar LBAM) using postharvest oils (Taverner and Bailey 1995a). The bioassay data from this study suggest that control of mealybug would be possible with lower rates than required for the control of lightbrown apple moth larvae.

The dose response curves suggested that the two mealybug species responded differently to dipping in oil. Likelihood ratio testing showed that the lines were parallel, but, not equal. Parallel lines may indicate a quantitative difference, rather than a qualitative difference in the two species' response to CPD oil. Generally, insects are thought to respond to toxicants in direct proportion to their body weights. In this study, the surface area was estimated, rather than by weighing individual mealybug, but the two variables are likely to be highly correlated. As such, the difference in surface area of the Citrophilus and Longtail mealybug may be responsible for the quantitative trend in response of the two species usually associated with a variation in body weight. However, Robertson *et al.* (1981) suggested that responses do not vary as a simple function of body weight, and the significance of weight should always be tested. Further studies are needed to examine the relationship between dose and surface area in dip bioassays.

2.4.2 Comparison of Application Methods

Earlier laboratory bioassay studies (Riehl and LaDue 1952) of the susceptibility of citrus pests to spray oils have shown high variability in their response, and this was generally attributed to poor application techniques. Herron *et al.* (1995) showed that a Potter tower could be used for testing a range of pests with oils. However, large variations in some species still occurred, which they attributed to the suffocating, rather than a toxic, mode of action of oils. The low heterogeneity factor in this study suggests that the dip bioassay method produced a uniform oil

deposit on the adult mealybug. The dip bioassay method may be useful in assessing the relative efficacy of oils to different pest species.

2.5 Summary of Main Points

1. Lower molecular weight petroleum oils had the highest efficacy. The order of efficacy from lowest to highest was DC-Tron (C23)⇒ NHC5(C22)⇒ Citrole(C19) ⇒CPD(C15).

2. The increased efficacy of light petroleum oils in this study may be due to the dipping method and/or an alternative mode of action.

3. The two vegetable oils selected showed the lowest efficacy.

4. Bioassay results showed that mealybug adults of both species are susceptible to dipping in postharvest oil, CPD, at relatively low rates.

5. The dose response curves suggested that the species responded differently to dipping in oil. However, the difference in surface area of the Citrophilus and Longtail mealybug may account for only part of the quantitative difference in response of the two species.

6. Earlier laboratory bioassay studies (Riehl and LaDue 1952) of the susceptibility of citrus pests to spray oils have shown high variability in their response, and this was generally attributed to poor application techniques.

7. The low heterogeneity factor in this study suggests that the dip bioassay may be useful in assessing the relative efficacy of oils to different pest species.

Chapter 3

Toxicity of Selected Oils on Citrophilus Mealybug Sheltering on Oranges.

3.1 Introduction

Dip bioassays using Ampol's Citrus Postharvest Dip, CPD, on exposed mealybug resulted in high mortality at rates above 500ppm (Chapter 2). However, mealybug tend to settle in small grooves on the orange surface and under the calyx where they are at least partially protected. Experiments on lightbrown apple moth, which also conceals itself, showed much higher rates of oil are needed to penetrate beneath the calyx and cause mortality (Taverner and Bailey 1995a). An effective rate to control mealybug concealed on oranges needs to be determined.

Commercial trials using 30,000ppm of CPD showed high mortality of mealybug on naturally infested oranges (Taverner and Bailey 1995b). It seems likely that CPD at this rate will give good control but the numbers of mealybug on oranges in this trial were too low for statistical validation. Confirmatory testing using greater numbers at a fixed treatment dose can be used to estimate the true survival rate with greater confidence (Robertson and Preisler 1992).

The aim of the confirmatory bioassay in this study was to give greater confidence that the rate of 30,000ppm of CPD used in commercial trials will result in high mortality of mealybug. Smaller bioassays were performed to establish LC_{50} and LC_{90} estimates for CPD against mealybug sheltering beneath the orange calyx. Bioassays to assess the relative efficacy of other oils on concealed mealybug were also performed.

3.2 Materials and Methods

3.2.1 Oil Formulations

The details of the oils used are in Chapter 2. General specifications of CPD and DC-Tron are in Appendix 1.

3.2.2 Collection of Oranges and Mealybug

Navel oranges clipped from commercial trees to leave the calyx intact were placed in cool storage (5^0C) until use. Citrophilus mealybug for bioassays were obtained from a culture maintained at the South Australian Research and Development Institute. First instar mealybug were used to infest oranges as their small size allowed them to completely conceal themselves. The crawlers were obtained by placing ovisacs in a cage covered with a fine gauze mesh. The cage was maintained in the laboratory at 20 ∓ 2^0C, 60% humidity, natural light of 14h day:10h night.

3.2.3 Infesting Orange with Mealybug

Oranges were placed calyx end down on a bench top next to the gauze cage containing the ovisacs. The emerging crawlers moved through the gauze to infest oranges. Larger stages were excluded from the oranges by the fine gauze.

After 24hrs, the oranges were removed from the bench and placed in a rearing room (20-23^0C, 60% humidity, natural light of 14h day:10h night) for 24-48hrs to allow the mealybug crawlers to settle on the oranges.

3.2.4 Bioassay Procedure

Mealybugs that settled outside a 25mm diameter area around the calyx of each infested orange were removed. Pre-treatment counts of mealybugs were made inside the area

according to degree of concealment. Mealybugs under the calyx were viewed by gently lifting the calyx sepals with forceps. Three areas were defined as:

1) exposed on the rind (Open)

2) partially concealed on surface of the calyx (Partial)

3) fully concealed beneath the calyx (Closed)

Oranges with between 50 to 150 mealybug were selected for dipping, with the remaining oranges being discarded. The selected oranges were then dipped individually into 1000ml of stirred oil emulsion for 30 seconds. For the confirmatory experiment using CPD, each replicate contained 25 oranges dipped into a 30,000ppm (3%) CPD solution and five oranges dipped in water controls. The survival rate was calculated using a method by Couey and Chew (1986).

Another bioassay was performed using CPD at six rates, of 1,000, 2,500, 5,000, 10,000, 15,000 and 20,000ppm, to obtain responses up to 100% mortality. Quantal assay data from CPD oil dips were fitted to a Probit model using Polo-PC (LeOra Software 1987).

Infested oranges were also treated with other oils, which included DC-Tron, Citrole, AWH-96-04 and NHC5. The same experimental procedures were followed as above to compare relative efficacy. Percentage mealybug mortality was recorded and arcsine square root-transformed percentage data were analysed by one way analysis of variance, with mean separation by the least significant difference method (T) (at 0.05).

In all cases, the dipped oranges were left 24hrs in the laboratory before mortality assessment. Post treatment counts of dead and live individuals were recorded. Individuals were scored as dead if they failed to respond when prodded with a fine needle.

3.3 Results

3.3.1 Confirmatory Experiments using Postharvest Oil, CPD, against Concealed Mealybug

The natural mortality of mealybug was high due to the large numbers washed off during a water dip. Mealybug concealed under the calyx were less likely to be washed from the orange (Table 3.1). There is also a significantly lower mortality of crawlers under the calyx relative to exposed crawlers.

Confirmatory tests showed that the rate of 3% CPD gave high mortality of Citrophilus mealybug crawlers in open and concealed situations on oranges (Table 3.2). After correction for natural mortality (Busvine 1971), a total of 13,293 mealybug were treated with postharvest oil. Forty five percent, a total of 5,981 mealybug, were fully concealed under the calyx. One mealybug survived and based on the method of Couey and Chew (1986), the calculated survival rate for the treatment is 0.75 mealybug/10,000 treated; the upper 95% CL survival rate is 3.5 mealybug/10,000 treated.

Table 3.1 Natural mortality of first instar citrophilus mealybug at 3 different levels of concealment on oranges.

Group#	*Subjects tested (n)*	*Mealybug removed+ % (n)*	*% Mortality on oranges* % (n)*	*% Total Loss*
Open	1179	73.4 (865)	8.9 (105)a	82.3
Partial	204	54.9 (112)	17.7 (36)b	72.6
Closed	1315	52.0 (684)	0.2 (3)c	52.2

+Mealybug removed during water dipping.
* Values are the means of 4 replicates. Values within a column followed by the same letter are not significantly different according to ANOVA of the arcsine square root transformed data ($F=8.39$, $df=2$, $p<0.05$). Mean separation by LSD (T) method (0.05%).
# Exposed on the rind (open), partially concealed on the surface of the calyx (partial), fully concealed beneath the calyx (closed)

Table 3.2 Survival of first instar citrophilus mealybug in open and concealed situations on oranges dipped in 3%CPD oil.

	Control		*Treated*	
Replicate	*Subjects tested (n)*	*% Mortality on oranges*	*Subjects tested (n)*[a]	*No. of survivors*
1	872	5.2	5,254	1
2	514	3.3	2,014	0
3	415	9.9	2,030	0
4	897	3.9	3,995	0
-			13,293	1

[a] Adjusted for natural mortality.

3.3.2 Efficacy of postharvest oil, CPD, against concealed mealybug

A dose of greater than 10,000 ppm was required to achieve very high control (>99%) of concealed first instar mealybug (Table 3.3 and 3.4).

Table 3.3 Under calyx mortality of 1st instar Citrophilus mealybug dipped in 6 doses of CPD, and held at 20°C.

Dose (ppm)	*Log10 (Dose)*	*Subjects tested (n)*	*Mortality*
0	0.000	788	400
1000	3.000	745	424
2500	3.398	759	562
5000	3.699	684	650
10000	4.000	647	641
15000	4.176	613	613
20000	4.301	594	594

Estimates of the intercept, the slope, and the standard error are as follows:

Under calyx intercept=-8.080±1.066, slope=3.843±0.293 (Figure 3.1)

The natural mortality of mealybug under the calyx is estimated at 52% (Table 3.1). Observations of large numbers of mealybug in the water solution after dipping suggest the major factor causing natural mortality is the action of the water removing mealybug during dipping, rather than the water causing mortality "in situ". High natural mortality resulted in high expected mortalities even at low doses. Large chi-squared goodness of fit values suggest a high heterogeneity in the bioassay data, probably due to the variation in numbers of mealybug washed off during dipping.

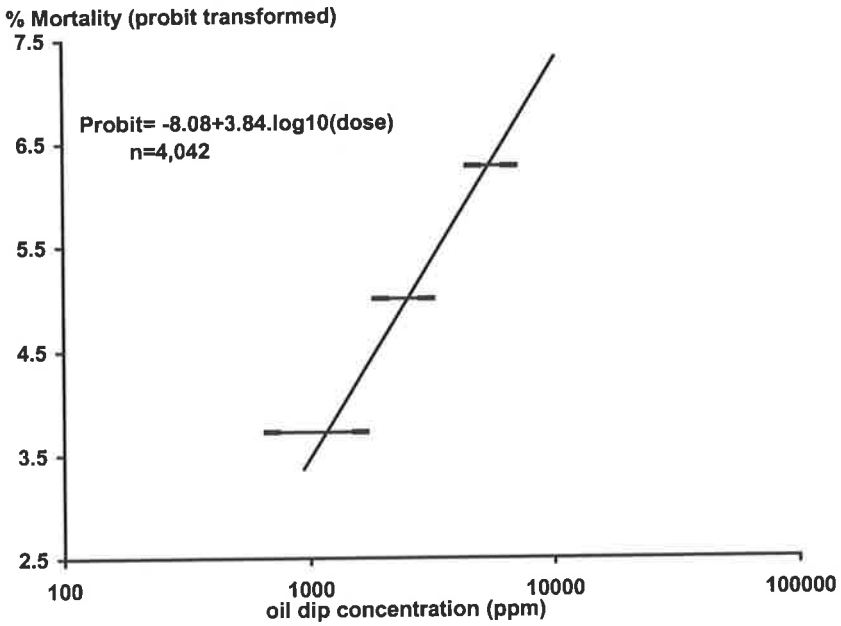

Figure 3.1 Under calyx mortality-dose response regression and selected 95% confidence limits of LC_{25}, LC_{50} and LC_{75} for first instar Citrophilus mealybug to postharvest oil dip, CPD

Table 3.4 Under calyx observed and expected mortality, deviation, and probability of response of first instar citrophilus mealybug dipped in 6 doses of CPD.

Dose (ppm)	*Observed Mortality (n)*	*Expected Mortality (n)*	*Deviation*	*Probability of Response*
0	400	409.5	-9.5	52.0
1000	425	408.9	15.1	54.9
2500	562	573.8	-11.8	75.6
5000	650	642.0	8.0	93.9
10000	641	643.6	-2.6	99.5
15000	613	612.6	0.4	99.9
20000	594	593.9	0.1	99.9

$\chi^2 = 6.858$, df=4. heterogeneity factor =1.71

Estimates of the lethal dose and their 95% confidence limits are as follows:

Under Calyx Mortality

CPD	Dose (ppm)	Lower Limit (.95)	Upper Limit (.95)
LC50	2,530.5	1,953.4	3,006.7
LC90	5,453.1	4,711.4	6,580.2
LC99	10,197	8,105.6	14,847

3.3.3 Comparison of Selected Oils against Concealed Mealybug

A bioassay performed to determine the relative efficacy of other petroleum spray oil and a vegetable oils against mealybug indicated a high level of control, but not high enough to satisfy quarantine (Table 3.7). All oil tested showed significantly higher levels of mortality than the control (water only) when assessed at 30,000ppm ($F=123.4$, $df=3$, $p<0.05$). The (C19-NR) petroleum spray oil, Citrole, produced significantly higher mortality than DC-Tron (C23-NR) petroleum spray oil.

Table 3.7 Mortality of first instar Citrophilus mealybug infested oranges dipped in water for 3 different oil formulations at 30,000ppm.

Oil	*n*	*% Mortality**
water	533	16.5a
Citrole	463	98.5b
DC-Tron	542	91.0c
AWH-96-04	495	96.4bc

* Values are the means of 10 replicates. Values within a column followed by the same letter are not significantly different according to one way analysis of variance of the arcsine square root transformed data. Mean separation by LSD method.

3.4 Discussion

3.4.1 Confirmatory Experiments using Postharvest Oil, CPD, against Concealed Mealybug

Postharvest oil, CPD, at a rate of 30,000ppm has proved to be highly effective in the control of citrophilus mealybug crawlers (first instar), with a 95%CL mortality rate of >99.95%.

The oranges used in the bioassay were artificially infested with large numbers of mealybug. Under these circumstances, large numbers were washed off during the dipping process. The mealybug appeared to settle by 24 hours, but would not be as well established as on field-infested oranges. In this study, oranges were infested after harvest and the quality of the oranges deteriorated quickly during storage. As such, a longer settling time was considered impractical. Further experiments using large numbers of field infested oranges are required to determine the level of mealybug washed off commercial consignments of fruit. Field-infested oranges would also allow a comparison of the efficacy against a range of instars concealed under the calyces.

A low natural mortality for mealybug remaining under the calyx suggests that the area is generally protective. Mealybug crawlers can tightly wedge themselves under the calyx to escape exposure. The high mortality rate of crawlers treated with CPD indicates the oil penetrates under the calyx effectively. These results and the data from exposed bioassays (Chapter 2) suggest that a 3% rate would also cause very high mortality in larger mealybug instars. Earlier commercial trials by Taverner and Bailey (1995b) also demonstrated very high mortality of mealybug.

3.4.2 Efficacy of Postharvest Oil, CPD, against Concealed Mealybug

Lower rates than used in the confirmatory experiment are effective in the control of mealybug. The rate of 30,000ppm was determined by the need to control lightbrown apple moth, an actionable pest in the USA. The LC_{99} value estimate for dipping oranges infested with mealybug crawlers was significantly lower at 11,853ppm of CPD oil, with an upper 95%CL of 15,234ppm. A rate of 15,000ppm may be sufficient when mealybug are the target pest requiring control. Further tests are required using concealed mealybug of all stages

including confirmatory experiments at 15,000ppm with the most resistant stage on infested oranges.

The bioassays supported earlier work that pests are more difficult to kill when concealed on the orange (Taverner and Bailey 1995a). The LC estimates for dipping oranges infested with mealybug crawlers were much lower than corresponding LC values for exposed adult mealybug (see chapter 2). For instance, the exposed adult Citrophilus mealybug LC_{90} value estimate was 375ppm of CPD oil, with an upper 95%CL of 523ppm, compared to an LC_{90} value for concealed mealybug crawlers of 5,453ppm, with an upper 95%CL of 6,580ppm. It is most likely that the higher rates allow the oil to penetrate more easily into the small crevices where the smaller mealybug are sheltering. Alternatively, a longer dipping time may allow the oil to penetrate under an orange calyx. However, the time is limited commercially by the large orange volume throughput expected from a packing shed. The recommended immersion period for postharvest fungicide dips of citrus is 30 seconds.

3.4.3 Comparison of Selected Oils against Concealed Mealybug

The three selected oils resulted in survival rates higher than similar experiments with CPD oil. The difference in mortality may be due to a combination of factors including the variable toxicity of oils and their physical ability to reach the sheltering mealybug. The ability to penetrate beneath the calyx would vary due to the properties of the oil and its formulation characteristics. It may be affected by the molecular weight of the oil, the viscosity of the paraffin components of the oil and/or the surfactants used. All of these factors could affect the level of oil "creep" under the calyx. Further experiments using oils that vary only in one aspect of the above factors would be needed to assess their effect on the mortality of sheltering mealybug.

Interestingly, the vegetable oil, AWH-96-04, also caused higher mortality of sheltering mealybug than DC-Tron. This is in contrast to the higher efficacy of DC-Tron on exposed mealybug bioassays (Chapter 2). The high efficacy of the vegetable oil probably reflects a significantly greater coverage of the vegetable oil over the orange compared to the other two petroleum oils. Further work with vegetable oils to increase efficacy is warranted.

3.4.4 Development of Oils as Postharvest Treatments for Mealybug

The presence of mealybug on export citrus has repercussions for growers and exporters. They become obvious in transit and cause costly delays due to re-packing of infested fruit. In order to minimise the risk of poor out-turn, exporters set internal standards at sorting. In some seasons, when there is a high field incidence of mealybug, large numbers of oranges will be sorted out before packing. This affects the grower's returns and places greater reliance on chemicals to achieve very high field control of mealybug.

Currently, there is no treatment applied during the packing of oranges to reduce the incidence of mealybug. CPD has the potential to protect citrus export markets, reduce rejection at packout, minimise re-packing costs and improve grower returns.

3.5 Summary of Main Points

1. Postharvest oil, CPD, at a rate of 30,000ppm has been shown to be highly effective in the control of Citrophilus mealybug crawlers (first instar), with a 95%CL mortality rate of <99.95%.

2. The oranges used in the bioassay were artificially infested with large numbers of mealybug. Under these circumstances, large numbers were washed off during a water dip contributing to a high natural mortality rate.

3. A low natural mortality for mealybug remaining under the calyx (0.5%) suggests that the area is protective compared to exposed surfaces of the orange (33.4%).

4. Lower rates than used in the confirmatory experiment may be effective in the control of mealybug. The LC_{99} value estimate for dipping oranges infested with mealybug crawlers was significantly lower at 11,853ppm of CPD oil, with an upper 95%CL of 15,234ppm.

5. A comparison of three oils showed a vegetable oil (AWH-96-040) and spray oil (Citrole) to be superior to another petroleum spray oil (DC-Tron) for the control of sheltering mealybug.

Chapter 4

The Toxicity of Selected Petroleum and Vegetable Oil Dips to Exposed Lightbrown Apple Moth.

4.1 Introduction

The expanding international trade in fresh horticultural produce has increased the risk of the transport of insects on the surface of fruit. International quarantine regulations and inspection require fruit to be free of live pests. Lightbrown apple moth (LBAM), *Epiphyas postvittana* Walker (Lepidoptera: Tortricidae), a leaf-roller pest of many horticultural crops in Australia and New Zealand is a quarantine pest on exports of Australian navel oranges to the United States of America (USA). The calyx of an orange provides a protective shelter for a range of small arthropods. LBAM spins a hydrophobic silken domicile that enhances their survival during the normal washing process prior to packing. All fruit entering the USA must be free of LBAM and detection of live larvae presently results in fumigation with methyl bromide. Methyl bromide treatment reduces the quality of navel oranges and delays marketing. Fumigation has resulted in substantial financial losses to growers (Cain D., Chief Executive Officer Citrus Board of South Australia, private communication) and methyl bromide will soon be banned (Anonymous 1992). An alternative disinfestation treatment is needed to remove surface-dwelling pests from fruit.

In this study, a C15 alkane, Ampol's CPD postharvest dip was evaluated as a postharvest treatment against LBAM on citrus fruit. My interest stemmed from historical use of petroleum oils for the control of arthropods (de Ong 1927; Chapman *et al*, 1944; Riehl and Carman 1953; Riehl and Jeppsen 1953; and Trammel 1965) and a desire to use food-grade

products in packinghouses. The relative efficacy of petroleum spray oils, vegetable oils and a C15 alkane were compared as dips against different LBAM life stages.

4.2 Material and Methods

4.2.1 Lightbrown Apple Moth Colony

The LBAM used in the experiments were collected from a laboratory culture maintained, since 1993, at the South Australian Research and Development Institute, Waite Precinct (Adelaide). Singh, Clare and Ashby (1985) described the rearing procedure.

4.2.2 Oil Formulations

The details of the oils used are in Chapter 2. General specifications of CPD and DC-Tron are in Appendix 1.

4.2.3 Larval Bioassays

All oils were tested against fifth and third instar lightbrown apple moth. The larvae were selected after the desired developmental period (at 20°C; 12 days for 3rd instar, 21 days for 5th instar) and were treated with petroleum and vegetable oils at a single rate (5,000ppm) to determine their relative susceptibility. For dose-mortality bioassays, 6 rates were selected to give a range of mortalities for each oil. The rates selected for DC-Tron were 1000, 2500, 5000, 10000, 25000 ppm and for the Postharvest oil, CPD, rates were 250, 500, 750, 1000, 2500 ppm. The controls were dipped for in water only and the experiment was replicated 4 times. The procedure was as follows: groups of 10 larvae were collected from media pots using fine camel hair brushes. The larvae were transferred into a fine wiremesh spherical cage ("tea-infuser") 400mm in diameter. The closed cage containing the larvae was suspended in a 500ml beaker of well-stirred emulsion for 30 seconds. After treatment, the larvae were placed into rearing media within a plastic container. The container was sealed

and placed in a rearing room (20±2°C, 55± 5%RH, and natural lighting of 14h day:10h night). Mortality assessed hourly for the first 4 h and finally at 24h from dipping. Larvae were counted as dead if they did not move after repeated prodding with a needle. Larvae were recovered and placed in 80% alcohol for 12-24 h. The head capsule width of the larva was measured on a representative sample of larvae (15/treatment), using a stereo-microscope with an eyepiece graticule, to confirm the developmental stage.

4.2.4 Egg Bioassays

Egg masses laid on plastic cups (Polar cup, Adelaide, SA) were treated with a single dose of the petroleum and vegetable oils. The plastic cups (dimensions: 100mm high, 70mm at the top and tapered to 50mm at the base (capacity=285ml))had vertical ribs on the sides, which were prefered ovipostion sites for LBAM. The procedure for obtaining egg masses was as follows: Unmated pairs were placed in plastic cups (2 pairs per cup) with a cotton wool wick soaked in 10% honey solution as a food source. The moths were held in a rearing room at 20± 3°C, 55± 5% RH and under natural light (14h day:10h night). The moths began laying eggs by 24h and were removed after 72h. The individual egg masses were trimmed from the plastic using sturdy scissors. Small egg masses and large egg masses with many eggs laid on top of each other were discarded. Egg masses containing between 30-60 eggs per mass were used in bioassays.

Eggs in different stages of development were treated to assess if susceptibility of lightbrown apple moth eggs to oil changes with age. The ages of the eggs were 2, 7 and 10 days old expressed as days at 20°C from first observed egg laying until treatment. The developmental stages green, yellow and black-head were used to describe the age of the eggs for 2, 7 and 10 days old, respectively. Individual egg masses of each developmental stage were dipped for 30 seconds in a single dose (1,000ppm) of each oil to compare the relative efficacy.

Control eggs were dipped in water only and each treatment was replicated 6 times. After treatment, egg masses were placed on their edges in petri dishes to allow complete draining of excess oil. The petri dishes were lined with lightly moistened filter paper and placed in a rearing room at 20± 3°C, 55± 5% RH and under natural light (14h day;10h night). Egg development was checked every 2 days until hatching was completed.

In a second series of experiments, egg masses were dipped for 30 seconds in increasing oil concentrations of 50, 100, 500, 1000, 5000ppm. Dose responses were calculated for each age group. Eggs 2, 7 and 10 days old were treated with postharvest oil, CPD. The procedure was as previously described.

4.2.5 Pupal Bioassays

Lightbrown apple moth pupae were treated with petroleum and vegetable oils at a single rate (5,000ppm) to determine their relative susceptibility. For dose-mortality bioassays, 6 rates were selected to give a range of mortalities for each oil. The controls were dipped in water only and the experiment was replicated 4 times. The procedure was as described in larval bioassays in section 4.2.3.

After treatment, pupae were placed in a petri dish lined with lightly moistened filter paper and placed in a rearing room at 20± 3°C, 55± 5% RH and under natural light (14h day:10h night). Pupal development was checked until emergence was completed.

4.3 Results

4.3.1 Larval Bioassays

4.3.1.1 *Relative Efficacy of Oils against LBAM Larvae*

Bioassays of oils applied as dips to third instar LBAM larvae assessed the relative efficacy of a range of oils at a single dose of 5,000ppm. The results showed that the lower molecular weight petroleum oils had highest efficacy (Table 4.1). The control mortality was zero (n=40).

LBAM larvae treated with CPD were immobilised rapidly, with black necrotic areas and dehydration obvious within 2-4 hours from exposure (Figure 4.1). By 24 hours, NHC5 also caused very high mortality. However DC-Tron showed significantly lower levels of mortality. A comparison of the two vegetable oils showed AWH-96-04 had much higher mortality when compared with Eco-oil. All oils resulted in some mortality by 4 hours compared to no mortality in the control larvae.

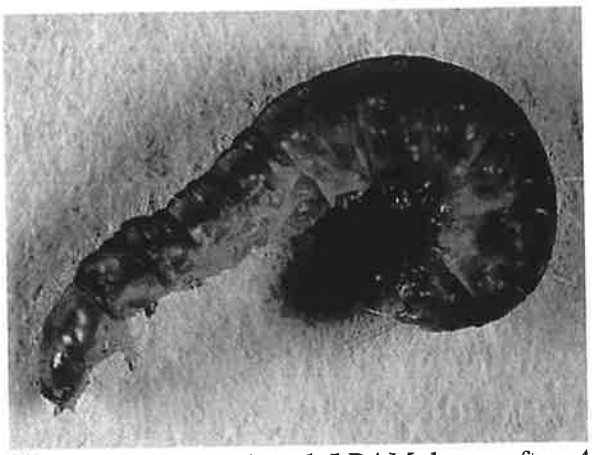

Figure 4.1. Dehydrated LBAM larva after 4 hours exposure to CDP dip.

Table 4.1 Mortality of 3rd instar lightbrown apple moth larvae dipped in 6 different oil formulations at 5,000ppm, and held at 20°C for 24hrs.

Formulation	*%Paraffin content*	*Carbon no.*	*Type**	*% Mortality(SEM)* 4hrs+	24hrs+
CPD	98+	15	p	100 (0.0)a	100 (0.0)a
Citrole	90+	19	p	80.0 (0.0)b	100 (0.0)a
NHC5	90+	22	p	42.5 (4.8)c	97.5 (2.5)a
DC-Tron	70+	23	p	22.5 (7.5)d	25.0 (7.5)b
AWH-96-04	-	-	v	37.5 (4.8)c	65.0 (9.6)c
Eco-oil	-	-	v	5.0 (2.8)d	10.0 (4.1)b

+Values are the means of 4 replicates. Means within a column followed by the same letter are not significantly different ($p > 0.05$, least significant difference)
* denotes type of oil; p=petroleum oil, v=vegetable oil

4.3.1.2 *Dose-Response Regression*

LBAM larvae were more tolerant to DC-Tron (Table 4.2), than to postharvest oil, CPD (Table 4.3). A dose response was also estimated for CPD against fifth instar LBAM to assess the influence of larval weight on tolerance to postharvest oil. Larger LBAM larvae showed more tolerance to oil than third instars (Tables 4.3 and 4.4).

Table 4.2 Mortality of 3rd instar lightbrown apple moth larvae dipped in 6 doses of DC-Tron, and held at 20°C for 24 hrs.

9n

Dose (ppm)	*Log10 (Dose)*	*Mortality (n)*	*Sample Size (n)*
0	0.000	0	20
1000	3.000	3	40
2500	3.398	9	40
5000	3.699	11	40
10000	4.000	16	40
25000	4.398	29	40

Table 4.3 Mortality of 3rd instar lightbrown apple moth larvae dipped in 6 doses of CPD, and held at 20°C for 24hrs.

Dose (ppm)	*Log10 (Dose)*	*Mortality (n)*	*Sample Size (n)*
0	0.000	0	20
250	2.398	4	40
500	2.699	26	40
750	2.875	27	40
1000	3.000	38	40
2500	3.398	40	40

Table 4.4 Mortality of 5th instar lightbrown apple moth larvae dipped in 6 doses of CPD, and held at 20°C.

Dose (ppm)	*Log10 (Dose)*	*Mortality (n)*	*Sample Size (n)*
0	0.000	0	20
1000	3.000	2	30
2500	3.398	13	37
5000	3.699	22	30
10000	4.000	55	60
25000	4.398	30	30

Estimates of the intercept and the slope, with standard error, are as follows:

3rd instar Larvae

DC-Tron intercept=-0.542, slope=1.364±0.223 (see figure 4.1)

CPD intercept=-6.210, slope=4.189±0.560 (see figure 4.2)

5th instar larvae

CPD intercept=-5.457, slope=2.975±0.369 (see figure 4.3)

The expected mortality, deviation from observed mortality, and the probability of response are shown in Tables 4.5, 4.6 and 4.7. The probit regressions for DC-Tron and CPD against fifth instar LBAM had low χ^2 values (Table 4.5 & 4.6). However, the dose response of third instar LBAM to CPD oil had a high χ^2 value (Table 4.7) suggesting high heterogeneity and a poor fit to the linear model.

Table 4.5 Observed and expected mortality, deviation, and probability of response of 3rd instar lightbrown apple moth larvae dipped in 6 doses of DC-Tron.

Observed Mortality (n)	*Expected Mortality (n)*	*Deviation*	*Probability of Response*
3	2.937	0.063	0.073
9	7.275	1.725	0.182
11	12.374	-1.374	0.309
16	18.610	-2.610	0.465
29	27.023	1.977	0.676

chi squared goodness of fit =1.852, 3d.f. heterogeneity factor = 0.62

Table 4.6 Observed and expected mortality, deviation, and probability of response of 3rd instar lightbrown apple moth larvae dipped in 6 doses of CPD.

Observed Mortality (n)	*Expected Mortality (n)*	*Deviation*	*Probability of Response*
4	4.885	-0.885	0.122
26	21.539	4.461	0.538
27	31.918	-4.918	0.798
38	36.509	1.491	0.913
40	39.950	0.050	0.999

chi squared goodness of fit = 6.681, 3 d.f. heterogeneity factor = 2.23

Table 4.7 Observed and expected mortality, deviation, and probability of response of 5th instar lightbrown apple moth larvae dipped in 6 doses of CPD.

Observed Mortality (n)	*Expected Mortality (n)*	*Deviation*	*Probability of Response*
2	1.886	0.114	0.082
13	13.475	-0.475	0.319
22	21.248	0.752	0.771
55	55.537	-0.537	0.952
30	29.871	0.129	0.996

chi squared goodness of fit = 0.3243, 3 d.f. heterogeneity factor = 0.11

A comparison of the LC_{95} estimates for DC-Tron and CPD on LBAM 3rd instar larvae show that CPD is almost 200x more efficacious (Table 4.8). A comparison of the LC_{95} estimates for CPD on LBAM 3rd instar and 5th instar larvae show that the larger 5th instar larvae are 10x more tolerant (Table 4.8).

Table 4.8 Estimates of the lethal dose and their 95% confidence limits (95%CL) for 3rd instar and 5th instar LBAM larvae dipped in DC-Tron or CPD.

Oil type	*Larval stage (instar)*	*LC_{50} (ppm) (95%CL)*	*LC_{95} (ppm) (95%CL)*
DC-Tron	III	11,587 (8,255-18,740)	186,310 (77,800-974,690)
CPD	III	474.1 (279.3-654.5)	1,171 (798.2-4,744.2)
CPD	V	3,270 (2,643.9-3,943.6)	11,682 (8,924-17,484)

The likelihood ratio (LR) test was used to determine if the two regression lines for CPD and DC-Tron against 3rd instar LBAM were equal. The LR statistic estimated that the two lines were unequal, ie. the slopes and intercepts were not the same (chi squared=180.51, df=2: p=0.00). The LR test for parallelism estimates that the lines slopes of the lines were not equal (chi squared=25.97, df=1: p=0.00). The different dose response and slope of the 2 regression lines can be seen in Figure 4.1.

A comparison of Citrole and NHC5 against third instar larvae shows both oils to be similar in efficacy to CPD at two selected doses. Using these estimates and the dose response data for CPD and DC-Tron to compare petroleum oils the efficacy of DC-Tron is lowest, with the remaining oils NHC5(C22), Citrole(C19) and CPD(C15) unable to be separated with these data.

The likelihood ratio (LR) test was used to determine if the two regression lines for CPD 3rd instar and 5th instar LBAM were equal. The LR statistic estimated that the two lines were unequal, ie. the slopes and intercepts were not the same (chi squared=132.40, df=2: p=0.00). The different dose responses of the 2 regression lines can be seen in Figure 4.1.

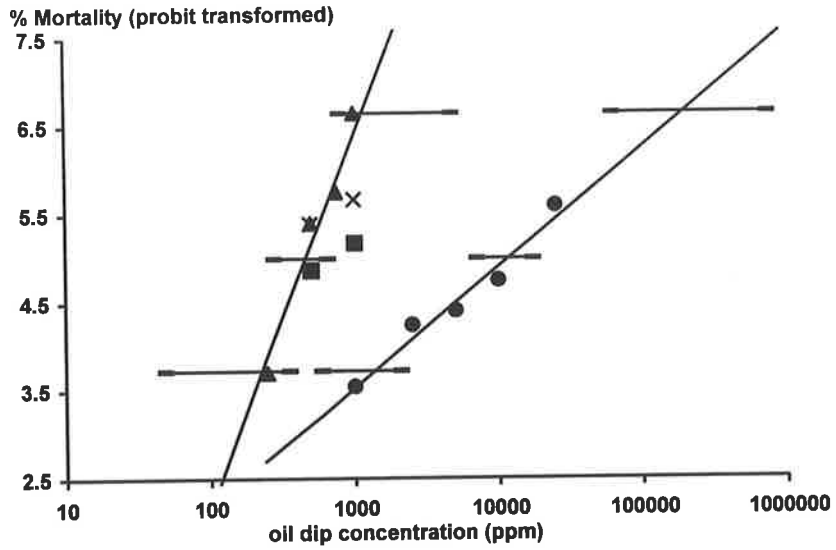

Figure 4.1 Dose response regression and 95% selected confidence limits of LC_{25}, LC_{50} and LC_{75} for 3rd instar LBAM larvae exposed to postharvest oil dip, CPD(▲) and DC-Tron (•), after 24hrs exposure, plus two doses of NHC5(■) and Citrole(×).

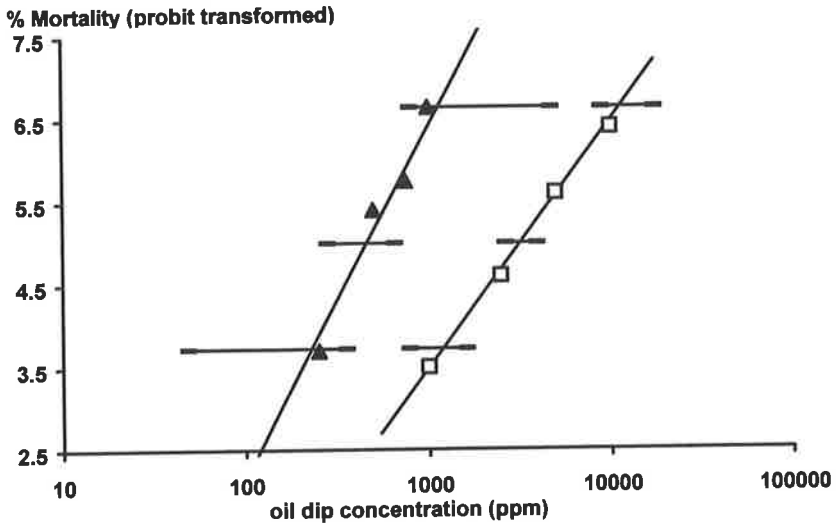

Figure 4.2 Dose response regression and selected 95% selected confidence limits of LC_{25}, LC_{50} and LC_{75} for 3rd instar (▲) and 5th instar (□) LBAM exposed to postharvest oil dip, CPD, after 24hrs exposure.

4.3.2 Egg Bioassays

4.3.2.1 *Relative Efficacy of Oils against LBAM Eggs*

Bioassays of oils applied as dips to black-head stage LBAM eggs were conducted to assess the relative efficacy of a range of oils at a dose of 1,000ppm. Dipping eggs in 1,000ppm emulsions of any of the selected petroleum oils caused high mortality (Table 4.9). The two vegetable oils, produced significant mortality, but were less efficacious than the petroleum oils. All petroleum oils showed equal efficacy at 1,000ppm. Control mortality was low (5.4%).

Two petroleum oils, DC-Tron and CPD, were selected at a lower dose (500ppm) against eggs at different developmental stages. Seven day-old (yellow) eggs were less susceptible to CPD than DC-Tron (Table 4.10). Two (green) and 10 day-old (black) stage eggs were equally susceptible to both oils. Some 10 day-old eggs hatched less than 24 hours from dipping and larvae emerging from DC-Tron treated eggs died when exposed to oil residues remaining on the plastic disc.

Further work with CPD oil-dipped LBAM eggs showed differences in susceptibility at different incubation periods. Two (green) and 10 day-old eggs (black) were significantly more susceptible than 7 day-old eggs (yellow) when dipped in 100 or 1000 ppm CPD emulsions. However, eggs of all ages were 100% susceptible to 5000 ppm emulsions (Table 4.11).

Table 4.9 Mortality with standard error of the mean (SEM) of black stage lightbrown apple moth eggs dipped at 1,000ppm for 6 oil formulations, and held at 20°C until hatched.

