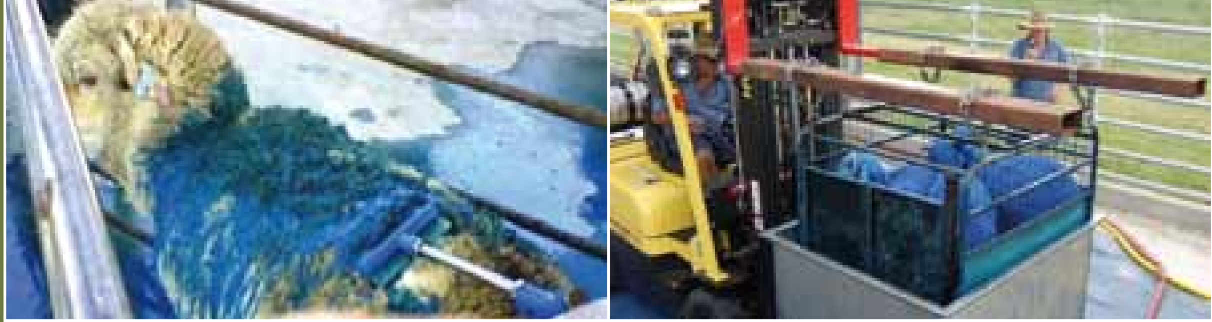

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Controlling Fly Strike and Louse Infections in Sheep with Tea Tree Oil

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Controlling Fly Strike and Louse Infections in Sheep with Tea Tree Oil

by Peter J James

December 2011

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Foreword

Tea tree oil, the essential oil of the native Australian plant *Melaleuca alternifolia*, has often been promoted as having insecticidal and insect repellent properties. However there is limited evidence in the form of scientifically conducted studies, with efficacy tested against a relatively limited number of pests. With increasing community eco-consciousness and concern about the use of artificial pesticides, there are growing opportunities for ‘natural’ products and a ready market for tea tree oil for use in ‘softer’ pest control technologies where efficacy can be demonstrated. In addition, tea tree oil has a potential advantage over other natural compounds in that levels of the major components, including those providing insecticidal activity, are stipulated under International Standard ISO 4730, helping to counter criticisms of variable efficacy often levelled at natural pest controls.

This study aimed to assess the effect of tea tree oil based formulations against two major ectoparasitic diseases in the sheep industry, flystrike and louse infestation, and to provide data to assist the assessment of the commercial feasibility of development of tea tree oil based ectoparasiticides. The results demonstrate insecticidal effects against both sheep lice and blowflies and repellent effects against adult flies and maggots. Dipping sheep in a Tea Tree Oil based formulation appeared to completely eradicate lice and suggests its potential use in sheep dipping formulations. Repellent and insecticidal effects against sheep blowflies, together with previously reported anti-microbial and wound healing properties, suggest significant benefits from the inclusion of tea tree oil in flystrike and wound treatment formulations. These effects occurred at concentrations of Tea Tree Oil that suggest the commercial viability of development of Tea Tree Oil based formulations for sheep parasite control and wound treatment and a potential new market for Tea Tree Oil.

This project was funded from industry revenue provided by P Guinane Pty Ltd. which was matched by funds provided the Australian Government through RIRDC’s Tea Tree Oil R&D program.

This report is an addition to RIRDC’s diverse range of over 2,000 research publications and it forms part of our Tea Tree Oil R&D program, which aims to support the continued development of an environmentally sustainable and profitable Australian tea tree oil industry that has established international leadership in marketing, value-adding, product reliability and production. Most of RIRDC’s publications are available for viewing, free downloading or purchasing online at www.rirdc.gov.au. Purchases can also be made by phoning 1300 634 313.

Craig Burns
Managing Director
Rural Industries Research and Development Corporation

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Abbreviations

ALK	Ethoxylated Castor Oil (Alkamuls OR/36®, Rhodia) and Ethoxylated Oleic Acid (Alkamuls A, Rhodia; Brenntag Pty Ltd, Notting Hill, Vic) combined in a ratio of 7:3
APVMA	Australian Pesticides and Veterinary Medicines Authority
GCMS	gas chromatograph, mass spectrometer
HLB	hydrophilicity-lipophilicity balance
PBO	piperonyl butoxide
SPME	solid phase micro extraction
TTO	tea tree oil

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Executive Summary

What the report is about

This report details the results of experiments conducted to assess the potential of tea tree oil (TTO) for use in the treatment of infestations of sheep lice (*Bovicola ovis*) and flystrike caused by sheep blowfly (*Lucilia cuprina)*. The report details a series of laboratory and animal studies which indicate significant potential for the use of tea tree oil in formulations for these purposes.

Who is the report targeted at?

The report is targeted at TTO producers and product manufacturers who produce and market tea tree oil and animal health products.

Background

Tea tree oil has documented insecticidal and repellent effects against a range of arthropods and potential for its use in 'natural' pesticides has been widely canvassed. A criticism often levelled at natural products is the potential for variable composition, but TTO composition is stipulated under international standard ISO 4730, providing confidence for consistency of effect. There is a growing recognition that the wool and sheep meat industries are well placed to capitalise on growing consumer attraction for natural products. To meet these needs there is an increasing demand for safe, environmentally benign, low residue pest controls and particularly those that can be accredited for use in organic production systems. Veterinary pesticides and repellents, and in particular those for use in the sheep and wool industries, represent a significant potential market for tea tree oil.

Aims/objectives

This project aimed to increase the market for tea tree oil by:

- Demonstrating the effectiveness of tea tree oil (TTO) formulations in controlling sheep lice (*Bovicola ovis*) at concentrations that make development of a commercial formulation economically viable.
- Demonstrating the effectiveness of a TTO based formulation in treating flystrikes and protecting wounds against new strikes.
- Providing data towards the assessment of the commercial feasibility of development of TTO-based sheep ectoparasiticides and that can support registration of TTO products suitable for use in conventional and organic production systems.

Methods used

A series of laboratory and animal studies was undertaken to develop formulations suitable for application to sheep and to test insecticidal and repellent effects against different stages of sheep lice and sheep blowflies.

Results/key findings

Sheep blowflies: TTO demonstrated insecticidal effects against all stages of sheep blowfly maggots. Formulations containing 1% TTO reliably gave 100% kill of 1st instar larvae and 2.5% TTO caused mortality of most 2nd and 3rd instar larvae in agar assays. However in experiments where 3rd instars were dipped in TTO formulations for 60 s, even 50% TTO gave only 46% kill. TTO was strongly repellent to blowfly maggots with most rapidly evacuating TTO treated areas. Killing maggots in strike wounds is undesirable to avoid septic effects from putrefaction products.

When mixed in formulations with some currently used larvicides TTO appeared to reduce insecticidal effect. In agar studies this was thought to be due to rapid exodus of maggots from the treated agar before a toxic dose of insecticide was acquired, but the reason for similar reduction in effectiveness in larval dipping studies is less certain. TTO also showed insecticidal effects, often by fumigant action against eggs, pupae and adults of *L. cuprina*.

TTO had strong repellent effects against adult flies. In laboratory studies with wool treated with 3% TTO, complete suppression of egg laying by adult *L. cuprina* was evident in for up 6 weeks.

With its documented antimicrobial effects and reputed wound healing properties, toxic effects against young larvae and eggs, repellent action against older larvae and repellent effects against gravid female flies it is considered that TTO could be a useful ingredient in flystrike or wound treatments.

Sheep lice: In laboratory wool dipping assays, 1% TTO formulation reliably gave 100% kill of lice. The majority of this effect appeared to be due to fumigant effects from terpinene-4-ol, the most abundant component of TTO. There also appeared to be fumigant and physical effects against lice eggs. In pen studies with sheep shorn two weeks previously, conducted according to Australian Pesticides and Veterinary Medicines Authority guidelines, dipping of sheep in both 1% and 2% TTO formulation appeared to completely eradicate lice. No lice were found on any of the treated sheep despite careful inspection of at least 40 fleece partings per animal at 2, 6, 12 and 20 weeks after treatment.

In studies of long wool efficacy, jetting sheep (high pressure spraying into the fleece) with both 1% and 2% TTO formulations reduced louse numbers by 94% in comparison to controls at two weeks after treatment. For the 1% formulation the reductions at 6 and 12 weeks after treatment were 94% and 91% respectively and for the 2% formulation, 78% and 84% respectively. Both formulations significantly reduced wool damage in comparison to controls.

Implications and recommendations for stakeholders

The results of this project indicate a number of potential roles for TTO in sheep ectoparasiticide formulations and possibly for use in other animal production systems. The apparent eradication of lice in the sheep dipping study with both 1% and 2% TTO formulation is particularly encouraging. In addition, TTO has properties that suggest substantial benefits for its incorporation in flystrike and wound treatments and protectants. Further assessment of market feasibility and studies by potential marketers to provide registration data for TTO-based formulations seems warranted.

Introduction

Consumers increasingly demand that food and fibre products are safe and produced by ethical and environmentally acceptable methods. The market for organic goods is booming, valued at US$33 billion for food alone in 2003, with forecast annual growth rates of between 10% and 30% (RIRDC 2006). The organic industry is the fastest growing retail sector in the European Union, Eco-labelling is becoming widespread and surveys in the UK show that only 20% of producers “don’t care” (Russell 2003). There is a growing recognition that the wool and sheep meat industries are well placed to capitalise on growing consumer attraction for natural products. To meet these needs there is a demand for safe, environmentally benign, low residue pest controls and particularly those that can be accredited for use in organic production systems.

Two of the main parasite diseases in sheep are flystrike, caused primarily by the Australian sheep blowfly, *Lucilia cuprina* and infestation with the sheep louse (*Bovicola ovis*). Most recent estimates put the cost of these two diseases to the sheep industry at $280m and $123m respectively (Sackett *et al.* 2006). Control of these two parasites relies significantly on the application of chemical insecticides. However the efficiency of control programs has been compromised by development of resistance to these insecticides in both sheep blowflies (Levot 1995, Levot and Sales 2002) and lice (Levot 1995, James *et al.* 2008) and there is an ongoing need to minimise chemical residues in sheep products (Pattinson, 1995; Savage, 1998; Williams and Brightling, 1999), reduce occupational exposure to chemicals (Murray *et al.*, 1992; Russell, 1995; Savage, 1998) and avoid environmental contamination (Littlejohn and Melvin 1991) from chemical parasiticides.

Tea tree oil (TTO), derived from the Australian native plant *Melaleuca alternifolia*, is well placed to capitalise on the move towards natural products. It has long been reputed to have insecticidal, acaricidal and repellent effects and recent studies have documented effects against a range of medical and veterinary pests including human lice (Heukelbach *et al.* 2008; Sammataro *et al.* 1998; Williamson *et al.* 2007), ticks (Iori *et al.* 2005), scabies mites in both humans (Walton *et al.* 2004) and swine (Magi *et al.* 2006) and house dust mites (Williamson *et al.* 2007).

Tea tree oil also has a range of other effects that could provide benefits when it is used in sheep parasite control preparations. Anti-microbial effects (Carson *et al.* 2006) could reduce bacterial and fungal spread in sheep dips and reduce diseases such as arthritis, cheesy gland, and dermatophilosis often associated with dipping. Antimicrobial effects together with suggested benefits in wound healing (Halcon and Milkus 2004; Woollard *et al.* 2007) may also aid in strike healing following treatment. In addition, Australian TTO has well established quality control procedures and composition of oil is regulated under International Organisation for Standardisation standard ISO 4730 (Oil of Melaleuca—terpinen-4-ol type), helping to counter criticisms of variable composition with consequent uncertain efficacy often levelled at natural pest controls.

This project was conducted to assess the possible use of TTO in formulations for the treatment of sheep flystrike and lice infestations, providing a potential novel new market for Australian tea tree oil.

Objectives

Increasing the market for tea tree oil by:

- Demonstrating the effectiveness of TTO formulations in controlling sheep lice (Bovicola ovis) at concentrations that make development of a commercial formulation economically viable.

- Demonstrating the effectiveness of a TTO based formulation in treating flystrikes and protecting wounds against new strikes.

- Providing data towards the assessment of the commercial feasibility of development of TTO-based sheep ectoparasiticides and that can support registration of TTO products suitable for use in conventional and organic production systems.

Methodology

Formulation

TTO composition

All TTO used in experiments reported in this paper was extracted from plants with a terpinen-4-ol chemotype (Homer, *et al.* 2000) from the north eastern region of New South Wales in Australia. Composition of the TTO used in these studies, measured by GCMS (NSW department of primary Industries Diagnostic and Analytical services laboratory, Wollongbar NSW), together with the acceptable range for TTO under ISO standard 4730, is given in Table 1.

Table 1: Composition of TTO used in the studies

Sample ID	**1232**	**ISO4730**
Lab No	**1886**	**RANGE %**
1. α-pinene %	2.3	1 - 6
2. sabinene %	0.4	tr – 3.5
3. α-terpinene %	10.0	5 – 13
4. limonene %	0.8	0.5 – 1.5
5. p-cymene %	2.3	0.5 – 8
6. 1,8-cineole %	1.7	tr – 15
7. γ-terpinene %	20.9	10 – 28
8. terpinolene %	3.5	1.5 – 5
9. terpinen-4-ol %	43.0	30 – 48
10. α-terpineol %	2.8	1.5 – 8
11. aromadendrene %	1.0	tr - 3
12. ledene %	0.7	tr – 3
13. δ-cadinene %	0.8	tr – 3
14. globulol %	0.2	tr – 1
15. viridiflorol %	0.1	tr - 1

Formulation development and rational

Formulation can have major effects on the efficacy of active ingredients. For practical usefulness and economic feasibility for use in parasite control TTO will need to be used at concentrations well below 100%, will likely need to be formulated for mixing in water and will need to provide effective parasite control once formulated. TTO is not miscible with water and emulsifiers and carriers were required both for laboratory assays and ultimately for animal application. In early laboratory experiments acetone, grapeseed oil and Tween® 80 were used but these were not considered suitable for field use and a number of other potential emulsifying agents and combinations were tested. Key characteristics that were required included the ability to form stable emulsions of TTO in water and the ability to readily wet wool (which is covered with water repellent lipid). The rate of evaporation of TTO from the emulsion was also assessed. It is generally considered that solubilisation is most efficient with emulsifiers with similar HLB (hydrophilicity-lipophilicity balance) to the agent being mixed. The HLB of TTO is 12 and agents with approximately similar HLB values were tested. These included Tween® 80, Triton®, Geropon® and various combinations of Ethoxylated Castor Oil (Alkamuls OR/36®, Rhodia) and Ethoxylated Oleic Acid (Alkamuls A, Rhodia). Grapeseed oil was also used as a carrier in some laboratory assays and assessed in combinations with some other agents. For assays with *L. cuprina* in which TTO was mixed in sheep serum Tween 80 was used as the emulsifier.

To assess the efficiency of wool wetting with different formulations a system similar to that described by Lipson (1976) was used. Briefly the weathered tip was trimmed from approximately 100 mg of wool to leave 3 cm in length. The wool was inserted into glass tubing which was glued in position through the screw top lids of plastic vials and then weighed. Test solution was added to the vials so that 1cm of the tube was immersed when the tops were screwed in place. After 1 minute the amount of fluid taken up by the wool was determined by reweighing the tube and wool.

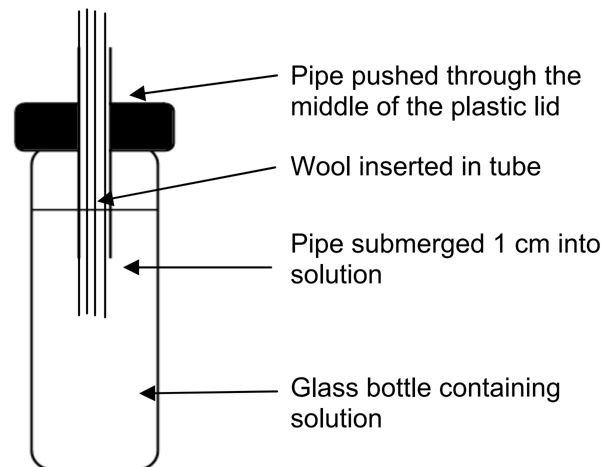


Figure 1: Apparatus used to measure wool wetting

Stability of solutions was assessed subjectively, noting the degree of separation over time and rate of evaporation was estimated by measuring the reduction in weight of emulsion with time. Some typical results for fluid uptake and evaporation are shown in Figures 2 and 3.

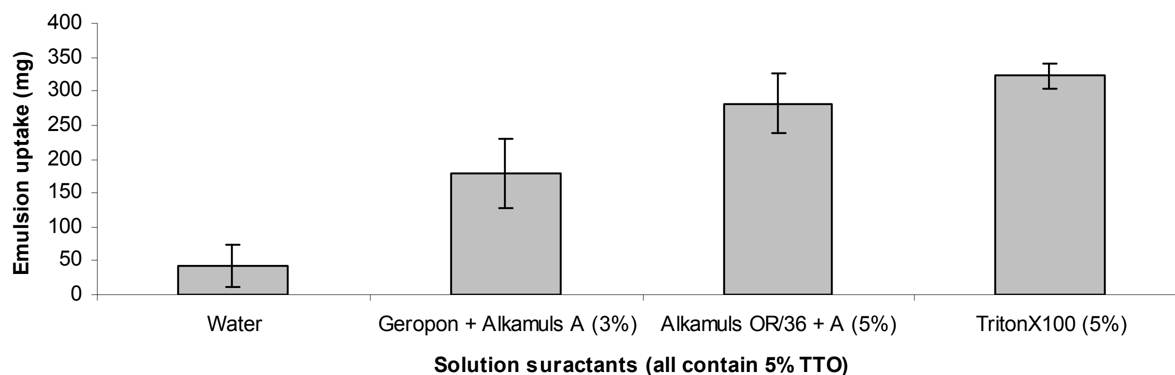


Figure 2: Amount of uptake of 5% TTO/water formulation into wool with different emulsifiers

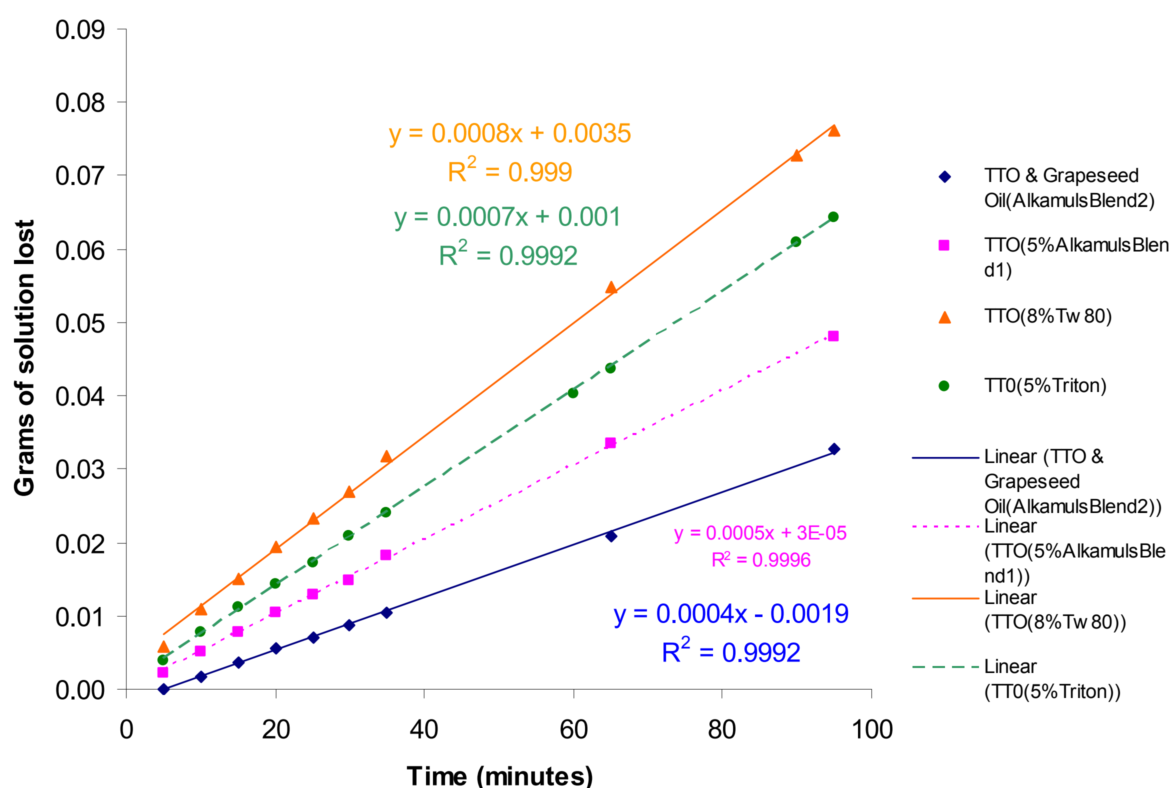


Figure 3: Rate of weight loss from 5% TTO solutions mixed in water with different emulsifiers

Ethoxylated Castor Oil (Alkamuls OR/36®, Rhodia) and Ethoxylated Oleic Acid (Alkamuls A, Rhodia; Brenntag Pty Ltd, Notting Hill, Vic). combined in a ratio of 7:3 gave an emulsifier blend (ALK) that produced stable emulsions of TTO in water, did not give unacceptable TTO loss on standing, provided good wetting of wool and was judged more suitable for animal application than some of the other solvent. The Alkamuls mixture (ALK) was used to emulsify TTO in most wool assays and in all animal studies.