Formulation	*Total eggs*	*Unhatched*	*% Mortality* (SEM)*
Water	268	13	5.5 (2.31)a
CPD	270	270	100 (0.00)b
Citrole	283	283	100 (0.00)b
NHC5	269	269	100 (0.00)b
DC-Tron	270	31	89.9 (5.56)b
AWH-96-04	274	134	54.4 (10.8)c
Eco-oil	265	185	26.4 (7.37)d

*Values are sums of 6 egg masses. Values within a column followed by the same letter are not significantly different according to one way analysis of variance of the arcsine square root-transformed percentage data. ($F=42.21$, $df=6$, $p>0.05$, least significant difference)

Table 4.10 Mortality of 3 stages of lightbrown apple moth eggs dipped at 500ppm for 2 oil formulations, and held at 20°C until hatched.

Stage	*Formulation*	*Total eggs*	*Unhatched*	*% Mortality (SEM)+*
green	Water	207	11	6.3 (3.66)
(2-day old)	CPD	239	231	96.0 (2.35)
	DC-Tron	236	236	100 (0.00)
Yellow	Water	207	14	5.2 (3.92)
(7-day old)	CPD	235	100	41.6 (2.94)
	DC-Tron	184	184	100 (0.00)
black	Water	227	19	8.99 (1.53)
(10-day old)	CPD	261	134	51.54 (11.67)
	DC-Tron	256	193	82.83 (15.63)

*Values are the sum of 6 egg masses.
ANOVA of arcsine square root-transformed percentage data: Mortality using different oils significantly different ($F=127.67$, $df=2$, $p<0.001$), egg stage is significant ($F=5.58$, $df=2$, $p<0.05$), and oil type by egg stage interaction is significantly different ($F=7.42$, $df=4$, $P<0.001$).

Table 4.11 Mortality of 3 developmental stages of lightbrown apple moth eggs dipped in CPD oil formulation.

Rate (ppm)	*Egg age (days)*	*Total eggs**	*Unhatched*	*%Mortality (SEM)*
0	2	253	34	13.4 (5.8)
	7	299	27	9.0 (3.2)
	10	248	29	11.7 (2.7)
100	2	264	175	66.3 (16.1)
	7	312	17	5.4 (2.2)
	10	263	135	51.3 (19.4)
1000	2	265	265	100.0 (0.0)
	7	301	73	24.3 (14.7)
	10	274	253	92.3 (1.2)
5000	2	248	248	100.0 (0.0)
	7	306	306	100.0 (0.0)
	10	316	316	100.0 (0.0)

*Values are the sum of 6 egg masses.
ANOVA: Mortality at different oil rates significantly different ($F=38.1, df=2, p<0.001$), egg stage is significant ($F=22.9$, $df=2$, $p<0.001$), and oil rate by egg stage interaction is significantly different ($F=4.61$, $df=4$, $P<0.005$).

4.3.3 Pupal Bioassays

Higher oil rates were required to kill LBAM pupae compared to eggs. Female and male pupae were equally susceptible to oils. LBAM pupae were more susceptible to Citrole and NHC5 compared to other selected oil formulations. DC-Tron was the least efficacious (Table 4.12).

Table 4.12 Mortality of lightbrown apple moth pupae dipped in 6 oil formulations at 15,000ppm, and held at 20°C until emerged.

Formulation	*Sex*	*% Mortality (SEM)*+	*% Cum. Mortality (SEM)#*
Water	Male	1.25 (0.630)	
	Female	0.75 (0.479)	1.00 (0.378)a
CPD	Male	6.75 (0.947)	
	Female	4.5 (2.062)	5.63 (1.133)b
Citrole	Male	10.0 (0.000)	
	Female	9.75 (0.250)	9.78 (0.125)c
NHC5	Male	9.75 (0.250)	
	Female	9.25 (0.479)	9.50 (0.267)c
DC-Tron	Male	1.75 (0.854)	
	Female	1.25 (0.630)	1.50 (0.500)a
AWH-96-04	Male	5.25 (0.750)	
	Female	6.00 (0.817)	5.63 (0.532)b
Eco-oil	Male	4.00 (0.707)	
	Female	4.00 (0.913)	4.00 (0.535)b

+Values are means of 4 replicates of 10 pupae each.
ANOVA: Mortality of alternate oils is significantly different ($F=34.00, df=6, p<0.001$), sex is not significant ($F=1.05$, $df=1$, $p>0.05$), and oil type by sex interaction is not significantly different ($F=0.57$, $df=6$, $p>0.05$).

#Values are means of the cumulative mortality (ie. sum of male and female). Values within a column followed by the same letter are not significantly different according to analysis of variance of data. ($p>0.05$, least significant difference).

4.4 Discussion

4.4.1 Larval Bioassays

C15 Ampol CPD was much more efficacious in dips against LBAM larvae than C23 Ampol D-C-Tron NR. These products are quite different and their acute effects on the larvae possibly relate to distinct physical characteristics due to their different molecular volumes. CPD is a homogeneous C15 alkane with a molecular weight of 212. It is liquid at room temperature (alkanes with ≥16 carbon atoms are waxes at room temperature), and has a molecular volume of 278 ml $mole^{-1}$ at 15°C. DC-Tron contains a vast range of molecules of different shapes and sizes. It contains no alkanes and its C23 nomenclature refers to its mean equivalent *n*-paraffin carbon number; its 50% distillation temperature is the same as the boiling point of a C23 alkane (Furness *et al.* 1987). It has an average molecular weight of 350 and most molecules are paraffinic (% $C_P \geq 60\%$) C15 mono-cyclic molecules with side chains. Their average molecular volume is 417 ml $mole^{-1}$ at 15°C.

Interestingly, the higher efficacy of the C15 alkane compared to DC-Tron can not be explained. Many field trials with petroleum spray oils have indicated that insecticidal effectiveness increased within a molecular weight range of 220-360 and the critical value for highly paraffinic oils was about 340 (Chapman *et al.* 1943; Riehl and Carmen 1953; Riehl and Jeppson 1953; Trammel 1965). Other factors must be contributing to the efficacy of the alkane in these tests. Many insects and their eggs are more effectively controlled by highly refined paraffin oils (Chapman *et al.* 1943; Chapman *et al.* 1944; Riehl and Carman 1953; Riehl and Jeppsen 1953). These authors did not explain why paraffinic oils are more effective against certain insects and eggs. Ebeling (1945) suggested that the relative persistence or oxygen permeability of the oil films may be factors. The light oils used in this study are highly paraffinic, but the contribution of paraffinicity to efficacy was not evaluated in this thesis study.

The application technique of dipping rather than spraying, the oil depositing characteristics of the formulations (Campbell 1972) and/or an alternative mode of action may all influence the relative efficacy of the oils.

LBAM larvae dipped in light paraffins appeared to die rapidly, with treated subjects becoming necrotic and dehydrated within 4h from exposure. Dehydration may be due to the oil affecting the permeability of the cuticle, leading to increased water loss (Wigglesworth 1941) or perhaps toxicity is at some other site causing loss of control of the water balance. The respiratory system is another potential site for dehydration. However, high water loss is contrary to the proposal that oil restricts gas exchange by covering the cuticle and blocking spiracles. LBAM larvae can tolerate very low levels of oxygen and mortality due to anoxia can take several days (Dentener *et al.* 1992, Whiting *et al.* 1991). Larvae dipped in light paraffinic oils showed necrosis within hours of exposure, well before suffocation would be expected. Suffocation may be the primary mode of action for heavier molecular weight spray oils (de Ong *et al.* 1927), but lighter oils appear to have some other mode of action.

The effects of emulsifiers on plant cuticles and waxes has been well described and reviewed (Gaskin 1995). However, although the effects of emulsifiers on plant and arthropod cuticles are probably similar (Hurst 1941) the different emulsifiers used in the two products are unlikely to have been primarily responsible for the effects of the latter on LBAM larvae. Although the emulsifiers may be different products, they are likely to be similar in their physio-chemical properties ie., non-ionic surfactants. They are a relatively small component of the total oil formulation (<5% by volume), and ,therefore, highly diluted in water emulsions. However, the role of emulsifiers, particularly in combination with oils, needs to be investigated.

A comparison of third and fifth instar larvae showed that the larger larvae were more tolerant to exposure in oils. The LC_{95} dose estimate was 10X higher for fifth instars compared to third instars exposed to CPD oil dips. Robertson and Preisler (1992) suggest that dose weight responses do not vary as a simple function of body weight, and the significance of weight should always be tested in specific areas where a relationship is needed.

The vegetable oils used in this study showed significantly different levels of efficacy against LBAM larvae. Vegetable oils are thought to have the same mode of action as petroleum oils, killing by suffocation (Davidson *et al.* 1991). The death of larva within 4h of exposure suggests a mode of action other than suffocation. Higher toxicity of some vegetable oils has been attributed to their fatty acid contents (de Ong *et al.* 1927). The mortality of LBAM larvae with vegetable oils selected in this study may be due to a chemical component in the oil, the dipping method, the oil depositing characteristics of the formulations and/or an alternative mode of action.

4.4.2 Egg Bioassays

The petroleum oils tested were effective ovicides at ≥ 1,000 ppm and more efficacious than the vegetable oils selected. C23 Ampol DC-Tron NR oil's high efficacy may be due to its persistence on the surface of the egg causing accumulation of toxic metabolites (Smith and Pearce 1948). A number of studies have revealed certain basic relationships between ovicidal efficacy and the physical and chemical properties of oils. Petroleum oil's effectiveness as an ovicide is inversely correlated with volatility and molecular weight (Pearce and Chapman 1952, Fiori *et al.* 1963). A molecular weight of >265 is considered necessary to obtain high egg mortality with highly paraffinic oils (Trammel 1965) and maximum efficacy is attained

at a molecular weight of 320; 50% distillation point (760mm Hg) of 354° C (Pearce and Chapman 1952). DC-Tron, with a molecular weight of 350, is well above the critical weight level, however, this does not explain the high efficacy of CPD, with a molecular weight of 212. CPD is an alkane, ie. %Cp=100, and efficacy increases with paraffinicity (Pearce & Chapman 1952). Fiori *et al.* (1963) suggested that the paraffin chains form a better seal over the egg and consequently interfere with gas exchange to a greater extent. The C15 alkane is likely to dissipate before it can interrupt gas exchange (Fiori *et al.* 1963) but would be expected to have more rapid penetration than C23 Ampol DC-Tron. Further studies are required to determine the similarity between its mode of action and reported effects of other oils on gas exchange through egg membranes, accumulation of toxic metabolites (Smith and Pearce 1948), hardening of membranes to prevent hatching, and interference with hormone or enzymes after they penetrate the chorion (Hoskins 1943).

The results also showed that susceptibility to oil varied with the age of eggs kept at 20°C. For C15 CPD, 2- and 10-day old eggs were more sensitive than 7-day old eggs. This is contrary to work on the eggs of oriental fruit moth, *Grapholita molesta* (Busck), and codling moth, *Cydia pomonella* L., which become less susceptible to petroleum spray oils at the most mature stages (Smith and Pearce, 1948; Riedl *et al.*, 1995). DC-Tron also becomes less toxic to LBAM eggs as they mature. However, in this study, the lower efficacy in 10-day old eggs may at least partially be due to egg hatch within 24h of dipping. Smith and Pearce (1948) showed that eggs rinsed of oil residues within 24h of application are ineffective, presumably due to insufficient time to interrupt gas exchange or accumulate toxic metabolites. Given this hypothesis, eggs hatching prior to 24h would result in the same outcome.

It is interesting to note that many larvae emerging from eggs treated with DC-Tron did not survive. This was probably due to larvae receiving a lethal dose from residues on the disc. The significance of this would need to be tested, as oil residues may be unnaturally high in this study. The oil would disperse less on a non-porous plastic compared to porous fruit or leaf surfaces (Riedl *et al.* 1995).

4.4.3 Pupal Bioassays

Pupae are the most tolerant stage to C15 CPD and C23 DC-Tron oils compared with eggs and larvae. Interestingly, the intermediate weight petroleum oils C19 Citrole and C22 NHC5 were more efficacious than the other selected oils. Their increased activity may be due to a combination of relatively high molecular volume, ie., increased persistence, and a high paraffin content, ie., lower viscosity. Alternatively, different emulsifiers are used in each product, which may have had some influence on the results. The mode of action needs to be investigated to determine the influence these characteristics have on efficacy.

4.5 Summary of Main Points

1. Petroleum oils can effectively control larvae when dipped in emulsions. The postharvest oil, CPD, a light alkane, was the most efficacious against lightbrown apple moth larva.

2. The higher efficacy of CPD is contrary to field trials with petroleum spray oils where the lightest fractions showed the poorest efficacy (Chapman *et al.* 1943; Riehl and Carmen 1953; Riehl and Jeppson 1953; Trammel 1965).

3. LBAM dipped in light paraffinic oils appeared to die rapidly, with treated subjects necrotic and dehydrated within 4 hours from exposure. High mortality of larvae dipped in light paraffinic oils occurred well before suffocation would be expected.

4. A comparison of third and fifth instar larvae showed that the larger larvae were more tolerant to exposure in oils. The LC_{95} dose estimate was 10X higher for fifth instars compared to third instars exposed to CPD oil dips.

5. The vegetable oils used in this study showed significantly different levels of efficacy against LBAM larvae. The death of larvae within 4h of exposure suggests a mode of action other than suffocation.

6. Oil dips would act as efficient ovicides. The mechanism for CPD needs to be investigated.

7. A comparison of CPD tolerance of egg stages showed that the yellow stage (7-day old) was more tolerant than other egg developmental stages.

8. The greater efficacy of DC-Tron on eggs, particularly 7-day and 10-day old eggs, compared to CPD is contrary to the trend of activity of those oils against larvae. This suggests that the mode of action of these oils may be different for eggs compared to larvae.

9. The intermediate molecular weight petroleum oils C19 Citrole and C22 NHC5 were more efficacious against pupae than the other selected oils. Their increased activity is not understood and the mode of action needs to be investigated.

Chapter 5

The Chronic Effects of Low Doses of Selected Oils on Lightbrown Apple Moth.

5.1 Introduction

It is a widely held view that petroleum spray oils kill solely by contact and consequently insects only die if present at the time of application (Davidson *et al.* 1991). Insects migrating to recently treated foliage appear unaffected, presumably due to the inability of the oil to transfer from the foliage in sufficient quantities to block spiracles and suffocate the insects.

Petroleum spray oils have essentially no residual value compared to synthetic pesticides, which has contributed to the favoured use of oils in many IPM programs (Davidson *et al.* 1991). However, a few studies have postulated that insects can be affected by oil residues, causing mortality weeks after exposure, reduced thrift (Baxendale and Johnson 1988, 1990) and reduced fecundity (Ebeling 1936). Oils are generally assumed to cause acute mortality by hypoxia, but the mode of action of any residual effects due to oils has not been reported.

The aims in this chapter were to determine the effects of selected petroleum and vegetable oils on the mortality of LBAM larvae over several weeks and the subsequent reproductive capacity of survivors. The larvae were treated either through incorporation of oil in the rearing media, directly dipped or sprayed with low doses of oil.

5.2 Materials and Methods

5.2.1 Lightbrown Apple Moth Colony

The larvae and eggs used in the experiments were collected from a laboratory culture maintained, since 1993, at the South Australian Research and Development Institute, Waite Precinct (Adelaide). Singh, Clare and Ashby (1985) described the rearing procedures.

5.2.2 Oil Formulations

The details of the oils used are in Chapter 2. General specifications of CPD and DC-Tron are in Appendix 1.

5.2.3 Residual Mortality at Low Doses

For residual mortality experiments, a single rate of 100ppm was selected to produce a very low mortality 24 h after exposure. Mortality was subsequently recorded over a period of two weeks to determine the residual effects of oils on LBAM larvae. Prior to dipping, groups of 15 larvae were collected from media pots of the desired development period (at 20^0C; 12 d for 3rd instar) using fine camel hairbrushes. The dipping procedure was as described for previous bioassays (Chapter 4, section 4.2.3). After 24 h, all larvae were scored for activity according to the criteria described by Firko and Hayes (1990, Appendix 3). Any larva dead or unable to right itself easily was discarded, with the 10 highest scoring larvae per replicate placed in new media pots. The containers were sealed and placed in a rearing room (20±2°C, 55±5%RH, and natural lighting of 14h day:10 night) and mortality assessed every 4-7 d until emergence was completed.

5.2.4 Reproductive Capacity at Sub-Lethal Doses

Reproductive capacity was assessed on adult moths that emerged after oil exposure as 3rd instar larvae. A single rate of 100ppm was selected to produce a very low mortality and the survivors were allowed to emerge and mate. The number of eggs produced by moths developed from oil-dipped larvae were compared to eggs produced by moths from water-

dipped larvae. Prior to dipping, groups of 10 larvae of the desired development period (at 20°C; 12 d for 3rd instar) were collected from media pots using fine camel hairbrushes. The dipping procedure was as described for previous bioassays (Chapter 4, section 4.2.3). The sexes were separated as pupae. After eclosion, virgin pairs were placed in plastic cups (Polar cup, Adelaide, SA), with a single pair per cup and a cotton wool wick soaked in 10% honey solution as a food source. The moths were held in a rearing room at 20±3°C , 55±5% RH and under natural light of 14h day:10h night. The mated moths laid eggs on the inside surface of the plastic cup until 72h after pairing, when the moths were removed. The plastic cups with egg masses were returned to the rearing room until complete emergence or desiccation of control eggmasses when numbers of eggs and percent emergence were assessed.

In another experiment, adult moths were sprayed with CPD oil to assess mortality and egg laying. Forty newly emerged moths of each sex were anaesthetised with CO_2, placed on a filter paper in a petri dish and sprayed with a 10,000ppm, 25,000ppm or 50,000ppm emulsion of CPD oil. Control moths were sprayed with water only. All spray applications were made with a Potter tower (Burkhard, Rickmanshire, England). Each treatment consisted of a 4ml oil emulsion sample (or water control) applied at 103 kPa with a 15 s settling time.

After treatment, virgin moths of the same sex and treatment were placed in small plastic cages. The moths were held in a rearing room at 20± 3°C , 55± 5% RH and under natural light of 14h day:10h night. Mortality was assessed 24 h after spraying. Those moths treated with water or 50,000ppm CPD were paired in 3 combinations: control male with control female, CPD treated male with control female, and control male with CPD treated female. There were five pairs per treatment. Each pair was placed in cups (Polar cup, Adelaide, SA), with a cotton wool wick soaked in 10% honey solution as a food source and held in the rearing room for 72h. The number of eggs and percent egg hatch was recorded for all treatments after control eggs hatched or desiccated.

5.2.5 Effect of Oil Contaminated Food on Larval Mortality

Oils were added to the normal rearing media of the established LBAM colony to determine the effects of feeding exposure to oil on the survival of LBAM larvae. The rearing media was made as described by Singh, Clare and Ashby (1985). Postharvest oils were added at 10% (vol/vol) to liquid media during preparation. Approximately 30gms of mixed media was then placed in 6 cm-diameter pots with lids, allowed to solidify, and then stored at 5°C for 7 d. Tests with Automate Dye Red B (Petrafin, Sydney) indicated that the mixing procedures resulted in even distribution of the oil in the media. On day 7, groups of 10 third instar larvae were placed in the media pots. The larvae were then reared at 20±3°C and 55±5 %RH under natural light. Mortality was assessed after 4h and 24h. After 14d, the number of larvae that completed pupation and emergence were recorded for all treatments.

5.2.6 Effect of Oil Contaminated Food on Larval Feeding and Development

Oils were added to the normal rearing media of the established LBAM colony to determine the effects of chronic exposure of oil on LBAM larvae feeding behaviour and weight gain. The rearing media were made as described by Singh, Clare and Ashby (1985). Postharvest oils were added at 1% (vol/vol) to liquid media during preparation to minimise mortality. The media were prepared as described previously in Section 4.2.5. Groups of 10 stage 3 LBAM larvae were weighed in Petri dishes and placed in the media pots. The larvae were then reared at 20±3°C and 55±5 %RH under natural light of 14h day:10h night. Each media pot and group of larvae was weighed after 3d, 7d and 15d when pupation and adult eclosion rates were determined. Each of the two treatments comprised four replicates.

5.2.7 Fluorescence Techniques

Confocal microscopy was used to determine the location of fluorescent oil in larval structures, particularly the tracheal system. Paraffins have no autofluorescence, so an oil soluble

fluorescent dye, Fluorescent Yellow FG (Morton Chemical Company, Chicago, USA), was added to the oil at a rate of 1ml/l. Emulsions were made using the fluorescent stock solution and decontaminated water. To assess oil penetration after dipping, the larvae were dipped in 1,000ppm fluorescent oil emulsion or water only as previously described. To assess oil distribution by indirect exposure the oil was incorporated into diet media. Diet media was prepared by adding 30ml of 1,000ppm fluorescent CPD oil emulsion to 300ml of liquid media as described in section 4.2.5. Larvae were exposed for 24h in media pots before preparation for microscopy.

All larvae were rinsed thoroughly in water to remove most of the surface oil and mounted on glass slides within a plasticine well. Glycerol was added and a glass coverslip pressed on the sides of the plasticine well until it rested against the cuticle. Fluorescent microscopy was used for imaging the intact larvae. A Bio-Rad MRC-1000 laser Scanning Confocal Microscope System in combination with a Nikon Diaphot 300 inverted microscope in fluorescence mode with excitation at 488/10 nm and emission at 522/32 nm was used. The images of the larvae were collected using a 20x NA 0.40 dry objective lens. The confocal intensity settings used to capture an image of fluorescent oil produced no autofluorescence image of tracheae from control larvae.

5.2.7 Data Analysis

Statistix 4.1 (Analytical Software 1994) was used for analysis of variance (ANOVA). Bartlett's Test was used to test the hypothesis of equal variances. If the hypothesis was rejected a square root transformation of the data was used before one way analysis of variance. Percentage data were arcsine square root transformed before analysis. The formula used was as follows:

$$Tvalue=180/\Pi \times (\arcsin(\sqrt{value/100}))$$

Mean separation was determined using the least significant difference method (T), where T is the Student's t-statistic from the degrees of freedom (df) associated with the mean square of error.

5.3 Results

5.3.1 Residual Mortality at Sub-Lethal Doses

Oil formulations at 100ppm did not show any effect on the development of third instar LBAM larvae through to adulthood. Mortality levels were low in all treatments and were not significantly different from controls (Table 5.1). Greater than 80% of larvae that survived oil treatment developed, pupated and emerged as adults.

5.3.2 Reproductive Capacity at Sub-Lethal Doses

Pairs of adults treated with oil as third instar larvae were allowed to mate. The egg laying activity was recorded, ie. total number of eggs and size of eggmasses. There were large variations in the number of eggs laid by controls, with the 5 females laying 225, 256, 11, 224 and 208 eggs, respectively. The large variances in treatments made very large differences necessary for significant mean separation (Table 5.2).

Control pairs, and pairs treated with the lighter molecular weight oils, CPD and Citrole , averaged more than 100 eggs per female laid over a 72h period. The higher molecular weight petroleum oils, NHC5 and DC-Tron, gave lower numbers of eggs. The vegetable oils produced very different responses, with a mean of 254.3 and 69.3 eggs per female for AWH-96-04 and Eco-oil, respectively.

Table 5.1 Post 24h mortality of stage 3 lightbrown apple moth larvae dipped at 100ppm for 6 oil formulations, and held at 20°C for 14 days.

Formulation	*%Larval mortality (SEM)*	*%Pupal mortality (SEM)*	*%Emergence (SEM)*
Water	2.5 (2.5)a	5.0 (2.9)a	92.5 (2.5)a
CPD	10.0 (4.1)a	5.0 (5.0)a	85.0 (6.5)a
Citrole	5.0 (5.0)a	7.5 (4.7)a	87.5 (2.5)a
NHC5	15.0 (2.9)a	2.5 (2.5)a	82.5 (2.5)a
DC-Tron	2.5 (2.5)a	7.5 (2.5)a	90.0 (4.1)a
AWH-96-04	7.5 (4.8)a	10.0 (4.1)a	82.5 (8.5)a
Eco-oil	2.5 (2.5)a	5.0 (2.9)a	92.5 (2.5)a

*Mean of 4 replicates of 10 larvae converted to percentages. Means within a column followed by the same letter are not significantly different according to one way analysis of variance ($p>0.05$). ANOVA: oil formulation does not significantly affect larval mortality ($F=1.73$, $df=6$, $p>0.05$) pupal mortality ($F=0.44$, $df=6$, $p>0.05$), and adult emergence ($F=0.84$, $df=6$, $p>0.05$).

The pattern of egg laying, ie., the number of eggs per egg mass, varied considerably between treatments. Egg treated with NHC5 and DC-Tron had significantly fewer eggs per egg mass than control females, with a mean of 37 eggs per mass. DC-Tron, NHC5 and Eco-Oil produced the smallest egg masses, and consequently, also laid the lowest overall egg numbers.

The fertility of eggs was not necessarily related to the total number of eggs laid. Control females and females treated with AWH-96-04 had the highest total egg numbers coupled with high fertility. However, treatment with light molecular weight petroleum oils, CPD and Citrole , gave high numbers of eggs which were predominantly infertile. DC-Tron had a profound effect, with females producing fewer eggs, which were also usually infertile (Table 5.2).

Table 5.2 Reproductive capacity of female lightbrown apple moth dipped as stage 3 larvae in 100ppm of 6 different oil formulations.

Formulation	*no. of eggs/♀* (SEM)*	*no. of egg/mass* (SEM)*	*% emergence# (SEM)*	*no. of viable larvae /♀* (SEM)*
Water	184.8 (70.1)ab	37.1 (7.57)a	78.9 (19.7)a	145.8 (35.6)a
CPD	190.8 (69.9)ab	14.6 (5.10)abc	23.1 (23.1)bc	44.1 (44.1)bc
Citrole	104.0 (77.7)abc	8.8 (5.77)abc	0.0 (0.00)c	0.0 (0.00)c
NHC5	30.8 (17.4)bc	7.6 (4.52)bc	51.5 (15.8)ab	15.9 (4.8)b
DC-Tron	7.2 (4.53)c	2.1 (1.35)c	2.4 (2.43)c	0.17 (0.17)c
AWH-96-04	254.3 (70.1)a	32.0 (8.27)ab	47.0 (26.2)abc	119.5 (69.6)ab
Eco-oil	69.3 (51.7)bc	6.0 (2.13)abc	0.0 (0.00)c	0.0 (0.00)c

*Means of at least 5 replicates. Means within a column followed by the same letter are not significantly different according to one way analysis of variance of the data ($p > 0.05$, least significant difference)

Means within a column followed by the same letter are not significantly different according to one way analysis of variance of arcsin square root-transformed percentage data ($p > 0.05$, least significant difference)

When adult male and female moths were sprayed with CPD using a Potter tower, they showed a high tolerance to oil sprays. No mortality occurred in either sex after 24 h from exposure at rates of 10,000, 25,000 and 50,000ppm CPD oil. Males treated at 50,000ppm were mated with control females, and treated females with control males to determine the effects of the oils on the reproductive capacity of each sex. A comparison of control-only pairs with the other treatments showed no significant difference in the total eggs per female or the number of egg masses per female (Table 5.3). However, the fertility of eggs laid by pairs with an oil treated adult were significantly lower than controls. Both sexes appear to be affected, with similar egg fertility regardless of the sex of the oil treated adult in the pair.

Table 5.3 Reproductive capacity of lightbrown apple moth adults sprayed with 50,000ppm of CPD oil.

Treated pairs	*No. of eggs/♀** (SEM)	*No. of eggmass/♀** (SEM)	*% Emergence#* (SEM)
control♂,control ♀	277.4 (30.5)a	11.8 (0.97)a	66.3 (17.3)a
5% CPD♂,control ♀	147.2 (52.3)a	14.6 (2.82)a	17.4 (17.4)b
control ♂,5% CPD♀	147.2 (54.5)a	14.2 (1.74)a	10.6 (10.6)b

*Means of at least 5 replicates. Means within a column followed by the same letter are not significantly different according to one way analysis of variance of the data ($p > 0.05$, least significant difference)

Means within a column followed by the same letter are not significantly different according to one way analysis of variance of arcsin square root-transformed percentage data ($p > 0.05$, least significant difference)

5.3.3 Effect of Oil on Larval Feeding and Mortality

LBAM larvae were introduced to rearing media containing different oil formulations to assess the effect on feeding and mortality. At 10% oil in media the effect was rapid with mortality occurring within 4h from exposure. Controls recorded no mortality. High mortality occurred on oil media within 24h of exposure. Exposure to CPD oil produced significantly higher mortality than the other heavier molecular weight petroleum oils (Table 5.4). No individual survived to adulthood in any oil treatment.

Table 5.4 Mortality of stage 3 larvae placed in media containing 10% oil for 3 formulations, and held at 20°C until emergence.

Formulation	*% Mortality (SEM)*		*% Emergence (SEM)*
	4 h	24h	
Water	0.0 (0.00)a	0.0 (0.00)a	90.0 (8.16)a
CPD	47.5 (17.3)b	85.0 (12.9)b	0.0 (0.00)b
NHC5	7.5 (5.00)a	30.0 (0.00)c	0.0 (0.00)b
DC-Tron	17.5 (5.00)c	45.0 (17.3)c	0.0 (0.00)b

Values are means of 4 replicates. Means within a column followed by the same letter are not significantly different ($p > 0.05$, least significant difference)

Confocal microscopy was used to trace the coverage of CPD oil over the larval cuticle and entry via the spiracles among larvae exposed only through their diet. Dead larvae showed that oil had been ingested and had lined the oesophagus (Figure 5.1). The surface of the head capsule was covered with oil and had penetrated through the head capsule up to 60μm deep (Figure 5.2). The larval cuticle was largely covered and oil had penetrated deep into the tracheal system to invade tracheoles of 1-2μm diameter. Although the larvae were extensively covered there were large sections of the abdominal tracheal system, including spiracular openings, clear of any oil (Figure 5.3).

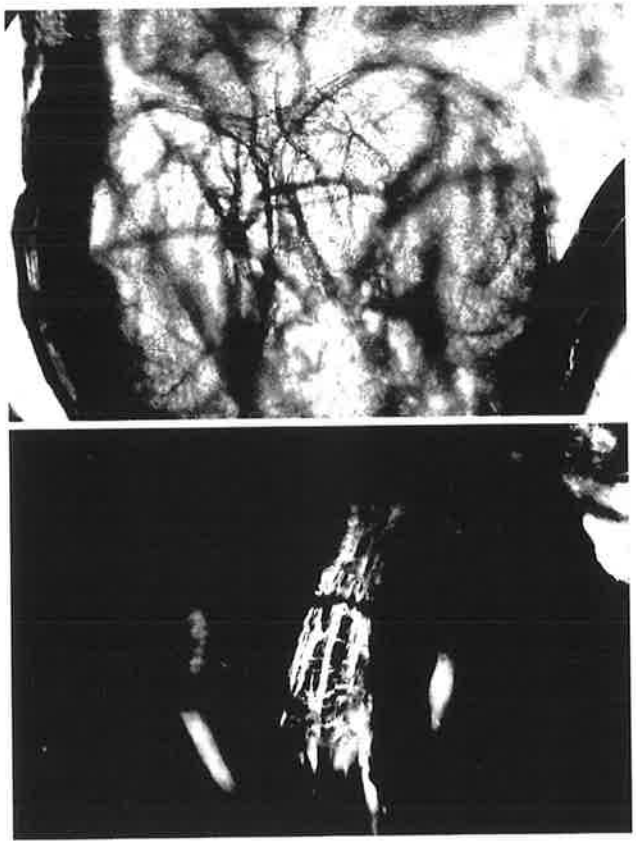

Figure 5.1 LBAM larval head capsule (dorsal view). Light transmission image showing capsule (top), and confocal image of fluorescent oil lining the oesophagus after CPD exposure (bottom).

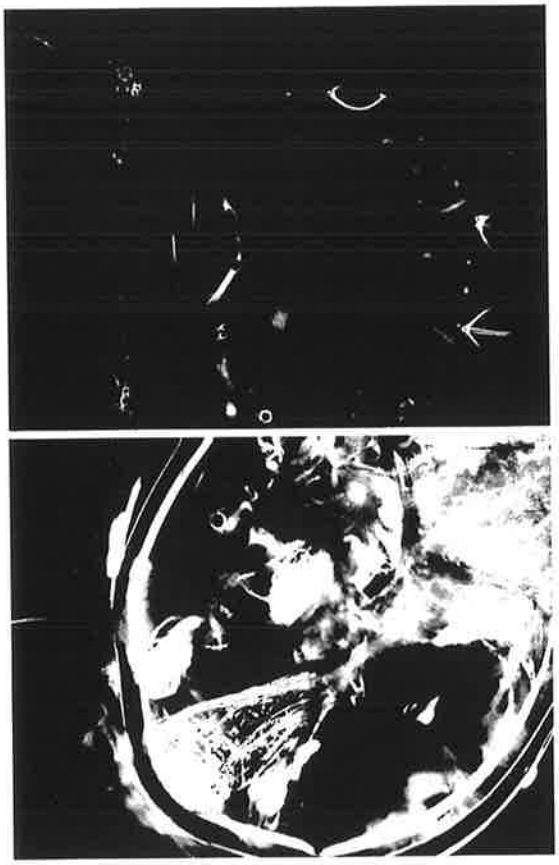

Figure 5.2 Confocal image of LBAM larval head capsule (dorsal view). Low autofluorescence without oil exposure (top), and high oil fluorescence after CPD oil exposure (bottom).

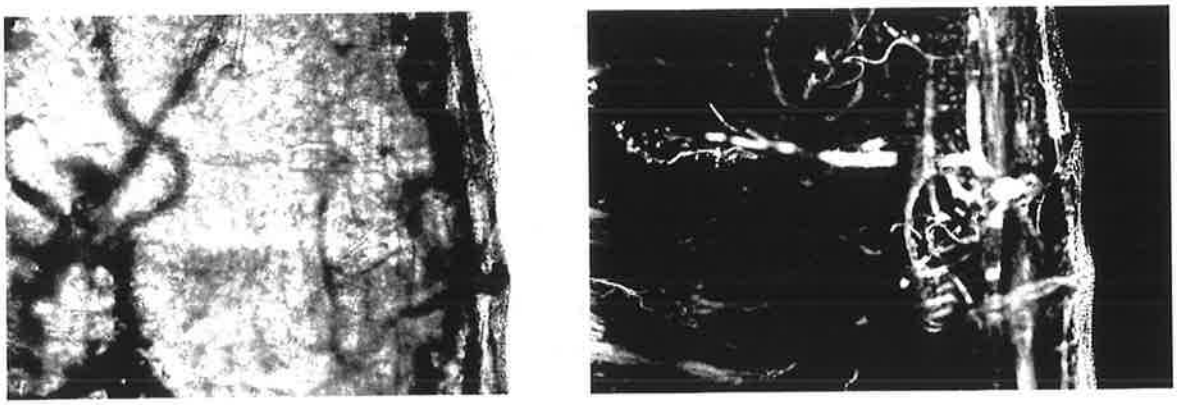

Figure 5.3 LBAM larva exposed to CPD contaminated food (longitudinal section of abdomen) light transmission image showing dark air-filled tracheae where oil has not penetrated (left), and confocal image showing fluorescent oil in some tracheal branches (right).

When larvae were exposed to a reduced dose of CPD oil (1%) incorporated into rearing media the levels of mortality after 15d exposure were not significantly different from controls, with a mean mortality of 17.5% and 12.5% mortality for oil exposed and control larvae, respectively (critical value for comparison is 13.2, $p>0.05$, LSD). However, larvae exposed to oil media did develop slower, with 35% pupated by 15d compared to 67.5% of control larvae (critical value for comparison is 16.9, $p<0.01$, LSD). Oil exposed larvae had a significantly lower body weight, appeared to avoid the food and ate significantly less than control larvae throughout their development (Table 5.5). Pupae that developed from larvae exposed to oil were also significantly smaller (oil=10.52mg/pupa, control=23.12mg/pupa; critical value for comparison is 4.63, $p<0.001$, LSD).

Table 5.5 Cumulative weight gain (mg) and food consumption (mg) of LBAM larvae after exposure to 1% CPD oil incorporated into media, and held at 20°C .

Treatment		*Days of exposure*		
		3d	7d	15d
H_2O	Avg. weight gain/larva (mg)	7.4	25.8	50.1
	Avg. food consumed/larva (mg)	32.1	97.3	203.2
CPD	Avg. weight gain/larva (mg)	3.6	17.9	43.2
	Avg. food consumed/larva (mg)	21.4	71.9	142.0

Values are means of 4 replicates. Differences between treatments in avg. larval weight gain significant on day 3 (critical value is 10.3, $p<0.001$), day 7 (critical value is 13.5, $p<0.05$) and day 15 (critical value is 7.65, $p<0.05$). Differences between treatments in food consumed significant on day 3 (critical value is 5.2, $p<0.01$), day 7 (critical value is 10.1, $p<0.001$) and day 15 (critical value is 7.65, $p<0.0001$).Critical value for separation of the means was determined by the least significant difference method (T).

5.4 Discussion

Petroleum spray oils are considered to give no residual control (Davidson *et al* 1995). However, in this study, some oils reduced the reproductive capacity of both male and female lightbrown apple moth. The mechanism to produce the effects described in this chapter are not known for any of the oils but it clearly is of interest. The heavier petroleum oils (C22 NHC5, C23 DC-Tron) had the largest affect on egg laying, presumably, because they were the least volatile and would affect the larvae over a longer period. Light paraffinic oils (C15 CPD, C19 Citrole) did not affect the numbers of eggs laid, but the fertility of eggs laid was low suggesting a mode of action more specific to egg viability, rather than general physiological depression. High volatility has classed them as unsuitable for field application (Ebeling 1950). However, although the oil quickly dissipates the effects on the insect are persistent. The vegetable oil, Eco-oil, also produced effects on fecundity and fertility. The mode of action of vegetable oils is poorly understood.

Few studies relate any residual effects to insects from the use of petroleum oils. Ebeling (1936) found that red scale, *Aonidiella aurantii*, treated with a dose of petroleum oil insufficient for suffocation resulted in scale continuing to die over several weeks. Surviving females had a reduced reproductive capacity, with large numbers of dead crawlers grouped about the female pygidium. This suggests an effect by oil on the physiological processes of the insect lasting several weeks. The oil used in Ebeling's field study was a light petroleum spray oil, with a 50% distillation point (760mm Hg) of about 325°C (based on 1932 California grade standards: Ebeling 1950; Davidson *et al.* 1995). However, it is unwise to compare modern highly paraffinic, narrow-range oils used in this study with the relatively crude, wide cuts available 60 years ago. Unfortunately, there have been no recent reports, to this author's knowledge, on the effects of narrow range oils on insect reproductive capacity.

The mechanism for the residual effects due to the alkane, which is more volatile, are more arcane. One explanation is that the dipping process used in this study may allow greater access into the tracheal system of insects than spray application. The oil would penetrate deeper where it may persist longer or affect different physiological processes.

Spraying a light paraffinic oil, CPD, affected the fertility of adult moths. The mechanism is unknown, but, both sexes were affected equally. As such, it is likely to be disrupting general physiological processes, rather than a highly specific event. DC-Tron was not tested but it is noted that the longevity and fertility of codling moth adults, *Cydia pomonella* L., were unaffected by a similar petroleum spray exposure (Riedl *et al* 1995).

Rearing LBAM larvae on oil contaminated media at low rates caused no mortality but effected the LBAM larvae through smaller size and slower development. This may have at least partially been due to feeding avoidance because larvae consumed smaller amounts of oil treated media. Baxendale and Johnson (1990) observed anti-feedant effects of petroleum oils on euonymous webworm, *Yponomeuta multipunctella,* but recorded no subsequent effect on development.

At higher doses in media, CPD produced larval mortality and confocal microscopy was used to examine the location of oil inside larvae. This technique was successful and should be used in preference to physical sectioning, which can alter the distribution of the oil during preparation.

Confocal microscopy of dead larvae revealed that CPD was ingested. However, it is not known if this contributed to mortality. Oil covered extensive parts of the larvae, particularly around the head and thoracic region, but there were still extensive areas of the abdominal tracheal system clear of oil. In this case, suffocation is unlikely to be the cause of mortality since the oil had not substantially blocked the tracheal system.

The oil was highly invasive and penetrated into the head, presumably through sutures in the head capsule. Oil, being lipophilic, could preferentially lodge in lipid containing tissues, including the nerve sheaths and lipids of the brain. The pharmacological effect of the absorption of hydrocarbons into lipid membranes is not clear. Hassell (1982) suggested that physical toxicity caused by inert substances, such as petroleum oils, may be due to their non-specific absorption on lipoprotein membranes resulting in the disruption of a highly specific event. This would not involve the formation of specific chemical attachments to receptors or active sites of enzymes.

The alkane, an organic solvent, would remove some surface lipids from the cuticular surface and tracheal linings. This is likely to cause immediate stress through water loss. Generally, oils high in unsaturated hydrocarbons have been implicated in "corroding" tissues (de Ong 1926). Modern narrow range spray oils low in unsaturates may still cause disruption of cell tissues, but, at a much slower rate. Oranges dipped in CPD oil, with no unsaturates, have recorded higher levels of ethylene production (Mark Hodgkinson, Caltex Australia Petroleum, pers. comm.) are consistent with increased cell disruption (Knoche and Noge 1991). It has been postulated that oils "open up" plant cellular membranes by displacing the membrane lipids (van Overbeek and Blondeau 1954) and that oils would also affect insect cell membranes in the same way (Corbett *et al.* 1984). Oils penetrating into areas with high lipid content would be susceptible to damage.

In addition, modern spray oils are formulated with emulsifiers which can have profound effects on biological tissues. The phytotoxicity of surfactants on plant cuticles and waxes has been well described and reviewed (Gaskin 1995). Given the similarity in structure of insect and plant cuticles (Hurst 1941) surfactants/emulsifiers probably affect insect membranes. Some of the residual effects of oil formulations may be due to the surfactant solubilising

membranes. Although the level of surfactants would be very low in emulsion (97% water; 2.9% oil, 0.1% surfactant) the oil may be acting as an efficient carrier of the surfactants, as well as, contributing to solubilisation itself.

The possible mechanisms proposed in this chapter involve general disruption but no specific event directly affecting reproduction has been implicated. It would seem that considerable work is required to understand the action of oils on insects that produce the residual affects described in this chapter. It is likely that the mechanism will vary according to the physical properties of the oil formulation used and may involve disruption at a number of different levels in the insect.

5.5 Summary of Main Points

1. Most LBAM larvae dipped in oil emulsions survived and continued to develop, but the reproductive capacity of both males and females was affected. The heavier petroleum oils (C22, C23) had the largest effect on egg laying. Light paraffinic oils (C15, C19) did not affect the numbers of eggs laid, but the fertility of eggs laid was low.

2. The dipping process may allow greater access into the tracheal system of insects where the oils may persist longer or affect different physiological processes.

3. Rearing LBAM larvae on media containing oil produced mortality if the dose was sufficient.

4. Confocal microscopy showed that the larvae were ingesting CPD. Suffocation is unlikely to be the cause of mortality since the oil had not substantially blocked the tracheal system.

5. CPD was highly invasive and penetrated into the head, presumably through sutures in the head capsule. Oil, being lipophilic, could preferentially lodge in lipid containing tissues, including the nerve sheaths and lipids of the brain.

6. Lower rates of oil insufficient to cause mortality had effects on the development of LBAM larvae. The larvae were smaller and developed slower on oil media. This may have at least partially been due to feeding avoidance because larvae consumed smaller amounts of oil treated media.

7. The oils and surfactants may be solubilising lipids of insect cuticles and cell membranes.

Chapter 6

The Mode of Action of Selected Petroleum and Vegetable Oils on Exposed Lightbrown Apple Moth: Anoxia.

6.1 Introduction

The most favoured theory explaining how oil kills insects is that the spiracles become plugged with oil leading to asphyxiation (Davidson *et al.* 1991). Early work by de Ong (1926) showed that lubricating oil entered the spiracles and penetrated a short distance into the tracheae of red scale, *Aonidiella aurantii* (Maskell), apparently resulting in suffocation. Investigations with various insects have shown that mortality caused by immersion in petroleum oils is an extremely slow process (de Ong *et al.* 1927; Ebeling 1945). Insects, including LBAM larvae, show a high degree of resistance to anoxia and will survive oxygen starvation for several days. The extended period taken to kill insects immersed in saturated oil is consistent with the effects of suffocation (Dentener *et al.* 1992; Whiting *et al.* 1991; Chapter 7).

The role of anoxia as the primary mode of action may be dependent on the method of application. Insects dipped in oil, and then exposed to the air, take much less time to die than continuously immersed insects. Ebeling (1945) suggested that the toxic properties of oil were expressed more rapidly in the presence of air and that desiccation, oxidation, vaporisation, or some other influence of the air increased the insecticidal properties of the oil.

The high and rapid efficacy of Ampol Citrus Postharvest Dip (CPD), a C15 alkane, against LBAM (Chapter 4) and mealybug (Chapter 2 &3) is difficult to reconcile with suffocation. A low molecular weight alkane should be rapidly expelled from the tracheal system of insects precluding anoxia (de Ong *et al* 1927).

The aims of this chapter were to determine the importance of anoxia as a mode of action for CPD and a range of spray oils. The period to mortality of LBAM larvae immersed in several petroleum and vegetable oils is compared. The influence of air on larval mortality after oil exposure is evaluated by continuous immersion, dipping and topical application. The oil distributions in larvae after different application methods are examined using confocal microscopy.

6.2 Materials and Methods

6.2.1 Lightbrown Apple Moth Colony

The LBAM larvae used in the experiments were collected from a laboratory culture maintained, since 1993, at the South Australian Research and Development Institute, Waite Precinct (Adelaide). The rearing procedure was described by Singh, Clare and Ashby (1985).

6.2.2 Oil Formulations

Ampol Research and Development Laboratories, Brisbane Qld, supplied a Citrus Postharvest Dip, Ampol C15 CPD, (an alkane with a carbon number of 15 ie.C15; paraffin content ie. %Cp>99%) and a commercial spray oil, Ampol C23 DC-Tron NR (narrow range oil with mean equivalent *n*-paraffin carbon number of a C23 alkane (Furness *et al.* 1987); %Cp<70%). General specifications of these oils can be found in Appendix 1. Total Solvents, Paris France, supplied two highly paraffinic oils, C19 Citrole NR and C22 NHC5 NR. An emulsifiable vegetable oil-based product, Eco-oil, used in agricultural spraying, was supplied by Organic Crop Protectants Pty Ltd, Lilyfield NSW, and an experimental vegetable oil-based postharvest dip, AWH-96-04, was supplied by the Victorian Chemical Company, Richmond Vic.

In addition, a medicinal paraffin, ALNOR 70, (Sigma, Adelaide SA) (Density >0.83, ASTM D-4052) was compared with CPD (Density = 0.787, ASTM D-4052) in immersion experiments.

6.2.3 Immersion and Dip (Thin Film) Larval Bioassays

Immersion and thin film experiments were conducted with a range of petroleum and vegetable oils to compare the relative period to produce anoxia in LBAM larvae. LBAM larvae (5^{th} instar) were immersed in CPD to determine mortality when exposed to a thick layer of oil. LBAM larvae were immersed in medicinal paraffin (ALNOR 70) and compared with CPD to assess the influence of oil density on pest efficacy. Light oils, like CPD, are expelled from the tracheae of insects, making them unsuitable as suffocants (de Ong *et al.* 1927). The influence of surfactants was assessed by immersing larvae in the C15 alkane used in CPD (ie., oil without surfactants) and comparing with CPD.

A filter paper was placed in the bottom of a 5.5cm diameter watch glass, and 1ml of oil was poured onto the filter paper. Groups of 10 5^{th} instar larvae were selected from the culture (at 20^0C, 21 days for 5th instar) and transferred onto watchglasses where they became covered with oil on contact with the oily filter paper. The larvae in the oil were placed in a rearing room (20±2°C, 55±5%RH, natural lighting of 14h day:10h night). Mortality was assessed every 30 minutes until 5 out of 10 larva in each group were dead. Larvae were counted as dead if they did not move after repeated prodding with a needle. The procedure is described by Ebeling (1945). The mean number of minutes required for the complete immobility of 5 out of 10 larvae (LT_{50}) was recorded.

In a second series of experiments, groups of larvae were placed on dry filter paper immediately after immersion in oil. Excess oil was absorbed by the filter paper and only a thin film of oil remained on the larvae. These larvae were assessed as for immersed larvae.

6.2.4 Topical Application Larval Bioassays

Small quantities of selected oils were topically applied to 5th instar LBAM larvae to determine mortality when the cuticle is exposed to a film of oil rather than total immersion. Groups of 10 larvae were selected from the culture (at 20^{0}C, 21 days for 5th instar) and either 2, 1 or 0.5µl of oil was applied to the cuticle of each larva within a group using a micropippette. Control larvae were dosed with water and all treatments were replicated 5 times. After oil application, the larvae were transferred into a filter paper in the bottom of a petri dish. The larvae were placed in a rearing room (20±2°C, natural lighting). Mortality was assessed at 4h and at 24h. Larvae were counted as dead if they did not move after repeated prodding with a needle.

6.2.5 Confocal Microscopy

Confocal microscopy was used to determine the location of fluorescent oil in larval structures, particularly the tracheal system. Paraffins have no autofluorescence, so an oil soluble fluorescent dye, Fluorescent Yellow FG (Morton Chemical Company, Chicago, USA), was added to oil at a rate of 0.01ml/l. Emulsions were made using the fluorescent stock solution and decontaminated water. Larvae were either dipped in fluorescent oil emulsions or water only as previously described. Larvae were rinsed thoroughly in 100ppm Triton-X to remove most of the surface oil, with a final rinse in water only. Immersed larvae were rinsed in liquid but never drained completely so as to retain complete liquid immersion during the entire washing process. Rinsed larvae were mounted laterally on glass slides within a plasticine well.

Glycerol was added and a glass coverslip pressed on the sides of the plasticine well until it rested against a section of the cuticle with spiracles.

Fluorescent microscopy was used for imaging the intact larvae. A Bio-Rad MRC-1000 laser Scanning Confocal Microscope System in combination with a Nikon Diaphot 300 inverted microscope in fluorescence mode with excitation at 488/10 nm and emission at 522/32 nm was used. The images of the larvae were collected using either a 40x or 20x NA 0.40 dry objective lens. The confocal intensity settings used to capture an image of fluorescent oil produced a faint autofluorescence image of the tracheae of control larvae. The first thoracic spiracle was used for comparisons of oil penetration into the tracheae. At least 3 larvae were examined per treatment.

6.3 Results

6.3.1 Immersion and Thin Film (Dipping) Tests

LBAM larva immersed in the oils without surfactants survived for a mean period in excess of 20h (Table 6.1). Larva dipped, blotted to remove excess oil and then exposed to the air died significantly quicker than immersed larvae. LBAM larvae were equally affected by CPD (Density = 0.787, ASTM D-4052) and medicinal paraffin (Density >0.83, ASTM D-4052) showing no influence of oil density on efficacy. The addition of surfactants to CPD did significantly influence efficacy against immersed larvae, with the LT_{50} value reduced from 28.5±1.6h to 9.8±0.48h, but, the addition of surfactants had no effect on the LT_{50} values for dipped larvae.

The next experiment compared the time-mortality response of a number of formulated oils (ie., with surfactants) against LBAM larvae in the absence (immersion) and presence (dipping) of air. The results showed immobilisation of immersed larva between 3 to 5h for most formulations (Table 6.2) which is much more rapid than expected for anoxia as supported by the 20h or more required for an LT_{50} for ALNOR 70 and CPD oil without

surfactants. Surfactants are present and may have an influence on the LT_{50} value. Larvae immersed in CPD took significantly longer to die than other immersed larvae, perhaps because the insect could restrict the movement of light oil and surfactants into the tracheae by active ventilation.

Table 6.1 Influence of oil density, surfactants and presence of air on the time-mortality (LT50) response of LBAM larvae (5th instar) following immersion or dipping in medicinal paraffin (ALNOR 70) and CPD with and without surfactants.

Oil type	*surfactants*	*Method*	*Mean LT_{50}{h} (SEM)**
ALNOR 70	-	immerse	20.8 (5.25)a
		dip	8.1 (3.32)b
CPD	-	immerse	28.5 (1.56)a
		dip	6.6 (1.28)b
	+	immerse	9.8 (0.48)b
		dip	6.0 (0.29)b

* Values are the means of 5 replicates of 10 larvae. Means within a column followed by the same letter are not significantly different ($F=3.08$, $df=5$, $p<0.05$, least significant difference (T)).

The LT_{50} estimates for dipping larvae in oils also showed immobilisation of immersed larvae in a period much more rapid than expected for anoxia, except for NHC5 (Table 6.3). The vegetable oils, AWH-96-04 and Eco-oil, caused the most rapid mortality. There is no obvious explanation for the range of responses between the oils but differences may relate to the individual formulations (ie., surfactant types and proportions).

The application technique (dipping or immersion) of oils produced a different response against LBAM for most oils suggesting a complex relationship between formulation and application method. Most importantly, regardless of the method, formulated oils immobilise larvae much more rapidly (LT_{50} of 3-10 h) than would be expected by anoxia alone (LT_{50} of more than 20 h).

Table 6.2 A comparison of oil formulations in the absence of air: Time-mortality response of LBAM (5th instar) to immersion.

Oil type	*Mean LT_{50} {h} (SEM)**
CPD	9.8 (0.15)a
Citrole	3.9 (0.22)b
NHC5	4.2 (0.45)b
DC-Tron	3.5 (0.68)b
AWH-96-04	4.3 (0.28)b
Eco-oil	4.0 (0.00)b

* Values are the means of 5 replicates of 10 larvae each. Means within a column followed by the same letter are not significantly different ($p < 0.05$, least significant difference).

Table 6.3 A comparison of oil formulations in the presence of air: Time-mortality response of LBAM larvae (5th instar) to dipping.

Oil type	*Mean LT_{50} {h} (SEM)**
CPD	6.1 (0.15)a
Citrole	7.0 (0.88)a
NHC5	19.0 (0.45)b
DC-Tron	5.8 (0.15)a
AWH-96-04	3.2 (0.07)c
Eco-oil	3.2 (0.00)c

* Values are the means of 5 replicates of 10 larvae each. Means within a column followed by the same letter are not significantly different ($p < 0.05$, least significant difference).

6.3.2 Distribution of Oil in Larvae after Immersion and Dipping.

The range of time mortality responses for different oils and application methods may be due to variation in the level of penetration of the oil. Greater penetration would displace a greater proportion of air from the tracheal system and reduce the available oxygen. Confocal microscopy was used to compare the distribution of two petroleum oils with distinctly different physical characteristics, CPD and DC-Tron, in the tracheae of larvae after continuous immersion or rapid dipping. Scanning the lateral surfaces of untreated larvae produced faint autofluorescence of the chitinous cuticular structures (Figure 6.1, top left). When larvae were immersed in CPD there appeared a strong fluorescence on the surface of the cuticle and within the spiracle, suggesting it had been blocked by oil (Figure 6.1, bottom left) but when rapidly dipped in oil, the spiracle was coated rather than filled (Figure 6.1, bottom right). Immersion in DC-Tron- produced similar results to CPD.

The oil distribution further into the tracheal system could be established because in untreated controls only faint autofluorescence shows in internal structures, such as the surface of tracheae, but cross section reveals no fluorescence inside tracheae (Figure 6.2). When larvae were immersed in DC-Tron, high intensity fluorescence occurs as the oil fills the tracheae near the surface (Figure 6.3, top left). The intensity of fluorescence decreased further down the tracheae and into smaller tracheal branches. Both CPD and DC-Tron deposited high amounts of oil at the surface but lower amounts of oil in smaller diameter tracheae.

Briefly dipping larvae in oil elicited a reverse distribution of oil within the tracheal system compared to immersion. Larvae dipped in DC-Tron showed oil in smaller tracheae but there was low fluorescence in the main tracheal branches and spiracles (Figure 6.3, bottom left). This is consistent with the larvae expelling the oil by respiratory movements from the spiracle and clearing the main tracheal branches. The low fluorescence appears to be due to

oil that has adhered to the spiracular wall and tracheae, coating rather than filling the spaces. Oil deeper in the tracheal system would be more difficult to clear. Overall, dipping in DC-Tron resulted in less oil in the tracheal system compared to immersion, but oil may still block the smaller tracheal branches.

Examination of larvae dipped in CPD also showed apparent extensive clearing of oil in the tracheal branches, but the oil also penetrated further into fine tracheoles where it could not be expelled (Figure 6.4, bottom left). Fluorescence of fine tracheoles was not detected in either control larvae (Figure 6.4, top left), larvae immersed in CPD or any DC-Tron treated larvae.

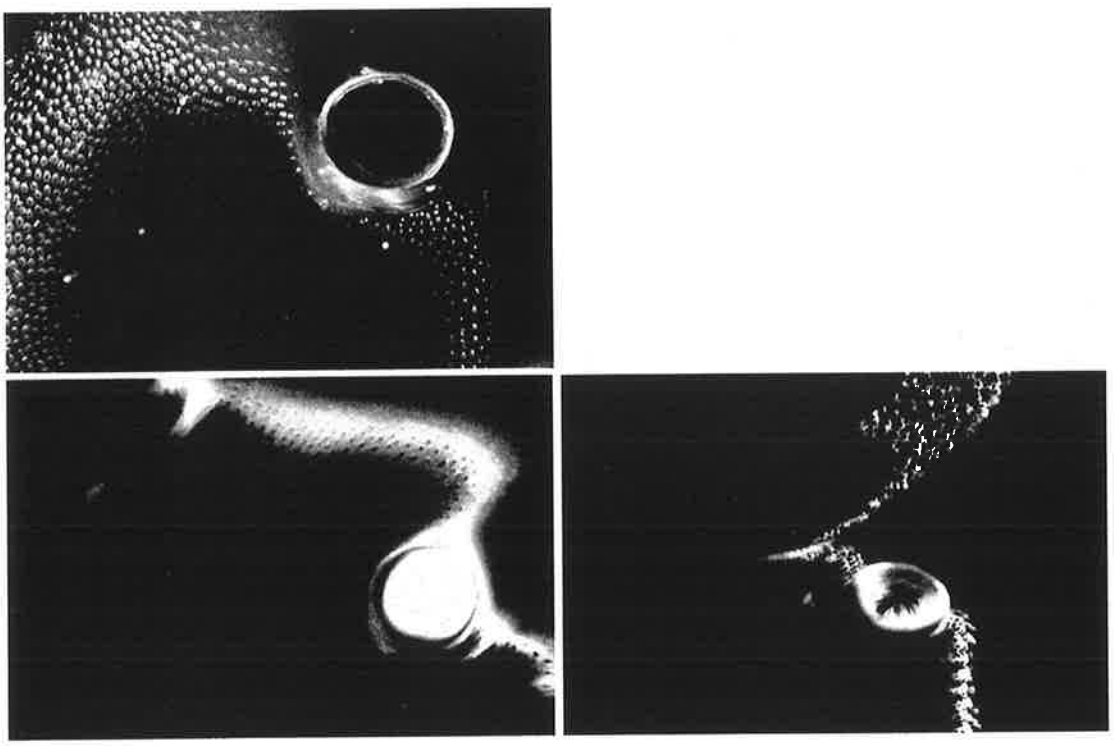

Figure 6.1 Confocal image of surface and 1st thoracic spiracle of LBAM larva; Autofluorescence of control larvae (top left), fluorescent oil-filled spiracle after immersion in CPD (bottom left), and fluorescent surface with clear spiracle after dipping in CPD (bottom right).

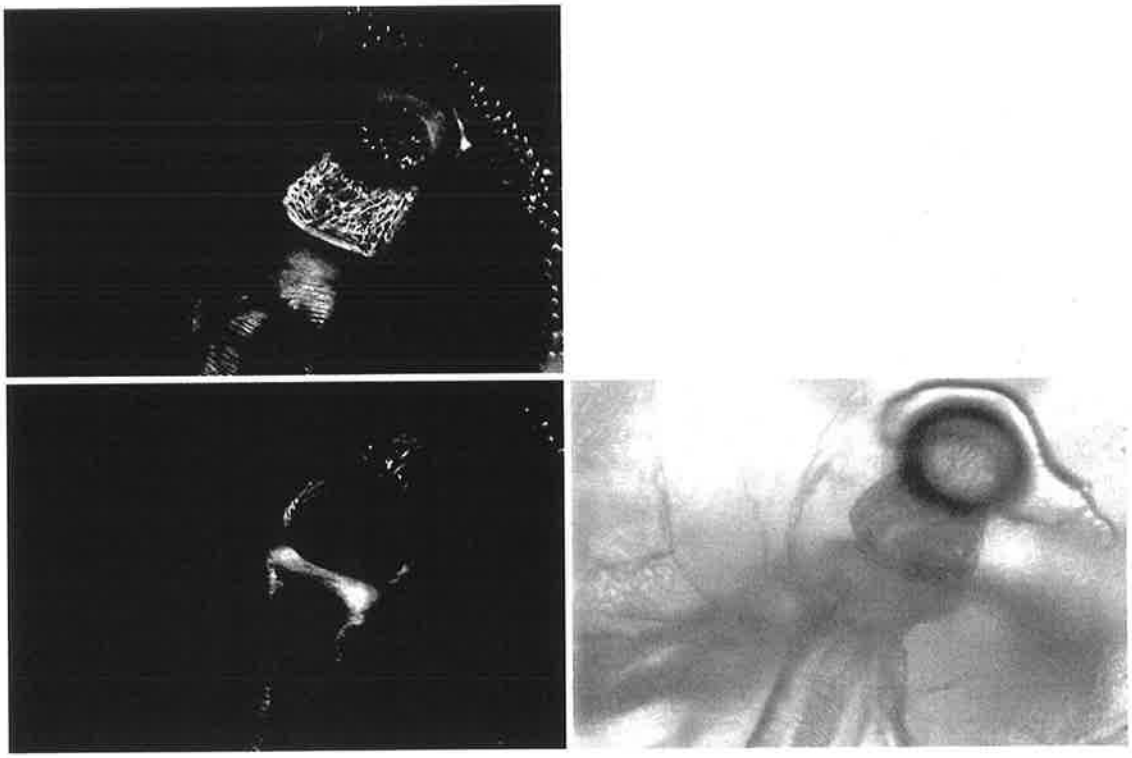

Figure 6.2 Natural fluorescence: 1st thoracic spiracle of untreated LBAM larva and associated structures (below surface shown in figure 6.1). Autofluorescence of tracheal surfaces (top left), and cross-section of tracheae shows no internal autofluorescence (bottom right). Light transmission image (bottom right) of tracheal branches.

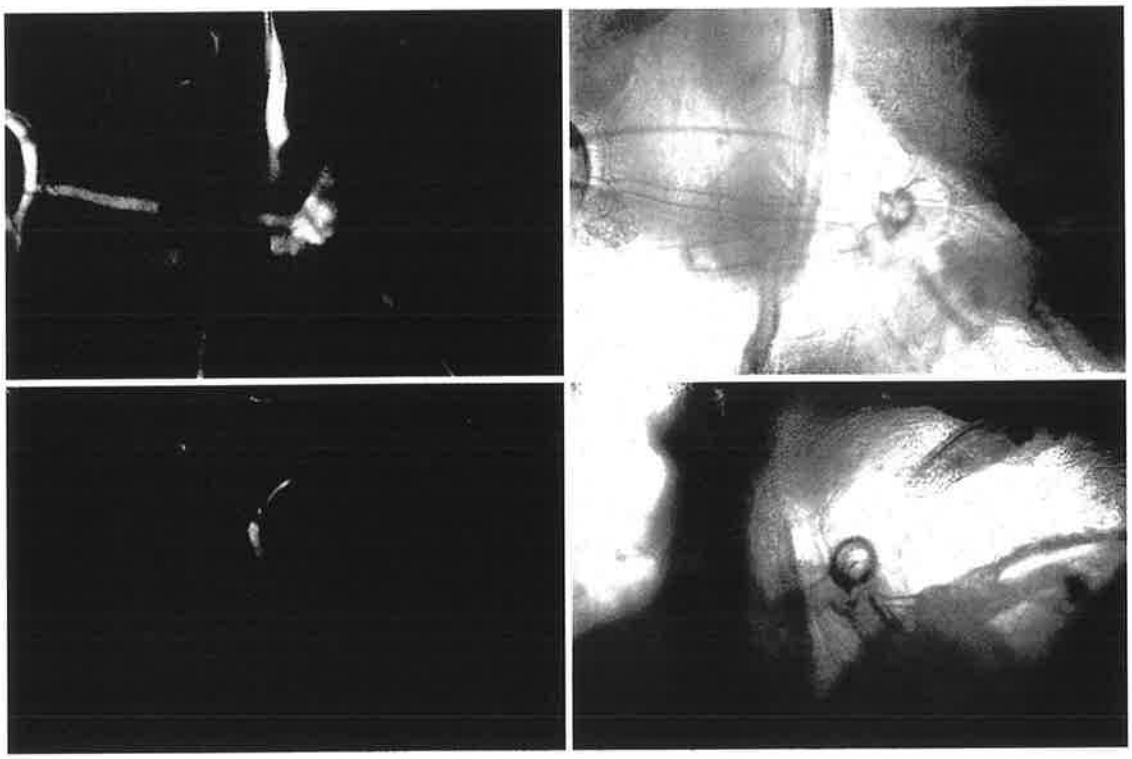

Figure 6.3 DC-Tron immersed (top), dipped (bottom) LBAM larvae showing 1st thoracic spiracle, and associated tracheal system. Oil fills the spiracle during immersion (top left) and coats the tracheae during dipping with only smaller tracheae remaining filled (bottom left). The respective light transmission images can also be seen (right).

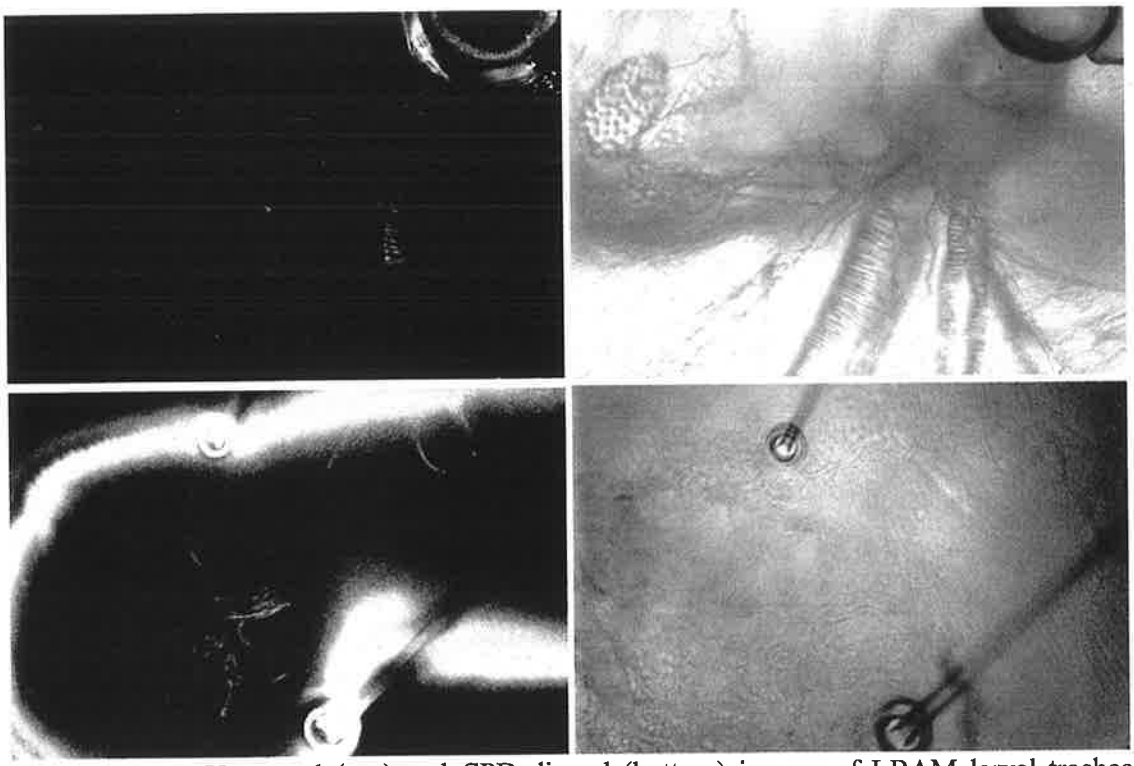

Figure 6.4 Untreated (top) and CPD dipped (bottom) images of LBAM larval tracheal system showing faint autofluorescence (top left) of the "air-filled" fine tracheoles visible in transmission (top right) and the strong fluorescence (bottom left) associated with "oil-filled" fine tracheoles invisible in light transmission (bottom right).

6.3.2 Topical Application Bioassays

Topical application of oils to larvae was used to establish the importance of the tracheal system as the site of action for oil. CPD was toxic to LBAM 5th instar larvae when applied topically at doses of 0.5µl, 1.0µl and 2µl per larva (Table 6.4). Control mortality in all 3 dose classes was zero compared with 28% and 32% mortality at the highest dose, 2µl/larvae, for CPD base oil and CPD, respectively. There were no significant differences in mortality with larvae exposed to oil with or without surfactants. A four fold increase in the oil dose (from 0.5 to 2µl) did not lead to a significant change in the level of mortality.

The distribution of oil after topical application was also observed by confocal microscopy. The oil largely covered the surface of the cuticle after topical application, but access to the tracheal system was limited to spiracles close to the point of application of the oil. The results of

topically applied CPD on LBAM larvae showed that there were 3 regions of exposure on any particular larvae:

1) Spiracles directly under the site of initial contact were filled with oil but very limited penetration of the tracheae was observed (Figure 6.5).

2) Spiracles at the edge of initial contact were coated with oil and there was greater penetration into the tracheae (Figure 6.6).

3) Spiracles well removed from the site of initial contact were clear of oil.

The blocking of spiracles reduced the penetration of the oil into the tracheal system. Oil that could flow into the tracheae and down the lipid lining without completely blocking the tracheae penetrated much deeper. Any particular oil-treated larva had a cuticle extensively covered with oil, but only spiracles near the point of application were filled or coated with oil. Complete blockage of the tracheal system was not necessary for some mortality to occur, which suggests again a mode of action other than anoxia.

Table 6.4 Mortality of LBAM larvae (5th instar) treated topically with CPD (with surfactants) and CPD base oil (without surfactants), at 3 doses.

treatment	*doses (µl)*	*% Mortality (SEM)**	
		4h	*24h*
CPD base	2.0	8.0 (3.7)	28 (4.9)
	1.0	6.0 (4.0)	24 (6.0)
	0.5	0.0 (0.0)	14 (5.1)
CPD	2.0	0.8 (3.7)	32 (3.7)
	1.0	0.4 (2.4)	34 (5.1)
	0.5	0.2 (2.0)	24 (2.4)

* Values are the means of 5 replicates of 10 larvae converted to percentages. ANOVA: After 24h, surfactant ($F=5.43$; $df=1$; $P=0.08$), dose ($F=2.55$; $df=2$; $P=0.14$), and surfactant by dose interaction ($F=0.26$; $df=2$; $P=77$) are not significant.

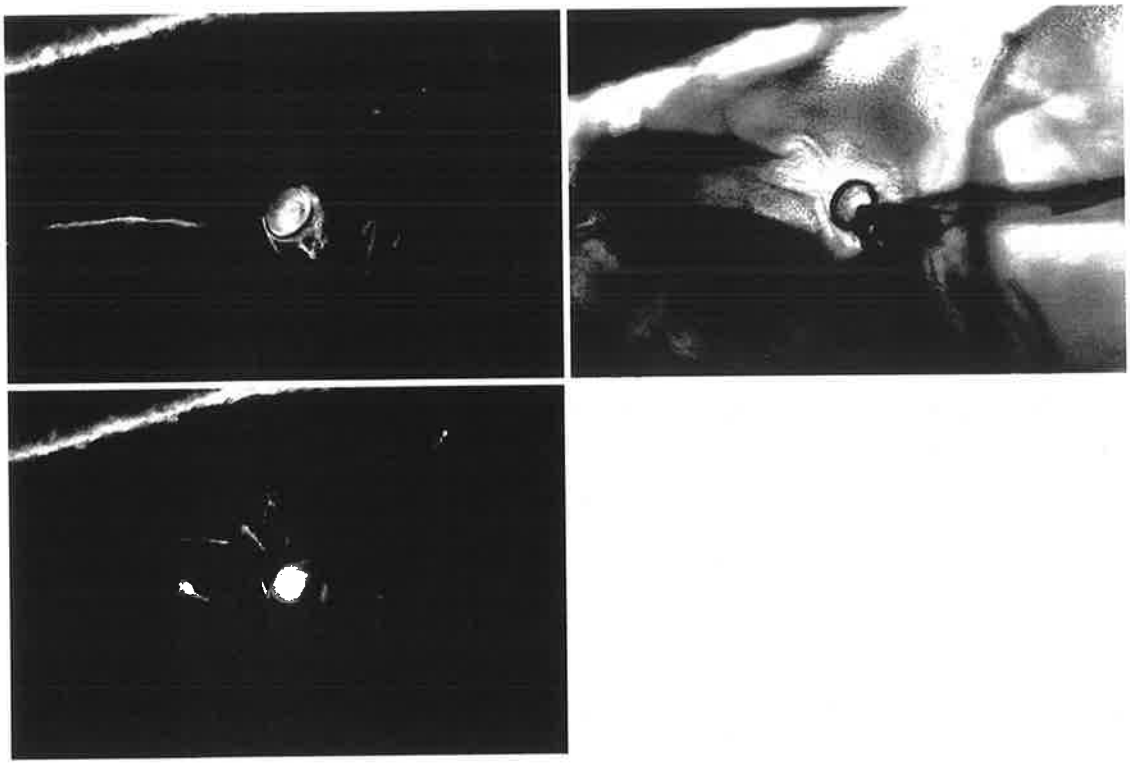

Figure 6.5 LBAM larval tracheae after topical application of 2µl of DC-Tron. Confocal image shows high fluorescence (top left) associated with an oil filled spiracle shown in transmission (top right). Focussing deeper shows limited penetration as low fluorescence occurs in tracheae connected to the spiracle (bottom left).

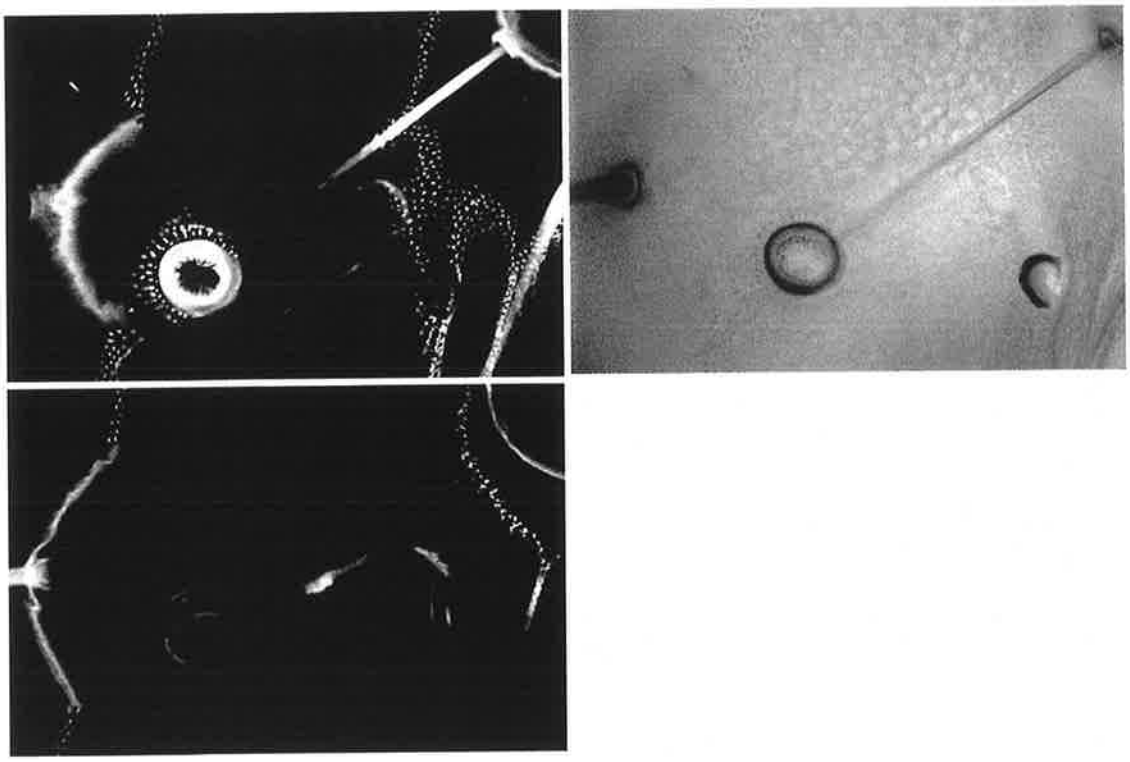

Figure 6.6 LBAM larval tracheae after topical application of 2µl of CPD. Confocal image shows high fluorescence (top left) associated with an oil coated spiracle shown in transmission (top right). Focussing deeper shows deep penetration as extensive fluorescence occurs in tracheae connected to the spiracle (bottom left).