Sheep blowfly

L. cuprina used for these experiments were of the LS insecticide-susceptible strain (Kotze *et al.* 1997) maintained in culture at the Agri-Science Queensland's Animal Research Institute laboratories at Yeerongpilly, Queensland.

Effects against larvae

First instar larvae – serum assay

Assays were conducted using a method adapted from Hughes and Levot (1987). Tea tree oil was diluted in sterile bovine serum containing 2% yeast and 0.5% monobasic potassium orthophosphate to give concentrations of between 0.1%, and 2%, following preliminary experiments to establish a dose range. Rolled pieces of chromatography paper (12 cm x 3 cm) were placed inside flat bottom glass vials (5 cm x 1.5 cm diameter) and one millilitre aliquots of serum with the desired concentrations of tea tree oil dispensed onto it. Small clumps of about twenty, 1st instar *L. cuprina* larvae were placed on the top of the treated chromatography paper and the mouth of the vial covered with filter paper secured with a rubber band. Vials were held at 29˚C in a foam rack over water in a plastic container with the lid resting on top, but not airtight. After 24 h, the larvae were washed into a Petri dish and numbers of live and dead larvae recorded. Four replicate tubes were used for each concentration tested.

Second and third instar larvae – agar assay

An agar based system was developed to test for likely effect in the resolution of strikes without the use of live animals. Meat liver agar (Merck) containing 5% bovine serum was added in 10 ml amounts to 75 ml (42 mm diam. x 56 mm height) plastic vials and allowed to set in a 1 cm layer in the base of each vial. Groups of ten, second instar larvae or feeding third instar larvae (not prepupal) were transferred from liver into each container and 1 ml of bovine serum added on top of the agar. Each container was covered with gauze and placed in an incubator to allow the "strike" to develop. After four hours the containers were removed from the incubator, checked to ensure that larvae had commenced feeding in the agar, and 1 ml aliquots of the test formulations applied to the surface of the growth medium. The vials were then covered with nylon mesh, held at 29˚C and 60% RH and numbers of dead and live larvae recorded at 24 h and 48 h. Concentrations between 0.1 % and 10% were tested but as there was poor effect at concentrations below 1% only the results for the higher concentrations are presented.

As TTO has potentially beneficial repellent and antibacterial activities it was also assessed against third instar *L. cuprina* in combination with other insecticidal compounds used in strike treatments. In the first study TTO was emulsified in water at 2.5% v/v with 2.5% ALK and boric acid (25 gL-1), diazinon (1 gL-1) or ivermectin (32 mgL-1). The concentrations of diazinon and ivermectin were those used in commercial flystrike treatment formulations. In a second experiment TTO and 2.5% ALK were mixed with cyromazine at 0.1 gL^{-1} and 1 gL^{-1}. The control in both experiments was 2.5% alkamuls in water. Following the application of treatments the vials were placed (uncovered) inside 600 ml round disposable food containers (95 mm diameter and 78 mm height) containing a layer of 2 cm of vermiculite , which were covered with gauze and placed in an incubator at 29˚C and 60% RH. This enabled the larvae to vacate the vials if repelled from the agar or when they finished feeding and to pupate in the vermiculite. The number of adult flies that had emerged was recorded after 14 days.

Third instar larvae – dipping assay

Groups of third instar larvae (n = 50 to 100) were removed from liver, placed into mesh bags (4 cm x 2 cm) and submerged for 60 s. in solutions containing the test compounds at appropriate dilution After removal the larvae were held on paper towelling within plastic food containers for 24 h before addition of vermiculite to an approximate depth of 2 cm to allow pupation to occur. They were held at 28˚C and the number of adult flies that emerged counted after 14 days. Following preliminary studies to

establish dose range, TTO concentrations from 5% to 50% emulsified in water with 8% ALK or 8% Tween80 were tested.

TTO was also tested in combination with diazinon, ivermectin and boric acid and in a separate experiment with cyromazine at the same concentrations as for the agar tests by this method. For these studies TTO and insecticide were emulsified in water with 2.5% ALK. The control was 2.5% ALK solution without TTO or insecticide. There were 3 replicates for each treatment in both experiments.

Larval repellence assay

Meat liver agar (Merck) mixed with 5% bovine serum solution, prepared as described above, was added to eight 90 mm diameter Petri dishes and allowed to set. TTO was mixed into the agar (without emulsifier) at concentrations of 0.5%, 2% and 5% (two Petri dishes for each concentration). Once set the agar from each dish was sectioned across the diameter and transferred to clean Petri dishes so that each new dish contained one half untreated agar, and one half tea tree oil spiked agar. The control dishes had untreated agar in both halves. Groups of 15 third instar larvae were added to each dish. Larvae were applied to the half of the dish with TTO agar in two of the four replicates for each concentration and to the untreated agar in the other two. A filter paper cover was secured over each dish with a rubber band and the dishes placed on a bench at 26˚C. The numbers of larvae in each half of the dish were counted after 2 h and the dishes then transferred to incubator at 28˚C for a further 22 h. Distribution of the larvae was recorded again and the dishes photographed at 24 h.

Adult flies

Toxic effects - topical application

L. cuprina females 7-10 days old were immobilised with CO_2 and 1µl of melaleuca oil diluted in butanone to the required concentration was applied to each fly's thorax with a micropipette. Three replicates of 20 flies (10 male and 10 female) were used for each concentration. Control flies were treated with butanone only. Groups of flies were held at 27°C in 540 ml plastic containers with gauze lids, with sugar and water provided. Mortality was assessed at 24 and 48 h after treatment. In separate studies, flies were treated with 0.15, 0.25 or 0.5 1µl of pure TTO and assessed using similar methods. In these studies control flies were treated with 0.5 µl grape seed oil.

Topical application – synergism with piperonyl butoxide (PBO)

Following a report of PBO synergising the effect of monoterpenoids in mosquitoes (Waliwitiya *et al.* 2008) two pilot studies were conducted to identify potential synergistic effects in *L. cuprina.* Preliminary studies determined that the maximum sub lethal dose of PBO for *L. cuprina* was 2% in 1 µl butanone or about 20 µg per fly. This concentration of PBO was used in both experiments.

Female flies were anaesthetised with CO_2 and 1µl of diluted PBO was applied to the dorsal thorax with a micropipette. The flies were then returned to their holding containers, with water and sugar provided, and held for one hour. After this time they were anaesthetised again and treated with 1µl of the required concentration of TTO in butanone. Cages were held in a temperature and humidity controlled room (27°C) between and following fly treatments.

In the first experiment the concentrations of TTO tested were 1%, 5%, 10%, 25% and 50%. There were two replicates of 20 flies for each concentration and mortality was assessed after 24 h. In the second experiment 5%, 15%, 25%, 35% and 50% concentrations of TTO were used. There were three replicates of 20 flies for each concentration and mortality was assessed after 24 and 48 h. Control flies were treated with PBO and butanone.

Fumigant effects

Adult flies were anaesthetised with CO2 and sorted to groups of 20 (10 male, 10 female) before being transferred to fumigation chambers (Figure 4). Fumigation chambers were constructed using 90 mm glass Petri dish lids containing 55 mm glass Petri dishes in which anaesthetised flies were introduced to the champers 21 mm x 21 mm glass coverslips placed outside of the 55 mm dishes onto which a drop of the test concentration was added. Two different types of studies were conducted. One to tested TTO presented as different concentrations of TTO in acetone in 60 µl droplets (0%, 5%, 25%, 50% and 100%) and the other used pure TTO presented in different size droplets (0µ, 1µl, 3µl, 6µl, 15µl). The dishes were held in an incubator at 27ºC and mortality recorded after at hourly intervals up to 6 h after introduction and again after 24 h. Three replicates were used for each concentration or amount of TTO.

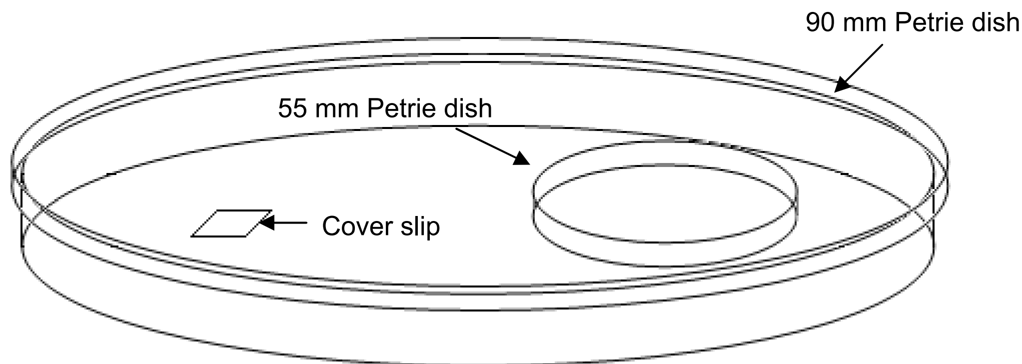


Figure 4: Fumigation chambers used for exposure of *B. ovis* and *L. cuprina* to TTO fumes

Repellent effects

L. cuprina used in the assays were 9-11 day old females that had been protein fed with sheep's liver 4 ays after emergence. Dissection and examination of a subsample of females prior to commencement of the assays confirmed that most were gravid. Flies were anaesthetised with CO2, sorted to groups of 40 and allocated to six 40 x 30 x 30 cm cages with perspex sides and mesh ends. They were provided with sugar and water and allowed to "acclimatise" overnight before commencement of the assay. Cages were laid out on a 1.5 m mesh platform with a bank of overhead fluorescent lamps positioned above to provide even lighting (Figure 5) and held at 28˚C and approximately 60% RH. Temperature and humidity were monitored and recorded with a data logger.

Oviposition stimulus was provide by way of a cotton plug, approximately 0.9 cm diameter and 3.7 cm length, fully submerged in a mix of 40 g homogenised liver and 30 ml water for 5 minutes (Figure 6). Test wool samples were arranged around the cotton plug and inserted into a 50 ml glass beaker, ensuring the top of the wool and plug were flush with the top of the beaker (Figure 6). This arrangement provided approximately 2 cm of wool between the plug and sides of the beaker on which flies could alight and stand to oviposit. Two beakers were positioned 20 cm apart, approximately 10 cm from each end of the cage. For the choice tests there was one control beaker and one test beaker in each cage arranged in alternate positions in the 6 cages. For the non choice tests there were either two test or two control beakers in each cage.

Test solutions consisted of 3% TTO emulsified with 3% Alkamuls solution. Emulsifier without TTO was used as the control. Wool for use in the assays was prepared by immersing approximately 3 g of wool 4-5 cm in length into the test solution for 30 s. Each sample was then drained of free liquid, allowed to sit for a further 30 seconds on paper towelling and then squeezed to remove all excess liquid. For tests of persistency of the repellent effect enough samples were prepared for the period of the study and held in their beakers in a cupboard at room temperature until used. Each preparation

was moistened with water sprayed from a plastic spray bottle held about 20 cm away prior to fly exposure to ensure moist areas for fly oviposition.

The test preparations were placed in each cage one hour before observations commenced (9 am on most days). The number of flies present on the wool in each preparation was counted at point observations at 10 min intervals for an hour at two hourly intervals until approximately 4 pm on each day (4 periods of 6 fly counts) in the 1 day experiments, and in two 1 h periods of 6 counts beginning at 9:00 am and 11:00 am in the two experiments to examine effectiveness over longer periods (6 days and 6 weeks). In addition, if flies were stationary, backed into a cavity in the fleece and probing with their ovipositor, or seen to be depositing eggs they were recorded as ovipositing. This corresponds to phase (iii) and (iv) in the description of the sequence of events in the oviposition behaviour of *Lucilia* spp given by Cragg (1956). At the conclusion of observations the preparations were removed from the cages and placed in a freezer to kill the eggs. Numbers of egg masses deposited were counted on the following day. As it was often difficult to identify individual egg masses, a scoring system was also used: 0 = no eggs; 1 = occasional or scattered eggs; 2 = one egg mass; 3 = 2-4 egg masses; 4 = large numbers of eggs (≥ 5 egg masses). Four different experiments were carried out. In the first two, repellent effects were assessed over one day in choice and non-choice situations. The later two studies examined persistence of the repellent effect in choice tests, the first using 3 cages over 6 days, and the second using 6 cages over 6 weeks.

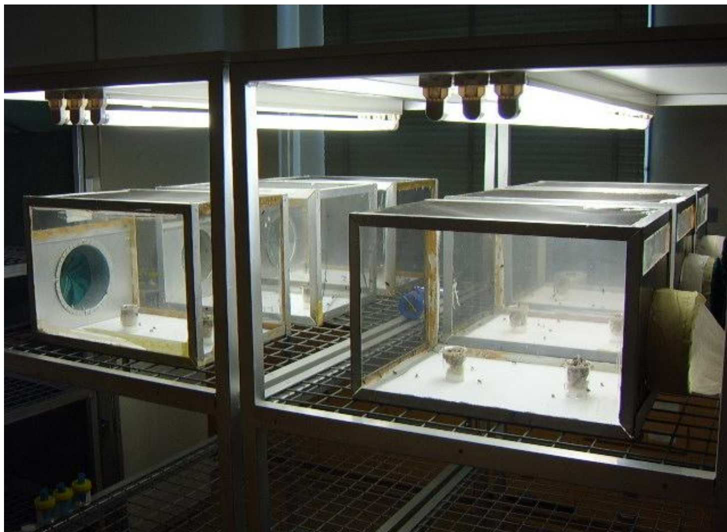


Figure 5: Cages and lighting used for *L. cuprina* repellence testing

Figure 6: Wool preparations used for repellence testing. Wool samples (treated or control) were arranged around the attractive cotton roll in glass beakers; attraction was provided by immersing the cotton roll in homogenised sheeps liver and water.

Eggs and Pupae

Fumigant effects

Fumigation chambers were constructed using 90 mm glass Petri dish lids containing 55 mm glass Petri dishes to hold either *L. cuprina* eggs or pupae and 21 x 21 mm glass coverslips placed outside of the 55 mm dishes onto which a 60 µl drop of TTO diluted to the required concentration in acetone was placed (Figure 4).

Eggs were collected by introducing a fresh piece of liver to a cage of gravid *L. cuprina* blowflies. Small, freshly collected clumps of eggs (about 40-100) were transferred to moistened 55 mm diameter filter paper (Whatman No. 1) inserted into the internal Petri dishes. Measured amounts of TTO were applied to the coverslips and the chamber immediately sealed by placing larger Petri dish base on its lid. Chambers were held in an incubator set at 29°C and 75% RH for 18 h. The number of emerged larvae was determined by counting numbers of larvae and empty egg shells under a dissecting microscope.

For tests against pupae, groups of 15, four day old pupae were placed on dry filter paper in the bottom of the 55 mm Petri dishes. TTO dilutions were dispensed onto the coverslips and the chamber immediately sealed as above. Two experiments were conducted, the first with concentrations of TTO from 0 to 15% and the second with concentrations from 25% to 100%. A treatment with a 600 µl drop of TTO was also included in the second assay. Chambers were held at 29°C and 65% RH for 7 d and the number of unecloded pupae, live flies and dead flies recorded at that time.

Sheep lice

Treated surface effects

Initially standard treated surface methods for lousicide susceptibility testing were used (Levot and Hughes 1989). Briefly, cotton squares were treated with 1 ml of test solution and held in place with 5 cm metal rings. Essential oils were applied with butanone or acetone as carriers and the squares left to dry overnight to avoid physical or solvent effects on the lice. However, after a number of trials this method was discarded as little difference was seen between treatments and controls in most assays. Drying overnight may have allowed for the evaporation of some key active components before the lice were exposed and reduced efficacy in comparison with that seen with other assay designs.

In order to reduce loss of volatile components a number of modified systems were also tested. The most successful of these used TTO diluted in grapeseed oil applied to 90 mm filter papers. A weight ratio of 1:1 oil to filter paper (≈ 0.57g of oil per paper) gave good dispersion over the paper without leaving excess oil in which lice could become trapped. In one such test, given as an example, groups of 10 lice were exposed to concentrations of 0%, 0.1%, 0.5%, 2.5% 10% and 20% TTO applied to 90 mm filter papers in glass Petri dishes. Lice were added to the test dishes once oil had dispersed on the filter paper and the dishes held uncovered in an incubator at 36°C and 65% RH. Dishes were inspected at 24 h and 48 h and lice assessed as dead (no response to a stimulus) or alive at each inspection.

Wool Dipping assays

As the previously described assay methods were considered unlikely to give a good indication of effectiveness in a practical context, a method that more closely simulated the situation on sheep was developed.

Test solutions, with different concentrations of TTO emulsified with ALK were prepared. Amounts of 400 mg of pesticide free wool were immersed 20 ml volumes of these solutions in 50 ml beakers for 60 seconds and agitated to ensure complete wetting. The wool was then taken out of the beaker, allowed to drain and a standard method of shaking seven times with a brisk wrist movement used to

remove excess fluid. The treated wool was added to 28 ml capacity flat bottom glass bottles and at least 20 adult lice, freshly collected from live sheep added to each bottle. In one experiment 20 adults and 20 nymphs (40 per bottle) were used. Usually there were two control groups, one with lice in dry untreated wool and one with lice in wool treated with emulsifier without TTO, and three replicates for each treatment or control. Assay tubes were held in an incubator at 36ºC and 65% relative humidity and usually assessed as alive or dead at 24 and 48 h after addition of the lice. Tests were also conducted to determine the effect of reducing the percent of emulsifier (ALK) from 5% to 1% and to assess the effect of including 200mg/L of the synergist piperonyl butoxide in the dipping emulsion.

Dried wool assays

To assess residual effect of TTO, 50 mg amounts of pesticide free wool were thoroughly wetted with 500 µl of TTO at the desired concentration solubilised in tap water with 2% ALK. Control wool was treated with ALK with no TTO. The wool was dried in a fume hood at room temperature for 1.5 h then inserted into 20 ml disposable flat bottom vials. Twenty lice were added to each and the vials placed in an incubator at 36ºC and 65% relative humidity. Numbers of live and dead lice assessed at 24 h and 48 h.