6.4 Discussion

Immersing LBAM larvae in highly refined paraffins, including a C15 alkane, resulted in a slow death, which is consistent with previous work using relatively wide-cut saturated oils (UR >98%) on other insects (de Ong 1926; de Ong *et al.* 1927; Ebeling 1936). These authors proposed that saturated petroleum oil kills by asphyxiation because oil is present in the tracheae and there is a prolonged period to death. Many insects, including LBAM larvae (Dentener *et al.* 1992, Whiting *et al.* 1991), can tolerate very low levels of oxygen and mortality due to anoxia and death can take several days. Anoxia appears to be the primary mode of action when LBAM larvae are immersed in oil. However, it can not be presumed that anoxia is the only mode of action that can be attributed to oil.

In this study, LBAM larvae briefly dipped in oils and exposed to air became motionless much more rapidly than continuously immersed larvae. This is consistent with work on oil-dipped Potato tuber moth, *Phthorimaea operculella* (Zeller), larvae that rapidly dried and shrivelled when exposed to the air (Ebeling 1945). Ebeling suggested that even the most highly refined oil kills by virtue of its toxicity many hours before death by suffocation alone could occur and that desiccation, oxidation, vaporisation, or some other influence of the air increases the insecticidal properties of the oil. He argued that if air is kept from the bodies of insects, their period of survival was greatly increased, but there was no experimental verification or explanations of the possible mechanisms associated with these propositions.

Topical application of oils to larvae established that complete blockage of the tracheal system is not necessary to cause death. The surface of the cuticle was largely covered by the oil after topical application, but, access to the tracheal system was limited to spiracles close to the point of application of the oil. Larval mortality occurred using this method of application without extensive blocking of the tracheal system. There were many spiracles free of oil blockage and anoxia is unlikely to be the mode of action. Oil penetration was greater where oil had not

blocked the spiracle but had presumably flowed down the tracheal lining. This effect on oil distribution may account for the rapidity of mortality in dipping compared to immersion and suggests that more extensive entry into the tracheal system enhances the effectiveness of the oil.

There was a change in distribution of oil in the tracheal system of larvae when dipped compared with immersion. The entry of DC-Tron and the C15 alkane into the tracheal system was rapid when larvae were immersed. Oil flowed into the tracheae apparently as far as the air pressure in the tracheae and the hydrostatic pressure in the fluid-filled tracheole branches allows, creating a 'plug' of oil. The ability of the oil to penetrate the tracheae is dependent on the surface tension of the liquid or, more properly, the interfacial tension between the liquid and the cuticle/tracheal wall. Mineral oils and aqueous solutions of wetting agents, with a surface tension of 50% of water are able to enter tracheae (Brown 1951). The rate of penetration into the tracheae was related to viscosity but penetration decreases in direct proportion to the diameter of the tracheae until a point where the oil advance was imperceptible (Brown 1951). High viscosity oils with a low surface tension, such as castor oil, showed negligible movement into tracheae (de Ong *et al* 1927). CPD and DC-Tron have low surface tensions and viscosities of 12.0 and 2.75mm^2/sec at 40°C, respectively. Both oils penetrated well into the tracheae after 15 minutes immersion.

When LBAM larvae were dipped in oil, this equated to 30 seconds immersion time before removal. Oil flowed into the tracheae, but the much shorter immersion time produced shallower penetration. This may be because on removal from the oil, the air pressure dynamics were altered and active ventilation expelled the oil from the tracheal system. Light oils can be expelled from the tracheae of red scale while still immersed (de Ong *et al* 1927) and it is likely that larvae will be able to expel oil by active ventilation after exposure to the air. This is supported by the tracheal system being coated rather than filled with oil after dipping. The difference between oils is that the C15 alkane is found deep in the tracheoles. No attempt was made to quantify any differences in depth of penetration during immersion, but due to its low viscosity the C15 alkane

would be expected to penetrate at a faster rate. If oil penetrates deeply into smaller tracheae then more energy would be needed for expulsion. In addition, after expulsion in the main tracheal branches some oil will still adhere to the wall. The lipophilic oil could flow along the lipids of the tracheal wall and deep into the tracheoles where it is unlikely to be expelled. Oil deep in the system may be causing anoxia or a rapid accumulation of carbon dioxide leading to narcosis. Carbon dioxide causes rapid narcosis, which can be lethal given exposure for a sufficiently long period (Brown 1951).

Alternatively, the oil may be diffusing into tissues associated with the tracheoles. Oil, being lipophilic would preferentially lodge in lipid containing tissues and cause direct disruption of function, including the nerve sheaths and lipids of the brain. The pharmacological effect of the absorption of hydrocarbons into lipid membranes is not clear. Hassell (1982) suggested that physical toxicity caused by inert substances, such as petroleum oils, may be due to their non-specific absorption on lipoprotein membranes resulting in the disruption of a highly specific event. This would not involve the formation of specific chemical attachments to receptors or active sites of enzymes. The presence of oil in nerves after dipping in C15 alkanes and any effect on nervous transmission is investigated in Chapter 7.

Petroleum spray oils and CPD are formulated with chemical adjuvants to improve deposit performance when mixed with water. Surfactants (soaps and detergents) are commonly used as emulsifiers, wetting agents and spreaders. LBAM larvae immersed in oil with surfactants died much more rapidly than would be expected by anoxia. The mechanism of toxicity is not known, but one explanation is that the surfactants are responsible for the toxicity. Surfactants have profound effects on biological tissues causing the disruption of cell membranes (Prasad 1989). Another possibility is that oil and surfactants act synergistically. Both are adjuvants and the combination may be translocating more efficiently to areas where significant disruption can occur. In order to get a response, such as nervous disruption, it is necessary to get the chemical

to the appropriate site of action. For instance, the oil may improve the surfactants access to tissues by the dissolving protective wax layers, or alternatively, a surfactant may allow deeper penetration of oil into the tracheal system by reducing the surface tension of the solution. The presence of surfactants with oil may have an influence on the mode of action of oil for field spraying or postharvest use, but, the dynamics of the emulsion, with a separate water and oil phase, would need to be taken into account.

The results of this chapter have demonstrated that at least some oils kill insects by a mode of action more rapid than anoxia. The determination of an alternative mode of action for oils will make it possible to develop more target-specific formulations. The dipping method has practical relevance to the use of oils for postharvest control of LBAM larvae. Petroleum spray oils are formulated to produce a thin oil film on infested foliage and on any pests present at application. Ebeling (1950) suggested that high volatility of light oils made them less effective in the control of insects. However, if we are not dealing with anoxia this may be a moot point. The resulting film from spraying may allow the toxic properties of the oil to proceed before asphyxiation if the oil has the appropriate physical properties and reaches an internal site of action, eg., nervous tissue.

6.5 Summary of Main Points

1 Anoxia appears to be the primary mode of action when LBAM larvae are immersed in oil.

2 LBAM larvae briefly dipped in paraffins became motionless much more rapidly compared to larvae immersed in paraffins.

3 Rapid mortality using topical application of oils without extensive blocking of the tracheal system, suggests that anoxia is unlikely to be the mode of action.

4 A comparison of application methods showed a difference in the distribution of oil in the tracheal system of larvae.. Both CPD and DC-Tron produced "plugs' of oil in tracheae dipping immersion. The difference between oils during dipping was that only C15 alkane was found deep in the tracheoles.

5 Oil deep in the system may be causing anoxia or a rapid accumulation of carbon dioxide leading to narcosis. Carbon dioxide causes rapid narcosis, which can be lethal given exposure for a sufficiently long period (Brown 1951).

6 After dipping, oil may be diffusing into tissues associated with the tracheoles. Oil, being lipophilic would preferentially lodge in lipid containing tissues, including the nerve sheaths and lipids of the brain.

7 LBAM larvae immersed in oil with surfactants died much more rapidly than would be expected by anoxia. The surfactants enhance toxicity.

8 Oil and surfactants may act synergistically. The combination may be translocating more efficiently to areas where significant disruption can occur. Oil may improve the surfactants access to tissues by the dissolving protective wax layers, or alternatively, a surfactant may allow deeper penetration of oil into the tracheal system by reducing the surface tension of the solution.

9 The findings of this study have implications for pest oil formulation. It is a paradigm that high volatility makes light oils ineffective in the control of insects. However, where the major mode of action is not anoxia this may be a moot point.

Chapter 7

The Mode of Action of Selected Petroleum Oils on Exposed Lightbrown Apple Moth: Narcosis and Nervous Disruption.

7.1 Introduction

Ampol Citrus Postharvest Dip (CPD), a C15 alkane, produces a rapid "knockdown" of LBAM larvae, a symptom associated with narcosis. Many narcotics are not the direct cause of death to the extent that the narcosis is completely reversible. However, it is symptomatically important and associated with disruption of the nervous system. Kerosene vapours have an effect on the nervous system of insects (Shafer 1911) after the vapours enter the respiratory system (Freeborn and Atsatt 1918). Ampol CPD is unlikely to have sufficient volatility to show fumigant action (Moore and Graham 1918a; Ferguson and Pirie, 1948). However, this has not been experimentally validated. Once oils have penetrated into the tracheal system they are not necessarily confined, and may diffuse into the haemolymph through the walls of the tracheae. Diffusion of dyed oils through tracheal walls and into the haemolymph has been observed in a number of insects (Roy *et al* 1943; Moore and Graham 1918b). Oil entering the haemolymph preferentially lodges in lipid-containing tissues in close connection with the tracheoles, including the nerve sheaths (Richards and Weygandt 1945). Petroleum oils may absorb onto lipoprotein membranes and cause the disruption of critical nerve processes.

This chapter aims to determine the narcotic effects of Ampol's CPD on LBAM larvae nervous tissue. Symptomatology of larvae dipped in CPD and DC-Tron or held in saturated atmospheres of oil are compared with larvae held at high CO_2 atmospheres. Oil penetration through the tracheal system and nervous tissue are measured using confocal microscopy. Nervous disruption by oils is

examined by measuring any changes in spontaneous electrophysiological activity from peripheral nerves of larvae exposed to oil.

7.2 Materials and Methods

7.2.1 Lightbrown Apple Moth Colony

The LBAM larvae used in the experiments were collected from a laboratory culture maintained, since 1993, at the South Australian Research and Development Institute, Waite Precinct (Adelaide). Singh, Clare and Ashby (1985) described the rearing procedure.

7.2.2 Oil Formulations

Two oils were compared; a light C15 alkane with low viscosity to enable deep penetration of the tracheoles and a more viscous commercial spray oil. Ampol Research and Development Laboratories, Brisbane Qld, supplied a Citrus Postharvest Dip, Ampol CPD (an alkane with a carbon number of 15, ie., C15; paraffin content ie. %Cp>99%) and a commercial spray oil, Ampol DC-Tron NR (a narrow range oil with mean equivalent *n*-paraffin carbon number of a C23 alkane (Furness *et al,* 1987); %Cp<70%). General specifications of these oils can be found in Appendix 1.

Ace Chemical Company (Camden Park SA Aust.) supplied n-Pentane (Cas no. 109-66-0; molecular weight, 72.15; density, ≈ 0.62; distillation range ≈ 34-37°C) which was used to assess the fumigant effect of low molecular weight paraffins on LBAM larvae.

7.2.3 LBAM Larval Mortality in Carbon Dioxide Atmosphere

The test apparatus to enclose larvae in a CO_2 atmosphere consisted of 5-litre plastic canisters with inlet and outlet lines. The inlet value was connected to a CO_2 cylinder with a needle value

gas regulator. The outlet hose opened through a water column to control backflow. Oxygen levels were measured using Figaro GS Oxygen Sensors series KE-25 (Adilam Electronics, Melbourne Aust.). Groups of 10 larvae were placed in canisters and $C0_2$ flow of 250 ml/min continued until O_2 levels declined to $<0.5\%$, when CO_2 flow was stopped. Both lines were clamped to maintain high CO_2 levels in the canisters. After exposure for pre-determined periods of up to 24h the larvae were removed and placed in a rearing room at 20±3°C, 55± 5% RH and under natural light of 14h day;10h night. The activity of the larvae was assessed at 5 mins, 1 h and at 24 h after exposure. Larvae were counted as moribund (at 5mins) or dead (at 1h and 24h) if they did not move after repeated prodding with a needle. Larvae were rated 0-10 for their responsiveness and the ability to right themselves using criteria developed by Firko and Hayes (1990). A description of these criteria is listed in Appendix 3.

7.2.4 LBAM Larval Symptomatology in Saturated Oil Atmospheres

Petroleum oils applied as contact insecticides may actually kill by the vapours entering the respiratory system. The lower range in the paraffin series, up to decane, shows moderate fumigant action and acts as narcotics (Ferguson and Pirie, 1948). LBAM larvae (5^{th} instar) were exposed to a saturated atmosphere of either n-pentane or CPD to determine symptomatology during 4h exposure. Groups of 5 larvae were placed in gauze cages and arranged on racks in plastic containers (260x190x60mm) above 50 ml of oil. Control larvae were placed in containers without oil. The treatments were replicated 3 times. All containers with larvae were placed in incubators at 20°C. After exposure for 4h the larvae were removed and placed in a rearing room at 20±3°C, 55±5% RH and under natural light of 14h day:10h night. The activity of the larvae was assessed at 30mins and 4h exposure, and 24h after exposure. Larvae were rated for their

responsiveness and the ability to right themselves using criteria developed by Firko and Hayes (1990).

7.2.5 Symptomatology of Oil-Dipped LBAM Larvae

LBAM larvae (5th instar) were dipped in either CPD or DC-Tron and the activity and coordination assessed during a 4h period. Larvae were dipped in either 100% oil or 10,000ppm oil emulsions as described in Chapter 4, Section 4.2.3. Controls were dipped in water. After dipping, all media pots with larvae were placed in a rearing room at 20±3°C, 55±5% RH and under natural light of 14h day:10h night. Symptoms were classified by the behavioural and physical changes following oil treatment and compared to controls. Larvae were also rated for their responsiveness and the ability to right themselves at pre-determined intervals of 30 mins, 1 h, 2 h, 3 h and 4 h using criteria (listed in Appendix 3) developed by Firko and Hayes (1990). Larvae were counted as dead (at 24h) if they did not move after repeated prodding with a needle.

7.2.6 Staining and Microscopy of Tracheal and Nervous tissue

7.2.6.1 *Histological Staining of Oil in LBAM Larvae*

An oil soluble red dye, Automate Dye Red B (Petrafin, Sydney), was added to CPD and DC-Tron to compare the distribution of oil in the tracheal system of dipped larvae. The oil soluble red dye was added to oil at a rate of 50ml/l. Emulsions were made using the red-dyed oil solution and decontaminated water. Larvae were dipped as previously described in chapter 4, section 4.2.3, in 15,000ppm dyed oil emulsions of either CPD or DC-Tron, held in air for 18h before dissection and examined by light microscopy. Dipped larvae were prepared for viewing the tracheae and

ganglia by rinsing thoroughly in water to remove most of the surface oil, pinning to a wax-coated dish, partial evisceration by dorsal dissection, and then flooding with decontaminated water.

7.2.6.1 *Fluorescent Staining of Oil in LBAM Larvae*

Confocal microscopy was used to determine the location of fluorescent oil in larval structures, particularly the tracheal system. Paraffins have no autofluorescence, so an oil soluble fluorescent dye, Fluorescent Yellow FG (Morton Chemical Company, Chicago, USA), was added at a rate of 1ml/l and 10ml/l to view tracheae and nerve ganglia, respectively. Emulsions were made using the fluorescent stock solution and decontaminated water. Larvae were dipped in fluorescent oil, emulsions or water only as previously described. Larvae dipped in fluorescent oil emulsions were held in air for predetermined exposure times of 10 mins and 2h before mounting on slides. Preparation for imaging the intact larvae involved rinsing larvae thoroughly in water to remove most of the surface oil and then mounting laterally on glass slides within a plasticine well. Glycerol was added and a glass coverslip pressed on the sides of the plasticine well until it rested against the cuticle. Preparation for imaging the nerve ganglia involved dissection and removal of the nerve ganglion. Ganglia were mounted on a glass slide in immersion oil with negligible fluorescence (Leitz) before adding a coverslip. Mounting in the immersion oil inhibited the desiccation of the ganglia.

A Bio-Rad MRC-1000 laser Scanning Confocal Microscope System in combination with a Nikon Diaphot 300 inverted microscope in fluorescence mode with excitation at 488/10 nm and emission at 522/32 nm was used. The images of the larvae and nerve ganglia were collected using a 20x NA 0.40 dry objective lens and 40x water lens. The confocal intensity settings used to capture an image of fluorescent oil produced a faint image of control larval tracheae.

7.2.7 Electrophysiology of Oil-Dipped Larvae

Spontaneous activity from peripheral nerves was measured in muscles of the larval body walls using methods described by Gunning *et al.* (1991). Fifth instar LBAM larvae were pinned to a plasticine-coated dish, eviscerated by dorsal dissection and the ventral body wall muscles flooded with saline. A suction electrode picked up activity from the peripheral nerves and the preparation was grounded using a stainless steel insect pin. The recording electrode was connected to a pre-amplifier (Ilesworth, UK 101A). The signal was fed into a MacLab® System (ADInstuments, USA). Nerve action potentials were recorded and displayed using MacLab® Scope v3.5 Software (ADInstruments, USA) on an Apple Macintosh computer.

Intact larvae were dipped in oil/water emulsions and then dissected as well as perfusing oil directly over isolated nerves. Dipping larvae in oil was performed to determine if oil could effectively translocate into the nervous tissue and elicit a response. The dipping method for oil emulsions was as previously described in Chapter 4, section 4.2.3. At least 4 larvae were dipped per treatment and controls were dipped in water only. Oils without emulsifiers were highly agitated in water to ensure adequate mixing during dipping. After 10-20 min from dipping, larvae were dissected and spontaneous nerve activity recorded over a 15 min interval. The number of action potentials (firings per min) were counted for at least two periods after each dissected larvae regained a stable resting state (> 5 mins after dissection). The frequency of nerve firing of all treated and untreated larvae were recorded and mean frequency (6 recordings per treatment) were analysed using one way analysis of variance to determine the effects of oil on spontaneous nerve activity.

7.2.8 Sensitivity of AChE Activity to Oil

A number of insecticides, such as organophosphates, exhibit their toxic action by inhibiting certain important enzymes of the nervous system, such as cholinesterases. Although oils do not mimic the molecular shape of neurotransmitters or other enzyme substrates, they may inhibit them in some other way. Acetylcholine esterase (AChE) activity against a substrate, acetylthiocholine (ATC), was used to test the enzyme sensitivity to CPD and DC-Tron oils. AChE solutions were prepared by adding 200µl of 0.1M pH 7.5 Sodium Phosphate Buffer, 0.01% egg albumin (0.1mg/ml) and 0.4 units of pure bovine AChE source (0.2mg/50µl) to sterilised 1.5ml microtubes. The microtubes were agitated and placed in ice until required. To 50µl of the AChE solutions either 1% CPD or DC-Tron (potential inhibitor) were added and incubated for 0, 30mins, 60mins, 90mins, 3h and 15h. Control solutions contained no oil. Substrate solutions consisted of 0.1ml of 2.25M ATC (substrate) solution, 0.5ml of DTNB (indicator) and 9.4ml phosphate buffer. After pre-determined incubation periods, 50µl of each AChE solution was added to separate rows of a microplate using a multichannel pipettor. Then 100µl of substrate solution was added to each well and the microplate placed in a Kinetic U.V. Max Microplate Reader set to 405nm, Kinetic L1 mode, 10s read interval and 2m run-time. The enzyme activity of AChE on ATC led to a reaction in DTNB, which produced a colour change. The strength of the colour change over time indicated enzyme activity.

AChE activity was measured by the mean optical density (mOD). Blanks using Oil only + substrate and Egg albumin only + substrate showed no significant change in optical density. Therefore, a comparison between treatments was made using the raw mOD/min data and was analysed using ANOVA.

7.3 Results

7.3.1 Symptomatology of LBAM Larvae in Carbon Dioxide Atmosphere

LBAM larvae held in an atmosphere of high CO_2 exhibit narcotic symptoms; a rapid loss of activity, inability to right themselves after 2h, and complete immobility after 8h exposure (Table 7.1). The symptoms were reversible, with larvae fully recovering activity, provided CO_2 exposure was <8h. After 8h exposure, larvae retained slight impairment of coordination, with a larval activity rating (LAR; Appendix 3) of 9.25∓0.25 (SEM). No mortality was recorded until 24h of continuous exposure in a CO_2 atmosphere (24h LAR=0.5∓0.29, 24h mortality=90%∓5.8).

Table 7.1 Larval activity rating (LAR) of Lightbrown apple moth larvae (5th instar) exposed to high CO_2 atmosphere for different periods.

CO2 exposure (h)	*Mean LAR+ (SEM)*	*Symptoms*
0	10.00 (0.00) a	Active, no visible effects
1	5.00 (0.00) b	Active, no attempt to right self
2	2.75 (0.75) c	Slight independent activity
3	3.50 (0.50) c	Slow writhing, no control
4	2.75 (0.48) c	Slight independent activity
8	0.00 (0.00) d	No activity
24	0.00 (0.000 d	No activity

+Values are the mean larval activity rating (LAR) of 4 replicates of 5 larvae. Values within a column followed by the same letter are not significantly different according to analysis of variance of data ($p>0.05$, least significant difference).

7.3.2 LBAM Larval Symptomatology in Saturated Oil Atmospheres

Volatile components of oil, which are liposoluble, are potentially narcotic. LBAM larvae held in a saturated atmosphere of n-pentane appeared very agitated, followed by ataxia and eventually paralysis, which is consistent with the succession of symptoms associated with narcotic vapours (Shafer 1911; Brown 1951). All larvae held for 4h in a saturated atmosphere were moribund and showed no recovery after being held for 24h in air (Table 7.2). In contrast, LBAM larvae held in a saturated atmosphere of CPD for up to 24h show no loss of coordination or mortality suggesting no direct fumigant action on the nerves.

Table 7.2 Larval activity rating (LAR) of LBAM larvae (5th instar) exposed to n-pentane and CPD atmospheres at 30 mins, 4 h and 24h after removal from 4h exposure.

Treatment	*Mean LAR+ (SEM)*		
	Exposure		*Recovery*
	30mins	*4h*	*24h*
n-pentane	1.33 (0.33) a	0.00 (0.00) a	0.00 (0.00) a
CPD	10.00 (0.00) b	10.00 (0.00) b	10.00 (0.00) b

+Values are the mean larval activity rating (LAR) of 3 replicates of 5 larvae. Values within a column followed by the same letter are not significantly different according to analysis of variance of data ($p > 0.05$, least significant difference).

7.3.3 Symptomatology of Oil-Dipped LBAM Larvae

The larval activity rating (LAR) of LBAM larvae dipped in CPD was greatly reduced compared to DC-Tron (Figure 7.1). DC-Tron dipped larvae had slightly reduced coordination associated with being "mired' in oil, whereas, CPD dipped larvae showed very rapid loss of coordination and reduced activity more consistent with narcosis. The symptoms were more rapidly induced than by CO_2 exposure (Table 7.1).

Symptomatology of CPD dipped larvae was as follows: Larvae on removal from the oil emulsion were very flaccid and showed no spontaneous movement. During the next 30 min the abdominal segments became swollen and paralysed. The anterior portion of the larva exhibited slow writhing when prodded and rapid twitching of prolegs. The cuticle began to darken after 2h exposure. Dehydration was associated with the large spiracular openings of the 1st thoracic and 8th abdominal segments and was pronounced 3h after application. By 4h, the haemolymph in some segments becomes blackened and there was no response to stimulation.

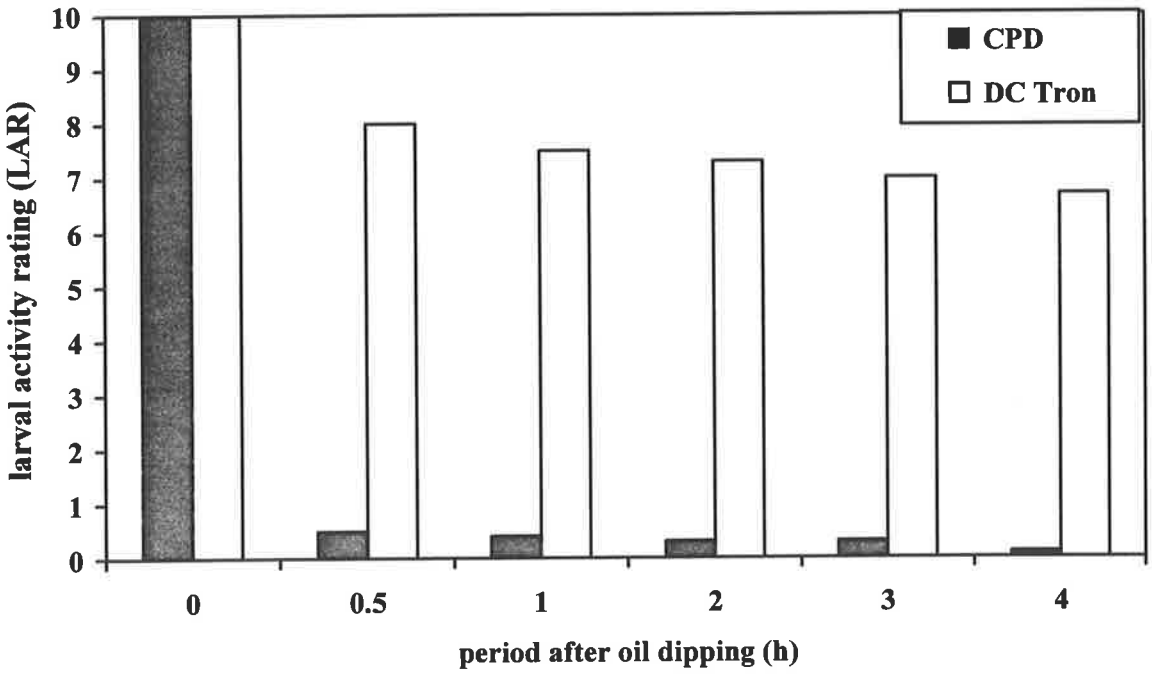

Figure 7.1 Activity of lightbrown apple moth larvae (5th instar) exposed to 10,000ppm CPD and DC-Tron.

7.3.3 Microscopy of Dyed Oil in Tracheal and Nervous tissue

Oil-soluble red dyes were used to compare the extent of penetration of DC-Tron and CPD into the tracheal system of oil-dipped LBAM larvae. Extensive staining of tracheae occurred in the larvae dipped in either oil, but CPD dipped larvae also showed staining in nervous tissues associated with the fine tracheoles. (Figure 7.2). All staining in DC-Tron larvae was confined to the tracheal system and did not appear to reach the tracheoles.

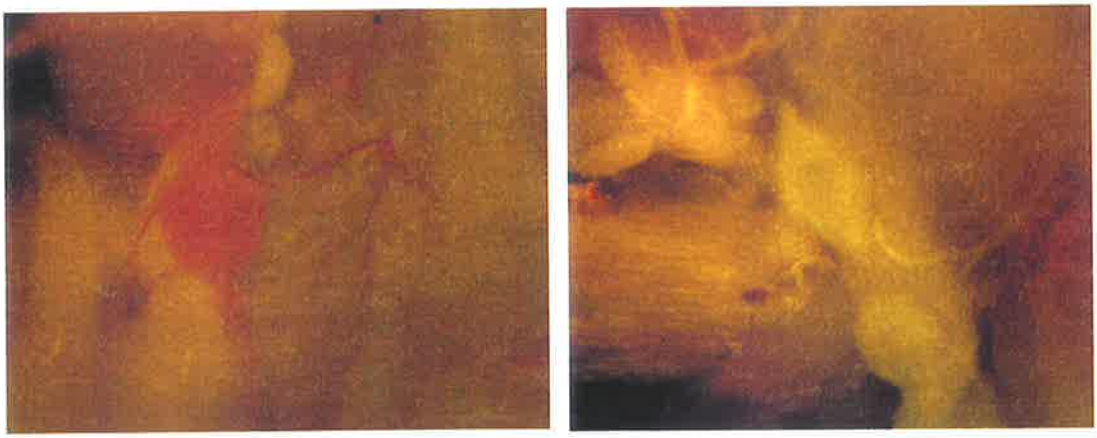

Figure 7.2 Dissection of LBAM larvae showing dyed oil penetrating into tracheoles and associated nervous tissue. Red stained nerve ganglia of CPD (left) are compared with unstained tissues in DC-Tron dipped larvae (right), 18h after dipping.

Confocal microscopy was used to determine the extent of penetration of CPD through the tracheal system and into associated tissues, such as the nerve ganglia. Initial settings were established that showed faint autofluorescence of main tracheae associated with chitinous structures, but fine tracheoles, seen in light transmission, produced no autofluorescence (Figure 7.3)

Confocal imaging of intact larvae dipped in fluorescent dyed oil showed that CPD penetrated extensively into the tracheal system (Figure 7.4). Cross-sections of tracheae revealed that the oil coated extensive sections of the tracheae (Figure 7.5) and fluorescence associated with very small tracheoles (1-2μm) suggests oil has flowed down deep into the tracheal system (Figure 7.6).

Tracheoles have a very strong association to certain tissues, including nerve tissue. Nerve ganglia removed from 5th instar LBAM larvae dipped in 15ml/l CPD emulsions revealed strong fluorescence in the tracheoles leading to ganglia and inside the ganglia themselves (Figure 7.7). Penetration into the nervous tissue was very rapid with fluorescence detected in ganglia 10 mins after exposure to oil dips. Interestingly, larvae continuously immersed for the same period, rather than dipped in CPD and then exposed to air, show no fluorescence in nerve ganglion (Figure 7.8).

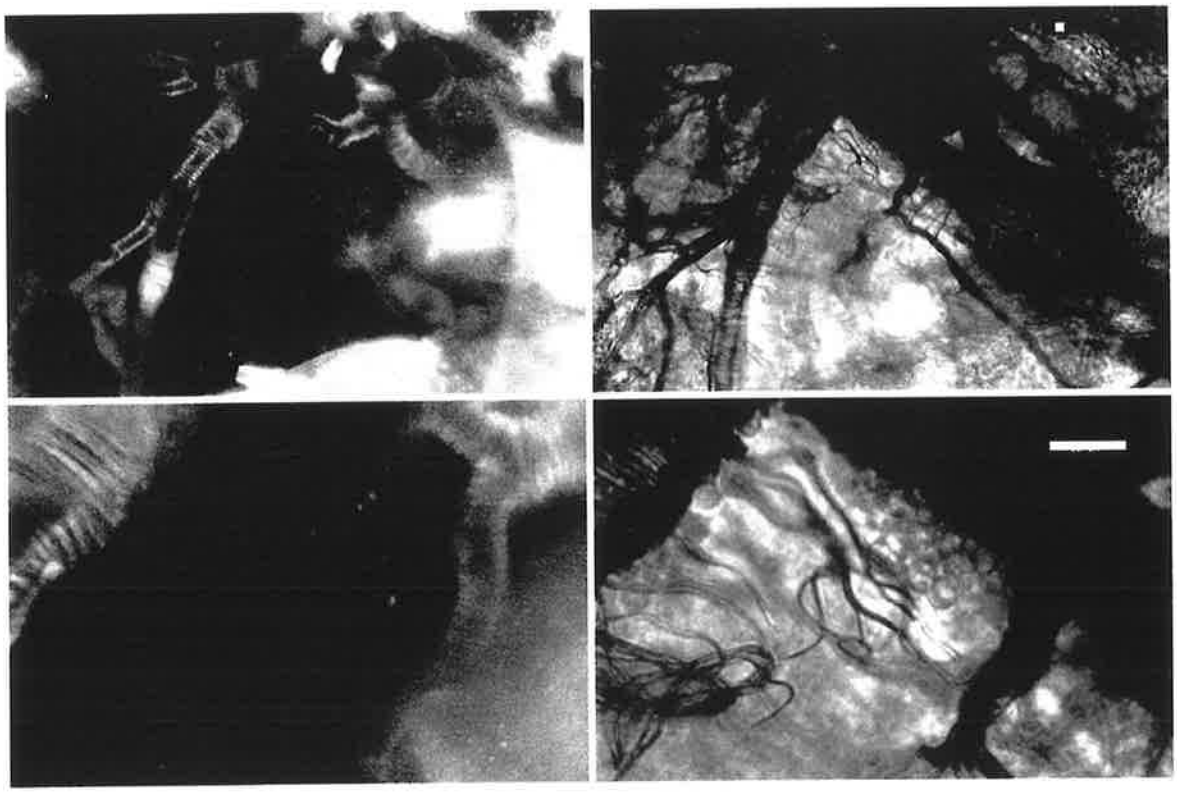

Figure 7.3 Untreated LBAM larval tracheae showing autofluorescence of tracheal surfaces (top left), and close-up of tracheae showing no autofluorescence of fine tracheoles (bottom left). Respective light transmission images (top & bottom right) show all tracheal branches (scale bars=10μm).

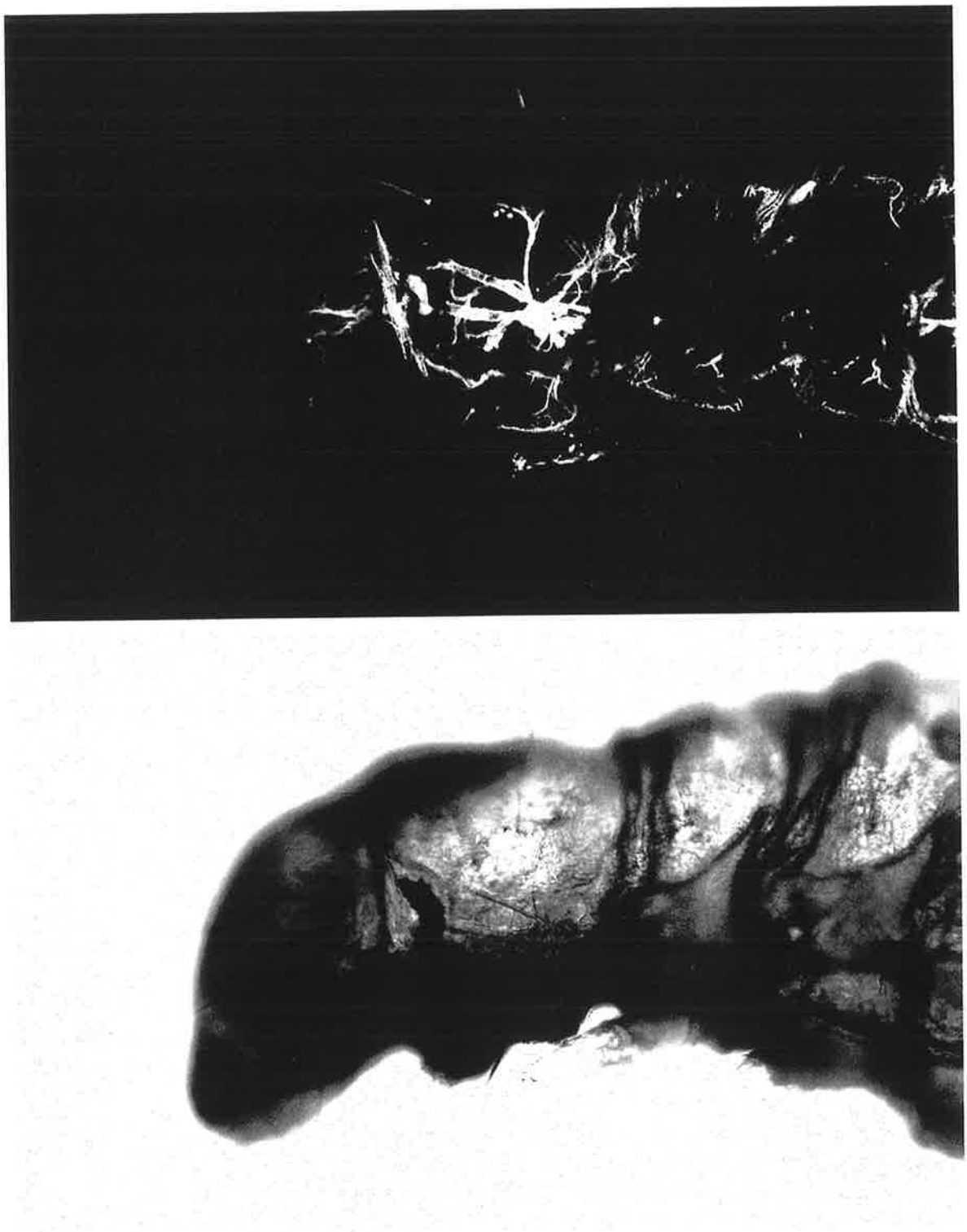

Figure 7.4 Lateral view of LBAM larvae dipped in fluorescent CPD showing extensive fluorescence of tracheae of head and thorax (top), and transmission image (bottom). (20x)

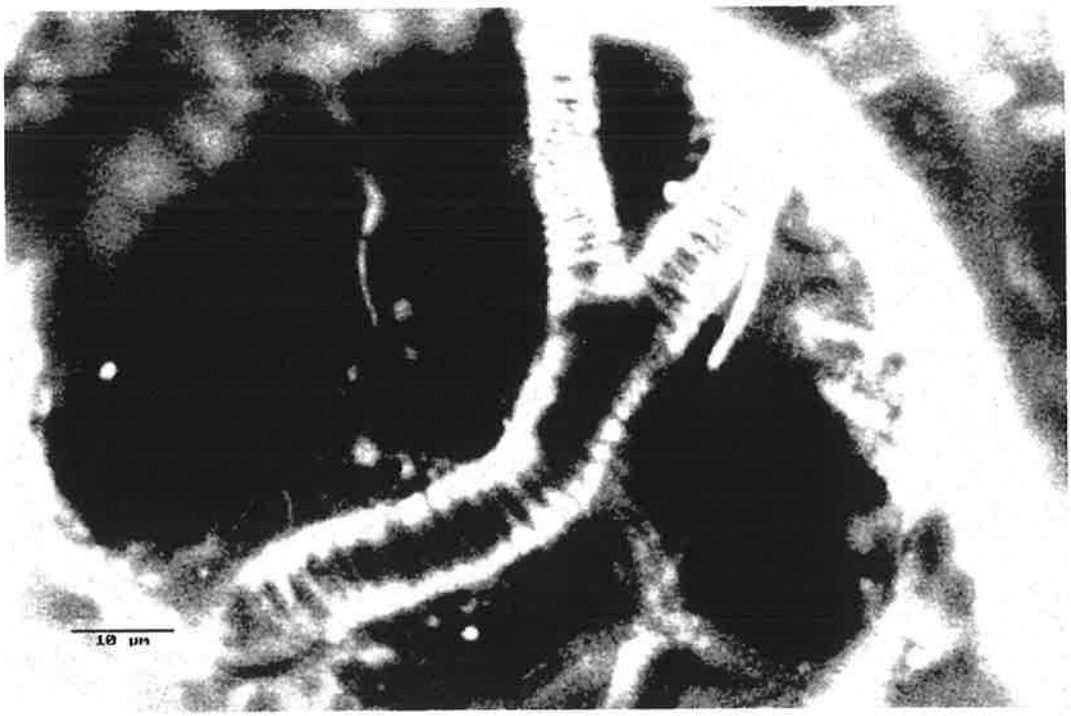

Figure 7.5 Confocal image of LBAM larva dipped in CPD showing cross-section of fluorescent tracheae. Strong fluorescence on the surface of the trachea indicates oil coating rather than filling the interior of the trachea. (scale bar=10µmm)