Fumigant effects against lice

Two methodologies were used for testing fumigant effects:

Fumigation chambers: Chambers were constructed as described for the fly studies (Figure 4). Test arenas consisted of 90 mm glass Petri dishes with uncovered 55 mm glass Petri dish bottoms inside to contain the lice. Lice were counted onto 55 mm filter papers, added to the small Petri dishes. Measured volumes of test solution were dispensed onto 21 mm x 21 mm glass coverslips placed within the large dishes but outside of the 55 mm dishes. Lids were applied to the large dishes which were held in an incubator at 36ºC and 65% RH. Lice were inspected at 1 h and 2 h or 3 h and the numbers of lice that were knocked down or dead and still moving were recorded. The small dishes containing lice were then removed from the larger dishes and returned uncovered to the incubator. After 24 hours they were re-examined to identify lice that recovered. In early studies of fumigant effect, acetone was used to solubilise TTO whereas in later studies 8% Tween 80 was also tested.

Wool fumigation method: To determine if the fumes of TTO applied to wool could cause mortality of lice not actually contacted by TTO the following apparatus was used. Glass tubing, 50 mm in length and 15 mm diameter, was glued into the screw tops of 80 mm x 27 mm diameter glass vials (Figure 7). Wool was weighed to 400 mg amounts and submerged in test solution for 30 s, removed and allowed to drain for 30 s and then added to the glass vials. Lice were contained in 20 mg of pesticide free wool held the tubes and prevented from contacting the treated wool by nylon mesh secured across the bottom end of the tube. In the first assay the top of the tube was covered with Parafilm® (American National Can, Neenah, WI), but in the second assay, the tubes were left uncovered. Concentrations of TTO between 0 and 2% emulsified in water with 1% ALK were tested, with two groups of controls, dry wool, and wool treated with ALK. There were 20 adult lice in each vial, 3 replicates for each treatment or control and mortality of lice was assessed after 24 h of exposure.

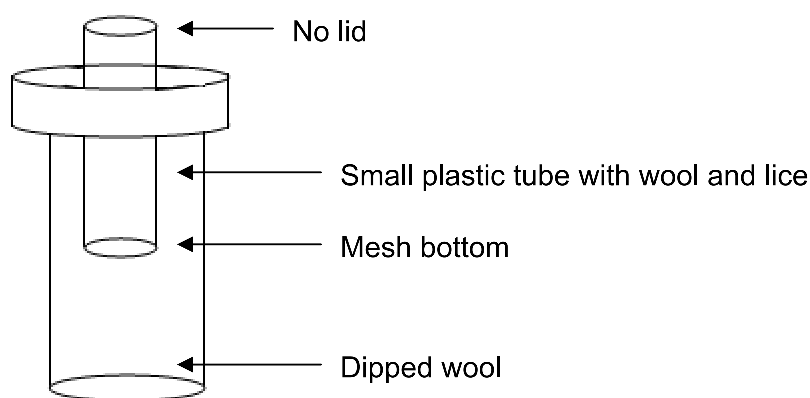


Figure 7: Apparatus used in wool fumigation assays.

Effect of TTO components in fumigation assays

Two experiments were carried out using fumigation chambers (Figure 4), with acetone as solvent, to investigate the relative effect of different TTO components. In the first experiment the components tested were terpinen-4-ol (2.15%), alpha terpinene at (0.5%) and gamma terpinene (1.045%). TTO at 7.5%, 5%, 2.5% and 1% of was included to give a basis for comparison All test substances were solubilised in 60 µl of acetone and the component concentrations equated to that which would be contained in 5% TTO (the minimum concentration at which 100% knockdown or death had been achieved in the previous assays). In the second experiment, terpinen-4-ol was tested at 2.15% as in the previous experiment, but also at 1.5% which corresponded to the minimum level of this compound allowable under ISO 4730 specification for TTO, terpinen-4-ol chemotype (Table 1). Limonene was tested at 0.04%, 1,8 cineole at 0.085%, alpha pinene at 0.115%, alpha terpineole at 0.14%. An additional treatment with all of the above components at the specified concentrations, except for terpinen-4-ol was also included. Acetone was used as the control in all assays. Lice were exposed in the chamber for 3 h, at which time knockdown/death was assessed. The Petri dish lids were then removed and the lice returned to the incubator for 24 h to check for recovery.

Lice eggs – dipping studies

A number of subsequent studies were attempted but difficulties in collecting enough eggs, and very low hatch rate in collected eggs in one study, meant that these experiments had to be abandoned. Results from the preliminary study are presented as they provide an indication of the likely effects of dipping on eggs and because the results match that which might be expected from the results of other experiments.

To provide louse eggs, wool containing lice was clipped from a heavily infested area on a sheep and placed in an incubator overnight. The next morning the clipped wool was inspected under a dissecting microscope and fibres with eggs that were solid and white and apparently healthy were separated from the rest of the wool. Forty viable eggs were available.

Separate pesticide-free wool was weighed to 400 mg lots and treated with 1%TTO emulsified with 1% ALK, with 1% ALK with no TTO, or left untreated. Treatment was conducted by immersing the wool in test fluid for 30 s and then allowing it to drain for 30 s. The wool was parted, 10 eggs placed in the middle and the wool folded back over the eggs. The preparations were placed in 28 ml McCartney bottles in an incubator at 36ºC and 65% RH and inspected over 11 days to allow time for viable eggs to hatch. There was one preparation with 10 eggs for the TTO/ALK treated wool, two preparations with 10 eggs each for the ALK treated wool and a further 10 eggs on untreated wool.

Lice eggs – fumigant effects

Lice were collected from a heavily infested sheep and held overnight in the laboratory on wool collected some weeks previously from an infested sheep, but with no live lice present. The wool was inspected the next morning for the presence of newly deposited eggs. Eggs, were carefully selected from the wool, leaving them with wool fibres when attached. Ninety eggs were counted to nine groups of ten on filter paper in 50 mm Petri dishes and added to fumigation chambers. Treatments were 60 µl of 100% TTO, 15 µl of 100% TTO added to coverslips in the fumigation chamber as previously described, or no TTO. There were three replicates for each treatment and control. Eggs were exposed in the fumigation chamber in an incubator set at 36°C and 65% RH for 24 h before being transferred to clean dishes, maintained at similar temperature and humidity. Hatching was assessed daily over 14 days.

Sheep studies

Dipping

Sheep: Louse counts were conducted on a mob of forty adult male castrate Merino sheep, infested with sheep lice (*Bovicola ovis*) and with bodyweight range of approximately 50-65 kg. The sheep had been purchased from an infested flock and had been running on pasture at the Centre for Advanced Animal Science at the University of Queensland, Gatton, Qld. for a number of months prior to commencement of the experiment. All sheep were drenched with Coopers Colleague® Broad Spectrum Sheep and Lamb Drench (Coopers Animal Health, 66 Waterloo Road, North Ryde NSW Australia) (19 g/L albendazone and 150 g/L pyraclofos) at rate of 1 m l/5 kg bodyweight two weeks before entry to the trial to control endoparasites. Treatment with this product had previously shown no effect on louse burdens. Eighteen sheep with moderate to heavy infestations of sheep lice were chosen from these animals and allocated to six groups of three, balanced for louse counts. Groups of three were randomly assigned to treatment (or control) with two groups for each treatment (control) (Appendix A). The sheep were shorn and groups assigned to outdoor pens 7.5 m x 7.5 m in dimensions with 1.3 m high closed sides located outdoors and open to weather, although part covered at one end by a 1.8 m strip of shade cloth. Sheep were left in the pens to ‘acclimatise’ until dipping, 2 weeks after shearing, and remained in the same pens for the duration of the study.

Dipping formulations: On the basis of the results from laboratory testing, and with the view to development of an economically competitive product, formulations containing 1% and 2% tea tree oil emulsified with 1% and 2% ALK respectively were chosen for testing in sheep studies. Methylene blue at 0.004% was also added to the dipping solution to check the thoroughness of wetting and identify any areas where poor coverage rather than lousicide failure may be responsible for poor results. Laboratory studies showed methylene blue to have no effect on louse survival.

Immersion dipping: Groups of 3 sheep were held in an open topped cage 0.9 m x 0.9 m x 1.4 m and dipped by lowering into a 1 m x 1 m x 1.2 m dipping vat, which immersed the bodies of all animals, for 1 min. The bath was not deep enough to cover the animal’s heads, so the wool on the head of each sheep was wetted by pouring over dipping fluid from a small pail. Following dipping, groups were returned to their respective pens where they remained except during inspection for lice or collection of samples. The same dipping fluid was used for both groups of 3 sheep for each concentration of TTO.

Figure 8: Apparatus used to dip sheep

Assessment of louse numbers: All louse inspections were conducted with the sheep standing in a holding crate with widely space horizontal bars. There were two assessors, one on each side of the sheep, for each inspection. Pre-shearing assessment of louse burdens was made by counting the number of adult lice and nymphs in 12, 10 cm fleece partings on each side of the sheep (24 parts). Partings were made in three rows, approximately 10 cm from the vertebral column, midway between the vertebral column and ventral midline and on the lower woolled areas with one parting on the shoulder/front leg, two on the flanks and one on the rump/backleg along each line. Post shearing assessments were made by inspecting 40 fleece parts (20 on each side). Partings were distributed on the front leg, flanks and backleg as noted above except that there were four lines from the vertebral column to the lower woolled areas as well as four parts down the underside of the neck and dewlap on each side. Post dipping inspections were made at 2, 6, 12 and 20 weeks post treatment.

Residues: A series of samples were collected to determine if residues of TTO components could be detected in the blood tissues and wool of sheep treated with TTO formulation. Tissue samples were collected 1 week after treatment. Blood samples were collected at 1, 3 and 6 weeks and wool samples at 2, 6, 12 and 20 w.

For collection of tissue samples the sheep were firmly restrained, a small area on the rump of each sheep closely clipped and a local anaesthetic administered. A small muscle biopsy (< 2 gm) was removed using forceps. Care was taken to peel back overlaying skin and sample only the underlying tissue. Following collection of the sample at least one stitch was inserted to close the wound and the animals closely supervised until healed. All muscle biopsies were conducted by a registered veterinarian. As no residues were detected in the first lot of samples, no further tissue samples were collected as agreed under our animal ethics approval**.** Blood samples (approx, 20 ml) were collected by jugular venipuncture using 18g hypodermic needles. Once the needle was inserted a small amount of blood was drawn off into one Vacutainer™, the first Vacutainer was removed from the needle and a second attached. Only the blood from the second Vacutainer was used to minimise the possibility of contamination from skin surface TTO. Wool samples were collected using scissors and animal clippers. Approximately 3 g samples were collected from each of the shoulder, mid back and rump, approximately 25 cm from the dorsal midline, on each sheep. All samples were stored at -25ºC until processed for analysis.

Jetting

Sheep: Eighteen adult male Merino wethers with heavy infestations of lice, managed prior to the experiment as described above were allocated to six groups of 3 sheep balanced for louse counts and randomly assigned to treatment (or control). There were two groups of 3 sheep for each treatment (control). Groups were randomly allocated to pens as described for the dipping study. Unfortunately

two sheep in one group of the 2%TTO-treated sheep contracted an unrelated disease (ovine posthitis) and were not present for the final louse count.

Figure 9: Jetting sheep with a Dutjet® jetting wand

Jetting: Jetting formulations containing 1% and 2% tea tree oil emulsified with 1% and 2% ALK respectively and 0.004% methylene blue to monitor the thoroughness of wetting were tested. Control sheep remained untreated. Jetting was conducted with a Dutjet® hand jetting wand (Figure 9) connected to centrifugal pump run to provide approximately 500 kpa at the handpiece head. Jetting was conducted slowly in two sweeps of the jetting wand from the poll to the tail along either side of the backline, taking care to allow time for the fluid to pool ahead of the jetting wand and run down through the fleece over the sides of each sheep. The time for the handpiece to deliver 4L of formulation was measured and an attempt made to deliver this quantity into the fleece of each sheep.

Asssessment of louse numbers and fleece derangement: Inspections were conducted and louse numbers assessed at 2, 6 and 12 weeks after treatment as described for the dipping study. Fleece derangement (signs of biting and rubbing at the fleece) was assessed on both sides of each sheep at the 6 and 12 week inspections using the scoring system described by James *et al.* (2007). Briefly, the scores were: 0, no rub; 1, suspect, but not sure; 2, light but obvious rub, fluffy tip or definite pulled fibres at some sites; 3, distinct but dispersed pulled strands (thicker than fluffy); 4, definite patches or areas of pulled strands, less than 20% of the fleece affected, no bare areas; 5, definite patches or areas of pulled strands, greater than 20% of the fleece affected; 6, grossly matted fleece, often with some areas rubbed bare.

Animal ethics: All animal management and experimentation in both the jetting and dipping studies was carried out according to criteria specified under Animal Research Institute Animal Ethics Committee approval SA 2009/02/282.

'Stripping' study (laboratory)

Stripping occurs when dipping chemicals is more soluble in wool grease than in water and so is removed from the dipping fluid at a faster rate than fall in dip volume, causing a progressive drop in the concentration of the active ingredient as more sheep are dipped. Stripping does not occur with all dipping chemicals. Whether or not dipping fluids "strip" during dipping is an important practical consideration when determining dip mixing procedure. When topping up stripping dips chemical must be added at a higher concentration than in the initial mix to compensate for reduction in the concentration of dip active. A 'bench top' simulation was designed to determine whether TTO was likely to strip from solution during dipping.

A stock mixture of 8L of 1% TTO emulsified with 1% ALK was mixed and stored in a sealed Winchester at 5ºC. This emulsion was used in all experiments. 'Dipping' was conducted with 10 g

wool samples in 500 ml beakers. Wool was weighed into 10 g lots and dipped using a vegetable masher adapted for the purpose (Figure 10). For each dipping the wool was placed in the beaker and immersed for 20 s during which time the wool was compressed gently against the bottom of the beaker 3 times. The wool was then compressed in the vegetable masher above the beaker allowing excess fluid to run back into the beaker. Wool samples were then reweighed and the post dipping weight recorded. Fifteen samples were dipped sequentially in each beaker of fluid and samples of dip fluid were taken after each lot of 3 wool samples had been dipped. Wool and fluid samples were stored at -25ºC until later chemical analysis for TTO components.

Figure 10: Apparatus used to drain wool in bench top dipping simulation.

TTO components were subsequently measured in both dipping samples and wool samples by GCMS as described below

GCMS analysis of TTO components

Determination of tea tree oil component residues in tissue and blood

Tissue and blood samples were sub sampled by taking an aliquot of 0.2 mg for tissue or 2 ml for blood and the subsample then extracted with hexane (2 ml). The hexane extract was chromatographed on a Gas Chromatograph/ Mass Spectrometer (GCMS) with the detector operated in the full scan mode. Components of tea tree oil were identified and quantified by comparison of retention time, mass spectrum and peak area by comparison with pure tea tree oil and with individual standards.

Determination of tea tree oil components in wool and dipping fluid

Wool samples were sub sampled by taking 200 mg of wool and placing in a 10 ml headspace vial which was then sealed with a silicon/ polytetrafluoroethylene septa. The sample was allowed to equilibrate for at least four hours (usually overnight). The headspace was sampled using a solid phase micro extraction (SPME) fiber (polydimethylsiloxane 100 µm) for 10 minutes. The fiber was desorbed into a GC inlet at 200ºC for one minute. The remaining GC conditions were the same as used for liquid extracts, described above. Dipping samples from the stripping study were diluted by $1x10^{-4}$ and analysed by the same method as wool samples.

Statistical analysis

Most experiments in this project were of relatively similar design, usually with a number of experimental subjects (various stages of *L. cuprina, B. ovis* or sheep) divided amongst treatments, with experiments replicated at least twice and sometimes with repeat measurements at different times. As such analysis was approximately similar for most experiments. In most instances one or two way analysis of variance was conducted, with appropriate transformation to normalise the data where necessary. A repeated measures analysis was conducted for the sheep jetting study. All analyses were carried out using GenStat v 11(Payne *et al*., 2007).

Results

Sheep blowfly

First instar larvae – serum assay

Mortality of 1st instar larvae at different concentrations of TTO is shown in Figure 11. A concentration of 0.9% TTO in serum was effective in giving 100% mortality in this assay and 1% TTO reliably gave 100% kill in three other assays (results not shown).

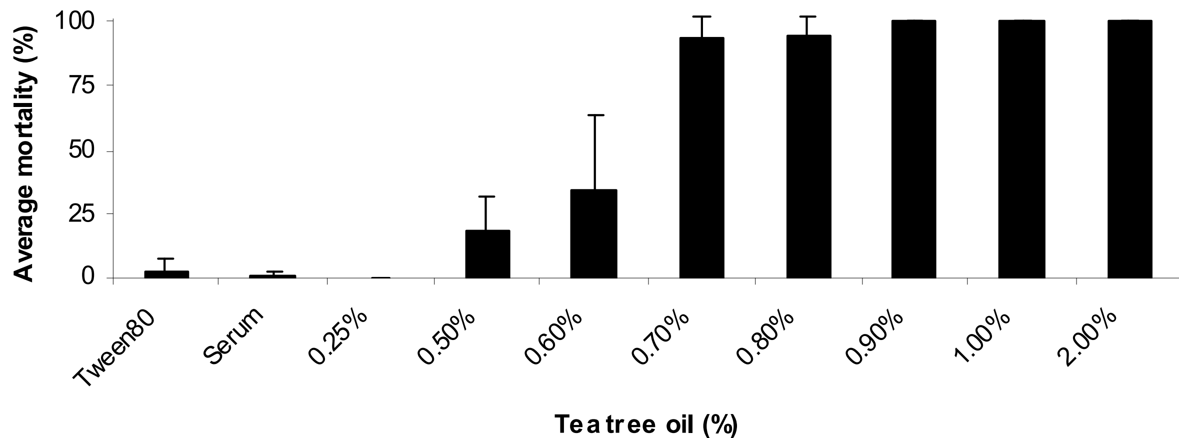


Figure 11: Mortality of first instar *L. cuprina* larvae after 24 h with different concentrations of TTO emulsified in bovine serum.

Second and third instar larvae – agar method

TTO at 2.5% gave 100% kill of second instar larvae, although l larvae out of the 30 total in the three replicates was still alive with 5% TTO at both 24 and 48 h. Higher concentrations were required to reliably kill third instars in this system (Figure 12).

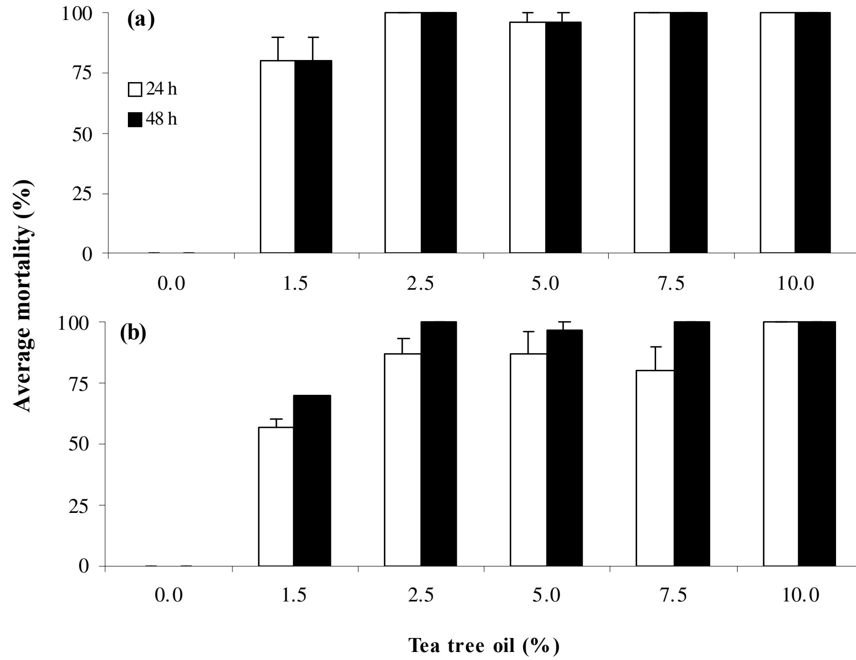


Figure 12: Mortality of (a) second instar larvae and (b) third instar larvae at different concentrations of Melaleuca oil in agar-based tests system.