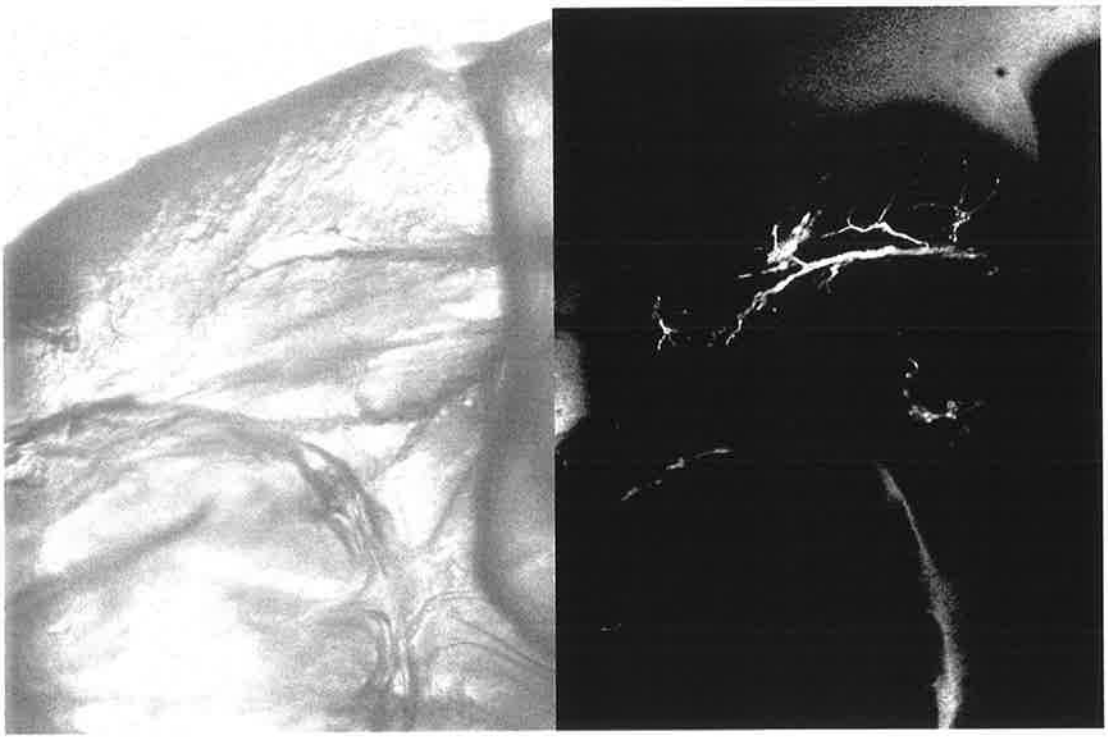

Figure 7.6 LBAM larva dipped in CPD showing strong fluorescence of fine tracheoles (right), and respective transmission image (left). (20x)

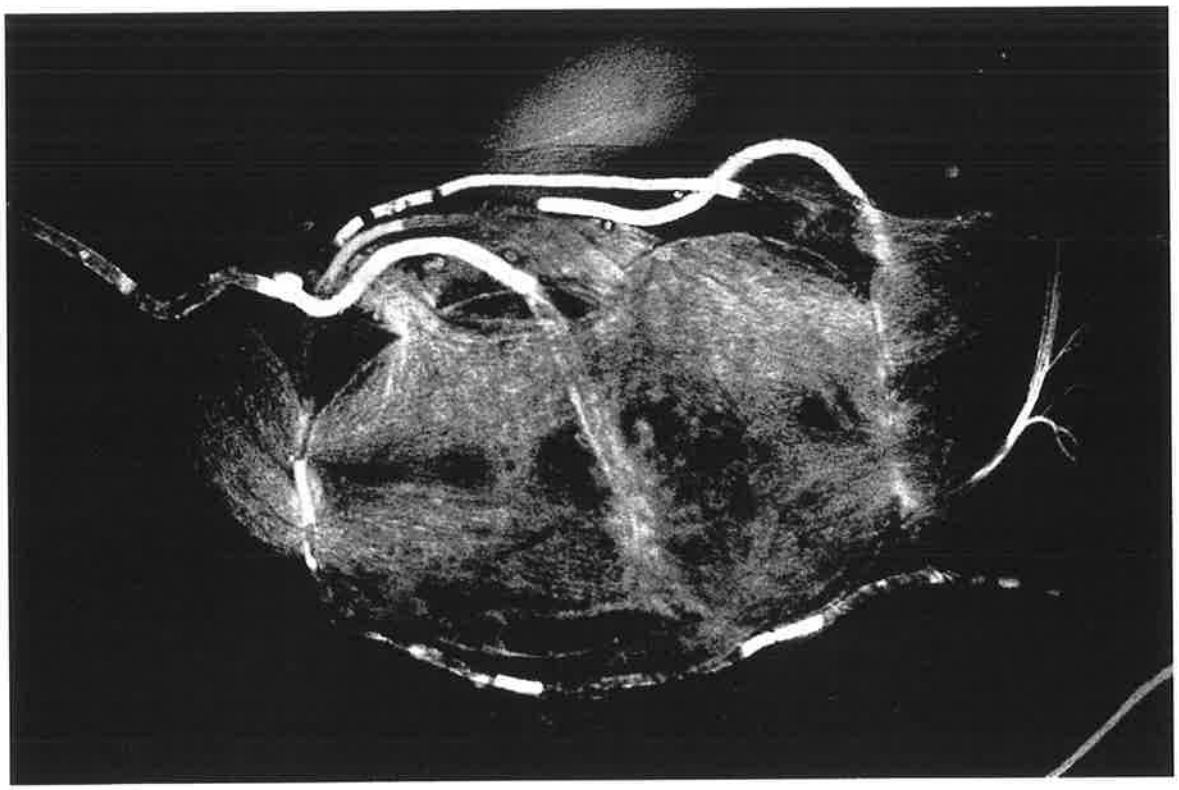

Figure 7.7 Nerve ganglion dissected from a 5th instar LBAM larva dipped in CPD (x20). Ganglion and associated tracheoles showing strong fluorescence related to the presence of fluorescent oil.

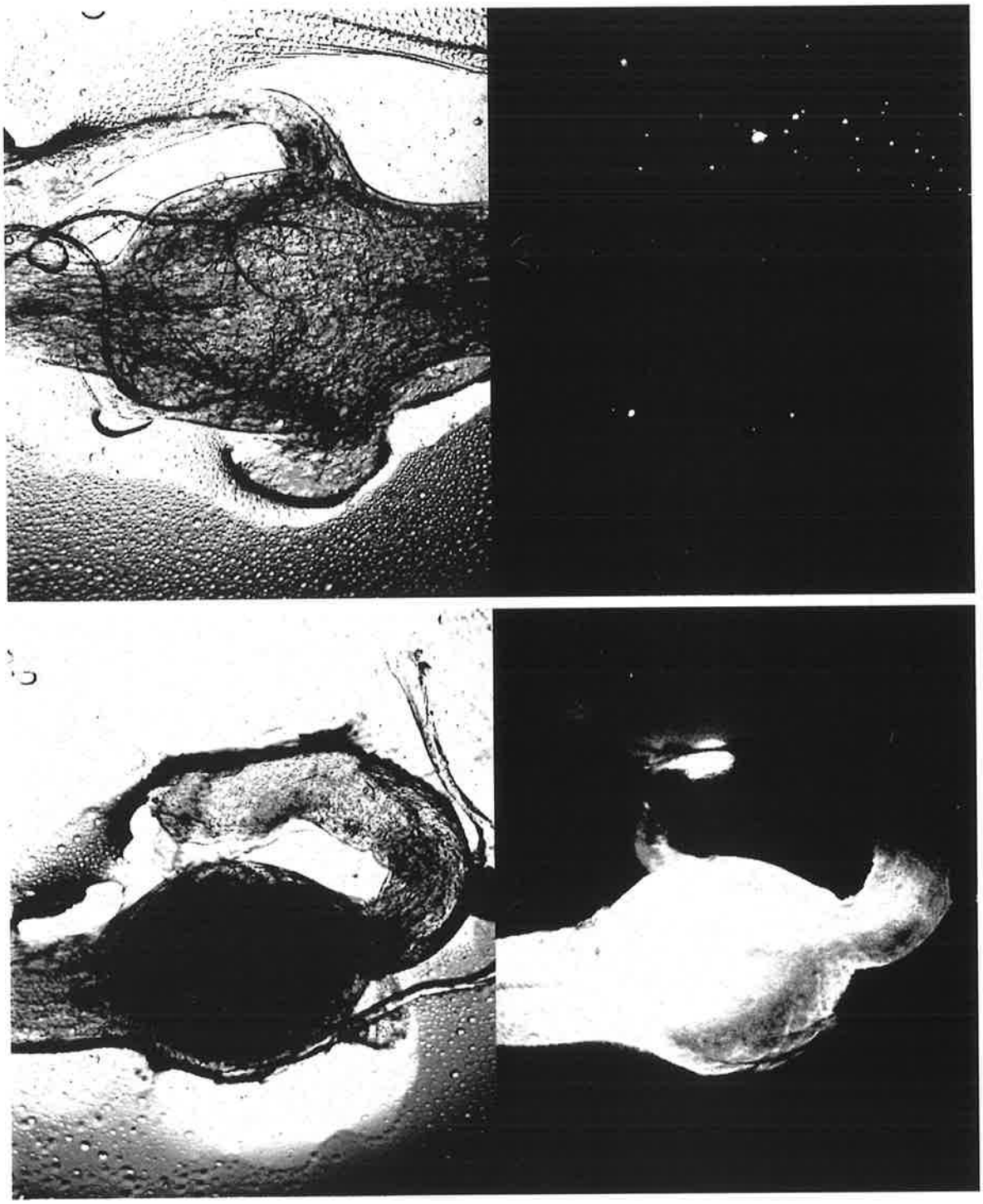

Figure 7.8 Comparison of ganglia dissected from LBAM larvae immersed (top) and dipped (bottom) in CPD (20x). Transmission images (left) show the ganglia. No fluorescence can be seen in the ganglion from immersed larvae (top right) compared with strong fluorescence in dipped larvae (bottom right).

7.3.4 Electrophysiology of Oil-Dipped Larvae

Spontaneous nerve activity was measured using the body wall tissue of oil-dipped and water dipped LBAM larvae. Nerve activity in untreated larvae was initially erratic but became more stable after 5 min. Perfusion of CPD directly into the body wall muscles of larvae induced an increase in activity 5 min after exposure (Figure 7.9). A comparison of larvae dipped in various concentrations of CPD shows an increase in the frequency of action potentials for concentrations above 200ppm when compared to control larvae (Table 7.3). The shape of the action potential in larvae dipped in CPD changes, with rapid multiple nerve firings and long trains of high amplitude spikes lasting many seconds (Figure 7.10). This was recorded in the peripheral nerves 20 min after intact larvae were dipped in CPD, demonstrating that oil rapidly translocated into nervous tissue to alter the pattern of activity.

Table 7.3 Frequency of nerve activity (action potentials min^{-1}) in peripheral nerves of 5^{th} instar LBAM larvae dipped in 4 concentrations of CPD and water only.

Treatment	*Frequency of nerve activity (action potentials min^{-1})[SEM]*
Water	88.33 [2.36] a
CPD 200ppm	94.00 [1.95] ab
CPD 1,000ppm	102.00 [2.14] c
CPD 5,000ppm	101.17 [3.06] bc
CPD 10,000ppm	100.83 [3.60] bc

*Mean of 6 recordings. Means within a column followed by the same letter are not significantly different according to one way analysis of variance ($p>0.05$, LSD)

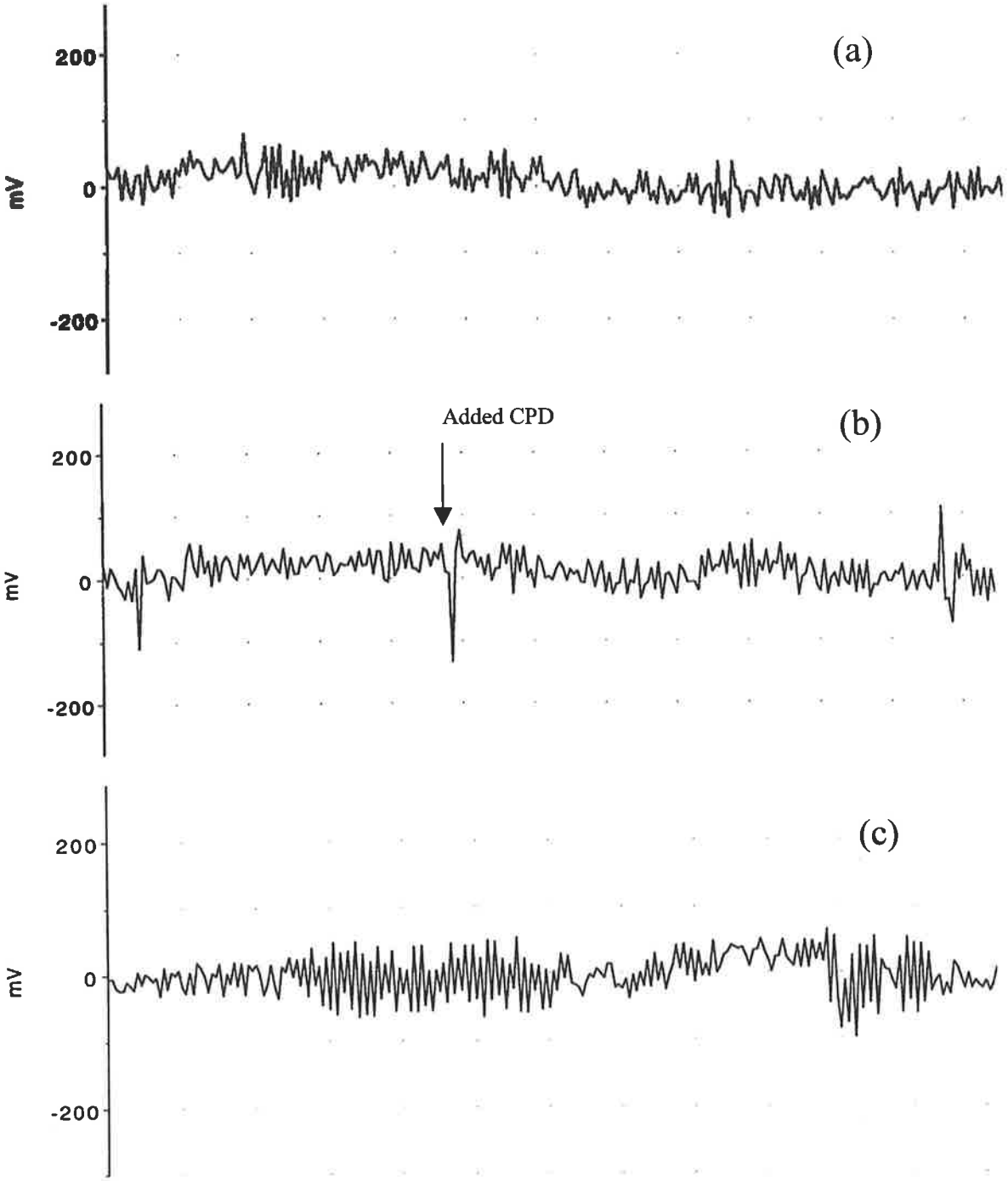

Figure 7.9 Spontaneous nerve activity of a dissected LBAM larvae resting and untreated (a), during perfusion of exposed peripheral nerves by 10,000ppm CPD (b) and within 5 minutes from exposure of CPD (c). Recordings show electrophysiological responses over a 60s period.

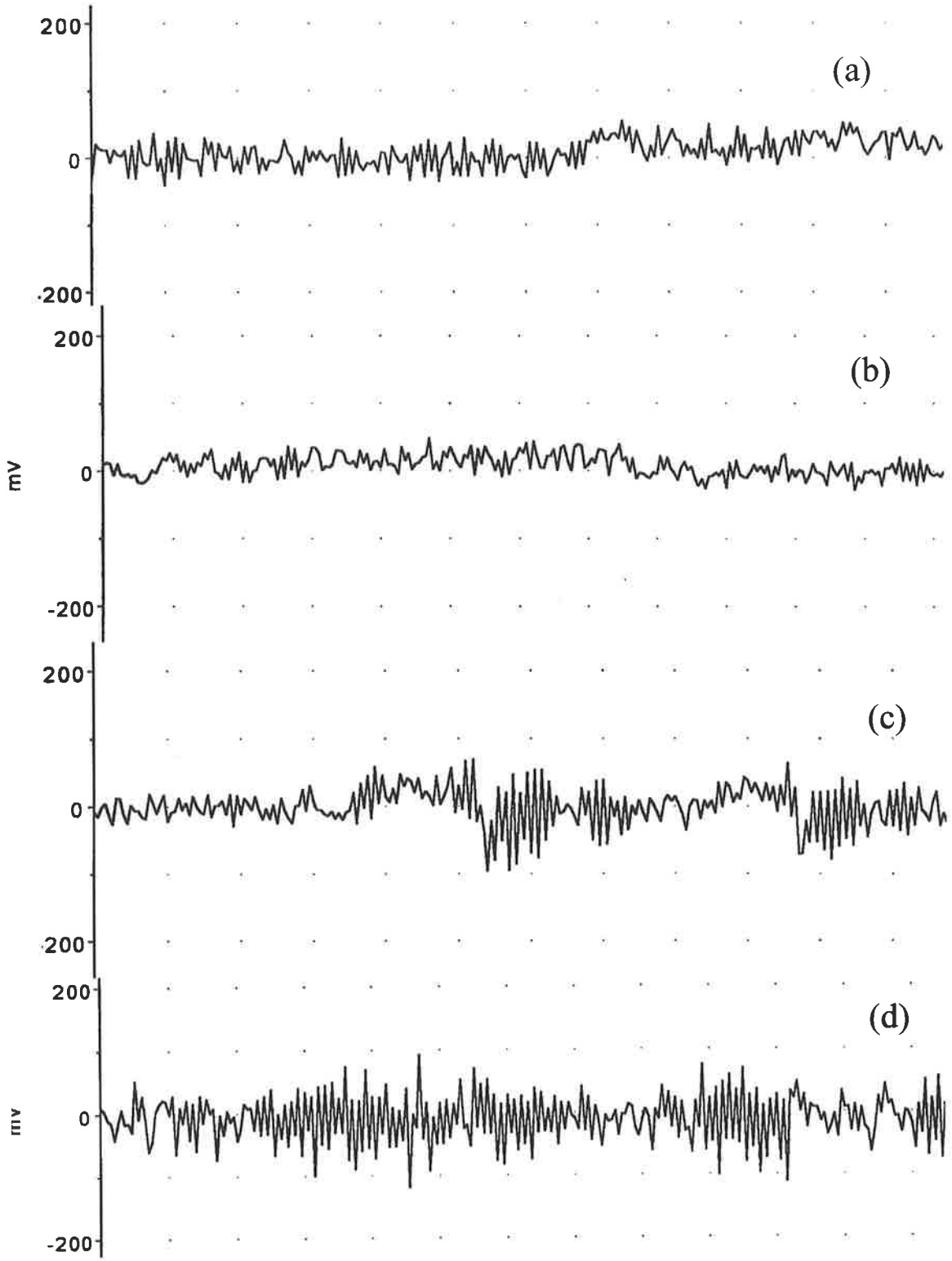

Figure 7.10 Spontaneous nerve activity of a dissected LBAM larvae resting and untreated (a), and between 15-20 minutes after larvae were dipped in 200ppm (b), 1,000ppm (c) and 10,000ppm of CPD (d). Recordings show electrophysiological responses over a 60s period.

CPD is predominantly a C15 alkane but also contains small volumes (<10% vol/vol) of non-ionic surfactants. To isolate the effects of each component, larvae were dipped in either C15 oil alone or surfactants alone to assess their individual effects. The deposit of the C15 alkane was difficult to control as emulsification could only be achieved by rapid agitation of the solution. Electrophysiological recordings suggested that larvae treated with the C15 alkane increased the frequency of action potentials compared with control larvae (Figure 7.11; a, b). High doses of surfactants (10,000ppm) also induced a response demonstrating that surfactants can reach and disrupt nervous tissue in dipped larvae (Figure 7.11; c). However, the surfactant levels in CPD are less than 10% of the total volume. Lower doses of surfactant (1,000ppm) that reflect the proportion of surfactant found in an efficacious dose of CPD had no effect on the frequency of the action potentials (Figure 7.11,d). It is therefore unlikely that the levels of surfactant in efficacious concentrations of CPD (10,000ppm) are primarily responsible for effects on the nervous tissue. Any synergistic interaction of the C15 alkane and surfactants has not been determined in these experiments.

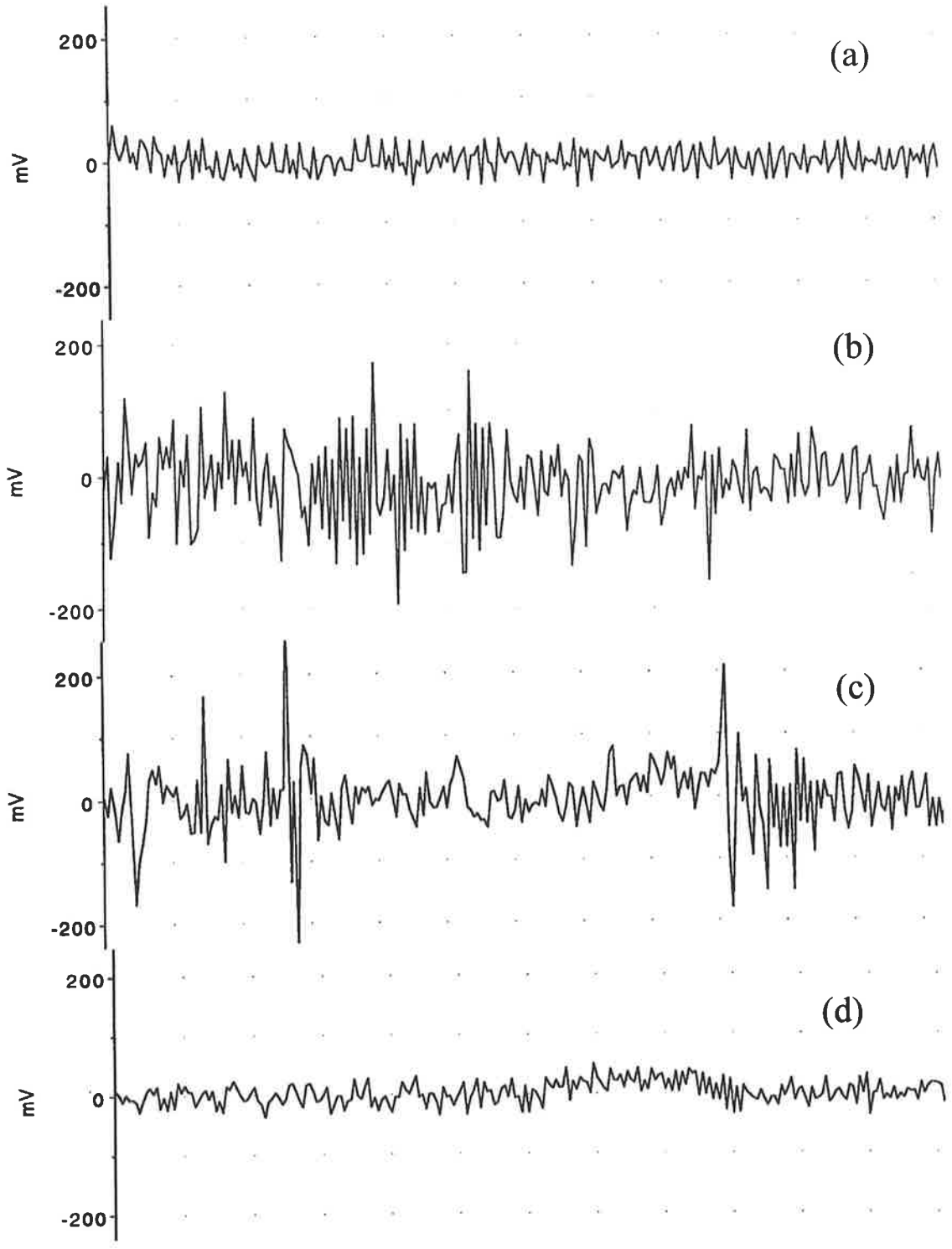

Figure 7.11 Spontaneous nerve activity of a dissected LBAM larva resting and untreated (a), and between 15-20 minutes after larvae were dipped in components of CPD; 10,000ppm C15 alkane (b), 10,000ppm surfactant blend (c) and 1,000ppm of surfactant blend (d). Recordings show electrophysiological responses over a 60s period.

7.3.5 AChE Sensitivity to Oil

A number of insecticides exhibit their toxic action by inhibiting certain important enzymes of the nervous system, such as cholinesterases. Oils do not mimic the molecular shape of neurotransmitters, as do organophosphates, but they may inhibit them in some other way. Acetylcholine esterase (AChE) activity against a substrate, acetylthiocholine (ATC), was used to test the enzyme sensitivity to CPD and DC-Tron oils. CPD and DC-Tron produces no inhibition of AChE for incubation times of 0h ($F=0.49$, $df=2$, $p>0.5$), 0.5h ($F=1.2$, $df=2$, $p>0.5$), 1h ($F=0.09$, $df=2$, $p>0.5$), 1.5h ($F=0.18$, $df=2$, $p>0.5$), 3h ($F=1.15$, $df=2$, $p>0.5$) and 15 hours ($F=1.48$, $df=2$, $p>0.5$) (Table 7.3).

Table 7.3 AChE activity (mOD/min) after incubation with CPD and DC-Tron over 6 different periods (0-15h).

Treatment	*AChE Activity (mOD/min) [SD]*					
	0h	0.5h	1h	1.5h	3h	15h
Control	273.57	257.57	248.27	253.43	265.33	246.43
	[9.19]	[31.12]	[2.10]	[20.50]	[3.35]	[6.76]
CPD	243.33	251.97	241.63	258.23	236.90	258.73
	[44.61]	[15.96]	[26.38]	[17.09]	[21.00]	[10.30]
DC-Tron	258.83	270.97	243.27	261.63	253.20	244.70
	[16.22]	[5.49]	[19.98]	[14.49]	[19.32]	[14.91]

7.4 Discussion

The symptomatology of larvae dipped in CPD is consistent with a rapid narcosis or neurotoxicity. The oil may induce these symptoms in one of the following ways:

1. Volatile oil fractions invade tracheal system, acting as a fumigant.
2. Oil blocking the tracheae leads to an accumulation of CO_2 in the larvae.
3. Oil liquid phase invades tracheal system and absorbs onto nerve membranes.

The first explanation, oil vapour invasion, is possible for very volatile oil fractions, such as n-pentane. However, fumigant effects are unlikely for the oils used in this study to control LBAM larvae. The C15 alkane did not produce any fumigant effects when larvae were exposed to oil vapours at ambient temperatures. This supports earlier work showing only the lower range in the paraffin series, up to decane, produced fumigant action in insects (Moore and Graham 1918a, Ferguson and Pirie 1948).

Narcosis is a decrease in cellular activity due to tissue anoxia, and in particular the reduction of oxygen to the nerve (Brown 1951). Narcosis in insects could be induced if the oil blocked the tracheae, causing an excess of CO_2. Confocal microscopy showed that CPD penetrated the tracheal system extensively, but that many tracheae are coated rather than filled with oil. Carbon dioxide in high concentrations causes rapid narcosis in LBAM but the period required for sufficient CO_2 to accumulate and induce narcosis has not been reported. Some mitigating factors to rapid narcosis from CO_2 accumulation would be the high solubility of CO_2 in mineral oil (Kubie 1927) and the ability of CO_2 to pass through a thin layer of oil (Shafer 1911). It is also important to note that a rapid knockdown associated with CO_2 accumulation, although symptomatically important, may not be the primary cause of death. Exposure to high concentrations rapidly affected the coordination of

LBAM larvae but the process was completely reversible even after several hours exposure. CO_2 exposure is unlikely to cause death unless it has been applied in high concentrations for prolonged periods.

In contrast to CO_2, saturated atmospheres of light paraffins, such as n-pentane, produce rapid and irreversible narcosis. It was not possible to remove the liquid oil from the tracheal system after dipping to verify reversibility but it seems unlikely that complete recovery would occur. Additional symptoms such as twitching of the prolegs, dehydration, and darkening of the haemolymph suggested other systems have been disrupted due to contact with the oil.

The most likely explanation, based on the results from this chapter, is that oil can cause disruption by direct contact with the nervous system. CPD, a non-volatile oil, induced narcotic symptoms in dipped LBAM larvae. Direct nervous disruption would require deep penetration of oil into the tracheoles and absorption onto nerve membranes. Confocal microscopy shows, that if the insects are dipped in CPD, then exposed to the air, a C15 alkane can rapidly move down into the nerve ganglion via the tracheal system. Importantly, larvae continuously immersed in oil, as opposed to dipped, do not have oil in their nervous tissue. This may explain, at least partially, the differences in tolerance between oil-dipped and oil-immersed larvae described by Ebeling (1950) and observed in earlier chapters.

The presence of oil in the ganglia affects the nervous activity of the peripheral nerves of larvae. The response of intact larvae dipped in CPD oil supported confocal observations that the oil rapidly moves down the tracheae into nervous tissue, but did not support the hypothesis of narcosis through oxygen starvation. The narcotic action of oils is generally associated with decreased activity through

anoxia (Brown 1951), but exposure to CPD induced a rapid onset of multiple nerve firing in peripheral nerves of LBAM larvae.

CPD is formulated as a mixture of a C15 alkane with low levels of surfactants (<10% vol/vol) to aid in emulsification. Exposure to the C15 alkane and surfactants separately induced repetitive firing demonstrating that oil and surfactants both contribute to a nervous response. Surfactants have traditionally been used by formulators to control oil deposit on sprayed surfaces, but may also be important in achieving translocation of the oil into nervous tissue of insects. This may involve a complex synergy as the surfactants may aid entry into the spiracles by controlling the deposit, while the oil may equally be assisting the translocation of the surfactants to nervous tissue.

Nervous disruption by surfactants is likely to be due to their surface activity and resultant cell disruption of nerve membranes (Gaskin 1995). The pharmacological effect of the absorption of hydrocarbons into phospholipid membranes is not clear, but is probably not due to a specific site, as is true for most insecticides (Hassell 1982). Thus, nervous disruption would not involve the formation of specific chemical binding to receptors or the active sites of enzymes, which is consistent with the lack of any apparent structural complexity or stereo isometry of the oils, especially compared to other insecticides. Assays using bovine AChE support this by showing no specific inhibition of that enzyme using high oil doses (1%) and long incubation periods (up to 15 h). It is more likely that oils are displacing protective lipids by their solvent action (van Overbeek and Blondeau 1954), and affecting nerve activity by increasing membrane permeability to ion exchange.

The effect of oils on arthropod nervous activity has important implications for the use of oils as insecticides. The formulation of insecticidal oils has been focused on using their physical characteristics to achieve efficacy by anoxia. Special purpose oils, such as CPD, use other physical characteristics of oils to achieve efficacy by an alternative mode of action. The increased excitability of nerves exposed to oils may also have a role in overcoming insecticide resistance to neurotoxins by

nerve insensitivity. A combination of a pyrethroid and light alkane could be part of a resistance management strategy for super kdr-type resistance in *Helicoverpa amigera* (Huber). A greater understanding of the range of symptoms caused by oils should lead to products that are more effective in this fashion.

7.4 Summary of Main Points

1. Oil induces narcosis in one of the following ways: Volatile oil fractions invade the tracheal system producing a fumigant action, oil blocks the tracheae leading to an accumulation of CO2 in the larvae, or oil in liquid phase invades tracheal system and absorbs onto nerve membranes

2. Volatile oil invasion is possible only for very volatile oil fractions, such as n-pentane. In contrast, the C15 alkane did not show any fumigant effects when larvae were exposed to oil vapours.

3. Oil blocking the tracheae can cause an excess of CO_2 and may indirectly induce narcosis. Larvae briefly dipped in CPD have tracheae coated rather than filled with oil. Additional symptoms, such as twitching of the prolegs, dehydration, and darkening of the haemolymph, suggested other systems have been disrupted due to contact with oil.

4. Oil can cause disruption by direct contact with the nervous system. Direct nervous disruption would require deep penetration of oil into the tracheoles and absorption onto nerve membranes. Confocal microscopy shows that given the right circumstances, ie., after dipping, a C15 alkane can rapidly move down into the nerve ganglia via the tracheal system.

5. Measurement of the spontaneous nervous activity of the peripheral nerves of larvae indicated that CPD has an effect on the nervous system. Exposure to the C15 alkane and surfactants separately induced repetitive firing suggesting that oil and surfactants both contribute an impairment of nerve function.

6. The pharmacological effect of the absorption of hydrocarbons into phospholipid membranes is not clear. It is probably due to non-specific absorption on lipoprotein membranes, especially given the structural simplicity of oils compared to most insecticides, rather than the formation of specific chemical attachments to receptors or active sites of enzymes. Assays using bovine AChE support this by showing no specific inhibition of that enzyme. It is more likely that oils are solubilising lipids (van Overbeek and Blondeau 1954), and affecting nerve activity by increasing membrane permeability to ion exchange.

7. The effect of oils on arthropod nervous activity has important implications for the use of oils as insecticides. The formulation of insecticidal oils has been focused on using their physical characteristics to achieve efficacy by blocking spiracles leading to anoxia. Special purpose oils, such as CPD, use other physical characteristics of oils to achieve efficacy by an alternative mode of action.

8. The increased excitability of nerves exposed to oils may also have a role in overcoming insecticide resistance to neurotoxic pesticides. A combination of a pyrethroid and light alkane could be part of a resistance management strategy for super kdr-type resistance in *Helicoverpa amigera*.

Chapter 8

The Mode of Action of Selected Petroleum and Vegetable Oils on Exposed Lightbrown Apple Moth Larvae and Mealybug: Desiccation.

8.1 Introduction

Mortality through desiccation is a significant threat to arthropods. Water can be lost through the cuticle, the respiratory system via the spiracles, the excretory system and the mouth.

Early studies on water regulation in insects suggested that petroleum oils increased cuticular permeability by dissolution of the thin lipid layer on the epicuticle (Wigglesworth 1945; Ebeling and Wagner 1959). Considerable work has been done on inert dusts to show they disrupt the waterproofing lipid layer, leading to desiccation of insects (Alexander *et al.* 1944; Kalmus 1944; Helvey 1952; Ebeling and Wagner 1959). However, water loss is not considered an important factor contributing to insect mortality from oils. Nonetheless, some insects may be more susceptible to this effect. Dissolution of the waxy coating of mealybug is likely to render them very prone to desiccation (Chapter 2, Figure 2.1). Lightbrown apple moth (LBAM) larvae dipped in a C15 alkane showed obvious dehydration a few hours after exposure (Chapter 4). Oil-induced water loss and subsequent desiccation may cause mortality in LBAM larvae in some circumstances. The physical properties of oils may influence water loss and a comparison of oils would clarify their relative effects.

The aim of this study was to examine the relationship between oil exposure and water loss in LBAM and mealybug, and to determine if weight loss *per se* contributes to arthropod mortality. Petroleum oils of different molecular weights were also used to determine the effect of volatility on larval weight loss.

8.2 Materials and Methods

8.2.1 Insect Colonies

Citrophilus mealybug (*Pseudococcus calceolariae* (Maskell)) adults were collected from cultures maintained on butternut pumpkins (*Curcubita pepo* L.). LBAM larvae and eggs used in the experiments were collected from a laboratory culture maintained, since 1993, at the South Australian Research and Development Institute, Waite Precinct (Adelaide). Singh, Clare and Ashby (1985) described the rearing procedure.

8.2.2 Oil Formulations

Caltex Australia Research and Development Laboratories, Brisbane Qld, supplied two oils; a homogeneous C15-alkane used for postharvest dipping of citrus, C15 Ampol CPD, and petroleum spray oil, C23 Ampol DC-Tron NR. The petroleum spray oil contains no alkanes and consists mostly of C15 mono-cyclic molecules with side chains. The C23 nomenclature refers to its mean equivalent *n*-paraffin carbon number; its 50% distillation temperature is the same as the boiling point of a C23 alkane (Furness *et al.* 1987). The specifications of both products are given in Appendix 1.

Total Solvents, Paris France, supplied two highly paraffinic oils, C19 Citrole NR and C22 NHC5 NR. The specifications of Total Citrole, a commercial petroleum spray oil, are given in Boutuorlinsky *et al* (1996) and Herron *et al* (1998). An emulsifiable vegetable oil-based product, Eco-oil, used in agricultural spraying, was supplied by Organic Crop Protectants Pty Ltd, Lilyfield NSW, and an experimental vegetable oil-based postharvest dip, AWH-96-04, was supplied by the Victorian Chemical Company, Richmond Vic., Aust.

8.2.3 The Effect of Oil on Adult Mealybug Weight loss

Adult Citrophilus mealybugs were dipped in CPD and DC-Tron to compare their relative effect on weight loss. Adult mealybugs were individually weighed on a microbalance LM600 (Beckman-Riic Ltd. Glenrothes Scotland) and then dipped in 10,000ppm oil emulsions. The dipping procedure was as described in Chapter 2, section 2.2.2, and included six replicates.

After dipping, each mealybug was placed on filter paper in a separate petri dish. The petri dishes were placed in a climate-controlled laboratory at 20±3°C, 55± 5% RH and under natural light of 14h day;10h night. The water was allowed to dissipate and mealybugs were weighed hourly up to 4h and at 24h. Mortality was assessed at 24h from exposure. Mealybugs were counted as dead if they did not actively move after repeated prodding with a needle. Statistix 4.1 (Analytical Software 1994) was used for analysis of variance and pairwise comparisons of the mean mealybug weight loss for each treatment and controls (water only).

8.2.4 The Effect of Oil on LBAM Larval Weight loss

LBAM larvae were treated with selected petroleum and vegetable oils to assess their relative effect on larval weight loss. Initially, LBAM 5th instar larvae (at 20°C for 21 days) were dipped in different oil emulsions at a rate of 15,000ppm. The dipping procedure was as described in Chapter 4, section 4.2.3, and included four replicates.

After dipping, the larvae were weighed and placed in gauze cages. The cages were placed in a 56% RH atmosphere generated by a sodium bromide saturated solution (Rockland 1960) within an incubator (20°C and no illumination). The larvae were removed and weighed hourly up to 6h and at 24h. Mortality was assessed at 4h, 6h and 24h from exposure. Larvae were counted as dead if they did not move after repeated prodding with a needle.