Third instar – dipping assay

Even after dipping in 50% TTO, 36 % of larvae with ALK as the emulsifier and 54% with Tween80 successfully developed to adult flies (Figure 3). There was no significant difference between the two emulsifiers (p>0.05). A number of other similar assays were carried out with different carriers but all achieved similarly poor results (data not shown).

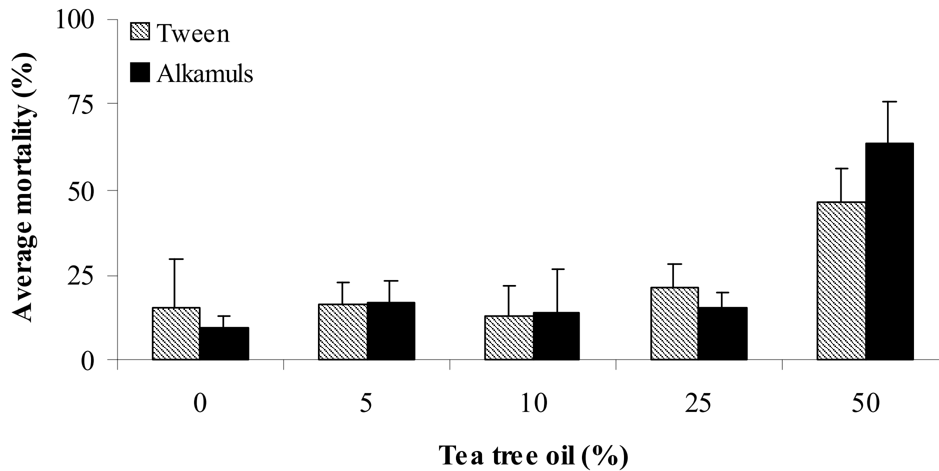


Figure 13: Mortality of third instar *L. cuprina* larvae following dipping in different concentrations of TTO for 1 minute with either 8% Tween 80 or 8% Alkamuls as emulsifiers.

Larval repellence (3rd instar)

Very few larvae were found in the TTO half of the Petri dish, regardless of if they were placed there initially (Table 2). By 24 h there was clear evidence of larval activity and feeding in the half of the dish without TTO whereas there was no indication of any larval feeding in the TTO treated agar. Occasional larvae observed on the TTO-treated half were usually on the surface rather than burrowing into the agar and may have been post feeding larvae searching for a site to pupate. In the dishes with untreated TTO in both halves, larvae were found on both sides of the plate and there was clear evidence of larval burrowing and feeding throughout the agar in the dish.

Table 2: Percent of larvae found in TTO-treated and untreated halves of meat liver agar in Petri dishes after 2 and 24 h (means for 2 dishes). There were 4 dishes with 10 larvae per dish for each concentration (total = 12 dishes) and larvae were initially placed on the untreated agar in 2 dishes [a] and the treated agar [b] in the other 2.

Time	TTO conc.	Initial untreated [a]		Initial TTO [b]		Total	
		Cont [c]	TTO [c]	Cont [c]	TTO [c]	Cont [d]	TTO [d]
2 h	**5%**	93.3	6.7	96.7	3.3	95.0	5
	2%	100.0	0	100.0	0	100.0	0
	0.50%	100.0	0	93.3	6.7	96.7	3.3
24 h	**5%**	86.7	13.3	90.0	10	88.3	11.7
	2%	90.0	10	96.7	3.3	93.3	6.7
	0.50%	100.0	0	93.3	6.7	96.7	3.3

[a] Initially treated agar
[b] Initially untreated agar
[c] Mean of 2 dishes.
[d] Mean of 4 dishes

Formulation of TTO with other insecticides

Agar method (3rd instar)

In the comparison of diazinon, ivermectin and boric acid with and without TTO, the repellent effect of TTO against larvae was again evident. Many of the larvae in the treatments with TTO had left the agar when the preparations were first examined at 24 h (Figure 14). Most of the larvae remaining on the agar in the treated vials were dead, presumably killed by the toxic action of the insecticides. The numbers of dead larvae remaining on the agar treated with TTO formulations with diazinon and ivermectin were higher than those with TTO/boric acid ($p<0.05$), presumably because of the more rapid action of these insecticides.

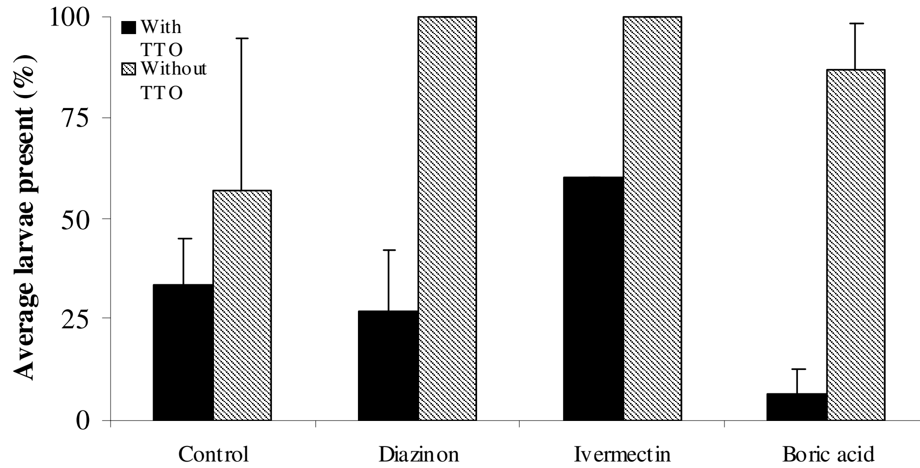


Figure 14: Number of dead or live larvae remaining on agar 24 hours after application of insecticide as single active ingredient or in combination with TTO

No larvae successfully pupated and emerged as adult flies in the diazinon, ivermectin or boric acid treatments without TTO (Figure 15a). In the experiment with cyromazine the average mortality was lower in those treatments with TTO (62 % and 78% compared to 84% and 90%, Figure 15b). Although a greater proportion of flies successfully emerged as adult flies in the treatments containing TTO than with insecticide alone, in no instance did more than 38% emerge, compared to 100% emergence in the untreated controls (Figures 4b, 5)

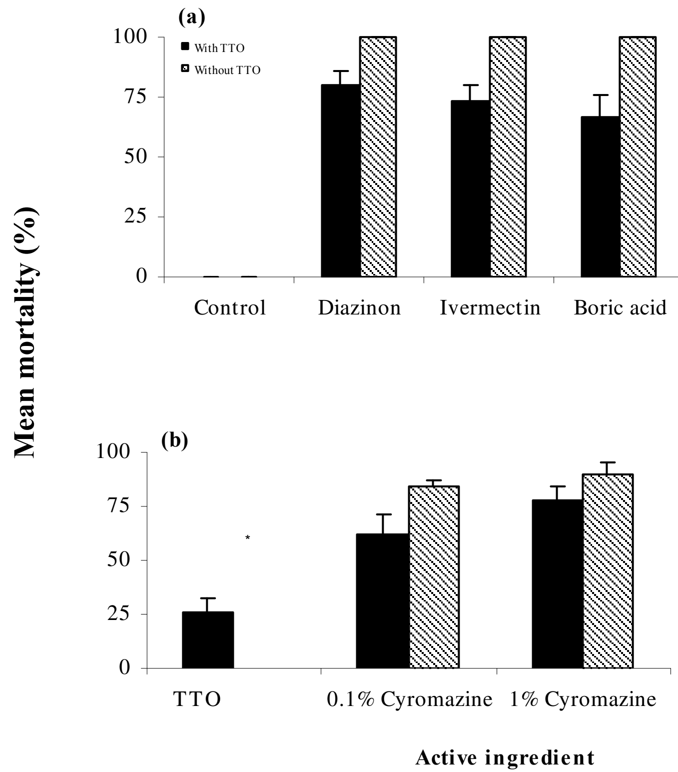


Figure 15: Effect of treatment of third instar larvae with insecticide as single active ingredient or in combination with TTO on the number of ecloding adult flies in agar assays: (a) diazinon (1 gL^{-1}), ivermectin (32 mgL^{-1}) and boric acid (25 gL^{-1}) (b) cyromazine (0.1 gL^{-1} and 1 gL^{-1}).

Dipping assay (3rd instar)

Mortalities were lower in the dipping studies than the agar assays, but as with the agar assays, in most instances addition of TTO to the formulation significantly lowered mortality in comparison with the equivalent formulation without TTO ($p<0.05$). The maximum kill of 83% was observed in the diazinon treatment without TTO.

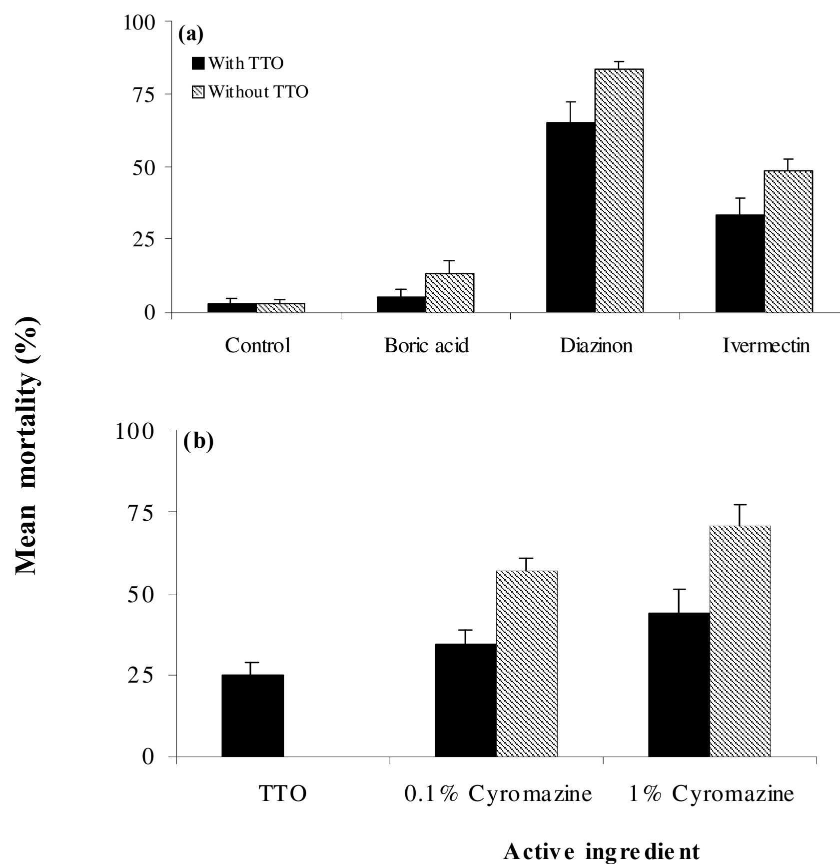


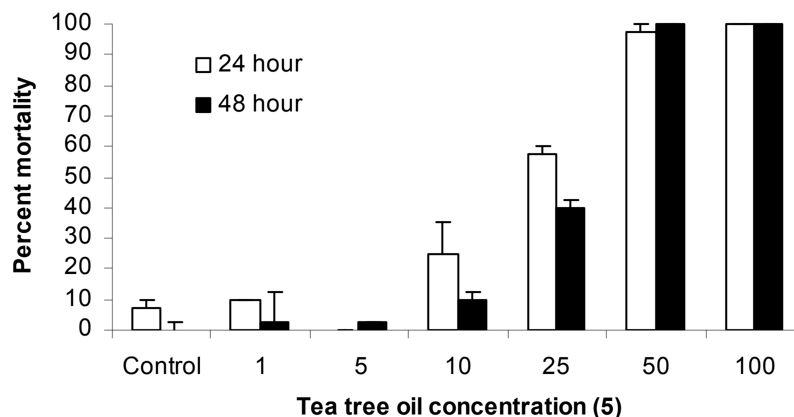
Figure 16: Effect of immersion of third instar larvae in insecticides with and without tea tree oil on the number of ecloding adult flies: (a) diazinon (1 gL^{-1}), ivermectin (32 mgL^{-1}) and boric acid (25 gL^{-1}) (b) cyromazine (0.1 gL^{-1} and 1 gL^{-1}).

Adult flies

Toxic effects - topical application

In this study and in other similar studies (data not presented) with 1 µl of topically applied formulation, at least 50% TTO was required to give 100% mortality in treated flies (Figure 17a). This represents a total amount of 0.5 µl of pure TTO. This was the amount also required when pure TTO was applied (Figure 17b).

(a) Concentration of TTO



(b) Quantity of TTO

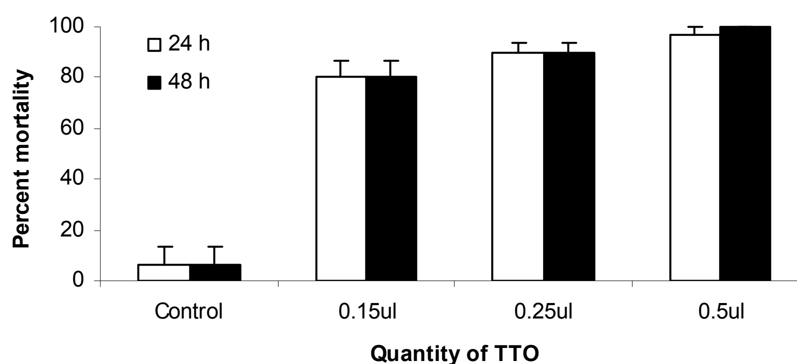


Figure 17: Effects topical application of tea tree oil on adult *L. cuprina*. (a) different concentrations in butanone, (b) different amounts of 100% tea tree oil. (6-09)

Topical application – synergism with piperonyl butoxide (PBO)

The results from the two experiments conducted with *L. cuprina* to assess the effect of PBO gave equivocal results. There was no detectable difference between mortality with and without PBO at high and low concentrations of TTO (Figures 18 and 19). However in the range from about 10% TTO to 35% TTO there may have been an effect. Mortality was significantly higher in the treatments with PBO than without PBO at 10% and 25% in experiment 1 (Figure 18) and at 35% in experiment 2 (Figure 19) ($P<0.05$). Further studies are required to clarify potential synergistic action from the addition of PBO.

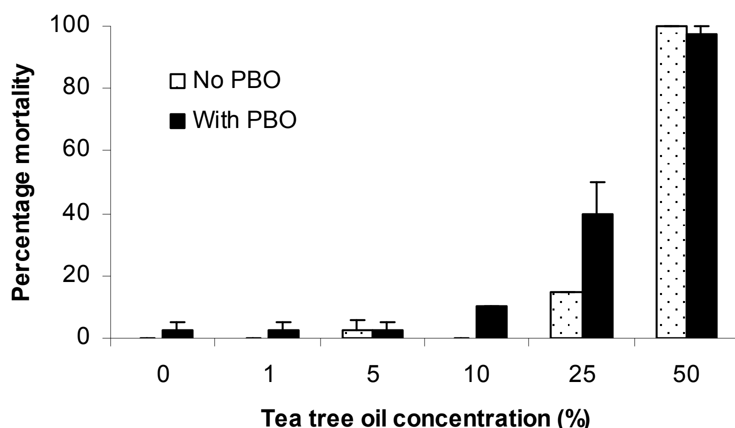


Figure 18: Mortality of *L. cuprina* females treated topically with and without 1 µl of 2% piperonyl butoxide and various concentrations of TTO (Experiment 1).

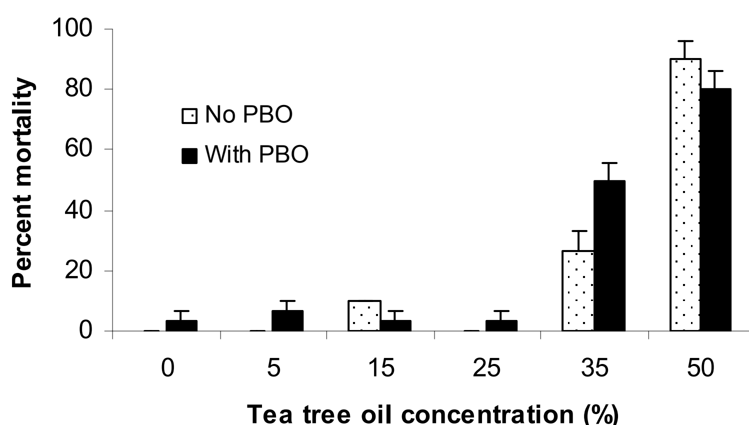
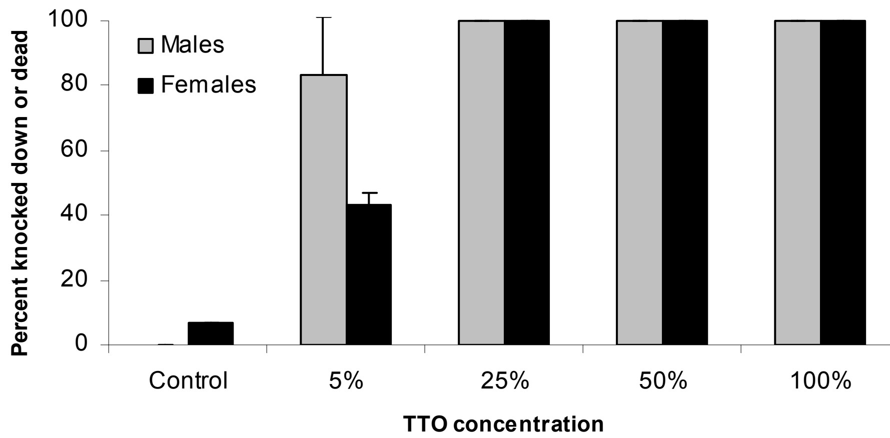


Figure 19: Mortality of *L. cuprina* females treated topically with and without 1 µl of 2% piperonyl butoxide and various concentrations of TTO (Experiment 2).

Adult flies - Fumigant effects

In the control treatments with acetone in the study with different concentrations of acetone, 2 flies were immobile at the initial 2 h inspection (Figures 20 and 21), but all were active at inspections at 3 h and 4 h. In the experiment with different quantities of TTO, in the controls all flies were active by the time of the first exposure at 0.5 h. Although some mortality was seen in some control groups at 24 h, it was generally low level and probably due to the effects of food and water deprivation. There were clear fumigant effects evident against *L. cuprina* even at low concentrations and amounts of TTO. With 5% TTO, 63% of flies were knocked down or dead at 2 h and this increased to 95% at 24 h, while with 1 µl of pure TTO 67% were knocked down or dead at 2 h and this increased to 97% at 24 h. There also seemed to be a sex effect with more males that females knocked down at low concentrations/amounts of TTO. No flies were seen moving in treatments with concentrations of 25% TTO or above following 2 h of exposure or at any time after that. Similarly in the experiments with different quantities of TTO, no flies were seen moving in treatments with 3 µl and above from the first inspection following 0.5 h of exposure to the last inspection at 24 h.