Petroleum oils of widely different molecular weights were used to examine the effect on LBAM larval weight loss. LBAM 5th instar larvae (at 20°C for 21 days) were selected and groups of 10 larvae were treated with DC-Tron and CPD oil at rates of 15,000ppm and 30,000ppm or 1,500ppm and 15,000ppm, respectively. Prior to dipping each lot were weighed. The dipping procedure was as described in Chapter 4, section 4.2.3, with four replicates. After dipping, the larval groups were weighed, placed in gauze cages and held in a constant temperature room (20±2°C, 60±5% RH and natural light). Each replicate was weighed at hourly up to 6h and at 24h. Statistix 4.1 (Analytical Software 1994) was used for analysis of variance and pairwise comparisons of the mean larval weights for each treatment and controls (water only).

8.2.5 Comparison of CPD and Chlorpyrifos Induced Weight loss

Some chemical insecticides induce a rapid water loss in treated insects (Ebeling 1974). The water loss from LBAM larvae dipped in oil was compared with chlorpyrifos treated larvae. Chlorpyrifos was chosen for comparison because the mode of action is neurotoxic inhibition of acetyl cholinesterase rather than due to the removal of surface lipids. In this experiment, LBAM 5th instar larvae (at 20°C for 21 days) were selected within the 25-35mg weight range and treated with either CPD at 1,000ppm or chlorpyrifos at a 250ppm (0.5ml/l Lorsban 500EC, 500gms/l chlorpyrifos). Prior to dipping, each group of 10 larvae was weighed. The dipping procedure was as described in Chapter 4, section 4.2.3.

After dipping, the larvae were weighed and placed on filter paper within a petri dish. The dishes were placed in a sodium bromide saturated atmosphere (Rockland 1960) of 56% RH within an incubator (20°C and no illumination). The larvae were removed and weighed at 1, 2, 3, 4, 5 and 6h. Larval activity was recorded at 4h according to the criteria described by

Firko and Hayes (1990; Appendix 3). Statistix 4.1 (Analytical Software 1994) was used for analysis of variance and pairwise comparisons of the mean larval weight of each treatment.

8.2.6 Effect of Humidity on Oil Dipped LBAM Larvae

Weight losses of LBAM larvae dipped in oil and held at different relative humidities were measured to assess the effect of oils on water loss and subsequent mortality. High larval weight loss was induced through low humidity atmospheres to assess if weight loss *per se* causes mortality.

After dipping, the larvae were weighed and placed in gauze cages. The cages were placed on racks in plastic containers (260x190x60mm) with atmospheres of 56%RH, 75%RH or 98%RH generated by sodium bromide, sodium chloride and potassium sulphate saturated solutions, respectively (Rockland 1960). A low humidity of 15 % RH was generated using silica gel (BDL Chemicals, Kilsyth Vic., Aust.). Humidities were measured using a hair hygrometer (Polytherm Haar). All containers with gauze cages were placed within an incubator (20^{0}C and no illumination). The larvae were removed and weighed at 1, 2, 3, 4, 5, 6 and 24h. Mortality was assessed at 4h and 24h from exposure.

8.3 Results

8.3.1 Effect of Oil on Mealybug Weight loss

An important observation in mealybug bioassays was the apparent relationship between weight loss and visual appearance in CPD dipped mealybug. Adult mealybug dipped in 10,000ppm CPD immediately had the white, waxy coating dissolved from their bodies (Figure 8.1) The rapid loss of waxy protection in mealybug dipped in CPD coincided with high weight loss during the first 2h exposure (Table 8.1). Mealybugs dipped in CPD were rapidly immobilised and did not recover (100% mortality), compared with 33.3% mortality

and no mortality in DC-Tron-treated and control mealybug, respectively. After 1h exposure, mealybug lost 0.85%, 0.87% and 7.0% of their initial weight for water, DC-Tron and CPD treatments, respectively. Mealybug dipped in water or DC-Tron did not show any change in appearance, but significant weight loss did occur in DC-Tron dipped mealybug compared to control-treated mealybug when measured several hours after exposure. After 24h exposure, mealybug treated with either oil recorded similar weight loss and greater losses than control mealybug (CPD caused a loss of 818.3 μg/mealybug; DC-Tron; 656.7 μg/mealybug; Control, 136.7 μg/mealybug; critical value for comparison is 495.4, LSD (T)).

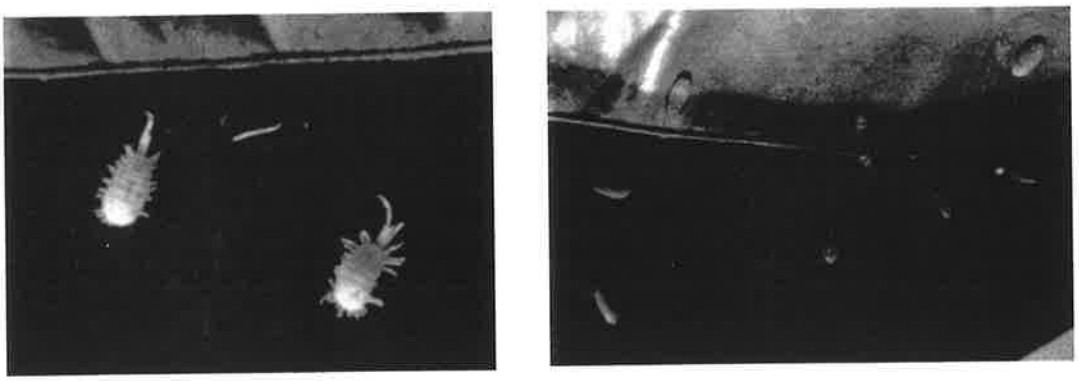

Figure 8.1 The loss of the protective waxy coating of mealybug before (left) and after (right) dipping in 10,000ppm CPD.

Table 8.1 Mean adult Citrophilus mealybug weight loss dipped at 10,000ppm for 2 oil formulations, and held at 20°C and 60%RH for 4 hours.

Treatment	*Initial wt[mg]* *(SEM)*	*Mean weight loss*+(μg/mealybug) *(SEM)*			
		1h	2h	3h	4hr
Water	2.95 (0.17)	25.0 a (6.7)	31.7a (7.9)	40.0a (8.3)	51.7a (9.8)
DC-Tron	3.57 (0.48)	31.7 a (16.4)	66.7a (20.3)	126.7a (28.9)	186.7ab (45.5)
CPD	3.52 (0.35)	245.0a (116.1)	351.7b (122.3)	385.0b (117.1)	450.0b (133.7)

+Values are the means of six replicates. Means within a column followed by the same letter are not significantly different ($p > 0.05$, least significant difference)

8.3.1 Effect of Oil on Larval Weight loss

LBAM larvae treated with CPD at 15,000ppm had significantly lower larval weights after 2h exposure than lower CPD dose (1,500ppm) and control larvae (Table 8.2).

Table 8.2 Progressive weight of 5th instar LBAM larvae dipped in 1,500ppm and 5,000ppm CPD oil and held at 20°C, 60%RH.

Treatment	*Progressive larval weight+ (mg)*							
	0h	*1h*	*2h*	*3h*	*4h*	*5h*	*6h*	*24h*
Water	21.0a	20.3a	19.7a	19.2a	18.8a	18.4a	18.4a	15.6a
1,500ppm	20.1a	19.2a	18.3a	17.6a	17.4a	17.1a	16.8a	13.8a
15,000ppm	19.8a	18.9a	17.8b	16.7b	15.7b	15.3b	15.0b	11.9b

+Means within a column followed by the same letter are not significantly different ($p > 0.05$, least significant difference)

Other oils tested, including vegetable oils (Eco-oil and AWH-96-04) did not demonstrate any desiccation of larvae 4h after exposure to 15,000ppm oil emulsions. However, several oil treatments did induce mortality (Table 8.3).

DC-Tron treated LBAM resisted desiccation at much higher dipping concentrations than CPD. Larvae treated with 15,000ppm or 30,000ppm DC-Tron showed no significant reduction in weight loss compared with the controls (Table 8.4). Larvae lost 14.3%, 15.4% and 14.4% of their initial weight 4h after exposure to 30,000ppm DC-Tron, 15,000ppm DC-Tron and water, respectively.

Table 8.3 Mean LBAM larval weight loss and % mortality of 5th instar LBAM larvae dipped at 15,000ppm for 6 oil formulations, and held at 20°C and 60%RH for 4 hours.

Formulation	*Mean Larval Weight(mg)*		*Weight loss*$^{+}$	*% Mortality*
	pre-treat	*After 4h*	*(SEM)*	
Water	34.35	28.87	4.05 (0.69)a	0.0a
Citrole	34.53	30.97	3.56 (0.38)a	45.0b
NHC5	35.48	31.70	3.79 (0.59)a	55.0b
DC-Tron	32.86	29.27	3.59 (1.04)a	0.0a
AWH-96-04	32.89	29.46	3.43 (0.34)a	10.0c
Eco-oil	33.24	29.34	3.92 (1.07)a	0.0a

+Values are the means of 4 replicates. Means within a column followed by the same letter are not significantly different ($p > 0.05$, least significant difference)

Table 8.4 Progressive weight of 5th instar LBAM larvae dipped in 15,000ppm and 30,000ppm DC-Tron oil and held at 20°C, 60%RH.

	Progressive larval weight (mg)							
Treatment	*0h*	*1h*	*2h*	*3h*	*4h*	*5h*	*6h*	*24h*
Water	31.9a	30.0a	29.0a	28.0a	27.3a	26.6a	25.6a	20.1a
15,000ppm	31.8a	29.5a	28.6a	27.7a	26.9a	26.4a	25.8a	19.3a
30,000ppm	31.4a	29.3a	28.5a	27.6a	26.9a	26.5a	25.7a	19.6a

Means within a column followed by the same letter are not significantly different ($p > 0.05$, least significant difference)

8.3.2 Comparison of Oil, CPD, and Chorpyrifos Induced Weight loss

Larvae treated with chlorpyrifos showed rapid weight loss compared with CPD treated and control larvae (Figure 8.2). Differences in weight loss between chlorpyrifos, CPD treated and untreated larvae occurred 4h after dipping. Chlorpyrifos treated larvae leaked internal fluids, which pooled around the treated individuals. In contrast, larva treated with CPD appeared to dehydrate without a collection of fluids and had a darkened cuticle within the first 2-4h from exposure (Chapter 4, Figure 4.1).

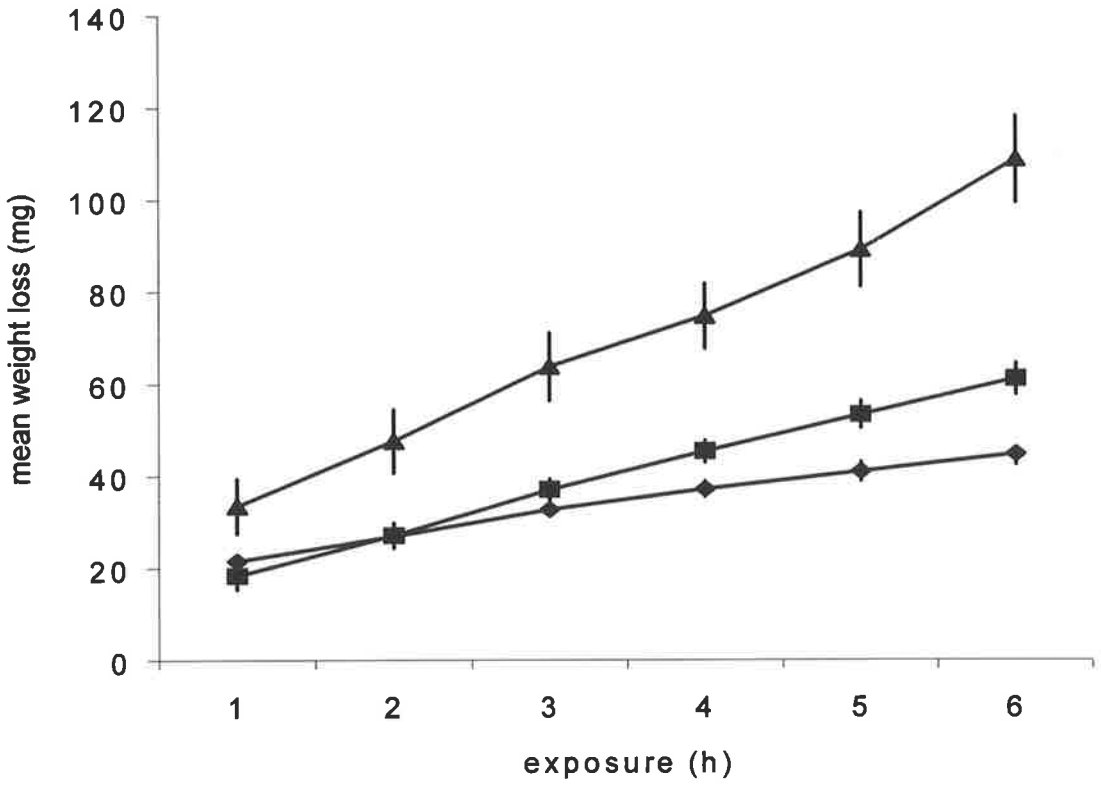

Figure 8.2 Mean weight loss of 5th instar LBAM larvae dipped in water (♦), 1,000 ppm CPD oil (■) and 250ppm chlorpyrifos (0.5ml/l Lorsban 500EC) (▲) and held at 20^{0}C, 55%RH

8.3.3 Effect of Weight Loss on Mortality of LBAM Larvae

Insects can normally control water loss under desiccating conditions. Relative humidity levels of between 15% to 98% had little effect on weight loss rates of untreated larvae. However, oil treated larvae showed a progressive increase in weight loss as humidity decreased (Figure 8.3). Slopes differ by a factor of 10, with oil dipped larvae recording much higher weight losses than the controls as the humidity decreased (oil dipped larvae; n=160, weight loss=-0.288*humidity +45.19, R^2=72.3, P<0.001; and control larvae; n=160, weight loss=-.0285*humidity +19.54,R^2=17.6, P<0.05).

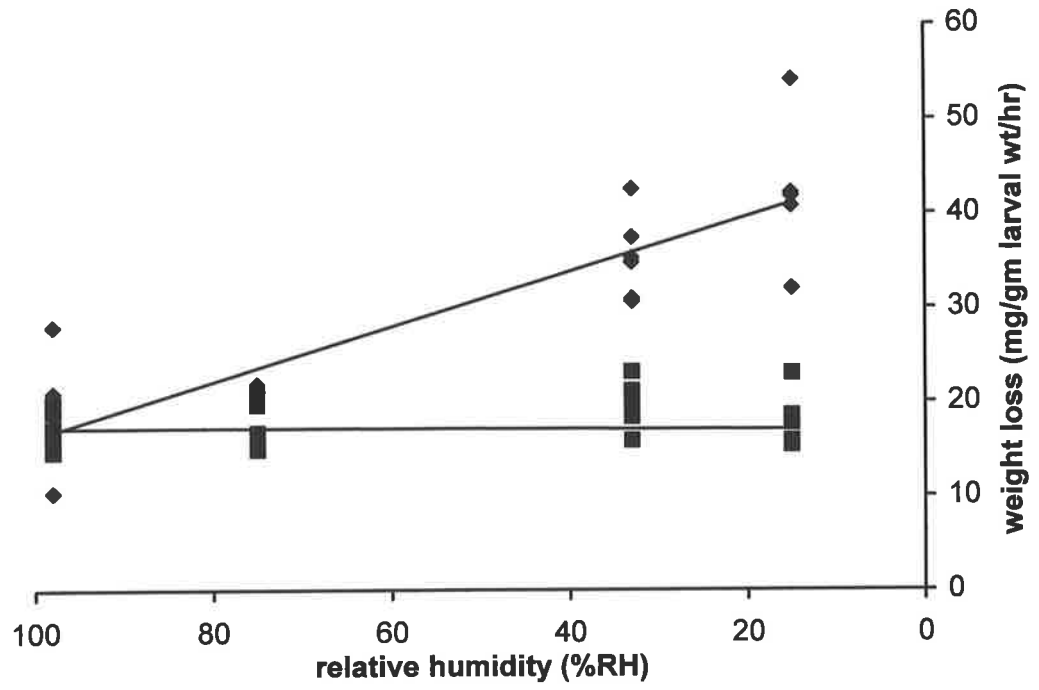

Figure 8.3 The relationship of Weight loss and humidity for 5th instar LBAM larva dipped in water (■) and 15,000ppm CPD oil (♦) after 6h.

Only oil dipped LBAM died, and died regardless of humidity levels (Table 8.4). Dead CPD dipped larvae at very high humidity (98%RH) lost weight (18.85mg/gm larva) similarly to surviving low humidity (33%RH) control larvae (19.46mg/gm larva). This demonstrates that

weight loss *per se* is not leading to mortality in oil dipped LBAM larvae at very high humidities.

Humidity showed a weak correlation with the mortality of oil-treated larvae (R^2=0.1873, $p<0.05$), with increased mortality after 6 h exposure in highly desiccating conditions. However, the influence of humidity on mortality was transient. After 24 hours exposure, all humidities led to similarly high levels of mortality of oil-treated larvae, with 100%, 92%, 88%, 92% and 100% mortality for 15%, 33%, 55%, 75% and 98%RH, respectively ($p>0.05$).

Table 8.4 Effect of 15,000ppm CPD oil dip on weight loss (mg/gm larval weight) and % mortality of 5th instar LBAM larvae at 4 humidities, held at 20^0C

Treatment -humidity	*initial weight (mg)*	*6h weight loss (mg/gm)*	*P level*[a]	*6h % Mortality*	*P level*[a]
Control-15%	27.2	17.5	0.002	0	<0.001
CPD-15%	26.7	27.9		60	
Control-33%	31.2	19.5	0.006	0	<0.005
CPD-33%	31.6	35.3		72	
Control-75%	26.0	16.5	0.012	0	<0.005
CPD-75%	25.9	21.0		60	
Control-98%	25.7	17.2	0.644	0	<0.005
CPD-98%	26.6	18.9		40	

a Least significant difference method for pairwise comparison of the means; difference between treatment and control significant when $p<0.05$.

8.4 Discussion

The role of desiccation in the mortality of insects may depend on the properties of the insect surface lipids as well as the properties of the oil. In this study, mealybug were more susceptible to oil induced weight loss than LBAM larvae. Mealybug dipped in CPD died, and were obviously affected by the loss of their waxy surface layer. Petroleum oils can affect water regulation by increasing the permeability of insects' cuticular lipids (Wigglesworth 1945, Ebeling and Wagner 1959). The outer layer of the cuticle, the outer epicuticle, is perforated by many channels that connect with the procuticle beneath and potentially make it a major site for

water loss (Locke 1965; Machin and Lampert 1985). The main function of the cuticular lipids, which cover the cuticle, is to minimise water transpiration. Oil with sufficient solvent power may physically wash away the wax layer or greatly alter its distribution. CPD dissolved the white waxy coating of dipped mealybug. Alternatively, oils may have become incorporated into cuticular lipids of insects. The water permeability of solid lipids is greatly increased by the addition of liquid hydrocarbons (Ebeling 1974). The introduction of liquid hydrocarbons into the cuticle may disrupt its waterproofing abilities by altering the molecular orientation (Wigglesworth 1945; Beament 1945) or modifying the phase behaviour (Locke 1965) of cuticular lipids.

The C15 alkane, CPD, has a greater influence on water regulation than other petroleum oils, such as DC-Tron. The reason CPD is more disruptive may be due to an alternative mode of action. Many chemical toxicants are highly desiccating (Ebeling 1974) but a comparison of CPD and chlorpyrifos suggests the mechanism to induce desiccation is not the same. Chlorpyrifos, a neurotoxic chemical, induced a very rapid loss of turgor, presumably by a gross disruption of internal functions, leading to a free pooling of liquid around the body. In contrast, the C15 alkane induced a slower desiccation as the internal fluids evaporated. Ebeling (1945) found a similar effect with potato tuber moth, *Phthorimaea operculella* (Zeller), larvae, which became "shrivelled" when dipped in kerosene. CPD and DC-Tron are quite different products and their effects on the larvae possibly relate to distinct physical characteristics. CPD is a homogeneous C15 alkane with a molecular weight of 212. DC-Tron is a mixture of C15 mono-cyclic molecules with side chains, which has an average molecular weight of 350. The low viscosity of CPD (2.75 mm^2/sec) compared to DC-Tron (12 mm^2/sec) allows it to penetrate deeply into the tracheal system. Disruption of the lipid lining of the tracheae would expose a large surface area to increased water loss. DC-Tron (50% distillation point at 101.33 kPa is 385°C) is less volatile than CPD (50% distillation point at 101.33 kPa is 385°C) and, therefore, more likely to persist on the surface of the cuticle and tracheae, acting as

a physical barrier to water loss. DC-Tron (417 ml $mole^{-1}$ at 15°C), with a larger molecular volume than CPD (278 ml $mole^{-1}$ at 15°C) would be slower to mix with or incorporate into solid lipids. The movement of larger molecules into surface lipids would be slower, but their eventual incorporation is likely to increase their free volume, and consequently, water permeability (Hurst 1940). Petroleum spray oils, such as DC-Tron, showed effects in mealybug but may need extended exposure to affect the cuticular permeability of LBAM larvae.

In addition, petroleum spray oils are formulated with surfactants, which may affect water movement across the cuticle. Water droplets appear more readily on the surface of the cuticle when insects are immersed in emulsive oils with various surface-active solutes, such as glyceryl monooleate, glycol oleate and glyceryl trioleate (Ebeling 1950). The role of surfactants also needs to be examined as they are considered to enhance the desiccating action of oils (Wigglesworth 1945, Ebeling and Wagner 1959).

Oils induce desiccation, but the role of weight loss in inducing larval mortality appears to be causative or symptomatic of the failure of other processes. It is unlikely that weight loss *per se* is the primary cause of mortality since control larvae held at very low humidities survived comparable weight loss to dead oil-treated larvae. However, accelerated weight loss may be a contributing factor to mortality during oil exposure. Interestingly, higher survival rates only occur in high humidity atmospheres during the first 6 hours after exposure to oil. After 24 hours, mortality of oil-treated larvae is similarly high regardless of humidity level. It is possible the oils produce a cascading effect with other mechanisms, such as suffocation, being induced after extended exposure.

The results of this chapter indicate that oil induced desiccation of LBAM larvae can influence mortality rates slightly. Perhaps, mealybug, with a demonstrably greater solubility of surface wax in oil would show a stronger relationship. The expression of the symptoms of desiccation probably depends on the relationship between the physical characteristics of the insect's lipids and the oil. Suppression of larval weight loss and mortality by high humidities suggests that the mechanism is a change in cuticular permeability rather than gross disruption of membranes. One

explanation is that light hydrocarbons either wash away or become incorporated into the cuticular lipids and tracheal lining, which could cause a loss of water from a large surface area. Considerable work is required to understand the influence of different petroleum fractions and surfactants on the structure and function of cuticular lipids.

8.5 Summary of Main Points

1. In this study, mealybug were more susceptible to oil induced weight loss than LBAM larvae. Mealybug dipped in CPD were obviously affected by the loss of their waxy surface layer.

2. The tracheal system should be considered as a potential site of water loss, as well as the surface cuticle. The tracheal lining has a vast surface area and therefore, may cause excessive water losses if disrupted by solvent oil.

3. CPD has a greater influence on water regulation than other petroleum oils, such as DC-Tron.

4. A comparison of the symptoms of poisoning by CPD and chlorpyrifos suggests the mechanism to induce desiccation is not the same.

5. CPD and DC-Tron are quite different products and their effects on the larvae possibly relate to distinct physical characteristics. The physical characteristics that would allow CPD to have a greater influence on water loss compared to DC-Tron are viscosity, volatility and molecular volume.

6. In addition, petroleum spray oils are formulated with surfactants, which may affect water movement across the cuticle and tracheae. The role of surfactants also needs to be examined.

7. Oils induce desiccation, but it is unlikely that weight loss *per se* is the primary cause of mortality. However, accelerated weight loss may be a contributing factor to mortality during oil exposure.

8. The effect of weight loss on larval mortality occurs during the first 6 hours after exposure to oil. Oils may produce a cascading effect with other mechanisms, such as suffocation, being induced after extended exposure.

Chapter 9

The Mode of Action of Selected Petroleum on Exposed Lightbrown Apple Moth: Solvents, Solubility and Cell Disruption.

9.1 Introduction

Unsaturated hydrocarbons in petroleum oils can rapidly dissolve cellular structures (de Ong 1926; de Ong *et al.* 1927). Saturated oils, such as paraffins, are considered to be much less surface active, but have been observed to disrupt plant cell membranes, probably by solubilising membrane lipids (van Overbeek and Blondeau 1954).

Insecticidal oils are formulated with surfactants to control oil deposition, but surfactants can also cause profound stress of plant tissues (Lownds and Bukovac 1989). The primary mechanism of surfactant phytotoxicity appears to be disruption and solubilisation of biological membranes (Gaskin 1995). It is likely that these surfactants would also solubilise insect waxes and cell membranes.

Disruption of membranes, whether by oil or surfactants, may be entirely due to oil solubilising membranes or may be assisted by secondary reactions. Ebeling (1945) found that insects dipped in oil died more quickly in the presence of air than when held submerged, and hypothesised that oxidation may be contributing to mortality. All aerobic organisms, including insects must cope with endogenous oxidative stress (Felton 1995). The solubilisation of the cuticular waxes and leakage of cell membranes may render insects more prone to lipid peroxidation.

This chapter aims to measure the acute toxicity to LBAM larvae of surfactants used in oil formulations. It is further intended to observe the effect of oils and surfactants on dissolving

cuticular lipids by using ammoniacal silver nitrate as an indicator. The role of oxygen will be determined by measuring the weight loss and mortality of oil-treated LBAM larvae in oxygen enriched atmospheres. Recording the mortality of larvae after feeding with high anti-oxidants diets will assess the hypothesis of oxidative stress.

9.2 Materials and Methods

9.2.1 Lightbrown Apple Moth Colony

The larvae used in the experiments were collected from a laboratory culture maintained, since 1993, at the South Australian Research and Development Institute, Waite Precinct (Adelaide). Singh, Clare and Ashby (1985) described the rearing procedure.

9.2.2 Oil Formulations

Ampol Research and Development Laboratories, Brisbane Qld, supplied a Citrus Postharvest Dip, Ampol C15 CPD (an alkane with a carbon number of 15, ie., C15; paraffin content, ie., %Cp>99%) and a commercial spray oil, Ampol DC-Tron NR (narrow range oil with mean equivalent *n*-paraffin carbon number of a C23 alkane (Furness *et al.* 1987); %Cp<70%). General specifications of these oils can be found in Appendix 1. Ampol Research and Development Laboratories also supplied separately the non-ionic emulsifier and oil-soluble humectant included in formulation of CPD. These two products were mixed at a ratio of 50:50 and are, hereto, referred to as the "surfactant blend".

9.2.3 Larval Bioassays using Surfactants

Insecticidal oils are formulated with surfactants to control oil deposition, but surfactants may also solubilise insect waxes and cell membranes. Bioassays were undertaken with the non-ionic emulsifier and oil-soluble humectant used in formulation of CPD to determine their influence on larval mortality. The humectant did not readily emulsify in water, so the surfactant blend

was used to allow emulsification in water. The water-soluble surfactant alone was then compared with the surfactant blend.

Bioassays tested the water-soluble surfactant and a "50:50" surfactant blend against fifth instar Lightbrown apple moth. The larvae were dipped in three concentrations, 1000ppm, 10,000ppm and 50,000ppm. The controls were dipped in water only and the experiment was replicated 4 times. The dipping and assessment procedure was as described in Chapter 4, section 4.2.3.

9.2.4 The Effect of Oils on the Cuticular Lipids of LBAM Larvae

Oils are solvents, which could dissolve the cuticular lipids and eventually disrupt lipid membranes. Ammoniacal silver hydroxide has been used successfully to establish the removal or disruption of insect cuticular wax by abrasion, solvency (Wigglesworth 1945) and absorption (Ebeling and Wagner 1959). The exposed polyphenols in the insect cuticle reduce, causing a black stain.

In this study, ammoniacal silver hydroxide was used to stain areas of LBAM larval cuticle where wax had been removed by exposure to oil or surfactant. A 5% ammoniacal hydroxide solution was prepared by adding 3ml of 3N NaOH to 100ml of 0.1N $AgNO_3$, then adding concentrated NH_4OH until the resulting precipitate dissolved. The method is described in Ebeling and Wagner (1959).

Groups of LBAM 5th instar larvae were dipped in 15,000ppm CPD, 1,000ppm surfactant blend, neat n-pentane and water to compare the effects of the oil, surfactants, a lipid solvent with the control, respectively. Treated larvae were held for 1h in petri dishes before rinsing in 100ppm Triton-X100. All groups were washed thoroughly with de-ionised water and placed in

a 5% ammoniacal silver hydroxide solution for 2h, 4h, 6h and 24h.. The cuticles were examined and where differences in staining were apparent, the larvae were photographed immediately on removal from the solution.

9.2.5 LBAM Oil Bioassays in Oxygen Enriched Atmospheres.

LBAM larvae were dipped in oil and held in enriched oxygen atmospheres to determine the effect on mortality. Oil dipped larvae were also compared with water dipped larvae to compare the effect of an enriched oxygen atmosphere on larval weight loss. LBAM larvae of the desired development period (at 20°C; 21 days for 5th instar) were treated with CPD and DC-Tron at 15,000ppm and 30,000ppm, respectively. Previous bioassays indicated these rates would induce LBAM larval mortality. Prior to dipping, each replicate (10 larva/replicate) was weighed. The dipping procedure was as described in Chapter 4, section 4.2.3.

After dipping, the larvae were weighed and placed into gauze cages. The experiment was a randomised block design with oil and water dipped replicates assigned to high oxygen (96%) or normal oxygen levels (34%). The cages were placed on racks in plastic containers and held in a constant temperature room ((20±2°C, 60±5% RH and natural light of 14h day:10h night). Enriched oxygen levels were maintained by flushing oxygen (Industrial grade oxygen; supplied by CIG, Adelaide) through the container until sufficient oxygen levels were obtained. The containers were sealed and oxygen levels were recorded throughout the experiment using Figaro GS Oxygen Sensors series KE-25 (Adilam Electronics, Melbourne). The larvae were removed, assessed for mortality and weighed at 4h. The larvae were placed for 24h in a rearing room at 20°C± 3°C, 55%± 5% RH and under natural light to allow recovery in air. Larval mortality was assessed at 24h. Statistix 4.1 (Analytical Software 1994) was used for analysis of variance and pair-wise comparisons of the means.

9.2.6 LBAM Oil Bioassays and Anti-Oxidants.

The role of oxygen free radicals in LBAM larval mortality was examined by adding a lipid soluble anti-oxidant, α- tocopherol (vitamin E), to the oil directly or the standard larval diet prior to oil dipping. Aucoin *et al.* (1990) demonstrated that tissue levels of vitamin E were substantially increased in tobacco hornworm, *Manduca sexta* L. , larvae fed with anti-oxidant supplemented diets. Initially, the Vitamin E was added directly to the oil at a rate of 5,000ppm in CPD to allow a rapid assessment. In this bioassay, groups of 5th instar LBAM larvae (at 20°C; 21 days) were treated with CPD/vitamin E at 15,000ppm in water. The treatment was replicated 4 times (10 larva/replicate) and the controls were dipped in 15,000ppm CPD only in water. The dipping procedure was as described in Chapter 4, section 4.2.3. Larvae were assessed at 1h, 4h and 24h and recorded as moribund (at 1h) or dead (at 4h and 24h) if they did not respond to repeated prodding with a needle.

LBAM larvae were also fed on elevated dietary levels of vitamin E by either raising the larvae on the high anti-oxidant diet or substituting the standard diet with high anti-oxidant diet 48h prior to treatment. For larva raised on anti-oxidant diet, egg masses were placed in pots filled with media containing 5,000ppm of vitamin E and hatched larvae allowed to develop according to the rearing procedure described by Singh, Clare and Ashby (1985). Control larvae were raised on media without vitamin E (standard media). Prior experiments established that larvae raised on a diet with >0.5% vitamin E avoided consuming their food and were obviously less thrifty than 5th instar larvae raised on standard diets. Larvae on these vitamin E diets also tended to be cannibalistic. Thus, CPD dip bioassays were restricted to larvae on diets with <0.5% vitamin E concentrations, where anti feedant properties were not apparent. At the desired development stage (4th instar at 20°C; 18 days) larvae were dipped in 5,000ppm CPD and assessed as described previously. For larvae with a substituted high anti-oxidant diet, 4th instar larvae (at 20°C; 18 days) were collected from standard media pots and

individually placed into the wells of Linbro® Tissue Culture multi-well plates (1.7cmx1.6cm) (Flow Laboratories Inc., McLean, Va., USA) to avoid cannibalism. To avoid diet rejection, larvae were starved for 24h before transferring diet cubes with varying amounts of vitamin E (0.01%, 0.1%, 1.0%, 5% and 10%) into wells. Control larvae were raised on standard media cubes. The tissue culture plates with larvae were placed for 48h in a rearing room at 20± 3°C, 55± 5% RH and under natural light of 14h day:10h night. Fifth instar larvae (at 20°C; 21 days) were dipped in 15,000ppm CPD and assessed as described previously.

9.3 Results

9.3.1 LBAM Bioassays using Surfactants.

The surfactants used in CPD, a combination of an oil-soluble humectant and a water-soluble emulsifier, are toxic to LBAM larvae when applied in dips (Table 9.1). However, very high doses of surfactant blend (50,000ppm) were required to induce significant mortality. Higher mortality occurred with larvae dipped in the surfactant blend compared to the water-soluble surfactant only (Table 9.1). This suggests that the oil-soluble component of the blend is contributing to the larval mortality to a much greater extent than the water-soluble component.

Table 9.1. % Mortality of LBAM 5th instar larvae dipped in water, water soluble surfactant and surfactant blend, after 24 hours exposure

Rate (ppm)	*Product*	*% Mortality(SEM)*+
0	Control	0.0 (0.0)a
1,000	Water soluble	0.0 (0.0)a
	Surfactant blend	0.0 (0.0)a
10,000	Water soluble	0.0 (0.0)a
	Surfactant blend	7.5 (4.8)a
50,000	Water soluble	7.5 (7.5)a
	Surfactant blend	45.0 (10.4)b

+ Values are the means of 4 replicates of 10 larvae converted to percentages. Means within a column followed by the same letter are not significantly different ($p<0.05$, LSD.)

9.3.2 The Effect of Oils on the Cuticular Lipids of LBAM Larvae

The ammoniacal silver hydroxide solution penetrated the lipid layer of untreated LBAM larvae and stained the exposed epicuticle, but staining should take place much sooner if the lipid layer is removed. Comparisons were made at 4h before significant staining of the cuticle occurred in untreated larvae (Figure 9.1). Untreated larvae showed slight darkening of the head capsule and appendages indicating the lipid barrier is thinner at these points. Darkening of the main tracheae connected to large spiracular openings indicated limited penetration of the tracheae. In contrast, larvae dipped in surfactant showed dark staining of the head and prolegs, as well as some dark patches on the cuticle.

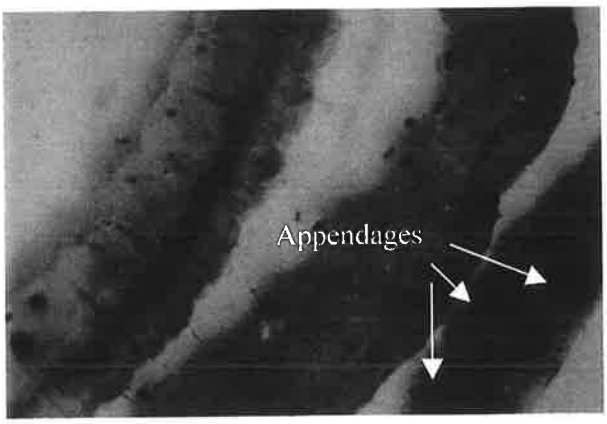

Figure 9.1 Untreated LBAM larval cuticles are largely unstained after 4h exposure in ammoniacal silver hydroxide solution. Ventral view shows dark staining of paired appendages.

Tracheal staining was more extensive than in untreated larvae and extended the length of the abdominal system (Figure 9.2). The surfactant removed the thin lipid layer coating the inside of the tracheae allowing staining. Larvae treated with n-pentane also showed dark head capsule and prolegs but no darkening of the tracheae. Treatment with CPD produced no staining in larvae, even of the surface cuticle, during the first 4h of exposure. CPD prevented the staining of the cuticle by 4h, suggesting that the oil had not removed the protective cuticular lipids.

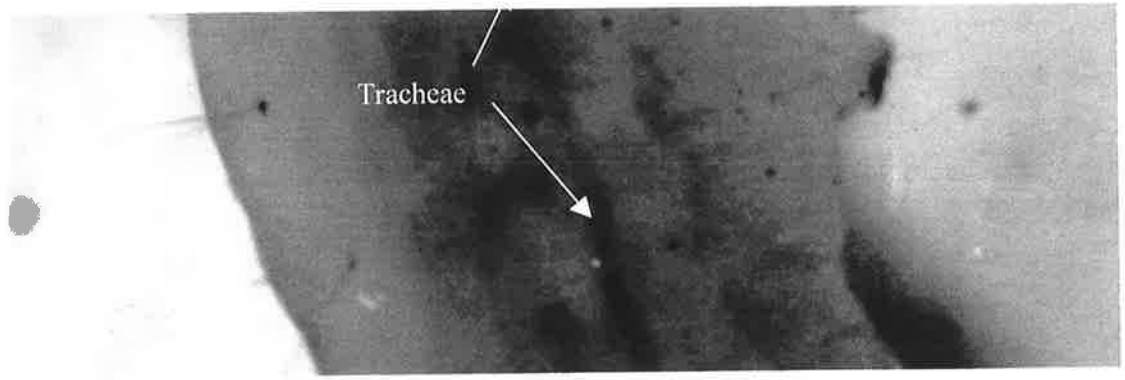

Figure 9.2 Staining of the LBAM larval tracheae after dipping in surfactant followed by 4h immersion in ammoniacal silver hydroxide solution.

9.3.3 LBAM Oil Bioassays in Oxygen Enriched Atmospheres.

High levels of oxygen *per se* had no influence on weight loss and mortality of 5th instar LBAM larvae. Larvae dipped in CPD and exposed to high oxygen atmospheres (96%) for 4h had significantly higher weight loss and mortality than larvae dipped in CPD and held in air (Table 9.2).