(a) 2 h of exposure



(b) 24 h of exposure

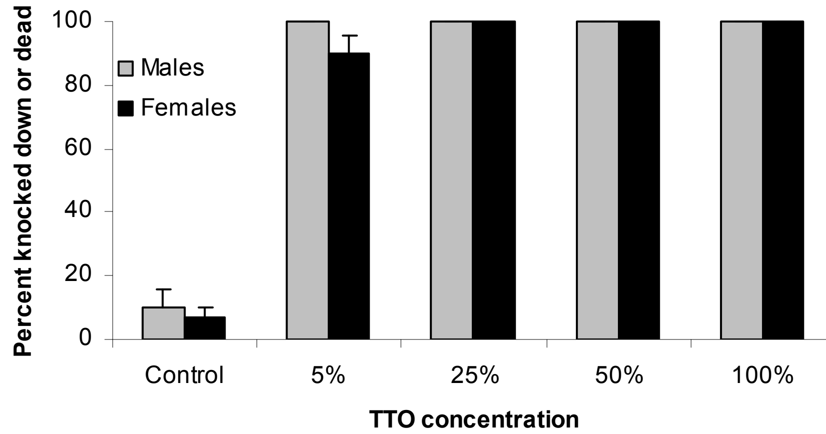
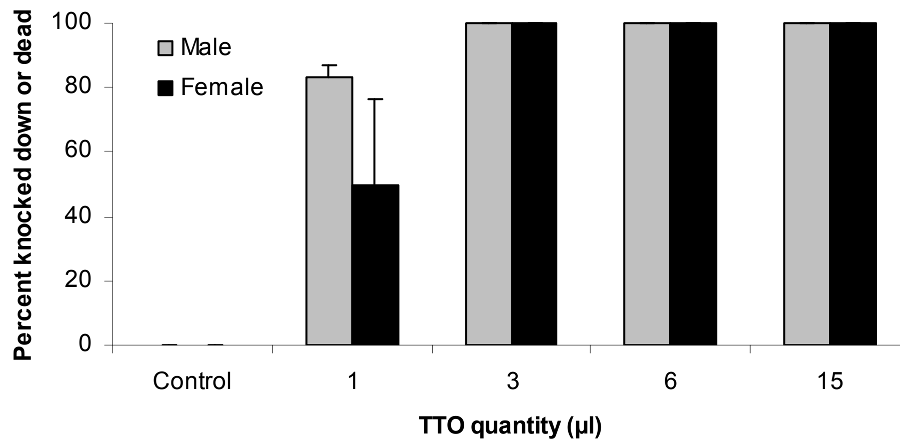


Figure 20: Fumigant effect: mortality of adult *L. cuprina* males and females exposed to 60 µl of different concentrations of TTO in acetone following 2 h and 24 h of exposure; (Controls exposed to acetone).

(a) 2h after exposure



(b) 24 h of exposure

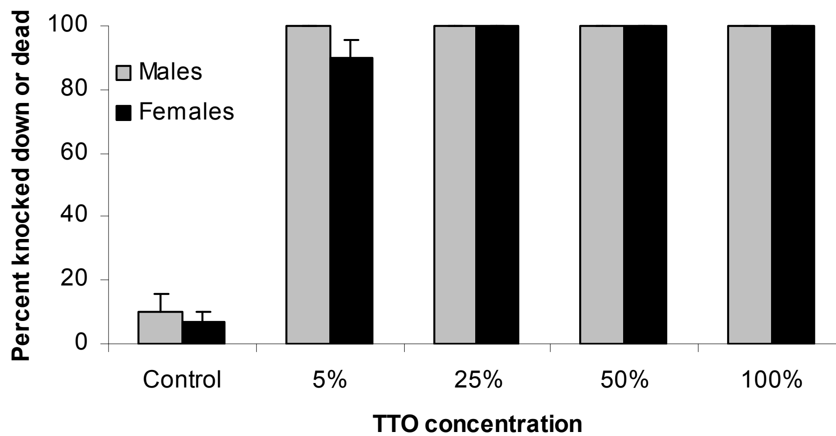


Figure 21: Mortality of adult *L. cuprina* males and females exposed to different amounts of pure TTO following 2 h and 24 h of exposure; (Controls exposed to grape seed oil).

Adult flies - repellency

Figure 22 shows the numbers of flies present on wool preparations and which were stationary and probing with their ovipositors or actually ovipositing at 24 inspections of the control preparations between 9:15am to 4:05 pm in the first choice test. No flies were seen resting on the TTO preparations at any observation during this period suggesting that TTO-treatment provided a strong repellent effect over this period. Table 3 shows the number of egg masses in the wool of the control preparations at the end of exposure. No eggs were found on the TTO –treated wool.

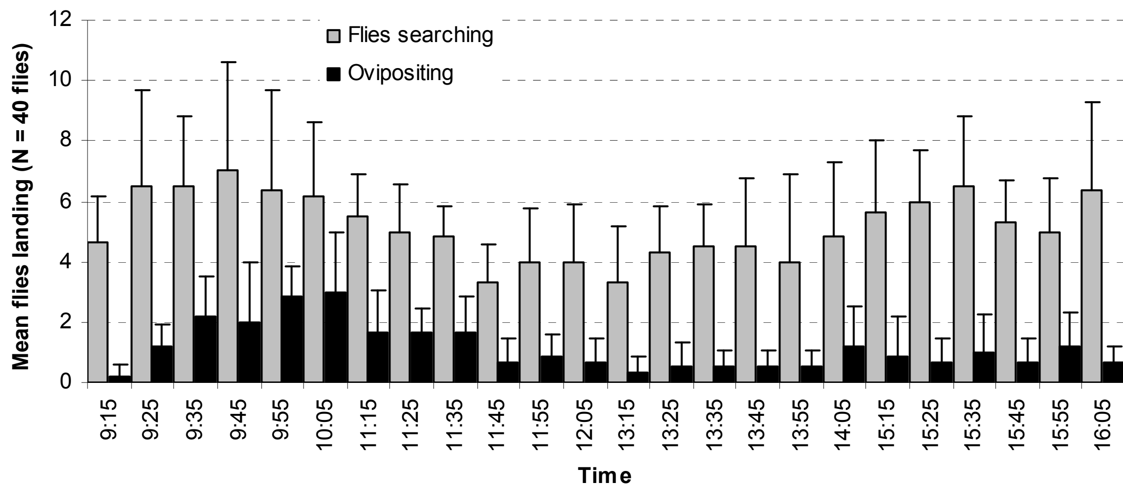


Figure 22: Mean number of flies present and exhibiting oviposition behaviour on control wool preparations baited with sheep liver homogenate – choice test. No flies were recorded on TTO- treated wool.

Figure 23: Sheep blowfly egg masses deposited on control wool preparation. No egg masses were deposited on TTO treated wool.

Table 3: Number of *L. cuprina* egg masses deposited on control and TTO-treated wool – choice test

		Egg masses estimated	
Cage	**Treatment**	**# egg masses**	Score
1	Control	28	4
	TTO	0	0
2	Control	22	4
	TTO	0	0
3	Control	21	4
	TTO	0	0
4	Control	19	4
	TTO	0	0
5	Control	23	4
	TTO	0	0
6	Control	29	4
	TTO	0	0

The non-choice testing confirmed that TTO is a very powerful repellent and oviposition deterrent for *L. cuprina.* Even though the flies were gravid, were provided with a powerful stimulus for both feeding and egg laying and were not given an untreated choice, only on 35 occasions were flies observed resting on TTO treated wool compared to 672 times on the control wool, a reduction of 95%. Flies that did land on the treated wool generally left soon after landing and although copious oviposition was observed on all control preparations, no ovipositing flies or egg masses were observed on TTO-treated wool.

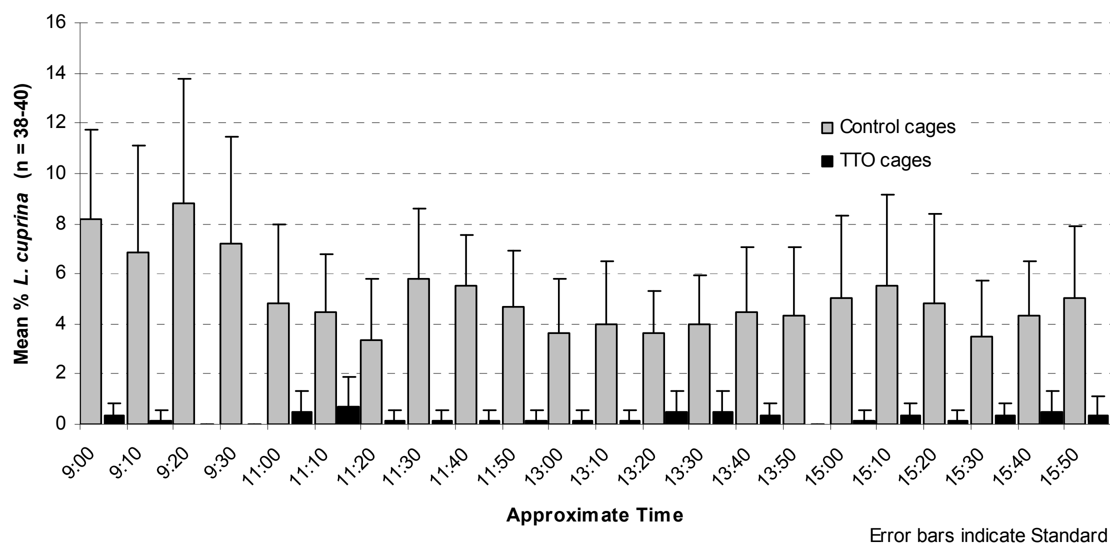


Figure 24: Mean number of flies recorded on control (± s.e.) and TTO-treated wool preparations baited with sheep liver homogenate – non-choice test. No flies were recorded on TTO- treated wool.

Table 4: Mean number of egg masses deposited on control and TTO- treated wool preparations – non choice test.

		Egg masses estimated	
Cage	**Treatment**	**# egg masses**	**Score**
1	Control	22	4
	Control	24	0
2	TTO	0	4
	TTO	0	0
3	Control	23	4
	Control	24	0
4	TTO	0	4
	TTO	0	0
5	Control	16	4
	Control	16	0
6	TTO	0	4
	TTO	0	0

In the first persistency study, conducted over 6 days, treatment with the 3% TTO formulation almost completely repelled flies. Only one fly was counted on TTO-treated wool at any of the observations (day 6) and no egg masses were found on any of the TTO-treated wool preparations.

In the second experiment conducted to examine persistency of effect, some flies were recorded on the wool at all observations from day 9 and later, although these flies remained on the wool surface for less than a few seconds in most instances. Consistent with earlier studies, no flies were counted on the

treated wool at observations on day 1. At 44 days the number of flies on the TTO preparations significantly increased in comparison to earlier times ($p<0.05$), although the numbers counted were still much lower than on the control preparations and there were no egg masses deposited on the TTO-treated wool. Unfortunately we had no treated preparations available for further tests and had to conclude the experiment after the 44 day test.

Table 5: Mean number of flies (±s.e.) counted on wool preparations (means across 3 cages, 12 observation times) and egg masses deposited on control and TTO- treated wool preparations up to 6 days after treatment with 3% TTO formulation – choice test.

	Flies on wool		Egg masses	
Day	**Control**	**TTO**	**Control**	**TTO**
1	5.7±0.28	0	12.7±2.0	0
2	6.4±0.0.2	0	12.0±1.5	0
3	7.6±0.3	0	13.3±0.7	0
6	5.8±0.6	0.03±0.03	7.0±0.6	0

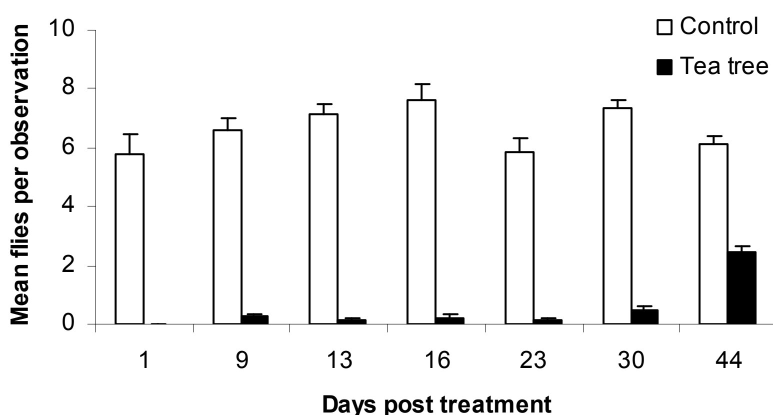


Figure 25: Mean (+s.e.) number of flies recorded on the surface of TTO-treated wool or wool treated with emulsifier only at different times after treatment. Bars represent the average of numbers of flies over 6 cages at 24 observation times over 1 day at the times after TTO-treatment shown in the x axis.

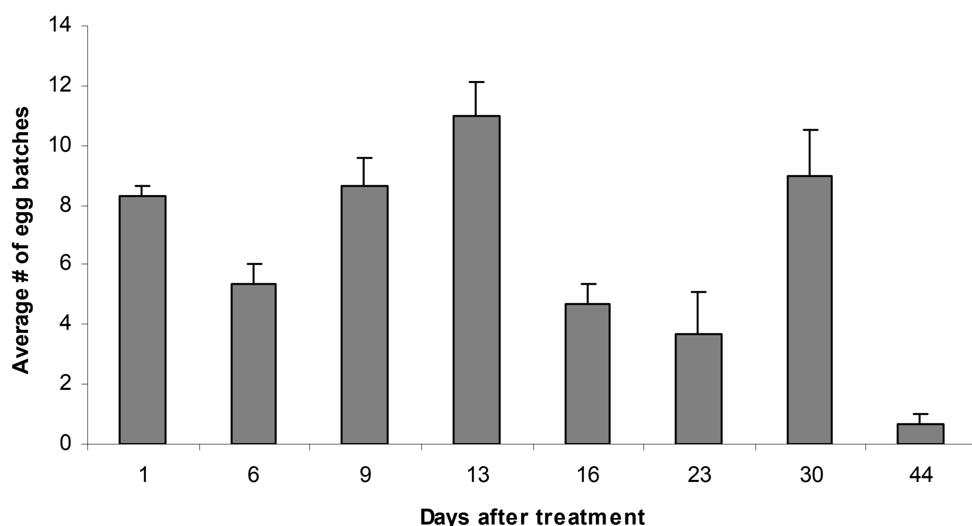


Figure 26: Mean (+s.e.) number of egg masses recorded on the surface of control wool preparations following one days exposure to flies up to 44 days after treatment. Bars represent the average of numbers of egg masses per cage over 6 cages. No egg masses were recorded on TTO treated wool.

Eggs and Pupae

Fumigant effect on eggs

A number of tests were conducted giving reasonably consistent results and confirming a fumigant effect of TTO on *L. cuprina* eggs. The results from these studies are summarised in Figure 27. Whereas the mortality induced by a concentration of 1% was not significantly different from controls (P>0.05), concentrations of 2.5% and above induced significant mortality.

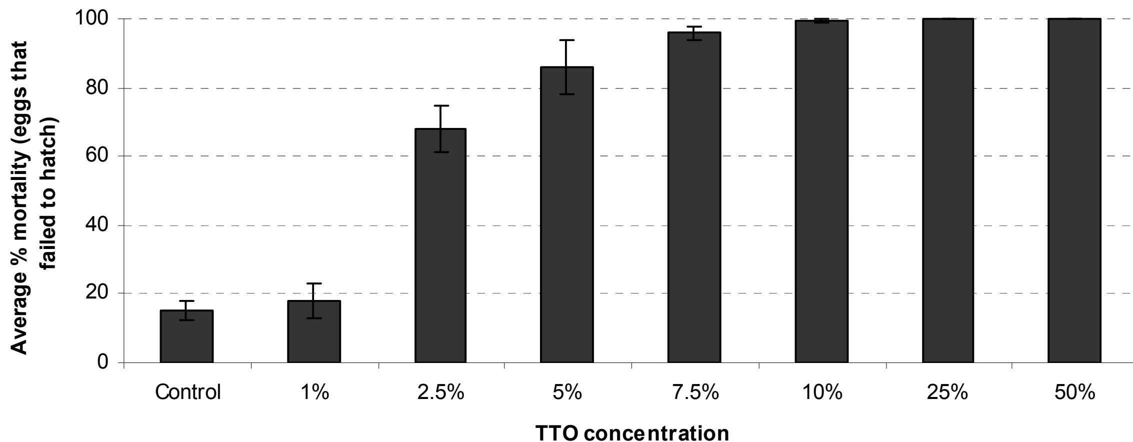


Figure 27: Mean percent mortality of *L. cuprina* eggs exposed to 60 µl of different concentrations of tea tree oil in acetone. (control = 60 µl acetone).

Fumigant effect on pupae

In the first experiment, exposure of pupae to concentrations of TTO up to 15% did not significantly (P>0.05) reduce the emergence of flies in comparison to controls and there was no relationship between pupal viability and TTO concentration within this range. In the second experiment where higher concentrations of TTO were used there was a significant reduction in numbers of flies emerging at concentrations of 75% and above (P<0.05) (Figure 28). However, even with 60 µl of 100% TTO the direct pupal mortality induced was less than 40%. Although pupae were not killed directly, most flies exposed to concentrations of 50% and above died during or soon after emergence. This may be because exposure in the pupal phase weakened the flies and affected their ability to emerge, or that the emerging flies were more susceptible to TTO fumes than pupae and that there was high enough vapour pressure of residual TTO present in the dishes to interrupt the eclosion process or kill the flies directly. Increasing the amount of 100% TTO to 600 µl markedly increased the direct toxic effect against pupae and no flies emerged from pupae in this group.

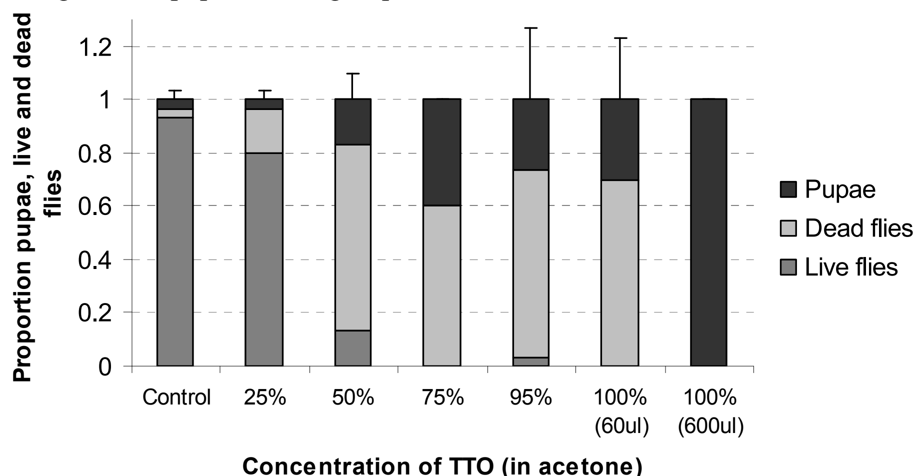


Figure 28: Proportion of *L cuprina* which were killed as pupae, killed as flies during or following eclosion or hatched to flies following exposure of pupae to fumigant action of different concentrations of TTO.

Sheep lice

Laboratory studies

Treated surface assays

Figure 29 shows the results of a typical contact assay conducted by a standard method (Levot and Hughes 1989) with butanone as the solvent for TTO. Clearly this method was not suitable for tests with TTO Drying overnight may have allowed for the evaporation of some key active components before the lice were exposed and reduced efficacy in comparison with that seen with other assay designs. Better effect was seen in tests with grapeseed oil as the solvent where the papers were not dried overnight before exposure. Careful choice of the quantity of test solution and using the dishes uncovered allowed immediate exposure of lice without high control mortalities. However, even in this system concentrations of at least 10% were required to give high louse mortality (Figure 30).

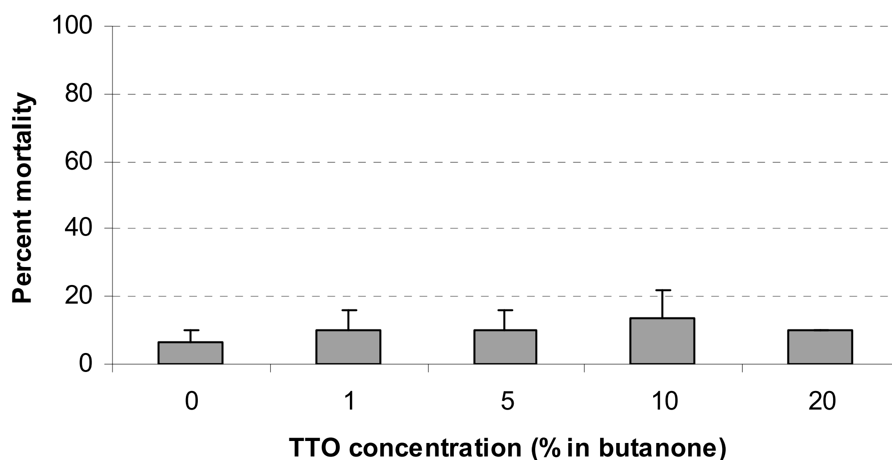


Figure 29: Mortality of lice exposed to cotton squares treated with different concentrations of TTO in butanone. Cotton squares were dried overnight in a fume hood prior to louse exposure.

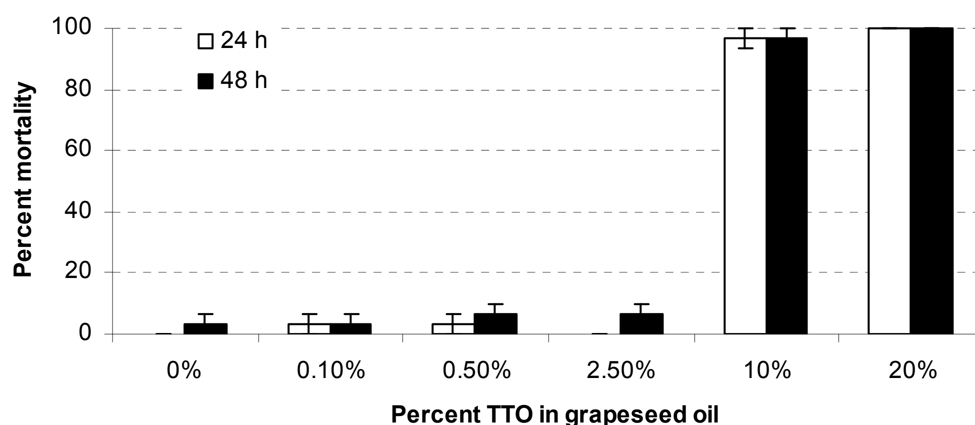
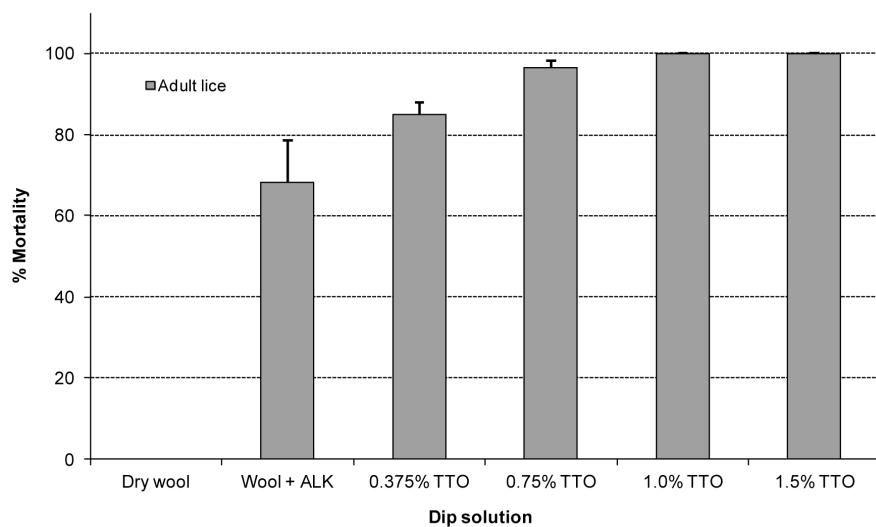


Figure 30: Mortality of lice exposed to filter paper treated with different concentrations of TTO in grapeseed oil. Oil was allowed to disperse on the filter paper, but papers were not dried prior to addition of lice

Wool dipping assays

In all wool dipping assays, including a number for which data is not shown, 1% TTO and concentrations above consistently gave 100% mortality of lice and were equally effective against nymphs and adult lice (Figures 31 to 33). In early assays TTO was emulsified with 5% ALK, but assays in which 1% ALK was tested with the aim of reducing ultimate cost of a formulation, suggested no reduction in efficacy (Figure 32).

Effect of synergism: Although only one preliminary study was carried out, the results from this experiment suggested little effect of PBO at any of the TTO concentrations tested (Figure 33). Again dipping wool in 1% TTO resulted in a complete kill of lice.



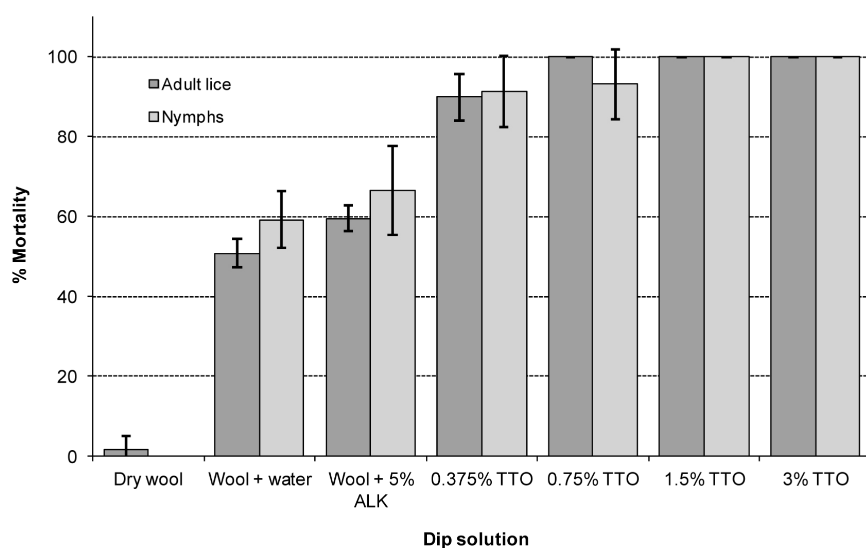


Figure 31: Percent mortality of lice at 24 h following wool dipping in different concentrations of TTO emulsified with 5% ALK in two assays.

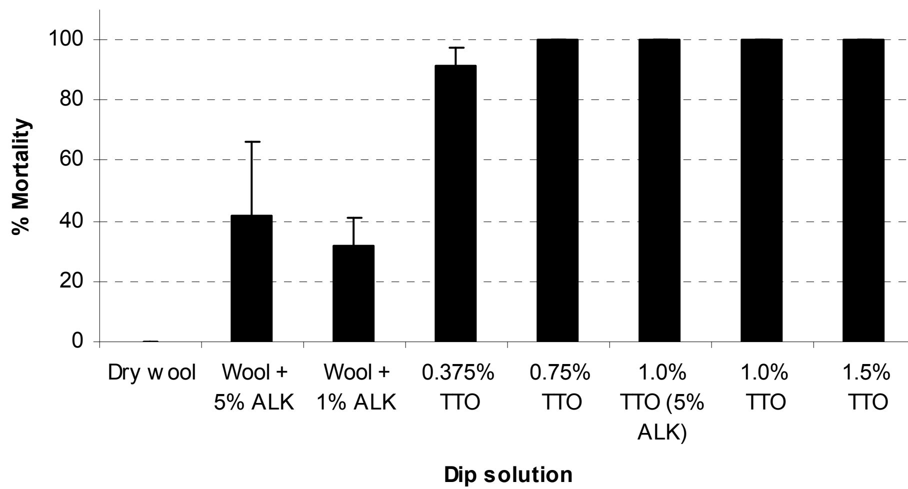


Figure 32: Percent mortality of lice following wool dipping in different concentrations of TTO emulsified with 1% ALK plus 1% TTO emulsified with 5% ALK.

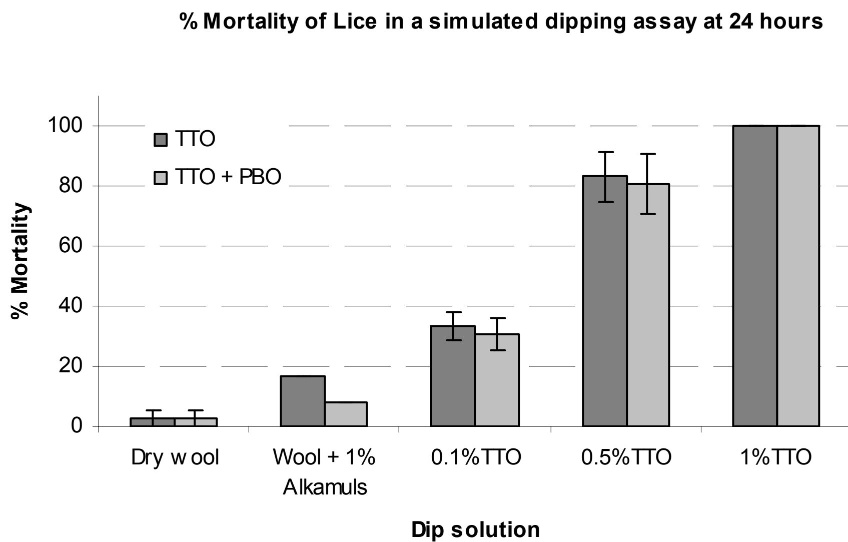


Figure 33: Effect of synergism with 0.02% piperonyl butoxide added to different concentrations of TTO (1% ALK) on percent mortality of lice in wool dipping assays

Dried wool assay

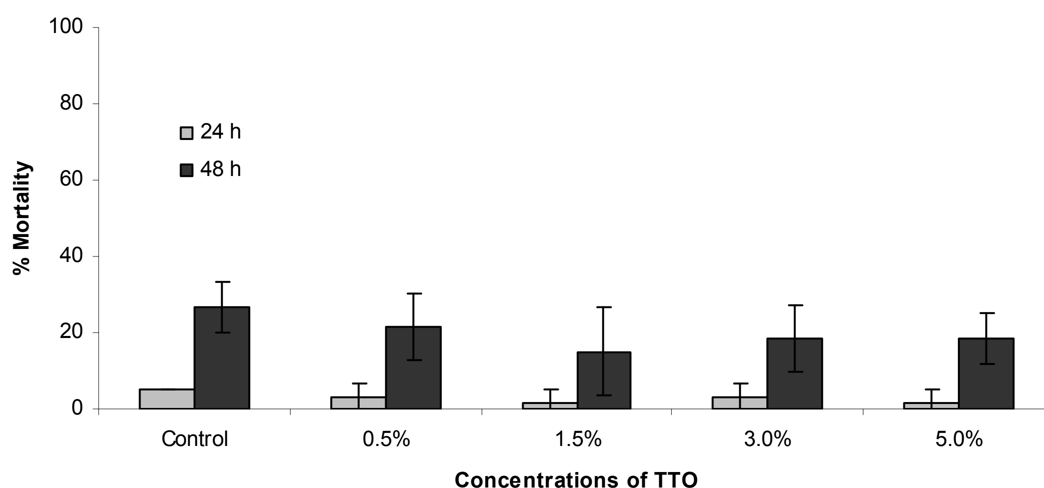


Figure 34: Mortality of lice exposed to wool treated with different concentrations of TTO and dried in a fume hood prior to lice exposure

The results shown in Figure 34 suggest very little residual effect from TTO when the wool was allowed to dry before lice were added. It seems that insecticidal effect is markedly reduced once the wool has dried. In some other assays conducted where wool was allowed to dry before lice were added (results not shown) there was more variability in results and some mortality in wool treated with higher concentrations of TTO. For example in one assay conducted as above, except that wool was dried on a bench top rather than in a fume hood, 3% and 7.5%TTO gave 57% and 70% mortality compared to 13% in controls. With the variability seen between replicates it was considered there was probably enough non-evaporated TTO left in some sections of the staple to cause this mortality and that additional drying would have further reduced insecticidal effect.

Fumigant effects

TTO demonstrated clear fumigant effects against lice. In the assays conducted with TTO either used at 100% or solubilised in 60 µl acetone, concentrations of 10% and above gave 100% mortality of lice (Tables 6 and 7). With 5% TTO (3 µg TTO), although all lice were knocked down at 1 and 2 h, 3 lice (5%) were able to recover overnight and at 1% (0.6µg) although most lice were knocked down when inspected at 1 and 3 h, by 24 h, 35% had recovered.

Table 6: Percent mortality (± s. e.) of lice exposed to fumigant effect of different concentrations of TTO in acetone

Treatment	TTO mass (µg)	Time after exposure[1]		
		1 h	3 h	24 h
Control (60 µl acetone)	0	0	0	2.3 (0.3)
25% (60 µl)	15	100	100	100
25% (120 µl)	30	100	100	100
50% (60 µl)	30	100	100	100
100% (60 µl)	60	100	100	100

[1]Lice were exposed to test solution up to 3 h then allowed to recover without exposure to 24 h
[2] n= 60 per concentration exposed in three replicates of 20 lice

Table 7: Percent mortality (± s. e.) of lice exposed to fumigant effect of different concentrations of TTO in acetone

Treatment	TTO mass (µg)	Time after exposure[1]		
		1 h	3 h	24 h
Control (60 µl acetone)	0	0	0	0
1% (60 µl)	0.6	11.7 (4.4)	4.4 (6.7)	35.0 (2.9)
5% (60 µl)	3	100	100	95.0 (5.0)
10% (60 µl)	6	100	100	100
25% (60 µl)	15	100	100	100

[1]Lice were exposed to test solution up to 3 h then allowed to recover without exposure to 24 h
[2] n= 60 per concentration exposed in three replicates of 20 lice

In a further study with lice exposed to similar amounts of TTO either in 100% droplets or emulsified at different concentrations in 60µl of Tween80, 6µg amounts of TTO and above, whether at 100% or in Tween, gave close to 100% knock down after 2 h of exposure. However, when they were inspected after removal from the test arenas for 22 h, a significant proportion had recovered (Figure 35). At 24 h there was marked difference in effectiveness of similar amounts of TTO presented in different forms with significantly more of the lice exposed to TTO emulsified in Tween recovering ($P<0.05$) (Figure 35). The difference appeared most marked with lower amounts of TTO. With 3µg of TTO 63% more lice recovered in the emulsified TTO treatment than with pure TTO, whereas with 6 and 15 µg the difference was approximately 25 %. This may have been because the vapour pressure of TTO was lower with emulsified than pure TTO and sufficient to knock down, but not to kill all lice over the two hours of exposure.

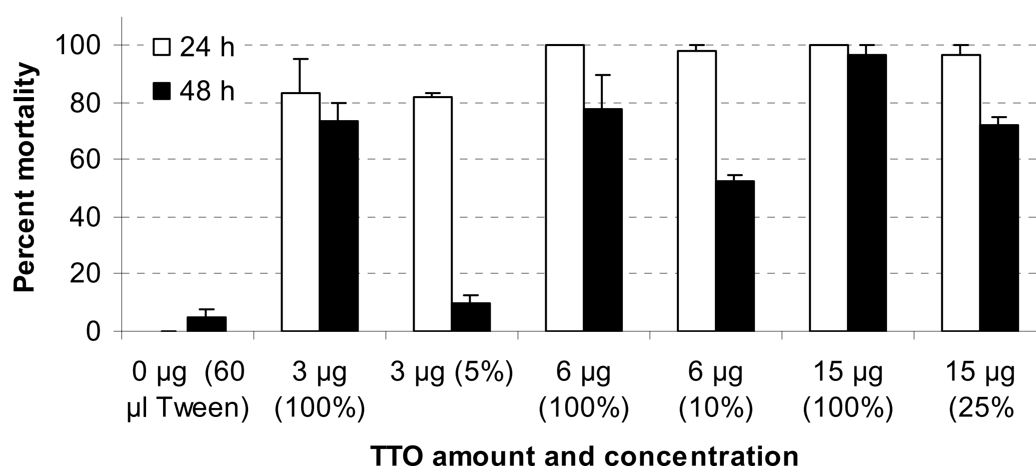


Figure 35: Percent mortality (± s. e.) of lice exposed to fumigant effect of different concentrations of TTO in 8% Tween 80.

Wool fumigation assays

Figure 36 shows the percent mortality in the first assay, with Parafilm covering the tube. The large standard error in the controls with ALK was due to 100% mortality of lice in one replicate. The wool containing the lice in this tube was quite damp to touch and it is thought that this could have caused high mortality in this replicate. Although the wool also felt damp in the other two replicates with ALK

but no TTO, mortality in these two replicates was only 5% and 15%. This compared to 100% mortality in the treatments exposed to 0.5%, 1 % and 2% TTO. In the second assay where the tubes were not sealed with Parafilm (Figure 37), no lice died in either the dry wool control or the wool treated with ALK but no TTO. Even though the tubes were not sealed 98% of lice were killed (1 found alive) in the 1% TTO treatment and 73% were dead with 0.5% TTO.

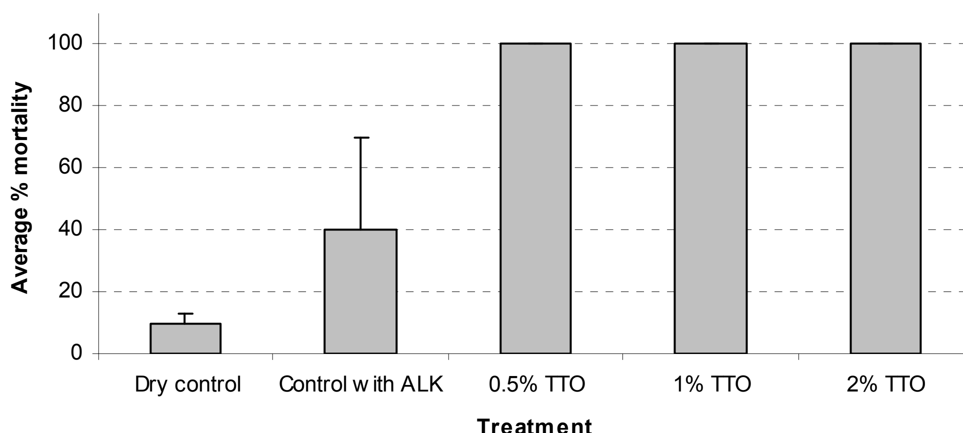


Figure 36: Mean percent mortality of lice (± s.e.) in wool fumigation assays. (In this assay the vials were closed with Parafilm.)

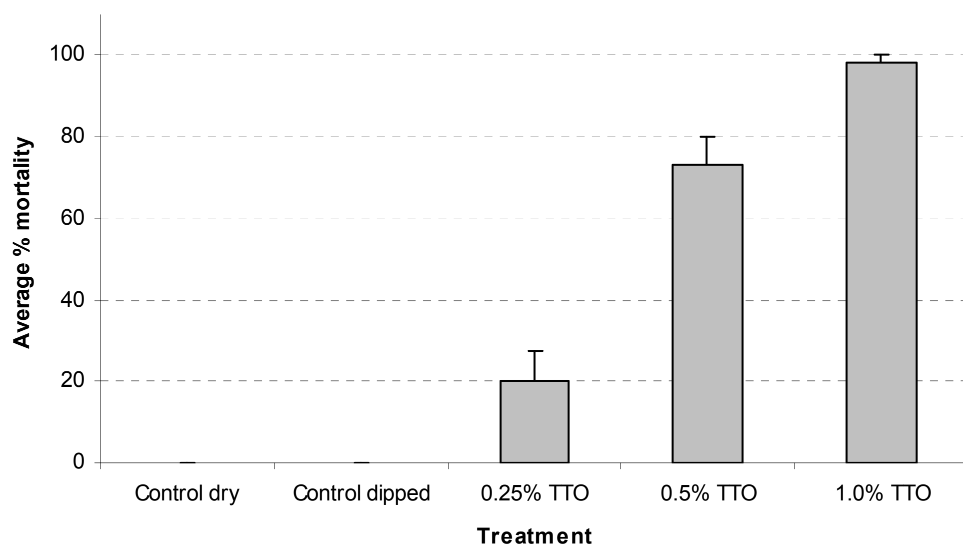


Figure 37: Percent mortality of lice (± s.e.) in wool fumigation assays (vials not closed with Parafilm.)