Table 9.2. Mean weight loss (mg/larva) and mortality of LBAM 5th instar larvae dipped in water and CPD (15,000ppm) after 4 hours exposure to air or high oxygen atmospheres

Atmosphere	*Oil*	*Initial wt (mg)*	*Final wt (mg)*	*Weight loss (SEM)*+	*% Mortality(SEM)*+
Air	-	16.0	14.7	1.3 (0.44)a	0.0 (0.0)a
	+	15.3	13.6	1.8 (0.31)a	27.5 (7.5)b
96% O_2	-	15.6	14.1	1.5 (0.24)a	0.0 (0.0)a
	+	15.2	12.4	2.8 (0.11)b	32.5 (8.7)c

+ Values are the means of 4 replicates of 10 larvae converted to percentages. Means within a column followed by the same letter are not significantly different ($p<0.05$, LSD)

All surviving CPD dipped larvae were allowed to recover in air after the 4h high oxygen exposure. Treatments were not significantly different after 24h recovery ($F=1.00$, $df=1$, $P=0.39$), with mortality levels of 30.0±10.8% and 42.5±2.5% for larvae initially exposed to high oxygen and air, respectively. Weight loss over the 24h period was also not significantly different ($F=0.92$, $df=1$, $p=0.41$), with a weight loss of 4.91±0.72mg/larva and 5.45±0.45mg/larva for larvae initially exposed to high oxygen and air, respectively.

In contrast to CPD, high levels of oxygen had no influence on the weight loss or mortality of larvae treated with DC-Tron. There was no significant mortality by 4h and similar weight loss in all treatments (Table 9.3). All DC-Tron dipped larvae were allowed to recover in air. Mortality recorded after 24h showed oil dipped larvae exposed to different oxygen levels were not significantly different ($F=1.42$, $df=1$, $p=0.32$), with mortality levels of 47.5±4.8% and 40±4.1% for larvae initially exposed to high oxygen and air, respectively. Weight loss over the 24h period was also not significantly different ($F=0.48$, $df=1$, $p=0.54$) with a weight loss of 2.16±0.22 and 2.51±0.35mg/larva for larvae initially exposed to high oxygen and air, respectively.

Table 9.3. Mean weight loss (mg/larva) and % mortality of LBAM 5th instar larvae dipped in water and DC-Tron (30,000ppm) after 4h exposure to air or high oxygen atmospheres

Atmosphere	*Oil*	*Initial wt (mg)*	*Final wt (mg)*	*Weight loss (SEM)+*	*% Mortality(SEM)+*
Air	-	19.5	18.7	0.8 (0.31)a	0.0 (0.0)a
	+	18.3	17.1	1.2 (0.34)a	12.5 (6.3)a
96% O_2	-	18.2	17.0	1.2 (0.37)a	0.0 (0.0)a
	+	18.4	17.5	0.9 (0.20)a	5.0 (2.8)a

+ Values are the means of 4 replicates of 10 larvae converted to percentages. Means within a column followed by the same letter are not significantly different ($p<0.05$, LSD)

9.3.4 The Effect of Anti-Oxidants on Oil-Induced Mortality in LBAM larvae.

An oil soluble anti-oxidant, vitamin E (α-tocopherol), was added to CPD to determine the effect on LBAM larval mortality. Larvae dipped in a mixture of vitamin E and CPD had significantly reduced 24h mortality when compared to CPD alone ($F=32.11$, $df=1$, $p=0.01$) (Table 9.4).

Table 9.4. % Mortality of LBAM 5th instar larvae dipped in 10,000ppm CPD with or without vitamin E (50ppm).

Treatment	% Mortality (SEM)+		
	1h	4h	24h
CPD - vitamin E	50.0 (14.1)a	65.0 (11.9)a	85.0 (5.0)a
CPD + vitamin E	15.0 (9.6)a	15.0 (8.7)a	42.5 (6.3)b

+ Values are the means of 4 replicates of 10 larvae converted to percentages. Means within a column followed by the same letter are not significantly different ($p<0.05$, LSD)

CPD treated 4th instar larvae raised on diets containing 0.25% vitamin E showed significantly lower mortality than larvae raised on standard diets at all assessment periods (Table 9.5). Differences between the larval groups were evident very early after dipping. Larvae raised on a diet with vitamin E were much more tolerant to CPD than standard diet larvae during the first hour ($F=243$, $df=1$, $p<0.001$), but the differences in tolerance had decreased by the 24h period ($F=14.45$, $df=1$, $p<0.05$).

Table 9.5. % Mortality of LBAM 4th instar larvae raised on a diet with or without vitamin E (0.25%) and dipped in 5,000ppm CPD.

vitamin E	% Mortality (SEM)+		
diet	1h	4h	24h
-	65.0 (5.0)a	75.0 (2.8)a	82.5 (2.5)a
+	20.0 (4.1)b	40.0 (8.2)b	50.0 (9.1)b

+ Values are the means of 4 replicates of 10 larvae converted to percentages. Means within a column followed by the same letter are not significantly different ($p<0.05$, LSD)

Larvae fed on a diet with vitamin E for 48h prior to dipping show no obvious differences in thrift when compared with larvae exposed to standard diet. After dipping in CPD, larvae exposed to a vitamin E diet for 48h showed more tolerance to oil than larvae fed on a control diet only (Table 9.6). This result shows that ingestion of vitamin E for even a short period before treatment (48h) tends to protect the larvae from the effects of CPD. However, a higher level of dietary vitamin E was required to induce tolerance over a 24h period. Larvae with a dietary vitamin E intake of 0.1% did not survive better than larvae on standard diet.

Table 9.6. % Mortality of LBAM 4th instar larvae exposed for 48h on a diet with or without vitamin E and then dipped in 5.000ppm CPD.

% vitamin E in diet	% Mortality (SEM)+		
	1h	4h	24h
0	95.0 (5.0)a	100 (0.0)a	100 (0.0)a
0.1	50.0 (13.7)b	65.0 (15.5)b	90.0 (4.1)a
1.0	42.5 (2.5)b	42.5 (11.8)b	77.5 (2.5)b
5.0	42.5 (4.8)b	62.5 (4.7)b	77.5 (6.3)b

+ Values are the means of 4 replicates of 10 larvae converted to percentages. Means within a column followed by the same letter are not significantly different ($p<0.05$, LSD)

9.4 Discussion

The surfactants used in CPD were toxic to LBAM larvae at high concentrations (10,000ppm). The primary mechanism of surfactant phytotoxicity appears to be disruption and solubilisation of biological membranes (Gaskin 1995). It is likely that surfactants would also solubilise insect waxes and cell membrane lipids. This would explain the higher toxicity of the oil-soluble humectant compared to the water-soluble surfactant. However, the relative importance of surfactants in the overall toxicity of oil formulations may be low. Surfactants are a very small component of CPD (<10% vol/vol), particularly when further diluted in water, and relatively high doses of surfactants were required to induce significant mortality. Alkanes may have sufficient mutual solubility of their own to disrupt cell membranes. However, this would not preclude a synergistic effect between the oil and surfactant, where the solubilising effect of both influence mortality.

Ammoniacal silver hydroxide, used to show the removal of the protective wax layer of the cuticle, showed that rather than removal of surface lipids, CPD appeared to give greater protection from staining. CPD either remained on the surface after washing or was incorporated in the cuticular wax. Exposure periods to oils of longer than 1h may be required to remove surface waxes. In contrast, the surfactant blend used in CPD was able to break down the protective coating, particularly the tracheal lining, but it seems that this effect is minimised in the oil formulation

Surfactants are known to increase the rate of water loss when applied to insect cuticles (Wigglesworth 1945) and water loss is symptomatic of a loss of lipid protection. In this study, the effect of oils and surfactants on the surface of the tracheae were closely examined due to observations of dehydration surrounding large spiracles of CPD dipped larvae (Chapter 6). The large surface area of the tracheal system presents a huge potential for water loss if the tracheal lining is disrupted. Larvae dipped in surfactant (1,000ppm) showed a loss of the tracheal lining and a potential for increased gaseous exchange. However, high concentrations of surfactants (10,000ppm) were necessary to induce mortality. Uncontrolled water loss from the tracheae would also require loss of the spiracular muscle's coordination of gas exchange. This may occur if the muscle's performance was affected by nervous disruption (Chapter 7).

Oils may cause water loss through cell disruption. Plant studies suggest that pure hydrocarbons solubilise cell membranes. The smaller the hydrocarbon molecule, the more the increase in water permeability of plasma membranes, but paraffins with molecular weights above dodecane (C12) were ineffective in producing acute effects (van Overbeek and Blondeau 1954). Light hydrocarbons solubilise into the plasma membrane "opening up" the structure by displacing fatty acids. The change in structure causes the cell to leak and when profuse cause the cell to collapse. Larger hydrocarbons penetrate much more slowly. However, when they eventually reach the plasma membrane, they cause larger displacement. The plasma

membrane, a double layer of fatty molecules stabilised by protein layers, acts as a colloid micelle. Any insect cell membrane with a similar structure would be solubilised. Oils entering the insect via the tracheae may persist for many hours or days eventually penetrating a susceptible membrane. This process would be similar to chronic toxicity in plants.

Ebeling (1945) postulated that the presence of air increased the toxicity of petroleum oils to insects and cited oxidation as a possible cause. In this study, high levels of oxygen increased the toxicity of CPD to LBAM larvae. However, no increase was evident in DC-Tron treated larvae. The heavier DC-Tron could cover the cuticle and inhibit gas exchange through the tracheal lining, which is consistent with the hypothesis of suffocation. If there is no gas exchange then the constituents of the atmosphere are irrelevant.

However, the increased weight loss and mortality of larvae dipped in CPD and held in high oxygen atmospheres seems to imply an effect on oxidation processes. The composition of hydrocarbons in the cuticular lipids has an important role on waterproofing (Hadley 1977; Toolson 1982) and perhaps, the tracheal lining also provides protection from oxidative stress. Anti-oxidants, as dietary supplements, were successful in reducing the toxicity of CPD and suggest that the oil may act as a pro-oxidant. The oil is unlikely to directly affect the enzymatic defences but may affect lipid peroxidation defence by altering the permeability of the tracheal surface waxes. After oil dipping, the water and oil emulsion initially restricts gas exchange, but as the water and volatile oil dissipate gas exchange would increase. Liquid hydrocarbons greatly increase the permeability of solid lipids (Ebeling 1974) and oils incorporated into the solid lipids of tracheal lining would increase the rate of water and gaseous exchange. Increased tracheal permeability coupled with a very high oxygen tension would lead to elevated levels of oxygen in the haemolymph. The incorporation of liquid hydrocarbons in the surface waxes may also lead to a leakage of long chain polar lipids from the waxes (Wigglesworth 1945). An excess of free unsaturated lipids and elevated levels of oxygen could

result in injury through an overproduction of reactive oxygen species (ROS) and lipid peroxidative attack.

Petroleum oils, whether by the oil or surfactants, affect water loss in insects. This may be due to solubilisation of membranes or may be assisted by lipid peroxidation. The results of this chapter suggest that CPD may contribute to acute toxicity by subjecting larvae to oxidative injury. However, most petroleum spray oils are relatively large molecular volumes (>350 ml $mole^{-1}$, at 15^0C) compared to CPD (278 ml $mole^{-1}$, at 15^0C). Larger hydrocarbons are less likely to be involved in acute toxicity effects, but may persist to penetrate biological tissues much more slowly. Incomplete spray coverage results in many insects receiving a dose of oil insufficient to induce suffocation. Solubilisation and oxidative stress caused by chronic exposure to sub-acute doses of oils may contribute to some of the residual effects, such as reduced fertility and fecundity, recorded in insects exposed to oils (Ebeling 1936; Chapter 5).

9.5 Summary of Main Points

1. The additives used to formulate CPD are toxic to LBAM larvae when used alone only at very high doses. The oil-soluble humectant/water soluble surfactant blend is more toxic than the water-soluble surfactant only.

2. Surface lipids are not removed after 1h exposure of CPD. Oil may remain on the surface or become incorporated in the cuticular wax. In contrast, the surfactant blend used in CPD was able to break down the protective coating, particularly the tracheal lining.

3. Oil can cause cell disruption in plants (van Overbeek and Blondeau 1954). Any insect cell membrane with a similar structure to plasma membranes, a double layer of fatty molecules stabilised by protein layers, would be at risk of solubilisation.

4. Ebeling (1950) concluded that the presence of air after dipping increased the toxicity of petroleum oils to insects and cited oxidation as a possible cause. In this study, high levels of oxygen exposure to LBAM larvae after dipping increased the toxicity of CPD. However, no increase mortality was evident in DC-Tron treated larvae held in high oxygen atmospheres.

5. CPD may effect the exchange of water and air across the tracheal lining and perhaps, increase oxidative stress. Anti-oxidants, as dietary supplements, were successful in reducing the affect of CPD and suggest that the oil may act as a pro-oxidant. The oil is unlikely to directly affect the enzymatic defences but may affect lipid peroxidation defence by altering the permeability of the tracheal surface waxes.

6. Increased tracheal permeability coupled with a very high level of atmospheric oxygen would lead to elevated levels of oxygen in the haemolymph. An excess of free unsaturated lipids and elevated levels of oxygen could result in injury through an overproduction of reactive oxygen species (ROS) and lipid peroxidative attack.

7. All aerobic organisms, including insects, must cope with endogenous oxidative stress. Exposure to oils may subject larvae to oxidative injury and contribute to acute toxicity. Pro-oxidants and oxidative stress are implicated in many long term pathologies and disorders. Oxidative stress may contribute to some of the residual effects, such as reduced fertility and fecundity, recorded in insects exposed to oils (Ebeling 1936; Chapter 5).

Chapter 10

The Mode of Action of Selected Petroleum and Vegetable Oils on Lightbrown Apple Moth Eggs: A Physical Barrier Interrupting Gas Exchange.

10.1 Introduction

Petroleum oils are considered to produce a lethal effect on eggs by mechanical interference with the normal gaseous exchange leading to an accumulation of toxic metabolites (Smith and Pearce 1948). The duration of oil exposure and the quantity of oil on the egg are critical to cause high mortality (Smith and Pearce 1948). Petroleum oils, with a molecular weight >320, which show little volatility over a 24h period are considered better ovicides (Pearce and Chapman 1952; Fiori *et al.* 1963).

Petroleum oils are effective against the eggs of tortricid moths, such as oriental fruit moth (Smith and Pearce 1948; Fiori *et al.* 1963) and codling moth (Riedl *et al.* 1995). A range of oils, including DC-Tron and CPD (Chapter 4) kills LBAM eggs. DC-Tron, with a mean molecular weight of 350, has low volatility, but CPD, with a mean molecular weight of 212, should be too volatile to interrupt gas exchange for a sufficient period to cause egg mortality (Fiori *et al.* 1963). An alternative mode of action, such as penetration of the egg chorion (O'Kane and Baker 1935) may be responsible for the lethal action of highly volatile oils.

The aims of this chapter were to determine the importance of gas exchange in egg mortality by measuring egg survival after rinsing oil deposits at various intervals, determining the volatility over a 24h period and examining the location of oil using confocal microscopy.

10.2 Materials and Methods

10.2.1 Lightbrown Apple Moth Colony

The LBAM eggs used in the experiments were collected from a laboratory culture maintained, since 1993, at the South Australian Research and Development Institute, Waite Precinct (Adelaide). Singh, Clare and Ashby (1985) described the rearing procedure. Egg masses were laid on plastic cups and cut into cards with single egg masses for bioassays.

10.2.2 Oil Formulations

Two oils were selected according to the volatility characteristics defined by Fiori *et al.* (1963) to represent effective and ineffective ovicides. Ampol Research and Development Laboratories, Brisbane Qld, supplied a Citrus Postharvest Dip, Ampol C15 CPD (an alkane with a carbon number of 15 ie. C15; paraffin content ie. %Cp>99%) and a commercial spray oil, Ampol C23 DC-Tron (narrow range oil with mean equivalent *n*-paraffin carbon number of a C23 alkane (Furness *et al.* 1987); %Cp<70%). General specifications of these oils can be found in Appendix 1. DC-Tron has a mean molecular weight considered necessary for an effective ovicide, whereas CPD is highly volatile and would be defined as an ineffective ovicide (Fiori *et al.* 1963).

10.2.3 Egg Bioassays

Smith and Pearce (1948) demonstrated that the removal of oil during the first 24h after treatment results in high degree of survival of the eggs of oriental fruit moth, Grapholita *molesta* (Busck). In this study, LBAM egg masses were treated with oil and then the oil removed from the egg surface by washing at various intervals to determine the influence of oil films on mortality. Egg masses were obtained and treated as described in Chapter 4 (section 4.2.4). Individual egg masses at the black-head developmental stage (10 days old) were dipped in a CPD or DC-Tron oil emulsion of 1,000ppm or 2,500ppm, respectively. The rates for each oil were selected to obtain high mortality of unrinsed eggs. Control eggs were dipped in water

only and each treatment was replicated six times. After dipping, egg masses were placed on their edges in petri dishes to allow complete draining of excess oil.

The rinsing method was adapted from Smith and Pearce (1948) where eggs were rapidly rinsed in low boiling point petroleum ether. In this study, eggs were thoroughly rinsed in 100ppm Triton-X100 in decontaminated water to remove residues. Preliminary work with eggs dipped in oil dyed with Automate Dye Red B (Petrafin, Sydney) and then rinsed with 100ppm Triton-X100 indicated high removal of coloured oil residues. The oil was removed by rinsing at intervals of 1h, 6h and 24h. One group of oil-dipped eggs remained unrinsed. The eggs were returned to petri dishes lined with lightly moistened filter paper and placed in a rearing room at 20±3°C, 55±5% RH and under natural light of 14h day;10h night. Egg development was checked every 2 days until emergence was completed.

10.2.4 Oil Deposit and Volatility

Fiori *et al.* (1963) concluded that oil volatility was a factor that affected ovicidal efficacy. Oil deposits must be maintained for 24h to effectively control oriental fruit moth eggs (Smith and Pearce 1948). In this study, oil deposits and the rate of dissipation were recorded for doses previously established as sufficient for high control of LBAM eggs: 1000ppm CPD and 2,500ppm DC-Tron in water. The oil deposits for these doses were ascertained by dipping pre-weighed 128 cm^2 aluminium foil sheets. The aluminium sheets were weighed on an analytical balance immediately after treatment, and then held at 23^0 C and 60% R.H. The sheets were placed on the points of four inverted entomological pins embedded in plasticine to allow air exposure to both sides and weighed at intervals over 24h. Aluminium sheets were used to allow comparisons with similar work by Fiori *et al.* (1963).

The oil deposit on foil could not be effectively measured until the water had evaporated. To allow a measurement of oil volatility during the first hour after dipping, oil was mixed with

acetone, rather than water. The acetone rapidly volatilised leaving a measurable oil deposit within minutes of application.

Automate Dye Red B (Petrafin, Sydney) was added to the oils for visual examination of the oil distribution and dissipation rates from dipped egg and aluminium surfaces.

10.2.5 Confocal Microscopy

Confocal microscopy was used to determine the location of fluorescent oil in eggs. Due to the absence of autofluorescence in the paraffin oil, an oil soluble fluorescent dye, Fluorescent Yellow FG (Morton Chemical Company, Chicago, USA), was added at a rate of 0.01ml/l. Emulsions were made using the fluorescent stock solution and decontaminated water. Egg masses were dipped in fluorescent oil emulsions or water only as previously described. Eggs were left for 30 mins after treatment before rinsing in 100ppm Triton-X100 (Ajax Chemicals, Adelaide, SA). Rinsed eggs were mounted in glycerol between two 24 x 40mm glass coverslips.

Fluorescent microscopy was used for imaging the intact eggs. A Bio-Rad MRC-1000 laser Scanning Confocal Microscope System in combination with a Nikon Diaphot 300 inverted microscope in fluorescence mode with excitation at 488/10 nm and emission at 522/32 nm was used. The images of the larvae and eggs were collected using either a 40x or a 20x NA 0.40 dry objective lens. The confocal intensity settings used to capture an image of fluorescent oil produced a faint autofluorescence image of control eggs.

10.3 Results

10.3.1 Oil Deposit and Volatility

The high initial weight of oil emulsions on aluminium foil is predominantly due to the presence of water. Visual examination of the foil held at 23^0 C and 60% R.H showed that initially there were high levels of water but by 1h the water had evaporated leaving only the deposited oil. After 1h, the DC-Tron oil residue was quite stable with minimal oil volatilisation during the 1h to 24h periods following dipping (Table 10.1). By contrast, the C15 oil deposit steadily dissipated over the first 3h from dipping. A concentration of 1,000ppm for CPD yielded an oil deposit of 2.34 $\mu g/cm^2$ after 24h, which was significantly lower than the 27.6 $\mu g/cm^2$ deposit recorded from dipping in 2,500ppm DC-Tron, even allowing for the 2.5x greater concentration of DC-Tron.

Table 10.1 Oil emulsion deposits ($\mu g/cm^2$) of CPD (1,000ppm) and DC-Tron (2,500ppm) in water on aluminium foil at various intervals from dipping

Treatment (dose)	Oil emulsion deposit ($\mu g/cm^2$)*					
	Period after treatment (h)					
	0	1	2	3	4	24
CPD 1000ppm	529.95	7.29	3.91	1.56	2.08	2.34
(SEM)	(66.99)	(1.58)	(0.45)	(0.45)	(0.69)	(0.45)
DC-Tron 2,500ppm	591.04	28.39	27.34	28.12	27.86	27.60
(SEM)	(115.76)	(6.40)	(6.81)	(6.64)	(6.60)	(6.69)

* Values represent the mean weights (SEM) of 3 replicates.

The above study did not distinguish between oil or water loss from the emulsion deposit during the first hour. Water loss would occur early, but light oil fractions, particularly from CPD,

would also dissipate within the first hour. The rapid volatilisation of acetone mixed with oil allowed a more accurate measurement of early oil dissipation than oil mixed with water. Importantly, it showed that the initial deposits of DC-Tron and CPD were similar, but the rate of dissipation was very different. The CPD oil deposit was reduced from 12.5μg/cm^2 to 2.3μg/cm^2 (82% loss) within the first hour compared with 19.5μg/cm^2 down to 17.2μg/cm^2 (12%) for DC-Tron (Table 10.2). After 1hr, the light oil fractions in CPD and DC-Tron had dissipated. The remaining deposit was negligible at 1 μg/cm^2 for CPD and quite stable at 17μg/cm^2 for DC-Tron.

Table 10.2 Oil deposits (μg/cm^2) of CPD (1,000ppm) and DC-Tron (2,500ppm) in acetone on aluminium foil at various intervals from dipping

Treatment (dose)	Oil emulsion deposit (μg/cm^2)*					
	Period after treatment (h)					
	0	1	2	3	4	24
CPD 10,000ppm	12.50	2.34	1.30	0.78	1.30	0.52
(SEM)	(0.90)	(0.45)	(0.69)	(0.45)	(0.69)	(0.52)
DC-Tron 10,000ppm	19.53	17.19	17.45	17.45	17.71	16.41
(SEM)	(1.19)	(0.90)	(1.14)	(1.14)	(1.14)	(1.35)

* Values represent the mean weights (SEM) of 3 replicates.

10.3.2 Egg Bioassays

Rinsing eggs in Triton-X100 produced no significant increase in mortality compared with unrinsed eggs ($F=0.29$, $df=3$, $p=0.58$). Oil dipped LBAM eggs rinsed in Triton-X100 after 1h exposure had higher egg viability compared to eggs rinsed after 24h exposure (Table

10.3). CPD dipped eggs showed no recovery of egg viability when rinsed 6h or more hours after oil exposure. C32 DC-Tron dipped eggs showed an intermediate response after rinsing at 6h for oil exposure (Table 10.3).

Table 10.3 Egg Mortality after removal of oil deposits of CPD (1,000ppm) and DC-Tron (2,500ppm) at various intervals from dipping

Rinse Time (h)	% corrected mortality* (SEM)	
	1000ppm CPD	2,500ppm DC-Tron
1	50.4(2.2) a	32.1(12.3) a
6	94.3(3.7) b	63.0(10.8) ab
24	100(0.0) b	82.9(10.0) b
No rinse	99.2(0.8) b	79.1(9.1) b

* Mortality adjusted using Abbott's Formula (Busvine 1971). Values are means of 6 egg masses. Values within a column followed by the same letter are not significantly different according to one way analysis of variance of the arcsine square root-transformed percentage data. ($p > 0.05$, least significant difference)

10.3.3 Oil Distribution on the Egg

LBAM lay their eggs in overlapping rafts (Figure 10.1). Confocal microscopy was used to examine the distribution of oil on the egg surface and to determine penetration into the interior of the egg. Untreated LBAM eggs (5 days old) showed a strong autofluorescence on the egg surface, but no internal fluorescence (Figure 10.2). After untreated eggs were rinsed with Triton–X100, the surface autofluorescence, was greatly reduced suggesting that Triton-X100 may have removed natural surface aromatics. Eggs dipped in 1000ppm CPD and

Figure 10.1 LBAM eggs laid in partially overlapping rafts shown in light transmission.

2,500ppm DC-Tron fluorescent oil and then rinsed in Triton-X100 showed a change in the distribution of fluorescence compared to untreated eggs. The autofluorescence of untreated eggs was a random "spotted" arrangement (Figure 10.2) compared to a lattice pattern on eggs treated with oil and rinsed (Figure 10.3). The location of the fluorescent oils coincides with the pattern on the egg surface, which can be seen in light transmission on the outer sheath (Figure 10.2, bottom right). Oil appears to have changed the distribution of fluorescence on the surface of the egg. Focusing into the egg reveals no internal fluorescence, suggesting oil does not penetrate the chorion after 4h of exposure (Figure 10.4). DC-Tron dipped eggs still showed a high intensity of fluorescence of the egg chorion 24h after exposure, indicating a significant deposit remained. The intensity of the fluorescence of CPD over the same period was very much reduced. However, increasing the sensitivity of the confocal settings showed a thin, continuous film remained (Figure 10.5).

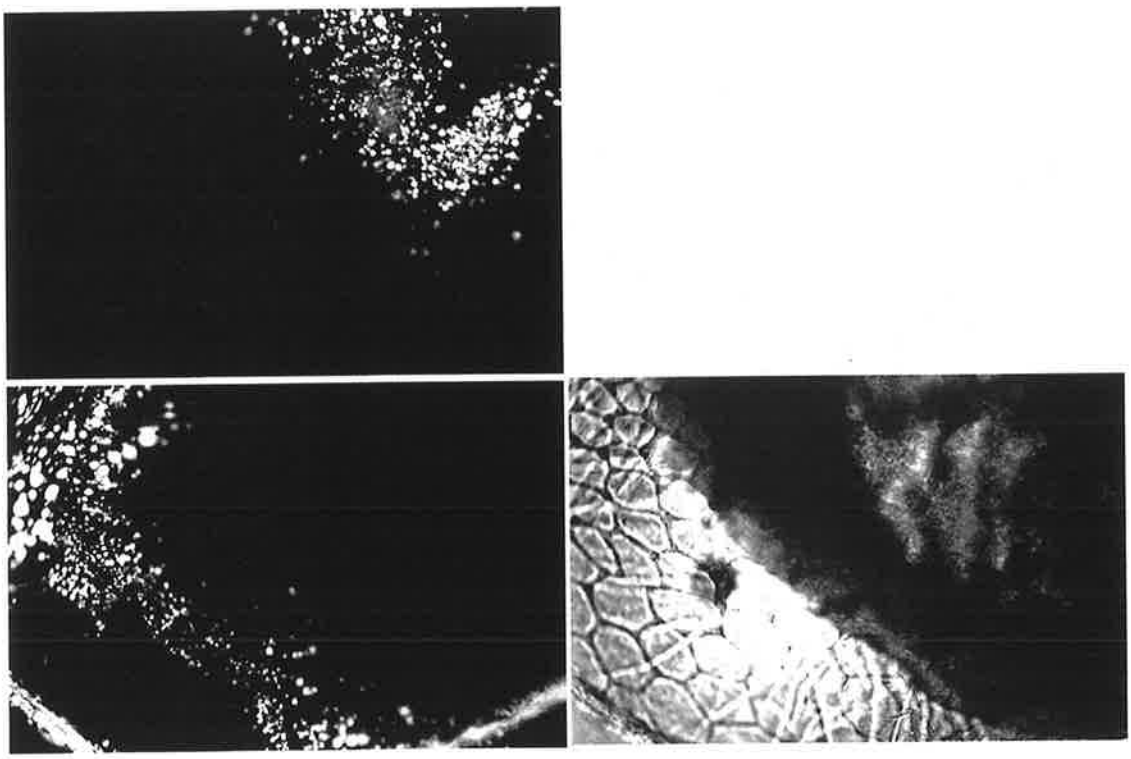

Figure 10.2 Confocal image showing high autofluorescence of untreated LBAM egg surfaces (top left). Focusing deeper into the interior of the egg (60 microns) showed no internal autofluorescence (bottom left); fluorescence was confined to the membrane sheath surface surrounding the egg (refer to transmission image, bottom right) (20x).

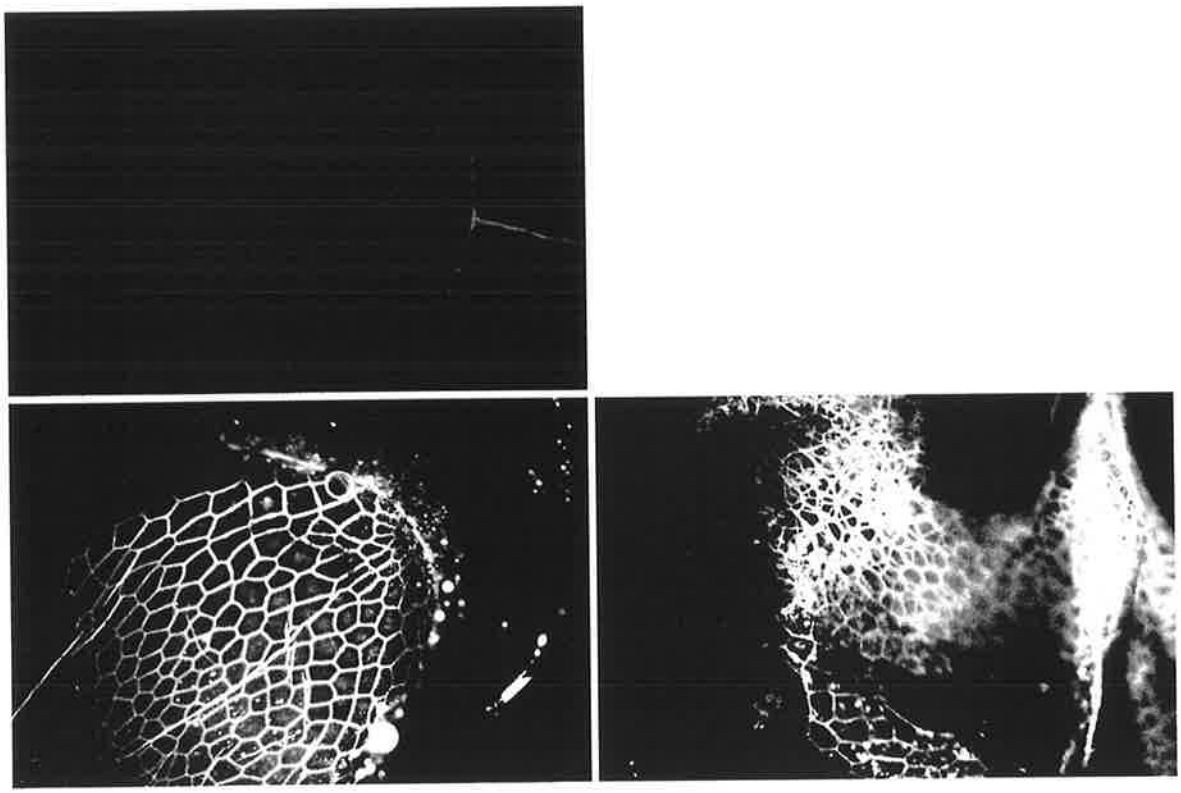

Figure 10.3 Confocal image of LBAM egg (20x) comparing the distribution and intensity of fluorescence of egg surface after rinsing in Triton-X100 only (top), rinsing with Triton-X100 4h after exposure to 2,500ppm DC-Tron oil dip (bottom left), and rinsing with Triton-X100 4h after exposure to 1,000ppm CPD dip (bottom right)

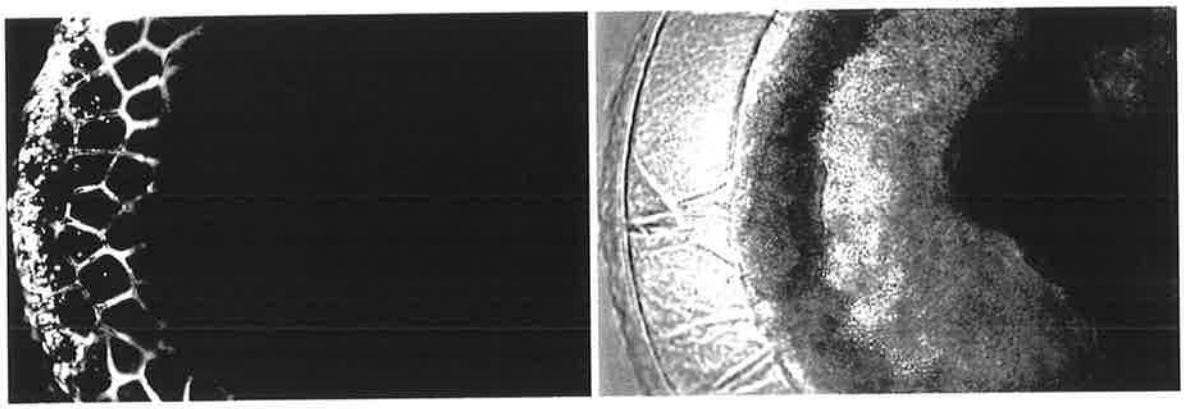

Figure 10.4 LBAM egg dipped in fluorescent CPD. Confocal image (x20) focusing deep into the interior of the egg (50 microns) shows no internal fluorescence (left), with fluorescence confined to the surface of membrane sheath around the egg (refer to transmission image, right).

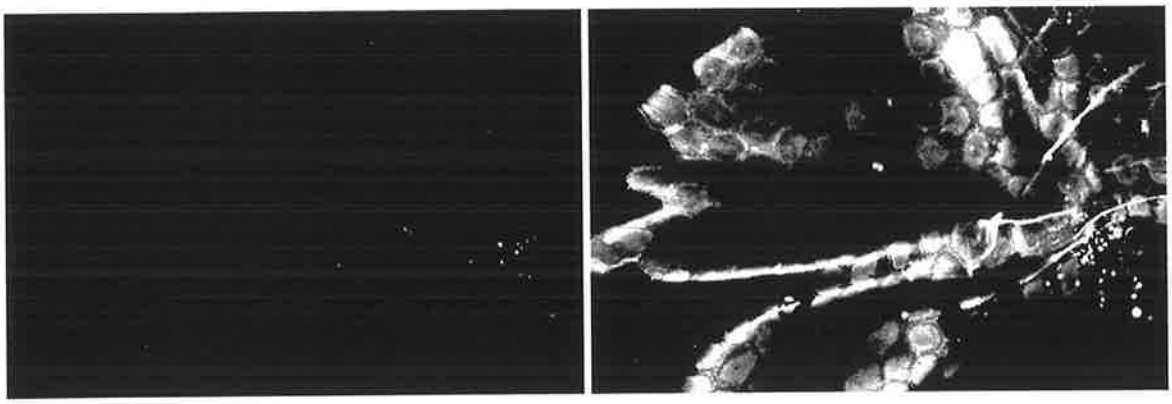

Figure 10.5 LBAM egg dipped in fluorescent CPD. Confocal image (x40) shows negligible fluorescence after 24h from exposure (left). Increased sensitivity of confocal settings (x4) shows a thin fluorescent film of oil (right).

Confocal imaging was also used to produce a cross-section of the egg (profiles; Figure 10.6) to determine the extent of an oil barrier on the egg chorion after rinsing and to indicate whether any oil penetrated into the interior of the egg.

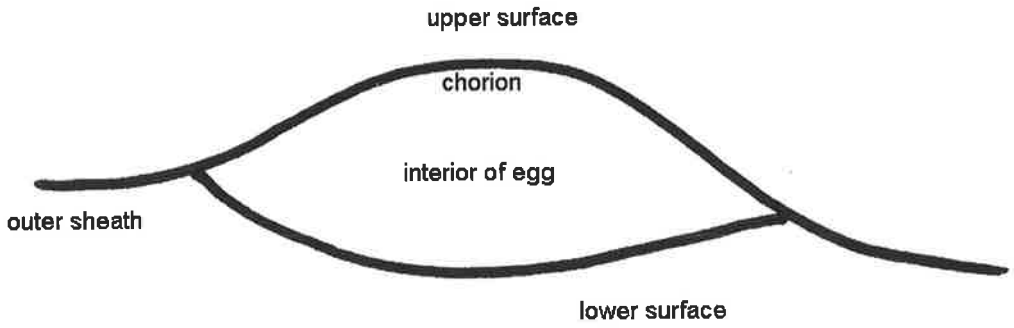

Figure 10.6 A diagram of a LBAM egg profile. The solid line represents the egg surface (chorion). A confocal image of an oil-dipped egg appears as the negative of this image.

Profiles were taken of eggs dipped in 1,000ppm CPD and 2,500ppm DC-Tron and then rinsed. The profiles showed no deep oil penetration into the egg's interior, but the oils show different distribution patterns after rinsing. DC-Tron formed a thin film of oil over the surface of the egg, which is punctuated with small gaps. Eggs were surviving after rinsing which suggests

that the thickness of the remaining oil deposit or the small gaps are sufficient to allow gas exchange (Figure 10.7, top right). The oil deposit remains stable over a period of 3-24h, which reflects the low volatility of the oil over that period (Figure 10.7, bottom right). In contrast, CPD produced a very intense band of fluorescence at 3h exposure (Figure 10.7, top left), which demonstrates that the oil was more difficult to remove from the egg by rinsing. The deposit was reduced to a very thin continuous film after 24h exposure (Figure 10.7, bottom left). This may reflect the dissipation of the more volatile oil rather than improved rinsing.