Effect of TTO components in fumigation assays

The two experiments conducted to examine the fumigant effects of TTO components against lice both suggested by far the majority of the effect is due to terpinen-4-ol. In the first experiment 7.5% and 5% TTO and terpinen-4-ol at an equivalent concentration to that found in 5% TTO both gave 100% knock down at 3 h (Figure 38). When examined at 24 h 10 lice (5 in each of two replicates) had recovered in the 5% TTO treatment whereas in the terpinen-4-ol treatment only 3 lice, all in one replicate had recovered. These numbers were not significantly different (P>0.05). Neither alpha terpinene nor gamma terpinene caused mortalities that were significantly different from the control (P>0.05).

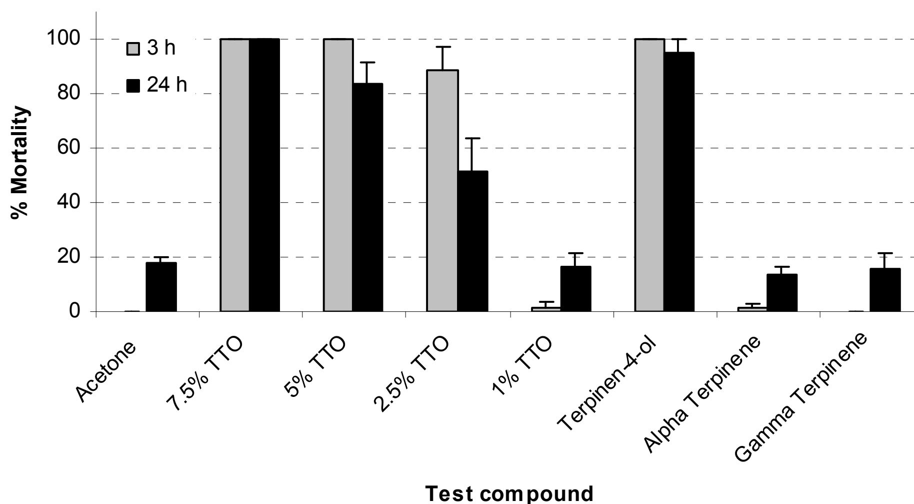


Figure 38: Mortality of lice exposed to the fumigant effects of different concentrations of TTO and the TTO components terpin-4-ol, alpha terpinene and gamma terpinene at 3 and 24 h after exposure.

Results were similar in the second experiment (Figure 39). The percentages knocked down or killed with 2.15% and 1.5% terpinen-4-ol were not significantly to that with 5% TTO at either 3h or 24 h, but all three of these gave mortality much higher than in the controls or other components (P<0.05). However mortality induced by the other components at 24 h either individually or in combination (without terpinen-4-ol) was significantly higher than in the control P<0.05). Alpha terpinene and gamma terpinene were not included in this combination, and the possibility remains that they could have interacted in mixture with some of the compounds tested in the second experiment. However the results suggest that terpinen-4-ol 1s responsible for the majority of the fumigant action of TTO against lice.

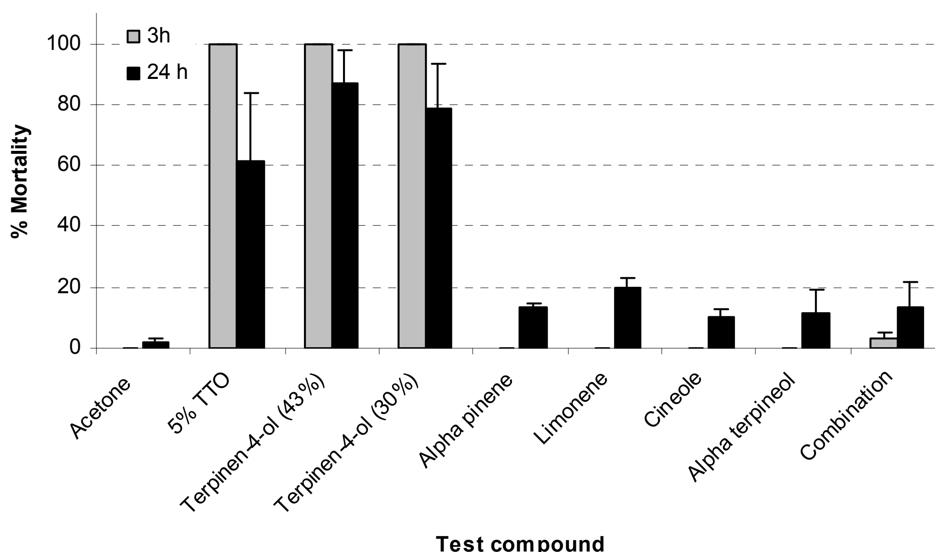


Figure 39: Mortality of lice exposed to the fumigant effects of 5% TTO, 2.15% and 1.5% terpinen-4-ol and some other TTO components at 3 h and after 24 h.

Effects against eggs

Dipping studies:

The results of this study are shown in Table 8. Egg hatch rate was 70% in the dry wool control and one egg apparently developed fully but died during hatching (as we regularly see in lice kept *in vitro*) . In the ALK treated groups, 60% hatched and a further 20% showed signs of development, In comparison, none of the eggs in the TTO treated group hatched or showed any signs of development. There appeared to be a physical effect on the chorion or outer layers of the egg in the TTO treated groups,

although not in the group treated with ALK only. This may have contributed reduction in viability. This requires further investigation.

Table 8: Hatching % of eggs placed in dry wool, in wool dipped in 1% ALK and in wool treated with 1%TTO emulsified in water with 1% ALK

Treatment	Number of eggs	Hatched	Unhatched	
			Developed	Undeveloped
Dry wool	10	7	1	2
ALK	20	6	2	2
TTO	10	0	0	10

Fumigant effects

Most eggs hatched in the absence of TTO in this assay and this was consistent across replicates (Figure 40). There was a clear effect of TTO on egg viability in comparison to controls (P=0.038), but difference in mortality between the two quantities of TTO tested was not significant (P>0.05), largely because of high variability amongst replicates within the two TTO treatments. In the 60 µl treatment, no eggs hatched in two replicates whereas in the third 60% of eggs hatched and in the 15 µl treatment , no eggs hatched in one replicate, 40% in the second and 80% in the third. The most likely explanation for the high variability is that some dishes were better sealed than others or that lids were momentarily dislodged during the early inspection, which may have led to differences in TTO vapour pressure or the period of time which it was maintained. These results indicate that TTO has fumigant effects against *B. ovis* eggs, in addition to possible physical effects on the egg chorion.

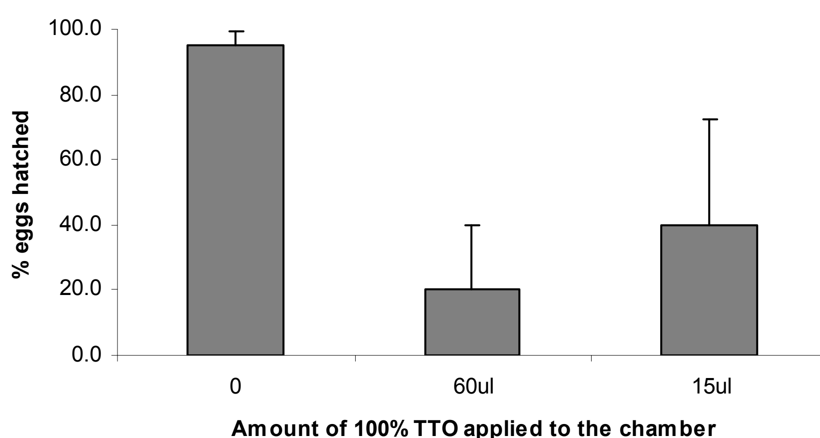


Figure 40: Percent hatch of lice eggs exposed to 0, 15 ul or 60 µl of TTO for 24 hours

Sheep studies

Dipping

Results

Mean and standard errors for pre-shearing counts per 10 cm fleece parting for each group of three sheep are shown in Figure 41. Counts in the different groups ranged from 11.4 to 13.7 per part which by convention would be considered heavy infestations of lice (>5 lice per 10 cm fleece part). Despite inspection of 40 parts on each sheep, no lice were found on any of the sheep following dipping in 1% or 2% TTO at any of the four post treatment inspections. It appears highly likely that dipping with TTO formulation completely eradicated lice in both treatment groups.

In the control group, all sheep remained infested throughout the study. Numbers of lice fell from a mean of 13.2 per part prior to shearing to 2.4 at 2 w after shearing. Reduction in lice numbers generally occurs after shearing and is due to both direct removal of lice during shearing and the effect of environmental exposure following fleece clipping. After this time louse numbers increased rapidly in the controls and a heavy infestation (mean count of 12.2 lice per part) was present at the final inspection 20 weeks after treatment. At the end of the study frequent rubbing and marked fleece derangement were evident in the control sheep (Figure 43) whereas no pruritic behaviour or fleece damage was observed in the treated groups.

One sheep in the 2% treatment was found dead in the pens following dipping. This animal was seen to get its head caught under the leg of another sheep in the small confines of the dip vat. Post mortem examination indicated that this animal had inhaled dipping fluid and had substantial fluid in its lungs. This is considered an artefact of the small cage dipping system used, and not due to any unsuspected toxic effects of TTO. No abnormal behaviour was observed in any of the other sheep.

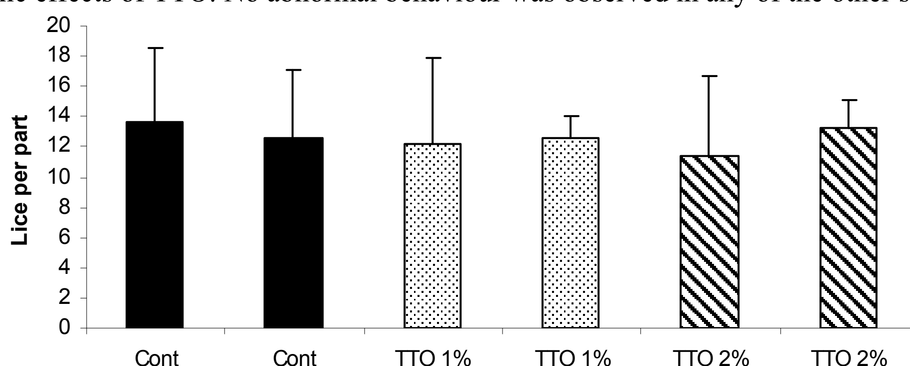


Figure 41: Mean (± s.e.) for counts of lice per 10 cm fleece parting on sheep in the 6 treatment and control groups at allocation

Table 9: Mean louse counts (± s.e.) for untreated sheep and sheep immersion dipped in 1% and 2% TTO formulation

Treatment	Time of inspection				
	Pre-shearing	2 weeks	6 weeks	12 weeks	20 weeks
Controls	13.2 (3.0)	2.4 (0.7)	2.5 (0.9)	3.7 (1.8)	12.3 (4.2)
1% TTO	12.4 (3.7)	0.0	0.0	0.0	0.0
2% TTO	12.4 (3.6)	0.0	0.0	0.0	0.0

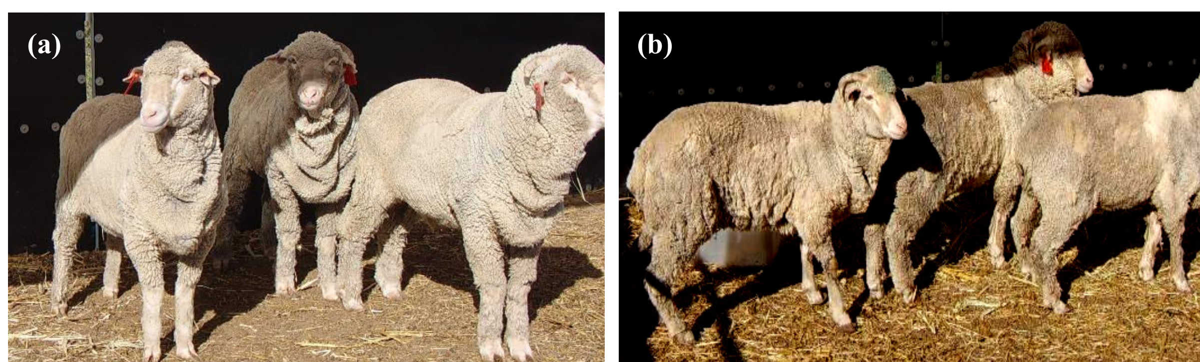


Figure 42: TTO-treated (a) and untreated (b) sheep at 12 w after dipping

Figure 43: Wool damage in an untreated sheep at the conclusion of the experiment at 20 weeks after dipping. No fleece derangement was evident in the TTO-dipped animals

Jetting

Mean louse counts for the different treatment groups of sheep at allocation are shown in Figure 44. Louse densities varied from 3.8 to 7.2 per part which by convention would be considered to be moderate (1-5 lice per part) to heavy (> 5 lice per part) infestations. The mean count for the first 2% TTO treatment group was approximately 60% higher than the other groups mainly because of a leverage effect of one sheep with very high counts. Louse density on this sheep was 16.5 per part at the start of the experiment, significantly above the mean of all sheep at the start of the study (5.0 per part) and well above the next highest count of 10.6 per part on one of the control sheep. This animal maintained relatively high counts through the experiment (3.9 per part at 2w. 7.9/part at 6 w and 12.4 per part at 12 w compared to counts of 0.8/part, 2.3/part and 4.0 per part respectively for the sheep with the next highest counts in the treated groups) and markedly affected results for the 2% TTO treatment.

Mean counts for the individual groups of 3 within each treatment over the course of the experiment are shown in Figure 45. Whereas louse numbers increased at each count for the two control groups, they dropped markedly following jetting (2 w count) in all treatment groups and then increased at the 6 w and 12 w counts. Jetting with these concentrations gave maximum reduction in louse numbers of 94% in comparison to controls (Table 10).

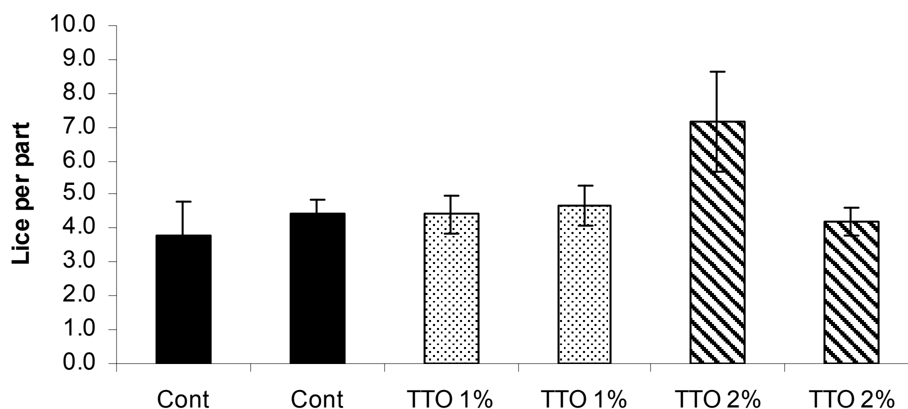


Figure 44: Mean (± s.e.) for counts of lice per 10 cm fleece parting on sheep in the 6 treatment and control groups at allocation in the jetting study.

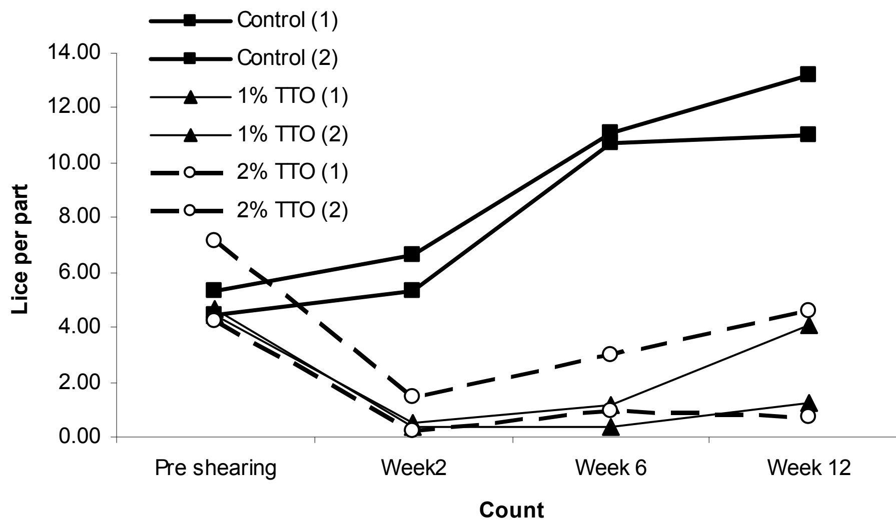


Figure 45: Mean counts of lice per 10 cm fleece part over 12 weeks in groups of sheep jetted with 1% or 2% TTO or left untreated (control)

Table 10: Percent reduction in louse counts at 2, 6 and 12 weeks after jetting (based on geometric means, calculated with the Henderson-Tilton formula).

Treatment		**Time after jetting**	
	2 weeks	6 weeks	12 weeks
1% TTO	93.5	93.9	78.1
2% TTO	93.5	91.1	84.0[a]

[a] Based on pre and post shearing counts for the 4 sheep remaining at 12 weeks

The distributions of rubscores in the different groups are shown in Figure 46. Scores were significantly lower in the treated groups than in the controls (P<0.05). Three of the six control sheep had maximum rubscores of 6 at both 6 and 12 w, which could be expected to be correlated with significant wool loss and reduction in wool quality. In the TTO-treated groups no sheep had reached score 6 at 6 w and only one sheep had reached this level by12 w. Unfortunately sheep were not scored for wool rub at the commencement of the study and many already had significant fleece derangement at this time. It was therefore not possible to separate wool damage that had occurred before the experiment began from that which occurred after treatment with TTO.

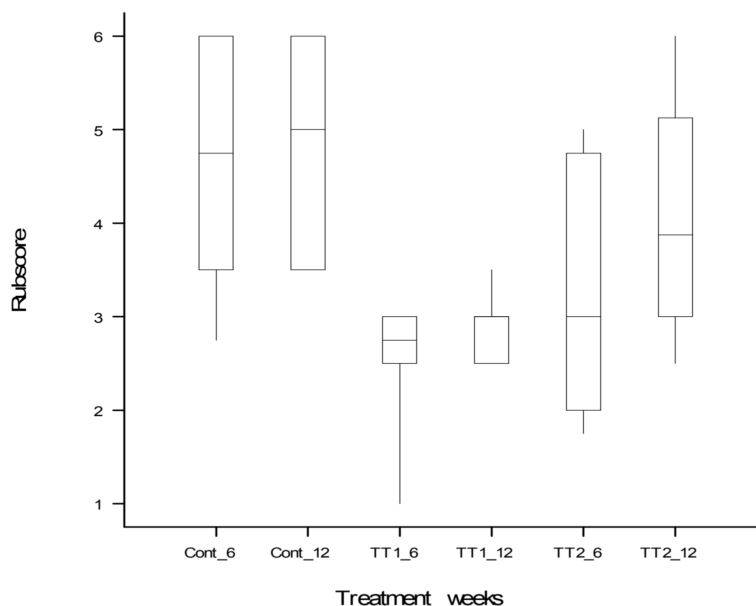


Figure 46: Mean wool rubscore in untreated sheep and sheep jetted with 1% TTO (TT1P) and 2% TTO (TT2P) formulation at 6 and 12 weeks after treatment. (Values for two sheep were missing in the TT2P group at 12 weeks.)

Residues

Tissue samples. Analysis of muscle sub-samples of approximately 0.2 g collected one week after dipping from the 6 control sheep, the 6 sheep treated with 1% TTO and 5 of the 6 sheep treated with 2% TTO found no evidence of the presence of any TTO components. As no residues were found in any sample no further tissue samples were collected, as agreed under our Animal Ethics approval,

Blood samples. GCMS analysis of blood samples collected 1 and 3 weeks after treatment from all sheep dipped in 1% and 2% TTO formulation found no indication of any TTO components.