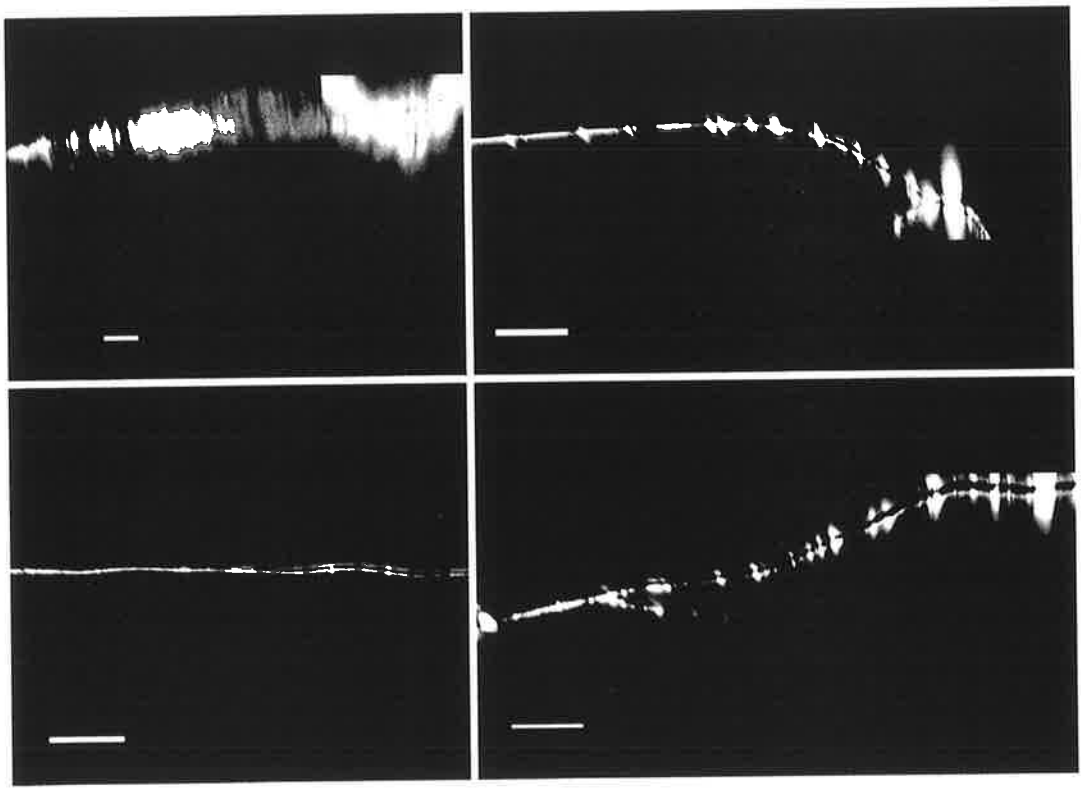

Figure 10.7 Confocal image showing cross section of LBAM egg chorion after treatment with oil, exposed for 30 mins and then rinsed in Triton-X. A comparison of eggs dipped in CPD; held for 3h (top left, 20x) and 24h (bottom left, 40x), and eggs dipped in DC-Tron, held for 3h (top right, 40x) and 24h (bottom right, 40x). (Scale bars = 25μm). [Note that flaring and "ghost images" are artefacts of the confocal procedure].

10.4 Discussion

CPD and DC-Tron act as ovicides when applied directly on lightbrown apple moth eggs. Petroleum oils are known to be effective against the eggs of other tortricid moths, such as oriental fruit moth (Smith and Pearce 1948; Fiori *et al.* 1963) and codling moth (Riedl *et al.* 1995), and are considered to produce a lethal effect on eggs by mechanical interference with the normal gaseous exchange (Smith and Pearce 1948). The quantity of oil and its persistence on the egg for at least 24 hours is considered critical to cause high mortality (Smith and Pearce 1948). Heavier oils, with a molecular weight >320, volatilise little over a 24h period and are considered better ovicides (Fiori *et al.* 1963).

The residue study on DC-Tron, with a mean molecular weight of 350, supports the physical barrier hypothesis. It showed low volatility over a 24h period and the effective dose residue remained above 27μg/cm^2 for the 24h period. This compares with effective dose residues of 23μg/cm^2 and 26.6μg/cm^2 in studies on oriental fruit moth (Fiori *et al.* 1963) and codling moth (Riedl *et al.* 1995), respectively. CPD, with a molecular weight of 212, would be expected to be an inefficient ovicide due to its high volatility (Fiori *et al.* 1963). However, CPD is able to effectively control LBAM eggs despite high oil losses and a residue of < 3μg/cm^2 after 24h exposure. The low CPD deposit on aluminium foil suggests that the mode of action on eggs is not a physical barrier inhibiting gaseous exchange.

Smith and Pearce (1948) showed that washing eggs before 24h exposure in a low boiling point petroleum ether or solvent, such as pentane, removed the oil film from the surface of the egg and restored viability. They concluded that penetration of the oriental fruit moth egg chorion did not occur. In this study, rinsing improved the viability of DC-Tron dipped eggs but not eggs dipped in CPD after 6h exposure. However, examination of oil dipped eggs demonstrated no oil invasion into the egg up to 24h after oil exposure. How does CPD kill LBAM eggs if it rapidly

dissipates from the surface and does not rapidly invade the interior of the egg? One explanation is that the deposit studies on aluminium foil do not reflect the actual deposit on the egg.

The deposit characteristics of CPD on eggs are probably different to non-porous aluminium. No attempt has been made to quantify the deposition and dissipation rate on the surface of the egg or whether different oils would have different deposition and dissipation rates on non-porous surfaces compared to egg surfaces. The chorions of many terrestrial insect species have large airspaces, which are joined by small holes or aeropyles (Miller 1974). It is possible that oils would fill some of these spaces. The degree of penetration would depend on the structure of the chorion and physical characteristics of the oil, such as molecular volume and surface tension. The structure of the LBAM egg chorion is not documented, but the lattice pattern of oil on rinsed eggs is similar to the outer plastron network described by Hinton (1963) (Figure 10.8). This structure would explain the profile of CPD treated eggs, which showed a thicker oil deposit than DC-Tron treated eggs after rinsing. Thicker deposits may be explained by the lighter CPD flowing into airspaces where it is more difficult to rinse and less likely to dissipate. Small amounts of CPD held in the middle layers of the chorion may inhibit gas exchange for longer periods. The oil would dissipate more slowly and smaller quantities would be necessary to block the aeropyles. Eggs dipped in CPD maintain a very thin continuous film after 24h exposure, but whether this is sufficient to inhibit gaseous exchange has not been determined by these experiments. The oil could also be solubilising tissues and causing irreversible damage to the surface of the egg. The importance of any change on egg surface and sub layers has not been investigated.

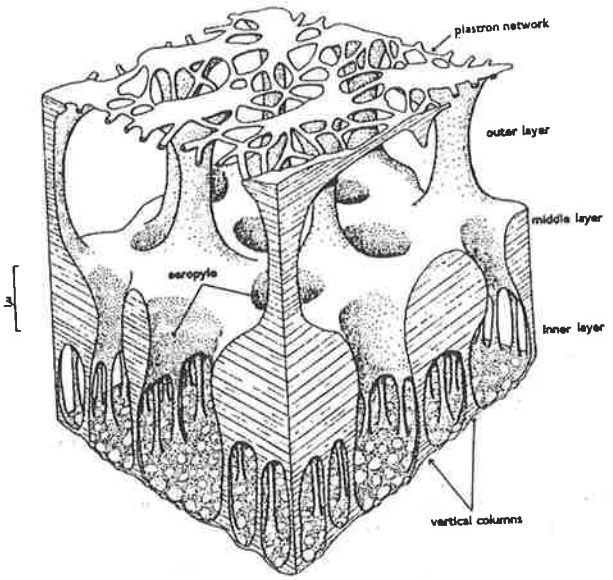

Figure 10.8 Diagram of the chorion between the hatching lines of *Calliphora erythrocephia.* (from Hinton 1963)

The effectiveness of petroleum spray oils as ovicides is commonly related to their persistence as a film on non-porous surfaces, such as aluminium foil (Smith and Pearce 1948; Fiori *et al.* 1963). The results of this study suggest that this approach is not appropriate for light alkanes. CPD does not have the physical properties associated with the persistent barrier hypothesis, nor does it rapidly penetrate the egg's interior. The success of CPD as an ovicide may relate to the ultrastructure of the egg chorion. The oil may be trapped in airspaces where it can either block gaseous exchange or disrupt the structure of the egg membrane. Confocal microscopy showed that oil and rinsing in Triton-X removed the 'spotty' distribution of egg surface aromatic compounds that cause autofluorescence. Microstructure analysis using electron microscopy could identify any physical changes to the egg surface, which would lead to reduced survival. It may also identify air spaces in the chorion of LBAM eggs as described in other insects' eggs (Hinton 1963). A greater knowledge of the structure of the egg chorion should give a greater understanding of the effectiveness of CPD.

10.5 Summary of Main Points

1. CPD, with a molecular weight of 212, would be expected to be an inefficient ovicide due to its high volatility (Fiori *et al.* 1963). However, CPD is able to effectively control LBAM eggs despite high oil losses and an oil residue of $< 3\mu g/cm^2$ after 24h exposure.

2. Oils used in this study did not penetrate the egg chorion at up to 24h after oil exposure.

3. One hypothesis to explain egg mortality caused by CPD is that oil may be trapped in sub-surface airspaces. Oil would dissipate more slowly and smaller quantities may be necessary to block the aeropyles.

4. Oils could also solubilise tissues and cause irreversible damage to the surface of the egg. Confocal microscopy showed treatment of LBAM eggs with oil removed the 'spotty' distribution of egg surface aromatic compounds causing autofluorescence. However, structural changes to the egg's surface after dipping to oils could not be demonstrated by this method.

5. Support for these hypotheses may come from microstructure analysis using electron microscopy to identify any physical changes to the egg surface, which would lead to reduced survival. It may also identify air spaces in the chorion of LBAM eggs as described in other insect's eggs (Hinton 1963).

6. The residue studies on DC-Tron, with a mean molecular weight of 350, showed low volatility over a 24h period and the effective dose residue on aluminium foil remained above $27\mu g/cm^2$ for the 24h period. This is comparable with effective dose residues of $23\mu g/cm^2$ and $26.6\mu g/cm^2$ in studies on oriental fruit moth (Fiori *et al.* 1963) and codling moth (Riedl *et al.* 1995), respectively.

7. Some authors have related the effectiveness of petroleum spray oils as ovicides to their persistence as a film on non-porous surfaces, such as aluminium foil (Smith and Pearce 1948; Fiori *et al.* 1963). This technique is not appropriate for light alkanes. CPD does not have the physical properties associated with the persistent barrier hypothesis, nor does it penetrate the egg's interior.

Chapter 11

The Mode of Action of Petroleum Oils on Selected Citrus Pests: Summary and Conclusions

11.1 Introduction

Insect control using petroleum oils has been based on the assumption that the mode of action is anoxia, ie., the oil blocks the tracheae and suffocates the insect. Many studies on petroleum spray oils against a range of pests indicate the greatest efficacy with higher molecular weight oils (Chapman *et al.* 1943; Riehl and Carmen 1953; Riehl and Jeppson 1953; Trammel 1965). Low molecular weight oils (<300) were thought to be ineffective because they can be expelled from the tracheal system of some insects (de Ong *et al.* 1927) or are too volatile to persist for long enough to suffocate (Ebeling 1950). Petroleum spray oils are, therefore, generally formulated using predominantly narrow range oils with a 50% distillation temperature equivalent to a C21 to C24 alkane (Furness *et al.* 1987) because they have demonstrated high pest efficacy and are reasonably safe to plants.

11.2 Comparison of Oil Efficacy

This study compared a number of petroleum and vegetable oils against citrus pests. However, the most enlightening comparisons were between Ampol C15 CPD, a C15 alkane, and Ampol C23 DC-Tron, a petroleum spray oil. Importantly, CPD was much more efficacious in dips against citrus pests than DC-Tron (Chapters 2, 3 and 7). These products are quite different and their acute effects on the insects possibly relate to distinct physical characteristics. The former is a homogeneous C15 alkane with a molecular weight of 212. DC-Tron is liquid at room temperature (alkanes with ≥16 carbon atoms are waxes at room temperature), and has a molecular volume of 278 ml $mole^{-1}$ at 15°C. It contains no alkanes and its C23 nomenclature refers to its mean equivalent *n*-paraffin carbon number. Its 50%

distillation temperature is the same as the boiling point of a C23 alkane (Furness *et al.* 1987). It has an average molecular weight of 350 and most molecules are paraffinic (% C*p* ≥ 60%) C15 mono-cyclic molecules with side chains. Their average molecular volume is 417 ml $mole^{-1}$ at 15°C.

CPD was more efficacious than DC-Tron against the active stages of LBAM (Chapter 4) and mealybug (Chapter 2 and 3). The alkane caused symptoms in treated insects, including rapid death that were inconsistent with anoxia. The oil dissolved the waxy coating of the mealybug exposing them to desiccation (Chapter 2). LBAM larvae were rapidly immobilised and showed obvious signs of dehydration 2-4h after dipping (Chapter 4).

Petroleum spray oils are considered to produce a lethal effect on eggs by forming a physical barrier over the chorion to prevent normal gaseous exchange (Smith and Pearce 1948). Oils with a molecular weight >320 volatilise little over a 24h period and are considered the best ovicides (Pearce and Chapman 1952). CPD, with a molecular weight of 212, by this reasoning should be an inefficient ovicide due to its high volatility (Fiori *et al.* 1963). However, CPD is able to effectively control LBAM eggs despite high oil residue losses and a residue of < 3μg/cm^2 after 24h exposure (Chapter 10). This suggests that the mode of action of CPD on eggs may not be a physical barrier inhibiting gaseous exchange across the egg surface.

Petroleum spray oils are considered to have no residual effects on insects (Davidson *et al.* 1991), however, LBAM larvae, which survived oil dips, suffered reduced fecundity and fertility among adult females. CPD produced high numbers of eggs but many were infertile. DC-Tron significantly reduced both the number produced and fertility of LBAM eggs (Chapter 5). The rate of dissipation of DC-Tron from exposed larvae would be slower than for the C15 alkane, and this may have contributed to its increased effects (Chapter 10). The

residual effects of CPD on LBAM are difficult to reconcile with its high volatility. Ebeling (1936) observed low fecundity of red scale, *Aonidiella aurantii* (Maskell), treated with a Grade 1 'light' petroleum spray oil (California grade standards (1932), specifications in Davidson *et al.* 1991), but did not propose a mode of action.

The effectiveness of CPD compared to DC-Tron can not be explained by the traditional paradigms that oils kill by suffocation and that higher molecular weight hydrocarbons are more efficacious. A comparison of petroleum oils is difficult because different emulsifiers are used in each product, and emulsifiers may have some influence on mortality (Chapter 6). However, the types of emulsifiers in both products are likely to be similar (eg., non-ionic surfactants) and the influence of emulsifiers on insect mortality would be minimised when applied as dilute water/oil emulsions (Chapter 9). Other factors must be contributing to the efficacy of the alkane and the chronic effects of both oils on insects. The application technique of dipping rather than spraying, the oil depositing characteristics of the formulations (Campbell 1962) and/or an alternative mode of action may all influence the relative efficacy of oils.

11.3 Model for the Mode of Action of Insecticidal Oils

It is proposed that petroleum oils can cause a range of symptoms in insects that will result in their eventual death. The effects on the active stages of insects can be divided into acute and chronic effects. Oil may produce a single effect, such as anoxia, but is more likely to produce a range of effects in any individual insect. These effects are unlikely to occur at the same time, leading to a cascading effect where nervous disruption can occur in minutes, desiccation after several hours, and anoxia after more than 24 hours. The expression and timing of these different modes of action are dependent on the physical properties of the oils, including the surfactants, and the application method used. A simple model of this

relationship is shown in a comparison of the symptomatology of two oils with different physical properties, CPD (Table 10.1) and DC-Tron (Table 10.2) when either applied as a dip or immersed.

The differences between these two oils reflect the differences in mode of action between traditional petroleum spray oils, such as DC-Tron, and a special purpose oil, CPD, on LBAM larvae. It is illustrative but not intended as a definitive comparison of all oils and their effects on all insects.

Modes of Action of CPD and DC-Tron on LBAM.

Anoxia appears to be the primary mode of action when LBAM larvae are immersed in oil (Chapter 6). However, the results of immersion experiments cannot be extrapolated to field or postharvest control of insects, where insects are sprayed or dipped. The distribution of oil in the tracheal system of larvae is different when dipped compared to immersed. During immersion, oil flowed into the tracheae, presumably as far as the air pressure in the tracheae and the hydrostatic pressure in the fluid-filled tracheole branches allow, creating a 'plug' of oil (Chapter 6). After removal from the oil emulsion, there was a change in the distribution of the oil in the tracheal system. DC-Tron remained in the upper tracheal branches only, but CPD was also found deep in the tracheoles. The penetration of oil into the tracheae is related to oil viscosity (Brown 1951). DC-Tron generally remains as a 'plug' of oil in the tracheae causing anoxia, but larvae dipped in CPD were coated on the inner wall with oil rather than 'plugged' (Chapter 6). CPD in the upper tracheal branches may be expelled by active ventilation, but any remaining oil would flow along the lipids of the tracheal wall and deep into the tracheoles where it couldn't be expelled. Surfactants in the formulation may allow deeper penetration of oil into the tracheal system by reducing the surface tension of the solution.

Oil penetration deep into the system induced symptoms associated with narcosis (Chapter 7). Narcosis can be induced in a number of ways. Petroleum vapours can cause narcosis, but CPD did not show any fumigant effects when larvae were exposed to oil vapours (Chapter 7). The oil blocking the tracheae and causing an excess of CO_2 probably induces narcosis. CPD may cause more rapid narcosis than DC-Tron because oil deep in the tracheoles would result in a much reduced tracheal volume for gas exchange than when only surface tracheae are 'plugged' with oil. Rapid knockdown associated with CO_2 accumulation, although symptomatically important, is unlikely to be the primary cause of death. Exposure of LBAM to high CO_2 concentrations is completely reversible even after several hours' exposure (Chapter 7). Additional symptoms in larvae such as twitching of the prolegs, dehydration, and darkening of the haemolymph suggested other systems have been disrupted due to contact with the oil (Chapter 4).

The most likely explanation for the acute toxic effects that CPD causes is disruption by direct contact with the nervous system. Direct nervous disruption would require deep penetration of oil into the tracheoles and absorption onto nerve membranes. CPD can rapidly move down into nerve ganglia via the tracheal system (Chapter 7). Importantly, larvae continuously immersed in oil do not show the presence of oil in nervous tissue (Chapter 7), and did not die rapidly (Chapter 6). This probably explains, at least partially, the differences in tolerance between oil-dipped and oil-immersed larvae described by Ebeling (1950) and observed in Chapter 6.

Measurement of the spontaneous electrophysiological activity of the peripheral nerves of larvae indicated that oil affected the nervous system (Chapter 7). The response of intact larvae dipped in CPD oil is consistent with the proposal that the oil rapidly moves down the tracheae into nervous tissue. Exposure to CPD induced a rapid onset of multiple nerve firing in peripheral nerves.

CPD is a mixture of a C15 alkane with low levels of surfactants (< 10% vol/vol) to aid in emulsification. Exposure to 10,000ppm of the C15 alkane and surfactants separately induced repetitive firing, suggesting that oil and surfactants can both affect nerves. The ratio of oil to surfactants has traditionally been used by formulators to control oil deposit but may also be important in achieving translocation of the oil into nervous tissue. This may involve a complex synergy as the surfactants may aid entry into the spiracles by controlling the deposit, while the oil may equally be assisting the translocation of the surfactants to nervous tissue.

The pharmacological effect of the absorption of hydrocarbons into phospholipid membranes is not clear, but is probably not due to a specific site, as is true for most insecticides (Hassell 1982). Thus, nervous disruption would not involve the formation of specific chemical binding to receptors or the active sites of enzymes, which is consistent with the lack of any apparent structural complexity or stereo isometry of the oils, especially compared with other insecticides. Assays using bovine AChE support this by showing no specific inhibition of that enzyme using high oil doses (1%) and long incubation periods (up to 15 h) (Chapter 7). It is more likely that oils are displacing protective lipids by their solvent action (van Overbeek and Blondeau 1954), and affecting nerve activity by increasing membrane permeability to ion exchange.

Petroleum oils can affect water regulation in insects by increasing the cuticular permeability (Wigglesworth 1945; Ebeling and Wagner 1959). The surfactants used in oil formulations may also enhance the desiccating action of oils (Wigglesworth 1945, Ebeling and Wagner 1959). CPD induced desiccation (Chapter 8), but the role of weight loss in inducing larval mortality may be either causative or symptomatic of the failure of other processes. It is unlikely that weight loss *per se* is the primary cause of mortality (Chapter 8). However,

accelerated weight loss may be a contributing factor to mortality during oil exposure. CPD has a greater influence on water regulation than DC-Tron. The low viscosity of CPD allows the oil to penetrate deeply into the tracheal system. Disruption of the lipid lining of the tracheae would expose a large surface area to increased water loss after the CPD dissipates from the upper tracheal branches. DC-Tron is less volatile and ,therefore, more likely to persist on the surface of the cuticle and tracheae acting as a physical barrier to water loss.

Water loss is symptomatic of a loss of lipid protection. The relationship between oil and a particular insect's lipid structure may vary. The solubilisation of the waxy cuticle of mealybug by CPD was dramatic and quite different from the effect on LBAM larvae (Chapters 2 & 9) . The physical specifications of the cuticular wax and oil would affect their mutual solubility. There are likely to be different responses to the same oil when the structure and composition of the cuticular waxes change. This could explain different responses to solubility with different insect groups. It is also possible that different instars or newly moulted individuals will show different sensitivities to oil depending on their lipid compositions.

In this study, particular emphasis was placed on the effect of oils and surfactants on the surface of the tracheae due to observations of dehydration surrounding large spiracles of CPD dipped larvae (Chapter 6). The loss of tracheal waxes by the solvent action of oils would affect water regulation. Loss of water through the tracheal system would be enhanced if the spiracular muscle's coordination of gas exchange could not compensate or the muscle's performance was affected by nervous disruption (Chapter 7).

Oils can cause cell disruption (van Overbeek and Blondeau 1954), with larger hydrocarbons penetrating cell membranes much more slowly. However, oils entering the insect via the

tracheae may persist for many hours or days eventually penetrating a susceptible membrane. This process may lead to the chronic effect in insects described by Ebeling (1945). In this study, LBAM larvae exposed to low doses of oil either by dipping or in the diet suffered reduced fertility and fecundity (Chapter 5). C23 DC-Tron had the largest affect on egg laying, presumably, because it was the least volatile and would affect the larvae over a longer period. C15 CPD did not effect the numbers of eggs laid, but the fertility of eggs laid was low suggesting a mode of action more specific to egg viability, rather than general physiological depression.

Ebeling (1950) postulated the presence of air increased the toxicity of petroleum oils to insects and cited oxidation as a possible cause. In this study, high levels of oxygen increased the toxicity of CPD to LBAM larvae. However, no increase was evident in DC-Tron treated larvae (Chapter 9). The heavier DC-Tron could cover the cuticle and inhibit gas exchange through the tracheal lining, which is consistent with the hypothesis of suffocation. If there is no gas exchange, then the constituents of the atmosphere are irrelevant. However, the effect on larvae dipped in CPD suggests an increase in oxidative stress. Anti-oxidants, as dietary supplements, were successful in reducing the effect of CPD, which supports the hypothesis that the oil may act as a pro-oxidant. The oil is unlikely to directly affect the enzymatic defences, but may affect lipid peroxidation defence by altering the permeability of the tracheal surface waxes. After oil dipping, the water and oil emulsion would initially restrict gas exchange, but as the water and volatile oil dissipate increased gas exchange would return. Oils incorporated into the solid lipids of the tracheal lining would increase the rate of water and gaseous exchange. Increased tracheal permeability coupled with a very high level of atmospheric oxygen would lead to elevated levels of oxygen in the haemolymph. Elevated levels of oxygen could result in injury through an overproduction of reactive oxygen species (ROS) and lipid peroxidative attack.

CPD and DC-Tron act as ovicides when applied directly on lightbrown apple moth eggs. Petroleum oils are known to be effective against the eggs of other tortricid moths, such as oriental fruit moth (Smith and Pearce 1948; Fiori *et al.* 1963) and codling moth (Riedl *et al.* 1995), and are considered to produce a lethal effect on eggs by mechanical interference with the normal gaseous exchange (Smith and Pearce 1948). The quantity of oil on the egg surface and it persistence for at least 24h is considered critical to cause high mortality (Smith and Pearce 1948). Heavier oils, with a molecular weight >320, volatilise little over a 24h period and are considered better ovicides (Pearce and Chapman 1952).

The residue studies on DC-Tron, with a mean molecular weight of 350, support the physical barrier hypothesis. It showed low volatility over a 24h period and the effective dose residue remained above 27μg/cm^2 for the 24h period. This is similar to effective oil residues of 23μg/cm^2 and 26.6μg/cm^2 in studies on oriental fruit moth (Fiori *et al.* 1963) and codling moth (Riedl *et al.* 1995), respectively. CPD, with a molecular weight of 212, would be considered an inefficient ovicide due to its high volatility (Fiori *et al.* 1963). However, CPD is able to effectively control LBAM eggs despite high oil losses and a residue of < 3μg/cm^2 24h after exposure. The low CPD deposit on aluminium foil suggests that the mode of action on eggs is not a physical barrier inhibiting gaseous exchange. How does CPD cause mortality in LBAM eggs if it rapidly dissipates from the surface and does not rapidly invade the interior of the egg (Chapter 10)? Presumably, it dissipates less rapidly from eggs than the aluminium foil used in residue studies.

The chorions of many terrestrial insects have a large airspace, which are joined by small holes or aeropyles (Miller 1974). It is possible that oils could fill some of these spaces. The degree of penetration would depend on the structure of the chorion and physical

characteristics of the oil, such as molecular volume and surface tension. The structure of the LBAM egg chorion is not known, but the lattice pattern of oil on rinsed eggs (Chapter 10) is similar to the outer plastron network described by Hinton (1963). The low viscosity of CPD would allow it to flow into airspaces where it is more difficult to rinse off and less likely to dissipate. Low deposits of CPD held in the middle layers of the chorion may inhibit gas exchange for longer as they would dissipate more slowly. CPD may also be more efficacious because smaller quantities would be necessary to block the aeropyles. Eggs dipped in CPD maintain a very thin continuous film after 24h exposure (Chapter 10), but whether this is sufficient to inhibit gaseous exchange was not investigated. The oil could also be solubilising tissues and causing irreversible damage to the surface of the egg. Support for these hypotheses may come from microstructure analysis using electron microscopy to identify any physical changes to the egg surface, which would lead to reduced survival. It may also identify air spaces in the chorion of LBAM eggs as described in other insect's eggs (Hinton 1963).

10.4 Conclusions

Most insecticidal oils are narrow range petroleum oils with a 50% distillation temperature equivalent to a C21 to C24 alkane (Furness *et al.* 1987). Oils are usually sprayed onto infested plant surfaces to kill insects on contact. The primary mode of action has been believed to be anoxia due to blocking of the insect's spiracles. The results of this thesis does not challenge the hypothesis that most insecticidal oils produced today induce anoxia, but contends that the preoccupation with anoxia as the primary mode of action for all oils has produced a limited range of oils for pest control. There are exceptions, such as the use of oils as ovipostion repellents (Shultz *et al.* 1983) or light oils to clog the stylets of sap-sucking disease vectors (Gibson and Cayley 1984), but there has been limited development of oils based on alternative modes of action.

Surfactants used in oil formulations to ensure emulsification can also affect pest efficacy. Surfactant concentration and type could be modified to enhance the ability of the oil to induce other physiological effects on the insect. Oil soluble surfactants could be used to decrease the surface tension of oils and allow them to penetrate deeper in the tracheal system. Some surfactants will cause more solubilisation in insect tissue but this must be balanced with any possible phytotoxic side effects.

Application method was very important in the expression of certain symptoms. This study compared immersion and dipping, but most insecticidal oils are applied as sprays. Oil dip bioassays using Sunspray Ultra Fine oil (viscosity, 68 s; 50% distillation point, 212.2°C; 10-90% distillation range, 18.3°C; UR, 94.0%) against European red mite, *Panonychus ulmi* (Koch), were much more efficacious than bioassays using Potter spray towers (Agnello *et al.* 1994). Spraying would result in the oil briefly covering the insect before running off to a thin film and may not achieve the complete contact of either dipping or immersion. Excellent spray coverage in the field would be critical to achieve the intimate contact associated with dipping. High volume sprays are considered necessary to achieve high pest efficacy though anoxia (Beattie *et al.* 1991; Herron *et al.* 1998). In the context of this study, it would be of interest to determine if high volume oil sprays lead to only tracheal blockage or other symptoms analogous to dipping.

Poor coverage with oil sprays would lead to partial exposure of oil but may still induce chronic effects. The use of petroleum spray oils in the field has resulted in insect mortality over a period of weeks (Ebeling 1945; Baxendale and Johnson 1990). Petroleum spray oils cause an extended population suppression of some pests that can not be explained by a single mortality event (James Altman, Pest Monitor, personnel communication). The role of

petroleum spray oils in the suppression of field populations by reduced fecundity after contact and feeding on oil residuals should be investigated.

The poor efficacy of light oils (<C19) as field sprays has been used to support the theory of anoxia, but poor application techniques and high volatility would also limit the penetration and contact with nervous tissue. A preoccupation with anoxia would have precluded the use of a light alkane for the postharvest control of surface pests. However, it has been precisely the physical specifications that would have classed it as unsuitable to produce anoxia that have allowed it to penetrate beneath the calyx, into the tracheae and produce other lethal effects. The question can be posed: How many more opportunities can we look forward to? Would spraying light alkanes under cool glasshouse conditions or in the field at night be efficacious? Would light alkanes applied with pyrethroid insecticides overcome super kdr-type resistance in *Helicoverpa armigera*? Would light alkanes be more efficacious against mealybug than traditional petroleum spray oils? Oils have the potential for much wider and effective use for insect control when formulation becomes more focussed on the conditions under which the oil is required to function and the modes of action on the pests.

Table 10.1 Comparison of the symptomatology of insects dipped or immersed in CPD

Product	*CPD*	
Application	*immersion*	*dipping*
Symptom -narcosis	-backpressure in tracheae limits oil penetration and creates oil "plug". -CO_2 accumulation from blocked tracheae. **Long periods of exposure are required for mortality. Shorter exposure regarded fully reversible, ie., no chronic effects.**	-air contact breaks oil 'plug' -low surface tension allows oil to flow deep into tracheae. -CO_2 accumulation from blocked tracheoles. **Long periods of exposure are required for mortality. Shorter exposure regarded fully reversible, ie., no chronic effects.**
-nervous disruption	-no direct contact with nerves as the hydrostatic pressure limits tracheal penetration **No contact or direct effect on nervous activity**.	- low surface tension allows oil to flow deep into tracheae. -physical absorption of oil onto nerve lipids. -surface activity of emulsifiers affect nerve cells. **Direct and rapid effects on nervous activity. Critical physiological processes disrupted by non-specific absorption. Chronic effects unknown.**
-desiccation	Cuticle -complete layer of oil stops water evaporation. -incorporation into cuticular waxes alters permeability to water. -surfactants disrupt surface lipids -polarity of surfactants aids in water droplets forming on cuticle. Tracheal Lining -hydrostatic backpressure limits oil penetration. **Rate of desiccation is unlikely to cause mortality before anoxia.**	Cuticle -volatile oil dissipates leaving the cuticle surface exposed to air. -incorporation of oil into cuticular waxes alters permeability to water. -surfactants disrupt surface lipids -polarity of surfactants aids in water droplets forming on cuticle (reduces interfacial tension). Tracheal Lining -low surface tension oil flows deep into tracheae. -incorporation of oil into tracheal waxes and the polarity of surfactants aids in water loss (as in cuticle). **Rate of desiccation more rapid due to volatility of the oil and effect on large surface area of the tracheal branches. Desiccation contributes to mortality. Chronic effects**

		unknown.
-cell disruption	Cuticle -mutual solubility of apolar alkane and cuticle weakens membrane integrity. -polar surfactants solubilise membranes. -acceleration of lipid peroxidation cycle leading to cell degeneration. Tracheal Lining -hydrostatic backpressure limits oil penetration. Nervous tissue -hydrostatic backpressure limits oil penetration. **Solubilisation of oils and surfactants limited to cuticle surface. Production of reactive oxygen species may be inhibited by exclusion of oxygen while immersed.**	Cuticle - mutual solubility of apolar alkane and cuticle weakens membrane integrity. -polar surfactants solubilise membranes. -acceleration of lipid peroxidation cycle leading to cell degeneration. Tracheal Lining -as above. Tracheal branches extensively lined by oil. Nervous tissue -as above. Oil diffuses from tracheoles into nervous tissue lipids. **Potential for solubilisation greater and more extensive than immersion. Cell leakage and increased atmospheric oxygen may enhance production of reactive oxygen species when oil dissipates. These processes can affect cells leading to acute and chronic effects.**
-anoxia	Full immersion leads to complete spiracular blockage and eventual anoxia. **Anoxia is a slow process as insects can metabolise anaerobically for long periods.**	-Active ventilation clears spiracular access to air. -Oil creep into some tracheoles may cause localised areas of anoxia. **Mortality due to anoxia unlikely due to volatility of the oil and expulsion from tracheae. Chronic effects due to localised oxygen starvation are not known.**

Table 10.2 Comparison of the symptomatology of insects dipped or immersed in DC-Tron

Product	*DC-Tron*	
Application	*immersion*	*dipping*
-narcosis	-hydrostatic backpressure limits oil penetration. -CO_2 accumulation from blocked tracheae. **Long periods of exposure are required for mortality. Shorter exposure regarded fully reversible, ie., no chronic effects.**	air contact breaks oil 'plug' - higher viscosity and surface tension than C15 alkane limits speed and depth of oil flow into tracheae -CO_2 accumulation from blocked tracheoles. **Long periods of exposure are required for mortality. Shorter exposure regarded fully reversible, ie., no chronic effects.**
-nervous disruption	-no direct contact with nerves as the hydrostatic pressure limits tracheal penetration **No contact or direct effect on nervous activity.**	-higher viscosity and surface tension than C15 alkane limits speed and depth of oil flow into tracheae. -high doses may be required for physical absorption of oil onto nerve lipids and surface activity of emulsifiers on nerve cells. **High doses required for direct effects on nervous activity. Critical physiological processes disrupted by non-specific absorption. Chronic effects unknown.**
-desiccation	<u>Cuticle</u> -constant layer of oil protects from water evaporation -slow incorporation of mono-cyclic hydrocarbons into cuticular waxes would eventually alter permeability to water. -polarity of surfactants aids in water droplets forming on cuticle. <u>Tracheal Lining</u> -hydrostatic backpressure limits oil penetration. **Rate of desiccation will be dependent on action of surfactant types. Rate is very slow with DC-Tron. Mortality due to anoxia occurs before**	<u>Cuticle</u> -non-volatile oil persists on the cuticle surface protecting water evaporation. -slow incorporation of mono-cyclic hydrocarbons into cuticular waxes alters permeability to water. -polarity of surfactants aids in water droplets forming on cuticle <u>Tracheal Lining</u> -low surface tension oil flows deep into tracheae. -incorporation of oil into tracheal waxes and the-polarity of surfactants aids in water loss (as in cuticle). **Rate of desiccation slow due to persistence of a film of oil.**

	any symptoms of desiccation.	
-cell disruption	Cuticle -mutual solubility of mono-cyclic hydrocarbons and cuticle weakens membrane integrity. -polar surfactants solubilise membranes. -acceleration of lipid peroxidation cycle leading to cell degeneration? Tracheal Lining -hydrostatic backpressure limits oil penetration. Nervous tissue -hydrostatic backpressure limits oil penetration. **Solubilisation of oils and surfactants limited to cuticle surface. Production of reactive oxygen species may be inhibited by exclusion of oxygen while immersed and slow solubility of large hydrocarbon molecules with surface lipids.**	Cuticle -mutual solubility of mono-cyclic hydrocarbons and cuticle weakens membrane integrity. -polar surfactants solubilise membranes. -acceleration of lipid peroxidation cycle leading to cell degeneration? Tracheal Lining -as above. Tracheal branches lined by oil. Nervous tissue -as above. Oil less likely to diffuse from tracheoles into nervous tissue lipids. **Potential for solubilisation greater and more extensive than immersion. Long persistence of oil and slow mutual solubility with surface lipids are more likely to contribute to chronic rather than acute effects through the production of reactive oxygen species.**
-anoxia	Full immersion leads to complete spiracular blockage and eventual anoxia. **Anoxia is a slow process as insects can metabolise anaerobically for long periods.**	-High viscosity inhibits clearing of spiracular access by active ventilation. -Partial clearing may result in only localised areas of anoxia. **Mortality due to anoxia. Expulsion of oil from tracheae unlikely due to viscosity of the oil Chronic effects of localised oxygen starvation is not known.**

Appendix 1 - Specifications of Petroleum Oils used in Bioassays.

Table 1

Property	*Test Method*	*C23 Ampol DC-Tron NR*	*C15 Ampol CPD*
Distillation temperature (^{0}C) at 101.33 kPa	ASTM[a] D-2887		
10% point		346	253
50% point		385	275
90% point		411	304
n-paraffin carbon number 50% point	HRGLC[b]	23.0	15.2
Mean molecular weight density 15^{0}C	ASTM D-4052	350	212
Density at 15^{0}C (g ml^{-1})	ASTM D-1298	0.8424	0.787
Viscosity; kinematic mm^2/sec at 40.0^{o}C	ASTM D-445	12.0	2.75
Pour point (^{0}C)	ASTM D-97	-15	+7
Maximum unsulfonated residue; % minimum volume	ASTM D-483	94	<99
Mean molecular volume 15^{0}C (ml mole^{-1})		417	278
Molecular types (%)			
Cp (paraffins)		70	100
Cn (naphthenes)		28	-
Ca (aromatics)		2	-

a American Society for Testing and Materials (1998)
b Furness *et al.* (1987).

Appendix 2 – List of Abbreviations

AChE	Acetylcholine esterase
ASTM	American Society for Testing and Materials
Cn	Mean carbon chain of n molecules (chain length)
CPD	Ampol's Citrus Postharvest Dip
DC-Tron	Ampol's commercial spray oil (C23 NR)
HCN	Hydrocyanic acid
HLB	Hydrophilic-lipophilic balance
HRGLC	High resolution gas liquid chromatography
IPM	Integrated pest management
LAR	Mean larval activity rating
LBAM	Lightbrown apple moth (*Epiphyas postvittana*)
mOD	Mean optical density
NR	Narrow range (oil classification)
PSO	Petroleum spray oil
SSU	Seconds Saybolt Universal (Oil Viscosity)
UR	Unsulfonated residues (oil purity)

Appendix 3 - Criteria to Assess Larval Tolerance

Score	*Ability to right itself*	*Activities and capabilities*
0	No	None
1	No	Minor activity after persistent probing
2	No	Minor activity, immediate response
3	No	Slight independent activity, responsive
4	No	Active, slow writhing, no control
5	No	Active, vigorous writhing, no attempt to right itself
6	No	Active, attempts to right itself
7	Yes	Active, rights itself with difficulty
8	Yes	Active, rights itself easily, but with adverse effects
9	Yes	Active, rights itself easily, minor effects only
10	Yes	Active, no visible effects

Each larva was assigned a score from 0 to 10 at each observation.

Table from Firko and Hayes (1990).

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