Wool samples. Figures 47 and 48 show changes in the relative concentration of some TTO components in the wool of sheep at different times after dipping in 2% TTO. For most compounds concentrations were at highest levels at the one week measure and then dropped relatively quickly to close to non-detectable levels by 12 weeks after treatment. It is notable however that there seemed to be a jump in the level of terpinen-4-ol from the 3 week measure where it was quite low to the 6 week measure where it was close to the 1 week levels in most sheep. It is tempting to suggest that this is an experimental artefact. However, the rump and shoulder samples were measured at different times by different operators, the same increase wasn't seen with other compounds and the increase in terpinene-4-ol at 6 w occurred in all sheep (Figure 48). Although sampling or experimental artefacts cannot be completely ruled out, at this time we believe the increase to be real. The sheep were exposed to 45 mm of rainfall the day before the samples were collected and this may have had an effect. At the time of preparation of this report we are analysing further samples from the 1% TTO treatment to confirm this increase.

Figure 47: Changes in the rump wool concentrations of some major TTO components over 12 weeks after dipping in 2% TTO

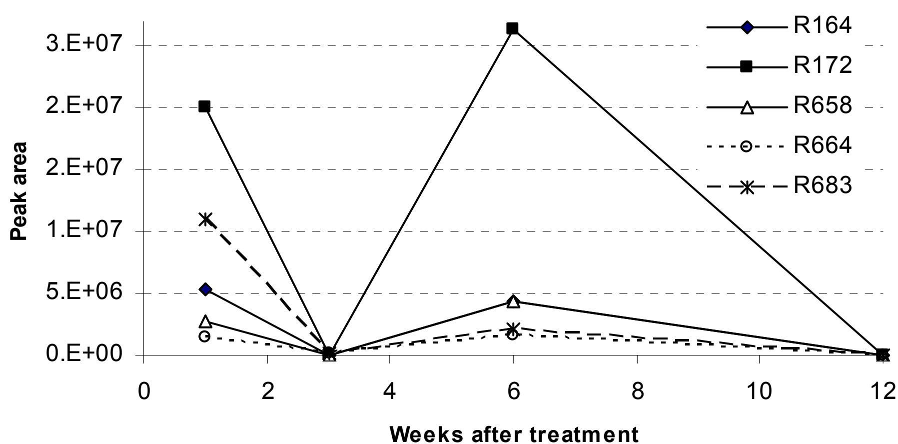


Figure 48: Changes in concentration of terpinene-4-ol in the shoulder wool of individual sheep dipped in 2% TTO at 1, 3, 6 and 12 weeks post dipping

'Stripping' study (laboratory)

Figure 49 shows the mean fall in volume of TTO formulation in the beakers after each three wool samples were dipped and Table 11 shows the reduction in concentration of TTO components from before dipping commenced until after the last (fifteenth) 10 g sample was dipped, retrieved and drained. At this stage the mean volume of wash had fallen by more than 60% from 500 ml to a mean of 195 (± 9) ml and the concentration of terpinene-1-ol had fallen by a mean of 49%.

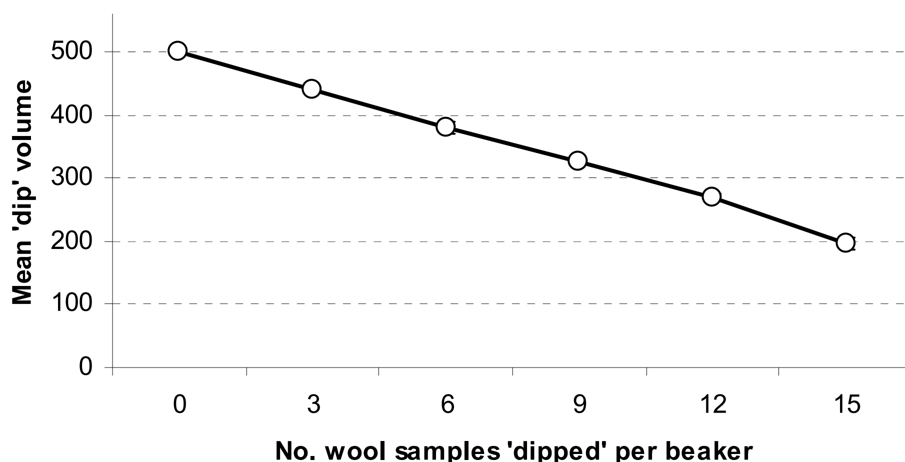


Figure 49: Fall in dip volume with progressive dipping of wool samples. (Initial dip volume = 500 ml; each wool sample = 10 g).

Table 11: Percentage reduction (± s.e.) in the concentration of some major TTO components in TTO formulation used in a simulated wool dipping study and in control fluid left standing on the bench top under equivalent conditions, but without wool dipping.

Treatment	Tea tree oil component			
	terpinen-4-ol	γ-terpinene-	α-terpinene	p-cymene
Control	1.9 (±6.4)	-1.4 (±2.1)	-1.9 (±5.6)	n.d.
Dipping	48.6 (±8.0)	54.4 (±0.9)	53.6 (±0.1)	12.5 (±5.3)

Discussion

Sheep blowfly

The results reported show that TTO has strong insecticidal and repellent effects against *L. cuprina* and generally at concentrations that suggest potential commercial feasibility of the development of flystrike and wound treatment formulations based on or including TTO.

Larvicidal activity of was confirmed with formulations containing 1% TTO consistently killing 100% of first instar larvae in serum assays. The mortality induced in later instars was dependent on the method of exposure. In the agar system, developed to approximately simulate flystrike and where feeding larvae were exposed to treated media for 48 h, 2.5% TTO killed second and third instars in most instances. However, in the larval dipping studies, where sheep were only exposed to TTO for 60 s much higher concentrations of TTO were required to give a good kill. In the agar assays, larvae were confined to the treated areas and this no doubt contributed to the high mortality achieved.

Killing the larvae in the wound is undesirable to avoid septic effects from putrefaction products. A good wound treatment should repel maggots from the wound before killing them Loeffler and Hoskins (1946). Our results suggest that TTO has a strong repellent effect against larvae and would stimulate maggots to move out of the struck area, aiding in the resolution of the strike. The demonstrated antibacterial properties of TTO (Carson *et al.* 2006) may also assist in preventing secondary infections.

The concentration of TTO required to obtain high mortality of larvae was much higher in the larval dipping studies, where larvae were only exposed to formulation for 60 s, than in the agar assays. The relatively poor kill with the larval dipping method was similar to that observed with many current commercial flystrike preparations in similar studies (Levot and Barchia 1995) and in terms of gaining successful resolution of the strike is probably not of concern. However, surviving larvae could contribute to future generations of flies, and sub-lethal exposure may increase selection for resistance (Levot and Hughes 1990). For these reasons and because of the many potential beneficial effects from including TTO in a flystrike treatment preparation, we also tested the effect of TTO in combination with chemical actives already registered for flystrike control.

In both the agar and dipping studies, inclusion of TTO with insecticide in the formulation reduced effectiveness in comparison with the chemical actives alone. In the agar test system with TTO, where the larvae were not confined to the 75ml plastic vials, they rapidly vacated the treated agar. This no doubt reduced contact with insecticide and consequent mortality. The reasons for reduced effect in the dipping studies are less clear. Possibilities are that detection of the repellent compounds by the larvae also causes them to reduce oral ingestion or stimulates spiracular closing and reduces uptake by this means. It is also possible that addition of essential oil to the formulation has physical effects on the uptake of the active ingredient through the larval cuticle.

Boric acid was included in these studies because it is accepted for use under many organic production standards and has been shown to be effective as an active ingredient in previously tested flystrike treatments (Johnstone 1951). The concentration of 2.5% used in our study was chosen on the basis of previous dose comparison studies. Boric acid at this concentration gave 100% kill in the agar assays when used as a single ingredient, but in common with the other insecticides, mortality was lower when it was used together with TTO. The 2.5% concentration used in our study was much lower than the 20% in the ‘Borocit’ formulation developed by Johnstone (1951) and a formulation containing higher concentration of boric acid together with TTO could provide a suitable flystrike treatment for use in organic systems.

Our main objective in formulation was to provide optimal dispersion and stability of TTO in an aqueous medium and to facilitate thorough wool wetting. A number of preliminary studies (data not

shown) were conducted towards this end with different solvents. Formulation is critical to achieving maximum effect against insects and quite large differences in effectiveness of flystrike preparations can occur with different formulations containing similar concentrations and type of active ingredient (Levot and Barchia, 1995). It is possible that a formulation developed to facilitate maximum larval wetting and uptake of TTO by larvae cuticle could enhance the effect of TTO-based flystrike formulations.

Resistance to organophosphorous insecticides, which are cholinesterase inhibitors, is now well established in sheep blowfly populations (Levot 1995). Mills *et al.* (2004) showed that TTO has anti-cholinesterase activity and hypothesised that its insecticidal effects may be attributable, at least in part, to this action. It is possible that OP resistance could reduce susceptibility to the insecticidal effects of TTO.

Our studies also demonstrated that TTO can have large and persistent repellent effects against adult *L. cuprina*. In the studies presented here oviposition was completely suppressed up to 44 days after wool treatment. This is much longer that the 2-3 weeks that may be required for wound healing .The period of repellency extended for much longer than we expected and we did not have enough wool preparations to conduct further tests at later times. There was indication that the repellent effect was starting to break down at the last observation, although the number of flies landing on the treated formulation was still only 40% of that on controls presented in a choice situation. *L. cuprina* generally undertakes a complex pattern of pre-oviposition behaviour and requires appropriate cues to be received by sensors on both the tarsi and ovipositor before egg masses are deposited (Barton-Browne 1977). It is possible that egg laying may have been suppressed for quite a bit longer that the period measured here. It should be noted, however, that the wool tested in this experiment was kept in a cupboard and therefore not subject to environmental effects such as rainfall, high temperatures and photo-degradation. These effects would be expected to reduce the period of repellency under field conditions.

We have also demonstrated that TTO can have direct insecticidal effects against the adult stage and eggs of *L. cuprina* which could contribute to the treatment and protection of flystrike wounds. It also has antibacterial effects (Carson *et al.* 2006) that could protect against secondary infections that can contribute to deaths from flystrike and has been suggested to aid wound healing (Halcon and Milkus 2004; Woollard *et al.* 2007). The combined effect of TTO in repelling flies, its toxic effects against eggs and first instar *L. cuprina* and toxic and repellent effects against later larval stages suggests that it could be a valuable 'natural' strike therapeutic or additive in flystrike treatments and wound dressings.

Sheep lice

The laboratory assays with sheep lice indicated two things. Firstly, in most treated surface experiments and wool assays where the surface or wool was allowed to dry before exposure TTO gave very poor effect, even at relatively high concentrations of TTO. Where treated surfaces were not allowed to dry before treatment, often high control mortalities resulted, making it difficult to differentiate the effects of TTO. Using grapeseed oil as carrier (less volatile than other solvents tried) and carefully choosing the amount of carrier to disperse through the filter paper without leaving excess fluid in which lice could become trapped gave better results. In this system there was low mortality in controls and close to 100% mortality with levels of TTO of 10% and above. It should also be noted, that as a lower volume of grape seed oil was used than acetone or butanone a lower total amount of TTO was used for a given concentration.

The wool dipping studies, which more closely approximated the conditions likely on a sheep, gave much better results. In assays conducted using this system, concentrations of 1% TTO and above consistently gave 100% mortality of lice. Usually in these experiments two different sorts of 'control' groups were used, groups with dry wool and groups in which lice were treated with the carrier, but

without TTO. This was considered to more closely reproduce conditions to which lice would be exposed on treated sheep. Very wet conditions and dipping in wetting agent alone are known to cause mortalities in sheep louse populations (Murray 1963, Heath *et al*. 1995). When sheep are treated by aqueous solutions of lousicide in practice, wetting no doubt contributes to control. In our wool assays, treatment with carrier and wetting agent gave mortalities significantly above the dry wool controls, but always significantly lower than with 1% TTO and whereas 1% TTO consistently gave 100% mortality, a large proportion of treated lice survived in the water plus wetting agent controls.

The assays for fumigant effect suggested that most, if not all, of the activity of TTO against sheep lice comes from fumigant activity. The assays suggested that the total quantity of TTO in the test chambers, which no doubt related directly to the vapour pressure of TTO components, was the most effective parameter determining effectiveness. However, the experiment in which pure TTO was compared to equivalent amounts of TTO formulated with Tween 80 showed significant effects of formulation on effectiveness, especially with amounts that were marginally, or not completely effective. When TTO was presented as pure TTO, many of the more volatile components probably volatilised rapidly providing a high vapour pressure in the chamber and higher mortality compared to the formulated TTO where more of the TTO remained with the formulation or took longer to volatilise with lower resultant vapour pressures in the chamber. Studies with a number of TTO components used in experiments at relative concentrations in proportion to which they were present in TTO suggested that by far the majority of this activity was due to terpinenen-4-ol.

The importance of vapour action probably also helps to explain the lack of effect observed in the treated surface assays with acetone or butanone and when wool was dried before lice were exposed. In this situation drying would have evaporated many of the vapour-active compounds with resultant acute reduction in insecticidal effects.

Although TTO was very active against adult lice and nymphs at relatively low concentrations, the apparent lack of persistent effect was of concern. Although the dense covering of fleece on sheep increases the difficulty of control because of problems in achieving good wetting and contacting all lice, with most sheep louse control formulations it also aids control by carrying high enough concentrations of insecticide for long enough to kill new nymphs as they emerge from eggs. With other species of lice (*Linognathus pedalis* and *L. ovillus*) that survive on the haired areas of sheep, (and often lice on other animal species that don't have wool) a second treatment is often necessary after all eggs have hatched, but before they develop to adults and start to lay eggs, to give good control. The direct ovicidal effects of TTO vapours and possible physical effects on the lice eggs, suggest that persistent effect may not be necessary to achieve good lice control with TTO formulations. Unfortunately a couple of subsequent assays with eggs failed (low hatch rate in controls in one instance and inability to collect sufficient eggs in a second) and these effects could not be followed up before the sheep were disposed of.

On the basis of the laboratory assays 1% TTO formulation was chosen for use in the animal tests. Experiments were designed according to guidelines specified by the Australian Pesticides and Veterinary Medicines Authority (APVMA). As insecticides are often less effective under the much less controlled conditions present in practical situations, a second formulation containing 2% TTO was also tested. When sheep were treated by immersion dipping both formulations reduced louse numbers to non-detectable levels. As no lice were found on any treated sheep in close examination of 40+ fleece parts on each sheep on four separate occasions, and as it seemed that there was unlikely to be any long term a persistent effect which could suppress population growth below detectable levels for the 20 week duration of the study, it seems highly likely that lice were in fact eradicated by both TTO formulations.

It is suggested above that ovicidal effects, either through fumigant action or physical effects on the chorion of louse eggs prevented survival of lice in the egg stage. Laboratory tests of TTO fumigant effects against lice, indicated that the total amount of TTO present and resultant vapour pressure of TTO components is critical to the degree of fumigant effect seen. Our formulation was designed to aid

wetting of the wool and dye marking suggested that this was successfully achieved with the wool taking up significant amounts of the TTO formulation. The dense covering of wool no doubt led to a high vapour pressure of TTO amongst the wool fibres, where sheep lice are found. Wool grease consists of a complex mixture of lipids (wax) and water soluble components (suint) and with the formulation used, no doubt absorbed significant amounts of the TTO formulation. The effect that this has on subsequent evaporation of TTO components is uncertain, but TTO could be smelt on the treated sheep for a number of weeks after treatment. With the dense covering of wool present it is possible that the vapour pressure of TTO components, particularly terpinene-1-ol, remained high enough amongst the wool fibres for the time that it took any surviving eggs to hatch (maximum 10 days) for mortality in hatching nymphs to also contribute to control.

An important consideration when determining optimal dip practice is knowledge of whether or not a dipping formulation "strips". Stripping occurs when active ingredient is selectively taken up in the wool grease at a greater rate than the water in which it is emulsified. This leads to a progressive fall in the concentration of active ingredient as more sheep are dipped and can ultimately lead to the concentration falling below effective levels. This can aid effectiveness by helping to absorb fluid into the fleece, particularly where treatment fluid is not recirculated as with jetting, but replenishment, usually at concentrations higher than the initial charge rate may be necessary to maintain the effectiveness of dipping where runoff fluid is returned to the dipping vat from draining pens. It was considered that with the low numbers of sheep treated in the animal studies, measurement of the dip wash was unlikely to indicate with any degree of confidence whether stripping occurred and so a bench top simulation was conducted. This study suggested that stripping occurred at different rates with different TTO constituents, but levels of the main active component fell by approximately 50% after reduction in dip wash volume of 60%. Lund *et al.* (2005) found that the concentration of diazinon, a compound used for sheep dipping over many years, dropped by more than 90% with a 25% fall in dip volume. Although it is difficult to compare these two studies, our results suggest that stripping occurs at a slower rate than with diazinon and should be readily managed by the design of appropriate topping up and replenishment procedures.

Long wool treatment with both 1% and 2% TTO formulations brought about reductions in louse numbers of up to 94%. To achieve the efficacy criteria required of a long wool louse treatment by APVMA in Australia, a product must achieve a reduction of 95% or greater in louse numbers for at least 30 days. However, registration guidelines also state that if a treatment can be shown to prevent further wool damage, registration may still be granted with lower percentage control. Although measuring wool damage was not included in the initial plan for the experiment, the scoring for fleece derangement conducted at 6 w and 12 w after treatment suggested a reduction in wool damage from TTO treatment. In addition, most lice survived in areas that were not marked with dye suggesting that coverage from jetting may have been a major limitation to the reduction in louse numbers achieved. The level of control may be improved with other methods or formulations that provide better wetting or coverage.

The residue profile of TTO components measured in wool samples suggests that TTO breaks down relatively quickly after application and this may enable its use as an emergency treatment closer to shearing than many other more residual pesticides. In addition, TTO is a plant based project and its use for lice control would seem to be in accord with the marketing image of wool as 'pure and natural'. Further trials with TTO towards fulfilling long wool registration criteria, possibly with other methods of application or formulations, would seem to be worthwhile.

Implications and Recommendations

The results of this project indicate significant potential for use of TTO formulations as sheep ectoparasiticides. Both insecticidal and repellent effects were demonstrated against sheep blowflies and sheep studies demonstrated probable eradication of lice when sheep were dipped in 1% and 2% TTO formulations. Further investigation of the commercial feasibility of development of sheep lousicides based on TTO and, depending on the outcome of these investigations, trials towards potential registration for commercial use seem warranted.

Insecticidal and repellent effects against *L. cuprina* larvae and adult flies also suggest considerable potential for use of TTO in flystrike treatments. These effects, together with the demonstrated antimicrobial and wound healing properties, and the perception of TTO as natural and soothing, also suggests possibilities for more widespread use in general veterinary wound treatments, particularly for treatment and protection of wounds resulting from husbandry practices such as tail docking, castration, mulesing and dehorning.

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Controlling Fly Strike and Louse Infections in Sheep with Tea Tree Oils

by P James

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Tea tree oil, the essential oil of the native Australian plant *Melaleuca alternifolia*, has often been promoted as having insecticidal and insect repellent properties. However, there is limited evidence in the form of scientifically conducted studies, with efficacy tested against a relatively limited number of pests. With increasing community eco-consciousness and concern about the use of artificial pesticides, there are growing opportunities for 'natural' products and a ready market for tea tree oil for use in 'softer' pest control technologies where efficacy can be demonstrated.

This report details the results of experiments conducted to assess the potential of tea tree oil for use in the treatment of infestations of sheep lice (*Bovicola ovis*) and flystrike caused by sheep blowfly (*Lucilia cuprina*). The report details a series of laboratory and animal studies which indicate significant potential for the use of tea tree oil in formulations for these purposes.

Tea tree oil producers and product manufacturers who produce and market tea tree oil and animal health products will find this research of interest.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report, an addition to RIRDC's diverse range of over 2,000 research publications, forms part of our Tea Tree Oil Program, which aims to to support the continued development of an environmentally sustainable and profitable Australian tea tree oil industry that has established international leadership in marketing, in value-adding, and in product reliability and production.

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